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Acquired copper imbalance in diabetes-induced cardiac disease: molecular mechanisms and reversibility

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Abstract

Diabetes is a metabolic disorder characterised by chronic hyperglycaemia, which results from disruption of several glucose-control mechanisms. In diabetic patients, it is well established that prolonged hyperglycaemia increases the risk of the cardiovascular complications, which are responsible for up to 80% of deaths and are the leading cause of the morbidity and mortality in T2DM.

Clinical studies on diabetic patients have shown that chronic hyperglycaemia can disrupt myocardial copper balance and cause elevated serum copper levels, particularly in those patients with cardiovascular complications.

It has also been shown that copper imbalance in the myocardium can be directly or indirectly involved in the pathogenesis of diabetes-induced cardiovascular disease. Recent studies from our group have shown that chronic treatment with a divalent Cu(II)-selective chelator, TETA, can ameliorate diabetes-induced disturbances in the regulation of copper homeostasis, and improve the structure and function of the heart in diabetic rats and humans.

In this thesis, I have examined the interplay of hyperglycaemia and copper levels on cardiomyocyte structure and function in both a cellular and an animal model. It was found that the copper balance in cardiomyocytes was altered by chronic hyperglycaemia, which elicited decreased cellular copper levels and increased copper sensitivity compared to controls. Based on the results, I have concluded that chronic hyperglycaemia-induced copper imbalance could be due to changes in the intracellular copper-transport pathways, with genes involved in antioxidant mechanisms and the mitochondrial pathway found to be suppressed in cardiomyocytes cultured with high glucose. In the STZ-induced diabetic rat model, some of these abnormalities were also found in diabetic myocardium.

In this thesis several of the abnormalities found in diabetic myocardium have been shown to be ameliorated with chronic TETA treatment. Although the mechanisms of drug action are not fully understood, we hypothesise that TETA treatment can improve cardiac function in the diabetic heart through the binding of excess free copper in the myocardium, which can lower copper-mediated oxidative stress and restore copper balance in the diabetic heart. These mechanisms can prevent further damage occurring and allow innate regenerative processes to take place in the myocardium to restore the structure and function of the diabetic heart.
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### Abbreviations

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<thead>
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<th>Definition</th>
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</thead>
<tbody>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>Actb</td>
<td>β-actin</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
</tr>
<tr>
<td>ATOX1</td>
<td>Antioxidant protein 1</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATP7a</td>
<td>ATPase copper transporter, alpha</td>
</tr>
<tr>
<td>ATP7B</td>
<td>ATPase copper transporter, beta</td>
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<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
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<tr>
<td>bpm</td>
<td>Beats per minute</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Ca²⁺</td>
<td>Calcium ion</td>
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<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
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<tr>
<td>cAMP</td>
<td>Cyclic Adenosine monophosphate</td>
</tr>
<tr>
<td>CAN</td>
<td>Cardiovascular autonomic neuropathy</td>
</tr>
<tr>
<td>CCO</td>
<td>Cytochrome c oxidase</td>
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<tr>
<td>CCS</td>
<td>Copper chaperone for SOD</td>
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<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
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<td>CTR1</td>
<td>Copper transporter receptor1</td>
</tr>
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<td>Cu</td>
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<tr>
<td>Cu⁺</td>
<td>Cuprous ion</td>
</tr>
<tr>
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<tr>
<td>CuSO₄</td>
<td>Cupric sulfate</td>
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<tr>
<td>DAT</td>
<td>N1,N10-diacyltriethylenetetramine</td>
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<tr>
<td>Dcytb</td>
<td>Duodenal cytochrome b</td>
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<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
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<td>Dimethyl sulphoxide</td>
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<td>Divalent metal transporter 1</td>
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<td>DNA</td>
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<td>D-penicillamine</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EC-SOD</td>
<td>Extracellular Cu/Zn superoxide dismutase</td>
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<td>EDHF</td>
<td>Endothelium derived hyperpolarizing factor</td>
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<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
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<td>Fas</td>
<td>Fatty acids</td>
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<td>Fe</td>
<td>Ion</td>
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<td>Free fatty acid</td>
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<td>FGF-2</td>
<td>Fibroblast growth factor-2</td>
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<td>GAPDH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>Gestational diabetes</td>
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<td>Graphite furnace atomic absorption spectrometry</td>
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<td>Glucose transporter type 1</td>
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<tr>
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<td>H₂O₂</td>
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<tr>
<td>IMS</td>
<td>Intermembrane space</td>
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<td>KH₂PO₄</td>
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</tr>
<tr>
<td>KHB buffer</td>
<td>Kreb-Henseleit bicarbonate buffer</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LVH</td>
<td>Left ventricular hypertrophy</td>
</tr>
<tr>
<td>MAT</td>
<td>N1-acetyltriethylenetetramine</td>
</tr>
<tr>
<td>Max</td>
<td>Maximum</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein-1</td>
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<td>MgSO₄</td>
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</tr>
<tr>
<td>Min</td>
<td>Minimum</td>
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<td>ml</td>
<td>Millilitre</td>
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<tr>
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</tr>
<tr>
<td>mmHg</td>
<td>Millimetres of mercury</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>MT1</td>
<td>Metallothionein 1</td>
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<tr>
<td>MT2</td>
<td>Metallothionein 2</td>
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<tr>
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<td>Murr1</td>
<td>Mouse U2af-rs1 region</td>
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<td>NDC</td>
<td>Nucleoporin</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>NO synthase</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>Superoxide anion</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum cutting temperature</td>
</tr>
<tr>
<td>OH¹</td>
<td>Hydroxyl radical</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>PARP-1</td>
<td>Poly-adenosine diphosphate-ribose polymerase-1</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF-β</td>
<td>Platelet derived growth factor beta receptor</td>
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<tr>
<td>PDH</td>
<td>Pyruvate dehydrogenase</td>
</tr>
<tr>
<td>PDK4</td>
<td>Pyruvate dehydrogenase kinase</td>
</tr>
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<td>PGI₂</td>
<td>Prostacyclin</td>
</tr>
<tr>
<td>PPAR-α</td>
<td>Peroxisome proliferator-activated receptor-alpha</td>
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<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin-angiotensin system</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
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<tr>
<td>-----------</td>
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</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPL13A</td>
<td>Ribosomal protein L13A</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Quantitative RT-PCR</td>
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<tr>
<td>Sco1</td>
<td>SCO cytochrome oxidase 1</td>
</tr>
<tr>
<td>Sco2</td>
<td>SCO cytochrome oxidase 2</td>
</tr>
<tr>
<td>SMCs</td>
<td>Vascular smooth muscle cells</td>
</tr>
<tr>
<td>SOD1</td>
<td>Copper/zinc superoxide dismutase 1</td>
</tr>
<tr>
<td>STEAP</td>
<td>Six transmembrane epithelial antigen of the prostate</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozotocin</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA box binding protein</td>
</tr>
<tr>
<td>TETA</td>
<td>Triethylenetetramine</td>
</tr>
<tr>
<td>TGF-1</td>
<td>Transforming growth factor-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue-plasminogen activator</td>
</tr>
<tr>
<td>U2AF</td>
<td>U2 auxiliary factor 35 kDa subunit</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>van Willebrand factor</td>
</tr>
<tr>
<td>β-MHC</td>
<td>β-myosin heavy chain</td>
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</table>
Chapter 1 General Introduction

1.1 Insulin and diabetes mellitus

Diabetes mellitus is a metabolic disorder associated with abnormally high blood glucose levels (hyperglycaemia) and specific forms of chronic organ damage, termed the complications, which mainly affect the heart (cardiomyopathy), arteries (arteriopathy), kidneys (nephropathy), retina (retinopathy) and nerves (neuropathy) (Kannel and McGee 1979; Greene et al. 1986; Ballard et al. 1988; Haidara et al. 2006).

Diabetes is caused by insufficient production of insulin coupled to inadequate sensitivity of cells to the action of insulin (Bergman et al. 2002; Rains and Jain 2011). Insulin is the major polypeptide hormone secreted by pancreatic beta cells after food intake that acts as a master regulator of the metabolic regulation of bulk fuels, including carbohydrates, fats and proteins (Saltiel and Kahn 2001; Marshall 2006); it exerts powerful anabolic effects on most tissues in the body, and also acts as a potent growth factor (Koontz and Iwahashi 1981; Hill and Milner 1985).

Insulin is the main hormone in the body that stimulates the clearance of glucose from the blood stream: it is thus the master hypoglycaemic (or glucose-lowering) hormone in the body. It lowers blood glucose levels by the insulin receptor-mediated stimulation of skeletal muscle cells to take up glucose and store it as glycogen and by suppressing glycogen breakdown (glycogenolysis) and release of glucose from the liver at the same time (Klip and Paquet 1990; Roden et al. 1996). Insulin also induces fat cells to absorb free fatty acids from the blood and convert them into depot fat made mainly from triglycerides (lipogenesis), and to suppress the breakdown of adipose-tissue lipids (lipolysis) at the same time (Kahn and Flier 2000; Klemm et al. 2001; McTernan et al. 2002).

There are four main forms of diabetes mellitus: type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes (GDM) and secondary diabetes (Kloppel et al. 1985; Galerneau and Inzucchi 2004; Kerner and Bruckel 2011). The last category comprises about 1% of all patients with diabetes and will not be considered further in this thesis.

T1DM is an autoimmune disease in which beta cells are destroyed by autoimmune processes (Kloppel et al. 1985; Atkinson and Eisenbarth 2001). T2DM is caused by insulin resistance in peripheral tissues or insulin secretory defects of the beta cells associated with the presence of
islet amyloid (Johnson et al. 1989; Stumvoll et al. 2005). Gestational diabetes occurs during some pregnancies in which pregnancy hormones are said to evoke insulin resistance (Galerneau and Inzucchi 2004). All forms of diabetes result in high blood glucose levels which can cause damage to many organs and lead to a group of diabetes-associated complications, including kidney failure, cardiovascular disease, nerve damage and microvascular damage.

1.1.1 Type 2 diabetes
Type 2 diabetes mellitus (T2DM) was previously designated ‘adult-onset’ or ‘maturity-onset’ diabetes. However, in association with the increase in obesity and reduced physical activity in many modern lifestyles, T2DM is now occurring in patients of progressively decreasing age (Cali and Caprio 2008). The development of T2DM is triggered by both environmental and genetic factors, leading to insulin resistance or reduced insulin sensitivity, combined with reduced insulin secretion (Johnson et al. 1989; Stumvoll et al. 2005). Impaired insulin function causes abnormalities in glucose homeostasis and thus results in hyperglycaemia. Currently, the exact molecular basis of insulin resistance is unknown and there is no known cure for T2DM. Patients with T2DM require long-term medication to manage their blood glucose levels and to limit the development of diabetes-associated complications.

1.1.2 Epidemiology
T2DM is the most common form of diabetes and accounts for about 90% of all cases globally. It currently affects more than 366 million adults and is a major health problem world-wide (Shaw et al. 2010; Guariguata et al. 2011; Whiting et al. 2011)). The incidence of T2DM has significantly increased in the last 30 years due to the changes in lifestyles associated with reduced physical activity and increased obesity. In the next 20 years, it is estimated that the number of adults with diabetes is expected to rise to 552 million with a 69% increase in developing countries and 20% in developed countries (Shaw et al. 2010; Guariguata et al. 2011; Whiting et al. 2011).

In New Zealand, the Ministry of Health has estimated that more than 200,000 people have diagnosed diabetes mellitus, and a further population of more than 100,000 people with undiagnosed disease (www.moh.govt.nz). Studies based on New Zealand populations have
shown that Maori and Pacific-Island groups have a higher prevalence of diabetes, around three times higher than among other New Zealand populations (Joshy et al. 2009). The incidence of diabetes has increased significantly among all ethnic groups in this country. It is thus important to gain a better understanding of this disorder to develop more effective strategies for its prevention and treatment.

1.1.3 Factors contributing to T2DM onset
The aetiology of T2DM is not fully understood. It is believed that T2DM is due to multiple processes including environmental factors, susceptible genes, poor diet, obesity, medications and infections. It is likely that different combinations of genetic and non-genetic risk factors act together to cause the disease in different individuals.

1.1.3.1 Genetic susceptibility
Several studies have provided evidence that genetic factors play a role in the development of T2DM. Family studies have shown that the lifetime risk for the offspring to develop T2DM is about 40% where one parent has diabetes, but increases to 70% if both parents have diabetes (Wagener et al. 1982). Studies have also revealed that first degree relatives of individuals with T2DM have a 3-fold higher risk of developing the disease compared to individuals with no diabetes history (Beaty et al. 1982). Furthermore, studies on twins have found that the concordance of T2DM in monozygotic twins is significantly higher than dizygotic twins, approximately 70% compared to 30% (Newman et al. 1987; Kaprio et al. 1992). These results suggest that genetic factors do influence the development of T2DM. Due to the genetic and environmental variations between different ethnic groups, it has proven hard to identify candidate genes for T2DM. Recent studies have identified several genes consistently associated with T2DM, including calpain-10, aryl hydrocarbon receptor nuclear translocator (ARNT), solute carrier family 30 member 8 (SLC30A8), transcription factor 7-like 2 (TCF7L2) and peroxisome proliferator-activated receptor-Gamma (PPAR-Gamma), which are involved in beta cell dysfunction and insulin resistance (Baier et al. 2000; Buzzetti et al. 2004; Parikh and Groop 2004; Frayling 2007; Rampersaud et al. 2007).
1.1.3.2 Environmental factors

Compared to genetic factors, environmental factors may play a bigger role in the development of T2DM. Environmental factors such as obesity (Mokdad et al. 2003), inactive lifestyles (Tuomilehto et al. 2001), poor diet (Hu et al. 2001), smoking and insufficient sleep (Mallon et al. 2005; Gangwisch et al. 2007) could impair glucose homeostasis and contribute to the onset of T2DM. Among all these factors, obesity is the leading risk factor for T2DM. It is believed that the increased incidence of T2DM in recent years has been mainly attributed to the rise in obesity. It has been estimated that in Western countries approximately 80% of all new T2DM cases are due to obesity (Lean 2000). The risk for diabetes has been reported to be about 2-fold in the mildly obese, 5-fold in the moderately obese and 10-fold in morbidly obese persons (Hartz et al. 1983; Scheen and Lefebvre 1998).

In addition to obesity, the distribution of body fat can also have an impact on T2DM risk. Individuals with a higher proportion of upper-body fat or abdominal fat have a higher risk of developing insulin resistance and T2DM (Ohlson et al. 1985; Meisinger et al. 2006). Since body weight is highly correlated with physical activity and food intake, an inactive lifestyle is another major risk factor in T2DM. Studies have shown that targeting diet and regular exercise can significantly reduce the risk of T2DM (Tuomilehto et al. 2001; Hu 2003).

Beside the postnatal environmental factors, several studies have suggested that the perinatal environment can also influence the risk of T2DM (Hales and Barker 2001; Sobngwi et al. 2003). Numerous studies have shown that low birth weight is associated with impaired glucose tolerance and T2DM later in life (Barker et al. 1993; Frayling and Hattersley 2001; Iliadou et al. 2004). In animal models, fetal under-nutrition can lead to an insulin deficiency disorder and over-nutrition can lead to insulin excess syndrome (Patel and Srinivasan 2002). However, the molecular basis of these observations remains unknown.

1.1.3.3 Interaction between genetic and environmental factors

As mentioned above, both genetic and environment factors can contribute to the development of T2DM. However, genetic and environmental factors can explain only part of the risk in the disease. For example, effects of diet on diabetes onset have been reported to differ between individuals (Corella and Ordovas 2005; Diego et al. 2007; Grarup and Andersen 2007; North et al. 2007). It is believed that interactions between genetic and environmental factors contribute to the variation between individuals. Although the mechanisms involved in
diabetes mellitus are not fully understood, it is well understood that environmental factors can alter gene expression to affect disease processes. On the other hand, intrinsic genetic variation can also determine how individuals react to the environmental factors. Therefore, understanding the interactions between genetic and environmental factors can help to improve the treatment strategies against the disease.

1.1.4 Pathogenesis of T2DM

T2DM is a metabolic disorder characterised by chronic hyperglycaemia, resulting from abnormalities and disruptions of several glucose control mechanisms. Insulin resistance, increased hepatic glucose production that is inadequately suppressed by insulin (or hepatic insulin resistance), and beta cell dysfunction are the three major defects present at the onset of hyperglycaemia in T2DM (Seltzer et al. 1967; Polonsky et al. 1988; Gerich 1998). These defects combine to result in abnormal glucose tolerance and the progressive development of hyperglycaemia.

Despite intensive research, the pathological mechanisms behind these abnormalities still remain poorly understood. It is believed that impaired glucose homeostasis occurs long before the onset of the disease. Genetic and environmental factors induce insulin resistance during the prediabetic state, in which reduced body tissue sensitivity or delayed responses to insulin can lead to impaired glucose tolerance (Gerich 1998; Groop 1999; de Lange et al. 2003). Current studies suggest that insulin resistance can be due to a variety of cellular and molecular defects including defects in receptor function, aberrant insulin receptors, signal transduction pathways, abnormalities in glucose transport and glucose metabolism although the relationship of many of these processes to the common form of type-2 diabetes are mostly unclear (Kolterman et al. 1981; Shulman et al. 1990; Minokoshi et al. 2003).

Insulin resistance is associated with reduced glucose uptake in both muscle and fat cells, reduced glycogen synthesis and increased hepatic glucose production in liver cells (Caro et al. 1989; Rossetti et al. 1993; Michael et al. 2000; Inoue et al. 2004). In order to maintain normal glucose homeostasis, beta cells will increase insulin secretion to compensate for the insulin resistance. As this abnormality progresses, a new equilibrium is reached with normal blood glucose and high plasma levels of insulin, which results in hyperinsulinaemia (Erdmann et al. 2008). T2DM is said to develop when continuous production of insulin causes beta cell ‘exhaustion’, a poorly-defined state, and leads to beta cell ‘dysfunction’,
another poorly defined state. Impaired beta cells can no longer produce sufficient insulin, resulting in hyperglycaemia (Kahn et al. 2001). Chronic hyperglycaemia can further impair insulin production and secretion by inducing glucose toxicity in beta cells, result in beta cell apoptosis which leads to reduced beta cell mass (Butler et al. 2003; Deng et al. 2004), but these states are thought to occur only after the onset of hyperglycaemia and cannot therefore contribute to the aetiology of the apparent beta cell dysfunction in type-2 diabetes.

Other potential molecular mechanisms including lipodystrophy, impaired incretin release, abnormal glucagon activity, increased absorption of fluid in the kidneys, and the loss of proper metabolic regulation of the central nervous system, are also considered to contribute to the pathogenesis of T2DM in some cases (Muller et al. 1970; Joffe et al. 2001; Bagger et al. 2011; D'Alessio 2011).

1.1.5 Diabetic complications

The diabetic complications are the major contributors to the increased morbidity and mortality in patients with T2DM (Kannel et al. 1974; Resnick et al. 2001). The detailed pathogenesis of the diabetic complications is not fully understood, but has been shown to be linked to prolonged hyperglycaemia. Diabetic patients with good glycaemic control have lower risk of developing diabetic complications (Turnbull et al. 2009). Loss of glycaemic control in diabetes patients can increase the risk of diabetic complications (Stratton et al. 2000; Duckworth et al. 2009).

Diabetic complications can be categorised into acute and chronic complications. The acute complications can occur at any time during the course of the disease and consist of diabetic ketoacidosis, non-ketotic hyperosmolar coma and hypoglycaemia (Selam 2000). Diabetic ketoacidosis and non-ketotic hyperosmolar coma are related to insulin deficiency, whereas hypoglycaemia is mainly due to excessively high insulin levels. Acute diabetic complications can lead to the development of a diabetic coma in the worst case scenario (Grimaud and Levraut 2001).

Beside the acute complications, prolonged hyperglycaemia can also damage the blood vessels thus inducing various kinds of vascular complications. Studies have shown that approximately 50% of diabetic patients have substantial vascular abnormalities at the time of T2DM diagnosis (Garber 1998; Grundy et al. 1999). Unlike the acute complications, chronic
diabetic complications cause permanent disabilities that are responsible for most of the morbidity and mortality associated with diabetes. About 50-80% of all individuals with T2DM die from cardiovascular disease, cerebrovascular disease and kidney failure (Casiglia et al. 2000). Chronic diabetic complications can be further sub-divided into macrovascular and microvascular complications.

1.1.5.1 Macrovascular complications

Macrovascular complications are due to prolonged hyperglycaemia-induced structural and functional changes in large arteries that lead to increased stiffness, abnormal pulse wave travel and systolic hypertension (Rahman et al. 2007). These complications can occur in blood vessels in any part of the body and cause heart attack, peripheral arterial disease, stroke and diabetic myonecrosis (Kannel and McGee 1979; Nathan 1993; King et al. 2005). Heart attacks are caused by blockage of blood vessels which are initially due to the development of atherosclerosis (Beckman et al. 2002; Falk 2006). Stroke is caused by damage to blood vessels in the brain or thromboembolism from extra-cerebral sites such as the carotid arteries or heart (Sloan 1987; Vilela and Goulao 2005). Peripheral arterial disease is due to the obstruction of blood supply to the legs that may lead to intermittent claudication (ischaemic muscle pain) and diabetic foot ulcers or gangrene (Jude et al. 2001; Dinh et al. 2009). Diabetic myonecrosis (diabetic muscle infarction) results from damage to muscle tissue caused by the loss of blood supply, and is usually found in the thigh (Reyes-Balaguer et al. 2005; Naderi et al. 2008).

1.1.5.2 Microvascular complications

Microvascular complications are due to prolonged hyperglycaemia-induced damage in small blood vessels or capillaries, resulting in reduced blood flow in affected body tissues. Diabetes-induced microvascular complications include neuropathy (nerve damage), nephropathy (kidney disease), retinopathy (vision disorders) and cardiomyopathy (heart disease) (Kannel and McGee 1979; Klein et al. 1985; Greene et al. 1986; Ballard et al. 1988; King et al. 2005).
1.2 Diabetes-associated cardiovascular disease

Chronic cardiovascular complications are the leading cause of morbidity and mortality in patients with diabetes. Cardiovascular disease is responsible for up to 80% of deaths in patients with T2DM (Winer and Sowers 2004). Although the relationship between diabetes and heart failure is not fully understood, several studies have shown that diabetes mellitus is an independent risk factor for the development of heart failure. Patients with diabetes have a higher risk of developing heart failure in both men and women compared with age-matched controls. In the Framingham heart study, it was shown that the risk was increased 2- and 5-fold in diabetic men and women, respectively (Kannel et al. 1974). It is believed that diabetes mellitus induces the development of both ischaemic and non-ischaemic cardiomyopathy (also known as diabetic cardiomyopathy), which leads to heart failure in diabetic patients (Fig 1-1).
Figure 1-1 Contributors and causes of cardiomyopathy in diabetes mellitus.
1.2.1 Diabetes-induced ischaemic cardiomyopathy

Diabetes mellitus is a well-known risk factor for ischaemic heart disease. A number of studies have reported that patients with diabetes commonly develop coronary artery disease and peripheral arterial disease (Kannel and McGee 1979; Feskens and Kromhout 1992). The risk of coronary artery disease and peripheral arterial disease is 2- to 4-fold higher in diabetic patients (Feskens and Kromhout 1992; Morrish et al. 2001). Furthermore, diabetes mellitus also affects the cerebral arterial circulation, which contributes to the 3-fold higher risk that diabetic patients have of developing a stroke (Bell 1994; Davis et al. 1999). Beside the early development of ischaemic heart disease, diabetes mellitus also worsens the outcome in established ischaemic heart disease, as patients with diabetes have an increased rate of myocardial infarction, re-infarction, stroke-related dementia, heart failure and death (Kjaergaard et al. 1999; Malmberg et al. 2000; Shindler et al. 2000). The increased risk of ischaemic cardiomyopathy among diabetic patients may be due to the acceleration of the atherosclerotic process (Garcia et al. 1974; Kannel and McGee 1979). It is believed that defective metabolic regulation in diabetic patients leading to chronic hyperglycaemia, dyslipidaemia and insulin resistance, alters the function of endothelial cells, smooth muscle cells and platelets, which accelerate the development and progression of atherosclerosis.

1.2.1.1 Endothelial dysfunction in diabetes mellitus

The endothelium consists of a monolayer of endothelial cells forming the inner layer of blood vessels (Sumpio et al. 2002). It provides a selective permeability interface between the vessel wall and circulating blood. The endothelium actively regulates vascular tone, vessel wall homeostasis, cellular adhesion, thrombosis, coagulation and vessel wall inflammation (Cines et al. 1998). Furthermore, it also interacts with other cell types and alters their function under different stimulation (Davies et al. 1988). It is believed that endothelial cell dysfunction is the first step towards the development of atherosclerosis in diabetes mellitus. Markers for endothelial dysfunction including von Willebrand factor (vWF), thrombomodulin, E-selectin, intercellular adhesion molecule 1 (ICAM-1), plasminogen activator inhibitor-1 (PAI-1), type IV collagen and tissue-plasminogen activator (t-PA), all of which are elevated in patients with diabetes mellitus (Jager et al. 2002; Stehouwer et al. 2002).

Under normal conditions, endothelial cells perform their multiple functions by synthesising and releasing multiple vasoactive mediators including autacoids, prostacyclin (PGI₂), nitric
oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and c-type natriuretic peptide (Gryglewski et al. 1988; Busse and Fleming 2003). Of these, NO and PGI$_2$ are the two major mediators released by the endothelial cells to induce vasodilation. NO is synthesised by NO synthase (NOS) through the conversion of L-arginine to L-citrulline and PGI$_2$ is synthesised by endothelial cyclooxygenase (Knowles and Moncada 1994). The release of NO and PGI$_2$ by the endothelial cells induces the formation of cGMP and cAMP, which contributes to the relaxation of vascular smooth muscle and thereby regulates vascular tone (Furchgott and Zawadzki 1980; Luscher 1990). Besides vasodilation, NO and PGI$_2$ can also inhibit abnormal platelet aggregation and vascular smooth muscle cell activity (Grodzinska and Marcinkiewicz 1979; Barrett et al. 1989; Rovati et al. 1995). NO and PGI$_2$ diminish vascular smooth muscle cell proliferation and migration by acting as antagonists of the growth-promoting action of Angiotensin II (Dubey et al. 1995). Furthermore, the release of NO can also inhibit leucocyte adhesion and prevent inflammation in the vessel wall (Kubes et al. 1991; Uyemura et al. 1996). These properties suggest that the levels of NO and PGI$_2$ produced by endothelial cells play an important role in blood vessel protection and inhibition of the development of atherosclerosis. However, several studies have shown that chronic hyperglycaemia in diabetic patients impairs the production of PGI$_2$ and NO which accelerates the atherosclerosis processes.

Hyperglycaemia reduces NO bioavailability in endothelial cells by interfering with the production of NO and enhancing its degradation. Impaired production of NO can be due to decreased protein expression of NOS, uncoupling of NOS protein activity and lack of substrate or cofactors for NOS (Asahina et al. 1995; Pieper et al. 1997; Wilcox et al. 1997). Under physiological conditions, endogenous antioxidant mechanisms maintain a balance between reactive oxygen species (ROS) and NO (Cai and Harrison 2000). Hyperglycaemia leads to the induction of ROS, such as superoxide anion, which can directly cause scavenging of NO and affect its bioavailability. Furthermore, the formation of ROS can also quench NO to form toxic peroxynitrite ion (Bucala et al. 1991; Koppenol et al. 1992). Peroxynitrite is an important mediator of LDL oxidation, and thus has a pro-atherogenic role that can attack many types of biological molecules (Griendling and FitzGerald 2003). The production of peroxynitrite leads to the oxidation of tetrahydrobiopterin, a co-factor for NOS, which results in shifting the function of NOS from an oxygenase that produces NO to a reductase that produces ROS (Milstien and Katusic 1999; Landmesser et al. 2003). The excess production of ROS will go back into the cycle to further reduce the production of NO. Beside the effects
on NO, peroxynitrite can also impair the production of \(\text{PGI}_2\) which affects the function of platelets.

Hyperglycaemia also stimulates the intracellular production of advanced glycation end (AGE) products, which enhances the polyol pathway and activates protein kinase C (Williamson et al. 1993; Cooper et al. 1998; Rahman et al. 2007). This leads to further induction of ROS and reduced NO bioavailability in endothelial cells. Production of AGE products can also promote endothelial expression of interleukin-6 (IL-6), vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) (Schmidt et al. 1995; Basta et al. 2002). These activities promote inflammation and activate endothelin-A receptors on vascular smooth muscle cells to induce vasoconstriction (Hopfner and Gopalakrishnan 1999). In conclusion, excessive formation of ROS can affect endothelial NO production and lead to endothelial dysfunction.

1.2.1.2 Vascular smooth muscle cell dysfunction

Vascular smooth muscle cells (SMCs) form the medial layer of the arteries and comprise the majority of the blood vessels (Owens et al. 2004). The main function of the vascular smooth muscle is to maintain an appropriate blood flow in blood vessels through contraction and relaxation (Lacolley et al. 2012). This activity is controlled by regulatory signals from the sympathetic nervous system and vasodilating NO from the endothelium, plus continuous flow of metabolic products (Luscher 1991; Webb 2003). Beside their function in vascular toning, vascular SMCs are also involved in vessel remodelling for long-term adaptation and repair after vascular injury (Owens et al. 2004). Under different conditions, vascular SMCs can change their cell morphology, expression levels of marker genes, proliferation and migration rates to perform different functions (Rensen et al. 2007). Dysfunction in vascular SMCs may accelerate the development of atherosclerosis in diabetes patients.

During the development of atherosclerosis, vascular SMCs proliferate and migrate to the nascent intimal lesion and lead to the formation of atherosclerotic lesions (Singh et al. 2002; Louis and Zahradka 2010). Diabetes alters the function of vascular SMCs and accelerates SMC proliferation and accumulation in atherosclerotic lesions. Vascular SMCs cultured from patients with T2DM, show enhanced migration (Suzuki et al. 2001). It is clear that hyperglycaemia plays an important role in vascular SMCs proliferation and migration,
although there is debate as to whether the effects are due to hyperglycaemia directly or its secondary downstream effects. Several in vivo studies have demonstrated that high levels of glucose can stimulate proliferation of SMCs (Natarajan et al. 1992; Yasunari et al. 1995; Watson et al. 2001). However, other studies have reported the opposite effects with high glucose levels having no effect or even showing an inhibitory action on SMC proliferation (Xia et al. 1995; Williams et al. 1997; Suzuki et al. 2001). Although the differences could be due to differences in experimental systems, some studies have suggested that the high glycolysis rate found in SMCs may protect them from hyperglycaemia-induced damage (Brand and Hermfisse 1997). High glycolysis rate increases glucose uptake in SMCs and could therefore minimise oxidative stress (Nishikawa et al. 2000; Brownlee 2001). However, the rate of glycolysis may be regulated by the endothelium; hyperglycaemia may be able to suppress this protective mechanism indirectly through altering the function of the endothelium (Morrison et al. 1976). In addition, hyperglycaemia can stimulate the proliferation and migration of SMCs by inducing the expression of growth factors and their receptors in SMCs, such as vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF-β), fibroblast growth factor-2 (FGF-2) and the platelet-derived growth factor-beta receptor (PDGF-β) (Mcclain et al. 1992; Inaba et al. 1996; Natarajan et al. 1997).

Hyperglycaemia also interferes with vascular SMC functions in relation to vascular tone in diabetes. As mentioned before, vascular SMCs require regulatory signals from the nervous system and NO from the endothelium to regulate vascular toning. Hyperglycaemia causes endothelial dysfunction and reduces the bioavailability of NO. Lack of NO supply from the endothelium to the vascular SMCs will lead to impaired NO-mediated vasodilation (Williams et al. 1996). Furthermore, hyperglycaemia stimulates the intracellular production of AGEs, which activates protein kinase C and nuclear factor β in vascular SMCs (Inoguchi et al. 2000). This leads to ROS induction and increases intracellular oxidative stress in vascular SMCs. In addition, increased protein kinase C activity can also promote inflammation and activate endothelin-1 molecules in vascular SMCs to induce vasoconstriction (Nugent et al. 1996). In conclusion, hyperglycaemia can alter the functions of vascular SMCs in multiple pathways and accelerate the development of cardiovascular disease in diabetes.
1.2.1.3 Impaired platelet function

Platelets are small anucleate discoid cell fragments which derive from megakaryocytes (Shi and Montgomery 2010). The main function of platelets is to circulate in the bloodstream and maintain vascular homeostasis (Ashby et al. 1990). During vessel damage, platelets perform a primary homeostatic role by adhering to the sub-endothelial surfaces and aggregating to form a plug at the site of injury to prevent internal bleeding (Angiolillo et al. 2010). They also activate the coagulation pathway for secondary homeostasis and different growth factors for wound healing. These platelet activities are regulated through different pro-aggregants and anti-aggregants binding to the specific receptors on the platelet surface (Stenberg et al. 1985; Ashby et al. 1990). Abnormal platelet activity will result in sustained thrombus formation and increase the risk of ischaemic heart disease (Willoughby et al. 2002).

Current studies show that patients with diabetes have impaired platelet function including increased platelet volume, higher expression of adhesion molecules on the platelet surface and increased release of vasoconstrictors (Sagel et al. 1975; Colwell and Nesto 2003; Top et al. 2008). These changes increase platelet adherence and aggregation. Although platelet dysfunction in patients with diabetes can be due to multiple causes, loss of sensitivity to anti-aggregants PGI\textsubscript{2} and NO is believed to be the major cause. As mentioned above, NO and PGI\textsubscript{2} are the two major mediators released by the endothelial cells to induce vasodilation. Under normal conditions, PGI\textsubscript{2} and NO are continually released by endothelium and act together to prevent platelet adherence to the endothelium, and platelet aggregation (Gryglewski et al. 1988). However, hyperglycaemia causes endothelial dysfunction resulting in impaired production of both NO and PGI\textsubscript{2} (Johnson et al. 1979; Gerrard et al. 1980; Harrison et al. 1980; Bucala et al. 1991). Beside the bioavailability of NO and PGI\textsubscript{2}, platelets from patients with diabetes have been shown to have diminished sensitivity and lowered response to PGI\textsubscript{2} and NO (Akai et al. 1983; Nolan et al. 1994). Without the effects of anti-aggregants, platelet activity would enhance and increase response to pro-aggregants, which leads to the increased production of platelet thrombi.

Hyperglycaemia can also enhance platelet adhesion and activation through inducing the expression of platelet surface proteins (Winocour et al. 1992; Watala et al. 1998). Platelets in patients with diabetes have increased expression of glycoprotein Ib, IIb-IIIa and P-selectin, resulting in amplification of platelet von Willebrand factor, which in turn forms an additional linkage between glycated protein and the collagen fibrils (Tschoepe et al. 1990; Vinik et al. 1991).
Moreover, insulin deficiency in diabetes could increase intracellular calcium concentration in platelets, leading to enhanced platelet degranulation and aggregation (Li et al. 2001). The release of granules can activate additional platelets and the coagulation cascade (Jurk and Kehrel 2005; Smith 2009). All of these changes will lead to abnormal platelet function, which accelerates the atherosclerotic processes and contributes to the development of cardiovascular disease in diabetes.

1.2.1.4 Abnormal coagulation and fibrinolysis

Coagulation and fibrinolysis are two important steps involved in the later stages of tissue repair. Coagulation is involved in fibrin clot formation and fibrinolysis is involved in the breakdown of fibrin clot (Nilsson 1987; Sidelmann et al. 2000). It is important to regulate the balance between coagulation and fibrinolysis, as altered balance may lead to haemorrhage or thrombosis. In patients with diabetes, hyperglycaemia induces blood coagulation activity to form a more compact fibrin network structure that is resistant to fibrinolysis which could lead to the formation of a thrombus (Carr 2001; Dunn et al. 2005; Dunn et al. 2006).

Coagulation consists of the intrinsic and extrinsic pathways. Each pathway is activated and regulated by different coagulation factors and enzymes (Macfarlane 1964; Nilsson 1987). Abnormal clot formation in diabetes patients is associated with elevated coagulation activity, which results from increased coagulation factors and reduced expression of anticoagulants in both pathways (Ceriello et al. 1988; Ceriello et al. 1990; Ceriello et al. 1995). An important protein that activates the extrinsic coagulation pathway is the tissue factor. It has been found that the level of circulating tissue factor is higher in diabetic patients than healthy controls (Krupinski et al. 2007; El-Hagracy et al. 2010). It is believed that increased production of tissue factor in diabetic patients is due to the formation of AGE products and oxidative stress induced by chronic hyperglycaemia (Vaidyula et al. 2006). Moreover, hyperglycaemia increases the level of plasma fibrinogen and thrombin, which influences the fibrin clot structure and stability (Ceriello et al. 1995; Barazzoni et al. 2000). Increased glycation of fibrinogen and high levels of thrombin could result in the formation of denser and less permeable clots which are resistant to fibrinolysis (Pieters et al. 2007; Wolberg and Campbell 2008; Andrades et al. 2009). Beside the changes in coagulation factors, hyperglycaemia also suppresses the anticoagulation activity by reducing the expression of anticoagulants such as anti-thrombin III and protein C (Ceriello et al. 1988; Ceriello et al. 1990). Protein C is an
anticoagulant involved in the defence mechanism against thrombosis which inhibits coagulation. However, the level of protein C was decreased in patients with diabetes, leading to impaired anticoagulation activity (Vukovich and Schernthaner 1986; Ceriello et al. 1990).

Fibrinolysis is a process that prevents blood clots from growing excessively and causing problems. The removal of clots is catalysed by the enzyme plasmin which cleaves fibrin into small fragment products, which will then be cleared by other proteases in the bloodstream. An important protein that inhibits fibrinolysis is plasminogen activator inhibitor-1 (PAI-1), which blocks the conversion of plasminogen into active plasmin (Saksela and Rifkin 1988). In diabetic patients, the concentration of PAI-1 is elevated (Juhan-Vague et al. 1989; McGill et al. 1994). It is believed that hyperglycaemia and elevated triglycerides in diabetes are the main causes of elevated PAI-1 levels (Wu and Yu 2004). In addition, endothelial dysfunction in diabetes may reduce the release of plasminogen activator, which may influence the production of plasmin. Abnormal fibrinolysis activity in vessel walls may contribute to thrombus formation and the development of atherosclerosis.

In conclusion, most of the abnormalities involved in coagulation and fibrinolysis are associated with hyperglycaemia. These changes tend to favour coagulation and impaired fibrinolysis leading to thrombus formation and increase cardiovascular risk in diabetes (Fig 1-2)
Figure 1-2 Alterations in coagulation and fibrinolysis pathway in diabetes mellitus.
1.2.2 Diabetic cardiomyopathy

Diabetic cardiomyopathy was first reported by Rubler et al in 1972 and was defined as the presence of abnormal cardiac function or structure in diabetic patients independently of other confounding risk factors such as coronary artery disease, hypertension or significant valvular disease (Rubler et al. 1972). Diabetic cardiomyopathy is characterised by left ventricular hypertrophy and diastolic dysfunction, which may lead to the development of systolic dysfunction. Several mechanisms are involved in diabetic cardiomyopathy including epigenetic changes, metabolic disturbances, apoptosis, myocardial necrosis and fibrosis, disordered copper metabolism, mitochondrial dysfunction and autonomic neuropathy. It is believed that all of these mechanisms are triggered by chronic hyperglycaemia.

1.2.2.1 Metabolic disturbances

In order to maintain cardiac function, cardiomyocytes obtain energy through glucose metabolism and oxidation of free fatty acids (FFAs). Fatty acids (FAs) provide 60-70% of the overall cardiac ATP supply with the rest being supplied by glucose and lactose (Neely and Morgan 1974). However, this metabolic balance is perturbed in the diabetic heart, leading to reduction in glucose usage and increased energy production from FFAs (Lopaschuk 2002; Taegtmeyer et al. 2002; Monti et al. 2004). It is believed that the increased reliance on FA oxidation as an energy source is due to depleted glucose uptake and increased circulating FFAs (Young et al. 2002).

The reduction in glucose metabolism in the diabetic heart results from impaired glucose uptake, glycolysis, and pyruvate decarboxylation (Stanley et al. 1997). In cardiomyocytes, glucose uptake is dependent on the transmembrane glucose gradient and glucose transporter types 1 (GLUT1) and 4 (GLUT4) (Kraegen et al. 1993; Luiken et al. 2004). Since the expression of GLUT4 is regulated by insulin, FAs reducing insulin action can indirectly affect the recruitment of GLUT-4 to the plasma membrane (Camps et al. 1992; Garvey et al. 1993; Carroll et al. 2005) and therefore reduce glucose uptake, glycolysis and glucose oxidation. Furthermore, increased FFAs in diabetes can also suppress glucose oxidation through receptor-alpha (PPAR-alpha) signalling pathways (Finck et al. 2002). Increased circulating FAs activate PPAR-alpha leading to transcriptional induction of enzymes involved in FA oxidation and increased expression of pyruvate dehydrogenase (PDH) kinase (PDK4), which further suppresses glucose oxidation by decreasing PDH activity (Wieland et
In addition, impaired pyruvate dehydrogenase can lead to accumulation of glycolytic intermediates and ceramide, enhancing apoptosis (Dyntar et al. 2001; Sparagna et al. 2004). Furthermore, increased FA dependence can also be due to increased FA oxidation. Unlike glucose, FFAs can be transported across the plasma membrane via both passive diffusion and protein-mediated transportation (Hamilton and Kamp 1999; Schwenk et al. 2010). Increased FFAs in the circulation simply increase FA transport into cardiomyocytes. As a result, cardiac tissues rapidly adapt to the increased intracellular FAs and promote FA utilisation.

Metabolic disturbances in diabetes can alter cardiac function and structure in multiple mechanisms. FA oxidation requires more oxygen to produce ATP than glucose oxidation. Increased reliance on FA oxidation as an energy source will decrease cardiac efficiency (ATP/oxygen consumption) (Peterson et al. 2004). FA-mediated ROS generation could lead to uncoupling of mitochondria, which reduces mitochondrial ATP production and further decreases cardiac efficiency (Boudina et al. 2005). Increased oxygen demand and decreased cardiac efficiency makes the heart especially vulnerable to damage following increased workload or ischaemia (An and Rodrigues 2006). As well, the toxic intermediates resulting from FA oxidation can lead to lipotoxicity (Rodrigues et al. 1998). Studies have shown that long-chain acyl CoA esters, intermediates produced by FA oxidation, favour the opening of $K_{\text{ATP}}$ channels on the cell membrane by reducing ATP sensitivity and therefore shortening the action potential (Liu et al. 2001). Impaired action potential could lead to reduction of $\text{Ca}^{2+}$ influx into cardiomyocytes and affect intracellular $\text{Ca}^{2+}$ homeostasis. Furthermore, impaired $\text{Ca}^{2+}$ homeostasis could lead to reduction of myocardial contractility. Finally, impaired $\text{Ca}^{2+}$ homeostasis could also affect the activity of the cardiac sarcolemmal $\text{Na}^+\text{-H}^+$ exchanger (NHE isoform1) which is involved in molecular mechanisms of hypertrophy, leading to a disproportionate increase in left ventricular mass (Darmellah et al. 2007; Darmellah et al. 2009).

1.2.2.2 Mitochondrial dysfunction

Mitochondria account for about 40% of total volume in cardiomyocytes and are the major source of cellular energy production (Saks et al. 2001). As described in the previous section, metabolic balance is disturbed in diabetes mellitus. Mitochondrial dysfunction may play a major role in metabolic imbalance and contribute to contractile dysfunction and ventricular
failure. Changes in mitochondrial morphology, remodelling in the mitochondrial proteome and decreased respiratory capacity have been found in animal models of diabetes mellitus (Bugger et al. 2009).

As mentioned above, elevated glucose and FFAs can lead to mitochondrial injury. Studies show that hyperglycaemia can induce the expression of carnitine palmitoyl-transferase 1 in mitochondria to increase FA uptake (Sharma et al. 2004). In addition, FAs can induce uncoupling of mitochondria through enhancing the expression and activity of uncoupling proteins (Boudina et al. 2007). Uncoupling mitochondria will result in reduced ATP synthesis and impaired energy supply (Boudina et al. 2005). In addition, increased FA uptake can also induce mitochondrial number and alter mitochondrial structure (Regan et al. 1977; Bugger et al. 2008). Furthermore, mitochondria are a major source of ROS production. Studies have shown that the ROS production in mitochondria was increased in diabetic patients (Ye et al. 2004). ROS generated by the mitochondria can induce oxidative damage to mitochondria. In order to prevent ROS damage, mitochondria have a protective mechanism to suppress the ROS production in cardiomyocytes (Ji 1999; Andreyev et al. 2005; Mari et al. 2009). However, animal studies showed that the levels of ROS scavenging proteins are reduced in diabetes (Johnson 2009). Deficiencies in the antioxidant defence system may allow ROS to cause further oxidative damage in mitochondria.

1.2.2.3 Myocardial fibrosis and collagen deposition

Early changes in cardiac structure including myocardial fibrosis and collagen deposition have been observed in diabetic cardiomyopathy. Studies of myocardial biopsies from diabetic patients have shown a significant increase in collagen deposition around the blood vessels and between the myofibers (Regan et al. 1977). Compared to non-diabetic patients, the proportion of collagen type III was also found to be increased significantly in diabetic patients (Shimizu et al. 1993; Kitamura et al. 2001). It is believed that myocardial fibrosis and alterations in collagen phenotypes account for myocardial stiffness and dysfunction in diabetic cardiomyopathy.

The development of myocardial fibrosis and collagen deposition in diabetic cardiomyopathy are related to the production of collagen cross-links (Ulrich and Cerami 2001; Aronson 2003). Collagen in myocardium first interacts with glucose to form a Schiff base. The
collagen-Schiff base complex can then rearrange to form glycated collagen. With further chemical modification, glycated collagen can generate AGE products and cross-link with collagen to form collagen-AGE products. Collagen AGE products are more stable and more resistant to proteolysis which could lead to development of myocardial fibrosis (Asbun and Villarreal 2006). In addition, AGE products can also interact with their receptors (RAGEs) to induce intracellular changes in extracellular matrix, which accelerates the development of myocardial fibrosis (Petrova et al. 2002). Additionally, elevated transforming growth factor-β1, a potent stimulator of collagen-producing cardiac fibroblasts, may also contribute to myocardial fibrosis and cardiac dysfunction in diabetes (Khan and Sheppard 2006; Bujak and Frangogiannis 2007).

1.2.2.4 Increased apoptosis and cell death

Apoptosis in cardiomyocytes has been identified as an essential process in the development of diabetic cardiomyopathy. Increased apoptotic cell death has been found in cardiomyocytes of patients with diabetes (Chowdhry et al. 2007; Kuethe et al. 2007). Apoptosis can cause a loss of contractile tissue, compensatory hypertrophy of myocardial cells and reparative fibrosis (Swynghedauw 1999; Engel et al. 2004). Studies have suggested that myocardial apoptosis can be induced by multiple mechanisms in diabetes (Murphy et al. 2003; Lim et al. 2004; Brand and Esteves 2005).

It is believed that hyperglycaemia and increased FFA levels in diabetes patients can induce cell death. Long-chain FAs can alter phospholipid composition of mitochondrial membranes to allow cytochrome c to detach (Murphy et al. 2003; Brand and Esteves 2005). Release of cytochrome c from mitochondrial membranes will activate the cytochrome c-activated caspase-3 pathway and initiate apoptosis (Jiang and Wang 2004). Hyperglycaemia can also induce apoptosis through the activation of the local renin-angiotensin system (RAS) (Lim et al. 2004). Studies have shown that the expression of angiotensin II receptor is elevated in diabetes (Sabbah and Sharov 1998). Since angiotensin II is recognised to have pro-apoptotic properties, an increase in angiotensin II receptor expression may enhance the effects of angiotensin II and induce cell death. Moreover, activation of RAS can increase oxidative stress and generate AGE products which lead to the formation of ROS. The formation of ROS can either directly alter gene expression by inducing apoptosis in cardiomyocytes, or interact with NO to form nitrotyrosine, which can cause oxidative DNA damage on
cardiomyocytes and activate caspase-3 to induce apoptosis (Frustaci et al. 2000). Clinical studies showed that inhibition of RAS could reduce ROS production and partially restore cardiac function in patients with diabetes (Brenner et al. 2001; Symeonides et al. 2007). In addition, hyperglycaemia can also increase apoptosis in cardiomyocytes by impairing cell survival mechanisms. Oxidative stress induced by hyperglycaemia can activates poly-adenosine diphosphate-ribose polymerase-1 (PARP-1) expression. Instead of their normal function in DNA repair and cell survival, over-activated PARP-1 can lead to cellular damage and cause apoptosis (Pillai et al. 2005; Sairanen et al. 2009). Lastly, increased resistance to another survival factor, insulin-like growth factor 1 (IGF-1) in diabetes may also reduce the survival of cardiomyocytes in diabetic heart (Kajstura et al. 2001; Yu et al. 2010).

1.2.2.5 Autonomic Neuropathy

The autonomic nervous system modulates the electrical and contractile activity of the myocardium through interaction of sympathetic and parasympathetic activity (Sztajzel 2004). One of the common forms of autonomic dysfunction found in patients with diabetes is cardiovascular autonomic neuropathy (CAN). The autonomic nerve fibres are damaged in CAN which causes abnormalities in heart rate control and vascular dynamics (Comi et al. 1990; Vinik and Ziegler 2007). This symptom is usually accompanied by a loss of the usual change in heart rate seen with normal breathing. CAN is one of the major causes of morbidity and mortality in diabetic patients and is associated with a high risk of cardiac arrhythmias and sudden death.

Hyperglycaemia can contribute to the development of CAN through multiple mechanisms including activation of the polyol pathway (Greene et al. 1988), activation of protein kinase C (Way et al. 2001), increased oxidative stress (Vincent et al. 2004), formation of AGE products (Brownlee 1992) and reduction in neurotrophic growth factors (Apfel et al. 1994). Through these mechanisms, hyperglycaemia can reduce the endoneurial blood flow and cause nerve hypoxia to alter nerve function (Cameron and Cotter 1997; Illa 1999; Vinik 1999). Altered nerve function will activate the genes involved in neuronal damage and induce cell death (Obrosova 2002; Pacher et al. 2002). An impaired cardiac nervous system will lead to a reduced cardiac ejection fraction, systolic dysfunction and decreased diastolic filling in patients with diabetes.
1.2.2.6 Epigenetic changes

Epigenetic mechanisms can regulate and modify gene expression in response to environmental signals without altering their DNA sequence. This allows the stable propagation of gene expression from one generation to another. However, long-lasting epigenetic modifications induced by hyperglycaemia and insulin resistance could contribute to the development of cardiovascular disease in diabetes.

Current studies have provided evidence to suggest that epigenetic factors might play an important role in regulating the interplay between genes and hyperglycaemia in diabetes (Liu et al. 2008; Villeneuve and Natarajan 2010). It is well known that chronic hyperglycaemia is a major factor in the causation of the diabetic cardiovascular complications. However, clinical studies have shown that diabetic patients with strict glycaemic control can still develop cardiovascular complications. It has been suggested that ‘metabolic memory’ stemming from prior hyperglycaemic level can lead to epigenetic changes in several target genes, resulting in permanent changes in gene expression which persist after return to normoglycaemia (Brasacchio et al. 2009). In addition, a study by El-Osta et al showed that vascular cells exposed to short-term hyperglycaemia can acquire long-term changes in the inflammatory gene promoter by chromatin remodelling (El-Osta et al. 2008), suggesting a possible role for epigenetic changes in diabetes and diabetic cardiomyopathy.

1.2.2.6 Disordered copper metabolism

Several studies have found that diabetic patients have higher copper (Cu) levels in their serum, especially in those with cardiovascular disease (Zargar et al. 1998; Viktorinova et al. 2009). It has been suggested that elevated serum copper levels in diabetic patients can be due to an imbalance in distribution between the intracellular and extracellular copper concentration (Walter et al. 1991). Hyperglycaemia can increase the copper levels in the extracellular matrix by reducing the copper-binding properties of caeruloplasmin and albumin (Islam et al. 1995; Argirova and Ortwerth 2003). Furthermore, AGE products produced in diabetes can also cross-link with long lived fibrous proteins to act as localised, fixed endogenous chelators to increase tissue binding to copper (Qian and Eaton 2000; Eaton and Qian 2002). The abundance of copper in the extracellular matrix might enhance the production of ROS through the Fenton reaction, resulting in oxidative stress and fibrosis. The role of copper in diabetes will be further discussed in the rest of this chapter.
1.3 Copper in biology

Copper is an essential nutrient required by all living organisms. Copper ions can exist in biology in two interconvertible redox states, oxidised or cupric (Cu$^{2+}$), and reduced or cuprous (Cu$^{+}$) ions. The redox properties of copper ions enable them to function as a cofactor for copper enzymes involved in fundamental biological process, including ATP synthesis, free radical detoxification and oxygen transport, which are required for cell growth, development and maintenance (Linder and Hazegh-Azam 1996). However, excess copper in the body can cause toxicity due to free, unbound copper ions which will react readily with hydrogen peroxide or superoxide anion to catalyse the production of highly toxic free radicals such as hydroxyl radical, that can cause damage to lipids, proteins and DNA (Kalyanaraman 1982). Therefore, copper homeostasis is tightly regulated to ensure adequate supplies without any toxic effects.

1.3.1 Copper homeostasis

Copper is an essential mineral which cannot be produced by the body. Copper intake in humans is primarily obtained from the diet. Food such as seafood, organic meats, nuts, and seeds are rich in copper. About 50% of the average daily dietary copper of (around 25 μmol or 1.5 mg) is absorbed from the stomach and the small intestine (Iakovidis et al. 2011). Absorbed copper in the portal circulation is bound to albumin and rapidly extracted by the liver in first-pass kinetics (Loudianos and Gitlin 2000). Hepatic copper is transmitted to the bloodstream via binding to the plasma protein caeruloplasmin, and redistributed throughout the body. The liver is the principal storage site for copper which contains 10% of the total body content of 1200 μmol (80 mg) (Iakovidis et al. 2011). Excess hepatic copper is excreted into the bile, resulting in a biliary copper pool that is not subject to reabsorption (Winge and Mehra 1990). Only small amounts of copper are found in urine, unless renal damage is present.

In order to prevent damage from copper toxicity or deficiency, copper homeostasis is tightly regulated by both absorption and biliary excretion. Studies have shown that the absorption efficiency for copper can vary according to the dietary copper intake. When the copper intake is less than 1 mg/day, the absorption efficiency is increased to almost 56% to prevent copper deficiency (Collins et al. 2005). However, the absorption efficiency will reduce to 12% when the copper intake is 7.5mg/day to avoid copper toxicity (Collins et al. 2005). Copper
excretion is the process most tightly regulated to maintain copper homeostasis. Studies have shown that the amount of copper being excreted in the bile is directly proportional to the size of the hepatic copper pool (Harris and Gitlin 1996; Ferenci 2004). Increased intracellular copper concentration in hepatocytes will activate the translocation of ATPase, which leads to copper excretion. The adaptations in absorption and excretion help to prevent damage caused by copper deficiency and toxicity.

1.3.2 Copper uptake into cells

Despite intensive research, the molecular mechanisms of copper uptake into the cells in humans have not been fully understood. Our current knowledge of the copper-regulatory pathways is largely based on studies in bacteria and yeast. In *Saccharomyces cerevisiae*, copper transport from the extracellular to intracellular space is mediated by two high-affinity copper transporter proteins yCtr1 and yCtr3, and a low-affinity copper transporter protein yCtr2 (Dancis *et al.* 1994; Knight *et al.* 1996). Deletion of yCtr1 and yCtr3 in yeast results in copper deficiency. In humans, a high-affinity copper-uptake protein hCtr1 has been identified and was found to be encoded by the *SLC31A1* gene (Zhou and Gitschier 1997). Ctr1 protein is expressed in all tissues, but the level of expression is variable, with highest expression in the liver (Klomp *et al.* 2002; Lee *et al.* 2002). Ctr1 is predominantly localised in intracellular vesicles which are closely apposed to the Golgi complex, but has been shown to recycle constitutively between the vesicular compartment and the plasma membrane (Klomp *et al.* 2002; Lee *et al.* 2002). Ctr1 protein is an integral membrane protein consisting of three transmembrane domains. The carboxy-termini (COOH-termini) of hCtr1 are exposed in the cytosol, while their amino-termini (NH$_2$-termini) are located at the extra-cytoplasmic side of the transmembrane (Klomp *et al.* 2003). The NH$_2$-terminus of Ctr1 contains a number of Met motifs that can serve as high affinity copper-binding sites and coordinate copper for subsequent transport (Puig *et al.* 2002; De Feo *et al.* 2009). Copper transport by hCtr1 is specific, energy-independent and stimulated by acidic pH and high extracellular K$^+$ concentration (Lee *et al.* 2002). Several *in vivo* studies have shown that overexpression of hCtr1 can result in enhanced copper uptake. In addition, animal studies using Ctr1 knockout mice display a defect in copper uptake and copper deficiency in body tissues (Kuo *et al.* 2001; Nose *et al.* 2006). Although the regulatory mechanisms by which Ctr1 modulates copper uptake are not fully understood, it has been suggested that Ctr1 can do this by altered
protein expression, changing its subcellular location or changing the conformation of its COOH-terminal tail (Petris et al. 2003; Kuo et al. 2006; De Feo et al. 2009; Wu et al. 2009).

Since the majority of extracellular copper is present as Cu$^{2+}$ and copper uptake by Ctr1 is specific for Cu$^+$, a metalloreductase is thus required to reduce Cu$^{2+}$ into Cu$^+$ to allow copper uptake into the cells. In *S. cerevisiae*, it has been reported that the reduction of Cu$^{2+}$ to Cu$^+$ is mainly catalyzed by the cell-surface copper/iron reductases FRE1 and FRE2 (Hassett and Kosman 1995; Georgatsou et al. 1997). In mammals, recent studies have found that copper metalloreductases such as STEAP (six transmembrane epithelial antigen of the prostate) protein family, APP (amyloid precursor protein) and Dcytb (duodenal cytochrome b) may be responsible for the copper reduction (Ruiz et al. 1999; McKie et al. 2001; Ohgami et al. 2006). After Cu$^{2+}$ is reduced to Cu$^+$, it binds to the metal-binding Mets motif in the NH$_2$-terminal domain of hCtr1 and is transported into the cytosol through a channel formed by the hCtr1 trimer (Aller and Unger 2006; Nose et al. 2006).

Although cellular copper uptake is mainly due to the activity of hCtr1, a low-affinity copper transporter, hCtr2 may also contribute to the cellular copper uptake. Studies using Ctr1 knockout mice have shown that their copper levels are not affected despite the lack of Ctr1, suggesting a role for hCtr2 in copper uptake (Lee et al. 2002). The hCtr2 is predominantly localized in intracellular organelles that are reminiscent of late endocytic and lysosomal compartments (van den Berghe et al. 2007). The structure of hCtr2 is very similar to hCtr1. Most of the molecular sub-structures that have been shown to be critical for copper transport activity in Ctr1 are conserved in Ctr2 (Eisses and Kaplan 2005). However, the high affinity copper-binding Mets motif in the NH$_2$-terminal domains of hCtr1 is absent from hCtr2 (Zhou and Gitschier 1997; Puig et al. 2002). The hCtr2 is also missing the characteristic His-Cys-His motif in the COOH-terminal tail of hCtr1. These differences in structure could result in lowered affinity of copper-binding by hCtr2. Recent studies have shown that cellular copper uptake mediated by hCtr2 *in vitro* is reduced by approximately 20-fold compared to hCtr1 (Bertinato et al. 2008).

Another candidate transporter involved in cellular copper uptake is the divalent metal transporter 1 (DMT1). DMT1 is the major iron(II) (Fe$^{2+}$) transporter mediating cellular iron uptake (Xu et al. 2004). However, several studies have demonstrated that DMT1 can also mediate copper uptake in intestinal cells when there is sufficient extracellular copper (Gunshin et al. 1997). Further studies have confirmed the role of DMT1 as an ATP-
dependent high affinity copper transporter in intestinal cells (Knopfel et al. 2005). Knockdown of DMT1 expression in Caco-2 cells has been shown to result in reduction of copper uptake, and enhanced DMT1 expression can increase copper uptake (Arredondo et al. 2003; Collins et al. 2005). In addition, it has been suggested that DMT1 can transport not only Cu\(^+\), but also Cu\(^{2+}\) directly into cells (Arredondo et al. 2003). However, the function, mechanism and overall contribution of DMT1 to cellular copper uptake, remains unclear.

1.3.3 Copper distribution within cells

Once within the cells, copper is either transported to specific cellular destinations by copper chaperones, or sequestered by metallothioneins for proper copper detoxification (Fig 1-3). Copper binds to copper chaperones which can end up in one of three known pathways: 1) delivered to mitochondria for respiration, 2) secreted through the Golgi, 3) transferred to copper/zinc superoxide dismutase 1 (SOD1). The intracellular distribution of copper is regulated in response to metabolic demands and changes in cell environment.

![Figure 1-3 Intracellular copper transport pathways in a human cell. Reproduced from (Lutsenko 2010) with modification.](image-url)
1.3.3.1 Copper delivery to mitochondria

As previously mentioned, copper is required for cellular respiration to generate ATP. In order to perform these functions, it needs to be delivered to cytochrome c oxidase (CCO) in mitochondria. CCO is a large transmembrane protein complex (Complex IV) which is the final electron acceptor in the mitochondrial electron transport chain (Capaldi 1990). CCO is a copper-containing metalloenzyme, which catalyses electron transfers from cytochrome c to molecular oxygen to create a transmembrane proton gradient for ATP synthase to drive ATP synthesis (Horn and Barrientos 2008). Previous studies have suggested that cytosolic copper is carried to CCO by copper chaperone hCox17 (Glerum et al. 1996; Beers et al. 1997). However, recent studies have shown that hCox17 merely carries copper within the mitochondrial inter-membrane space (IMS) (Maxfield et al. 2004; Cobine et al. 2006). It has also been suggested that cytosolic copper is carried by an unknown low molecular weight non-proteinaceous ligand into the IMS instead of hCox17 (Cobine et al. 2004; Cobine et al. 2006). In the IMS, Cu\(^{+}\) is bound by hCox17 and delivered either to hSco1/hSco2 complex or hCox11, which transfers two Cu\(^{+}\) ions to the Cu\(_{A}\) site on the Cox2 subunit, and one Cu\(^{+}\) to the Cu\(_{B}\) site on the Cox1 subunit of CCO, respectively (Fig 1-4) (Banci et al. 2008). Copper insertion in mitochondria provides stability and maturation of these subunits, and therefore allows CCO to perform its catalytic function.

Copper transfer in mitochondria requires reduced cysteine residues in both donor and accepting proteins. In vitro, hCox17 in the IMS exists in a partially oxidised form with two disulphide bonds and two reduced cysteine residues for metal binding (Banci et al. 2008). Copper-loaded Cox17 can simultaneously transfer Cu\(^{+}\) and two electrons to oxidised Sco1. This leads to the reduction of Sco1, enabling copper to bind to Sco1, while the metal-binding cysteine of Cox17 will be oxidised to form a third disulphide bond, which can be reduced by glutathione (Banci et al. 2008). In order to transfer copper to CCO, Sco1 needs to form a complex with Sco2, which appears to oxidise the copper-coordinating cysteine residue in Sco1 to release copper to CCO (Leary et al. 2009). Two other Cu\(^{+}\) binding proteins, Cox19 and Cox23, which have similar structures to Cox17 are found in the IMS; they may also be involved in copper transport into cytochrome oxidase (Nobrega et al. 2002; Barros et al. 2004). However, the mechanisms of copper transport within mitochondria remain largely unknown.
1.3.3.2 Copper delivery to the secretory pathways

In order to maintain intracellular copper homeostasis, it is important to balance the uptake, distribution and export of copper at the systemic and cellular levels. As mentioned previously, copper uptake is mediated by the copper transporters Ctr1 and Ctr2, whereas copper efflux is mediated by the P-type copper-transporting ATPases, ATP7A and ATP7B. Both ATP7A and ATP7B are polytopic membrane proteins with similar structure and function, but they are expressed in different tissues (Lutsenko et al. 2007). ATP7A is expressed in most tissues except in the liver, while ATP7B is predominantly expressed in liver (Bull et al. 1993; Chelly et al. 1993; Mercer et al. 1993; Yamaguchi et al. 1993). Their activity, post-translational modification and intracellular localisation are modulated by intracellular copper. ATP7A and ATP7B are located in the trans-Golgi network which uses the energy of ATP hydrolysis to translocate copper ions from the cytosol across cellular membranes (Yamaguchi et al. 1993; Hung et al. 1997). Copper bound to ATP7A and ATP7B will either be delivered to the secretory pathway or exported out of the cells. This transport process can prevent copper accumulating within the cells and contributes to the intracellular homeostatic control of copper.
Cytosolic Cu\(^+\) is carried to the secretory pathway by the copper chaperone ATOX1 (antioxidant protein 1), which is also known as HA1 (human atx homolog-1) (Wernimont et al. 2000; Anastassopoulou et al. 2004). Cytosolic Cu\(^+\) is bound by ATOX1 on an exposed surface and transferred through a series of inter-protein ligand exchange reactions to a metal-binding domain in the NH\(_2\)-terminus of ATP7A and ATP7B (Pufahl et al. 1997; Banci et al. 2007). Cu\(^+\) bound to ATP7A can be either delivered into the secretory pathway and incorporated into copper-dependent enzymes such as tyrosinase, lysyl oxidase, caeruloplasmin and others, or stored within vesicles and released into the extracellular environment through the fusion of the vesicles with the cell membrane, which allows copper to move across the enterocyte basolateral membrane and for delivery to the blood and other tissues (Chelly et al. 1993; Mercer et al. 1993; Vulpe et al. 1993). Cu\(^+\) bound to ATP7B in hepatocytes can be either incorporated into the copper-dependent ferroxidase caeruloplasmin (CP) for entry into the blood, or delivered to the bile canaliculi for biliary copper excretion (Walker et al. 2002; Bartee and Lutsenko 2007). In addition, recent studies have identified a small cytoplasmic protein COMMD1, which is able to bind to the NH\(_2\)-terminal copper binding region of ATP7B (van de Sluis et al. 2002; de Bie et al. 2007). COMMD1 may be involved in vesicular copper movement and excretion by hepatocytes (Liu et al. 2010). Mutations in genes encoding ATP7A and ATP7B result in metabolic disorders, known as Menkes’ disease (predominant copper deficiency, for example in the brain) and Wilson’s disease (Cu overload, particularly in the liver, brain and eye), respectively, whereas deletion of the Atox1 gene in mice results in perinatal mortality (Bull et al. 1993; Harrison and Dameron 1999; Hamza et al. 2001).

1.3.3.3 Controlling cytosolic copper

Superoxide dismutase 1 (SOD1) is a cytoplasmic copper/zinc (Cu/Zn) protein responsible for cellular antioxidant defence. It protects the cell against free ROS by using copper as a cofactor to catalyse the dismutation of superoxide into oxygen and hydrogen peroxide (Fridovich 1986). A copper chaperone, CCS is responsible for SOD1 activation by delivering Cu(I) to SOD1 and promoting oxidation of an intra-subunit disulphide bond (Brown et al. 2004; Furukawa et al. 2004). The CCS protein has three structural domains; domains I and III consist of a copper-binding motif and domain II structurally resembles SOD1 and mediates the CCS-SOD1 interactions (Lamb et al. 2001; Rae et al. 2001; Torres et al. 2001). Recent studies have suggested that domain I may be involved in binding cytosolic Cu(I), while
domain III translocates Cu(I) from domain I to the active site of SOD1 (Lamb et al. 2001; Rae et al. 2001; Torres et al. 2001). Copper-loaded CCS bound to Zn-loaded SOD1 promotes the formation of oxygen-dependent intra-subunit disulphide bonds in SOD1 allowing heterodimerisation between CCS and SOD1. Without copper and zinc binding and upon reduction of the disulphide bond, the SOD1 dimer is destabilized and exists as an inactive monomer (Forman and Fridovich 1973; Banci et al. 2006). The formation of the CCS-SOD1 heterodimer complex allows Cu(I) transfer from domain III of CCS onto SOD1 (Lamb et al. 2001; Torres et al. 2001). Although the mechanism of this process is not fully understood, recent studies using an electrospray ionisation mass spectrometry (ESI-MS)-based strategy have shown that the Cu(I)-binding affinity for SOD1 is 10-times higher than CCS, providing a thermodynamic driving force for copper transfer (Banci et al. 2010). In addition, cytosolic Cu(I) can also be inserted into SOD1 through a CCS-independent pathway. Human SOD1 is capable of being partially activated independently of ccs1 when expressed in yeast or flies (Carroll et al. 2004; Kirby et al. 2008). It is suggested that copper-binding in the CCS-independent pathway requires GSH, but the mechanisms of this pathway remain unknown (Carroll et al. 2006).

Although SOD1 is predominantly located in the cytoplasm, around 1 to 5% of SOD1 can be found in the mitochondrial IMS (Okado-Matsumoto and Fridovich 2001; Sturtz et al. 2001). SOD1 in mitochondria provides protection from superoxide that cannot be neutralised by the matrix-based SOD2. The presence of SOD1 inside the IMS is dependent on CCS, which is influenced by the oxygen level in cells (Kawamata and Manfredi 2008). High oxygen concentration promotes oxidative folding of CCS in the cytosol and retains SOD1 in the cytosol. Lower oxygen concentrations favour mitochondrial localisation of CCS and SOD1 translocation into the IMS. This suggests CCS not only functions as a copper carrier, but it can also act as an oxygen sensor and regulate SOD1 compartmentalisation (Kawamata and Manfredi 2008).

Beside antioxidant function, studies in S. cerevisiae have shown that the CCS/SOD1 complex can also be involved in the regulation of copper homeostasis. Copper acquisition in S. cerevisiae is mediated by a copper-dependent nuclear transcription factor, Mac1 through regulating the gene transcription of high affinity copper transporters such as Ctr1 and Ctr3 (Wood and Thiele 2009). CCS-activated SOD1 carries Cu(I) ion into the nucleus, catalysing the binding of Mac1 to its target genes, resulting in up-regulation of Ctr1 and Ctr3 transcription and increased copper uptake into the cells. Inactivation of either CCS or SOD1
impaired the ability to up-regulate the transcription of Mac1 target genes resulting in copper deficiency (Wood and Thiele 2009).

1.3.3.4 Copper-binding to metallothioneins

Metallothioneins (MTs) are the major intracellular metal-binding proteins that localise to the membrane of the Golgi apparatus (Ogra et al. 2006). MTs have a low molecular weights (~7kD), with high cysteine content (20-30%) that can co-ordinate zinc and copper atoms under physiological conditions (Kagi and Schaffer 1988). There are more than 16 isoforms of MTs which have been identified in humans, of which the four main isoforms are MT1, MT2, MT3 and MT4 (Binz and Kagi 1999). MT1 and MT2 are the two major forms which are expressed in most tissues, whereas MT3 and MT4 are specifically expressed in brain and stratified epithelia, respectively (Palmiter et al. 1992; Quaife et al. 1994; Kojima et al. 1998; Laukens et al. 2009). All MTs are composed of a single polypeptide chain with cysteine residues distributed in a fixed manner along its length. Each MT protein consists of two independent metal-binding domains (α and β) and can normally bind up to 12 Cu⁺ ions through the thiol groups of its cysteine residues (Chan et al. 2007). Several studies have demonstrated that increased intracellular copper concentrations can induce MT expression in mammals (Furst et al. 1988; Thiele 1992; Coyle et al. 2002). It has been suggested that MTs can regulate intracellular copper homeostasis and prevent cellular damage by scavenging free radicals, thus protecting against oxidative stress, and are also involved in copper storage, distribution and detoxification (Kelly and Palmiter 1996; Palmiter 1998; Sato and Kondoh 2002; Gold et al. 2008). Studies using MT knockout mice and cell lines have shown that impaired MT expression will reduce the capacity for copper storage and enhance the sensitivity to excess copper and oxidative stress (Park et al. 2001; Qu et al. 2002; Tapia et al. 2004). As well, impaired MT expression in MT-null cells also affects the response in mRNA expression of SOD1 and CCS following copper exposure (Tapia et al. 2004). Furthermore, studies on Menkes’ disease have shown that MT is essential for cell survival when the copper transporter ATP7A is dysfunctional (Kelly and Palmiter 1996). All these studies confirm that MTs plays important roles in regulating intracellular Cu homeostasis.
1.3.4 Copper-induced oxidative stress

Copper ions taken up from the diet are firmly bound to different copper-binding proteins and delivered to specific destinations. The amount of free copper ions existing in the body is very low and can cause damage to cellular components. Unbound copper ions can undergo redox cycling reactions in which a single electron may be accepted or donated. This action can induce oxidative stress by generating ROS such as superoxide anion ($O_2^{-}$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radical (OH$^-$) in biological systems (Simpson et al. 1988). It is believed that copper can generate ROS via free-radical-mediated pathways analogous to the Fenton reaction and the Haber-Weiss reaction shown below (Simpson et al. 1988; Kadiiska and Mason 2002).

$$\begin{align*}
O_2^{-} + Cu^{2+} & \rightarrow O_2 + Cu^+ \\
Cu^+ + H_2O_2 & \rightarrow Cu^{2+} + OH^- + OH^-
\end{align*}$$

ROS can cause oxidative damage to lipids, proteins and DNA, which can severely compromise cell health and viability (Kalyanaraman 1982). Defence mechanisms such as ROS-scavenging molecules and oxidative damage repair enzymes have been developed in evolution to protect against ROS (Guerin et al. 2001; Thorpe et al. 2004). However, excessive production of ROS catalysed by copper can overwhelm the cellular protective antioxidant defence and cause oxidative damage in the body. It has been shown that oxidative stress induced by copper is associated with numerous diseases including cancer, cardiovascular disease, diabetes, neurological disorders and chronic inflammation (Valko et al. 2007).

1.3.5 Copper and disorders of human health

Copper is an essential nutrient in human health that is required as a co-factor for a wide variety of important enzymatic reactions in the body (Linder and Hazegh-Azam 1996). Lack of copper will impair those enzymes’ activities and lead to various health disorders (Klevay 1998). On the other hand, overexposure to copper can cause copper toxicity which can lead to different physical and mental health problems. Therefore it is important to maintain copper
homeostasis to ensure tissue copper supply without any toxic effects. Since copper homeostasis is tightly regulated in the body, copper imbalance rarely occurs in humans. However, impaired copper balance can be found in humans, either due to genetic factors or environmental factors. It has been shown that copper imbalance can be directly or indirectly involved in the pathogenesis of numerous diseases.

1.3.5.1 Copper deficiency in humans
Copper deficiency in humans is rare and is found mainly in young babies or elderly patients with specific medical complaints. Copper deficiency in adult humans is mainly due to insufficient copper intake in the diet, unsupplemented total parenteral nutrition or malabsorption disorders (Cordano and Graham 1966; Karpel and Peden 1972). For infants, the cause of copper deficiency is similar to adults, but it can be also due to prematurity (low birth weight), preceding malnutrition and feeding of cows’ milk (low copper content) to premature infants (Graham and Cordano 1969; Sutton et al. 1985; Cordano 1998). Copper deficiency in humans can be diagnosed by low plasma copper (hypocupraemia) with low caeruloplasmin values in serum (Heller et al. 1978; Sutton et al. 1985). As stated above, copper is required for a wide variety of important enzymatic reactions involved in different body functions. Inadequate supply of copper will impair the synthesis and activity of copper-dependent enzymes, which can lead to major health disorders such as anaemia, neutropaenia and osteoporosis (Sutton et al. 1985; Jabbour et al. 2010; Lazarchick 2012). As well, deficiency of copper is also associated with the development of myocardial disease and neurological impairment in humans.

Besides environmental factors, genetic defects in copper metabolism can also cause cellular copper deficiency in humans. Menkes’ disease is an X-linked recessive neurodegenerative disorder caused by mutations in the \( ATP7A \) gene that encodes the copper-transporting ATPase, ATP7A (Menkes et al. 1962; Shim and Harris 2003; Tang et al. 2008). Mutations in \( ATP7A \) impair the absorption of copper in the gastrointestinal tract and reduce the copper transport across the enterocyte basolateral membrane to the blood circulation and other tissues. As a result, copper is accumulated within the intestinal mucosa and deficient in many other organs (Danks et al. 1972). Copper deficiency lowers the activities of cuproenzymes in the brain, which lead to neurodegeneration and severe brain damage in patients with Menkes’ disease (DiDonato and Sarkar 1997; Tapiero et al. 2003).
1.3.5.2 Copper toxicity in humans

Although copper is an essential nutrient required by the body, excess copper intake can be toxic in certain circumstances. In order to prevent copper toxicity, copper homeostasis is tightly regulated through intestinal absorption and biliary excretion. Since the amount of copper in the diet is relatively small, acute and chronic copper toxicity rarely occurs in human and is mainly due to accidents, occupational hazards and environment contamination. Exposure to excessive levels of copper can result in multi-organ failure and can be fatal if not treated (Gamakaranage et al. 2011).

1.3.5.2.1 Acute copper toxicity

Reports of acute copper toxicity are rare and it is mainly caused by accidental ingestion of copper or suicidal poisoning with high dosages. In humans, ingestion of more than 1g of copper can lead to toxicity and the estimated lethal dose is between 10 to 20g (Sinkovic et al. 2008). However, different individuals display quite different tolerances to toxic levels of copper exposure. Acute exposure to lower doses can lead to adverse responses in the stomach and cause vagal stimulation, eliciting a reflex response of nausea and vomiting (Wang and Borison 1951; Niijima et al. 1987). Exposure to higher doses of copper can lead to multi-organ failure and death. Reports of individuals who attempted suicide with high doses of copper have shown that excess copper can have deleterious effects on the gastrointestinal tract, blood, liver and kidneys (Oon et al. 2006). Copper can cause acute gastrointestinal symptoms including a metallic taste, nausea, vomiting and abdominal pain (Wahl et al. 1963; Gulliver 1991). Haemorrhagic gastroenteritis can also occur due to irritation and erosion of the gastric epithelium (Gulliver 1991). Copper in the circulation can cause damage to the erythrocytes and lead to intravascular haemolysis (Singh and Singh 1968; Valsami et al. 2012). Copper ions can also affect erythrocyte function by oxidising the haem ion in the erythrocytes to form methaemoglobin, which results in the loss of oxygen-carrying capacity (Yang et al. 2004; Sinkovic et al. 2008). Acute hepatic necrosis with jaundice and renal failure may also present in some patients and has been attributed to intravascular haemolysis, causing liver mitochondrial dysfunction (Nakatani et al. 1994) and kidney tubular epithelial damage (Chugh et al. 1977; Sinkovic et al. 2008), respectively. In some cases, acute copper toxicity can also cause cardiovascular collapse, hypotension and tachycardia, which is responsible for early fatalities or can occur later with other complications (Wahl et al. 1963; Deodhar and Deshpande 1968; Cole and Lirenman 1978).
1.3.5.2.2 Chronic copper toxicity

Chronic copper toxicity is mainly due to occupational hazards and environmental contamination. Compared to acute copper toxicity, the level of copper exposure in chronic copper toxicity is relatively lower. The main impact of chronic copper toxicity in humans is impaired liver function, as this organ is the primary site for copper storage. Similar to the effect of acute copper toxicity, excess copper accumulates in the liver causing oxidative damage; this leads to the development of liver cirrhosis with episodes of haemolysis, causing damage to renal tubules, brain, and other organs. Persistent exposure to excess copper can lead to serious health problems such as coma, hepatic necrosis, vascular collapse, and death. Lastly, studies showed that in young men with long-term high copper intake, it can result in alteration of the antioxidant status and affect immune function (Turnlund et al. 2004).

In humans, genetic defects in copper metabolism can also lead to increased copper levels and cause chronic copper toxicity. Wilson’s disease is an inherited autosomal recessive disorder caused by small mutations or deletions in the ATP7B gene on chromosome 13 that encodes the copper-transporting ATPase protein, ATP7B (Tanzi et al. 1993; Thomas et al. 1995; Lutsenko et al. 2007). Mutations in ATP7B impair the hepatic biliary copper excretion across the canalicular membrane reducing incorporation of copper into caeruloplasmin (Scheinberg and Gitlin 1952). As a result, copper accumulates in the liver leading to toxicity. The excess copper in the liver can cause oxidative damage and activate cell death pathways in hepatocytes, leading to the development of chronic active hepatitis, fibrosis and cirrhosis. Excess copper will eventually leak into the plasma pool and is accumulated in other extra-hepatic tissues including the brain and cornea of the eye (Tanzi et al. 1993; Thomas et al. 1995; Krajacic et al. 2006). Copper in the brain is mainly accumulated in the basal ganglia, particularly in the putamen and globus pallidus (Maeda et al. 1997; Alanen et al. 1999; Kozic et al. 2002). Copper overload in the brain can cause oxidative damage in these areas and lead to the development of neurological or psychiatric symptoms in patients with Wilson’s disease (Roberts and Schilsky 2008). Wilson’s disease is generally fatal and lifelong treatment is needed to prevent copper toxicity in affected tissues.
1.3.6 Role of copper ion in diabetic cardiomyopathy

Cardiac tissue demands a substantial amount of copper in order to sustain mitochondrial oxidative phosphorylation to generate large amounts of ATP for muscle contraction, peptide hormone biogenesis, oxidative stress protection, and other critical functions (Cederbaum and Wainio 1972; Medeiros et al. 1993). Defects in copper metabolism such as chronic copper deficiency and impaired intracellular copper transport can affect the copper supply to mitochondria and lead to cardiac dysfunction.

The role of copper in cardiomyopathy has been extensively investigated. It has been shown that copper deficiency can lead to the development of cardiovascular disorders such as cardiac hypertrophy, abnormal electrocardiograms, cardiac fibrosis and distorted cardiac myofibrils (Prohaska and Heller 1982; Elsherif et al. 2003; Mandinov et al. 2003). It is believed that cardiac hypertrophy in copper-deficient animals is due to alterations in mitochondrial expression and structure. Increased mitochondrial number, enlargement in volume, and extensive disruption of mitochondrial fine structure has been found in the copper-deficient heart tissue (Kopp et al. 1983; Medeiros et al. 1993; Mao et al. 1998). As well, copper deficiency can also impair mitochondrial function and reduce the production of ATP by decreasing oxidative phosphorylation. Animals receiving diets deficient in copper have decreased levels of cellular ATP and phosphocreatine, and elevated ribose 5-phosphate and phosphocholine levels (Kopp et al. 1983). Furthermore, electrophysiology studies in the copper-deficient heart have found abnormalities in cardiac contraction. Abnormalities in electrocardiographs such as aberrant ST segment, bundle branch block, supraventricular, ventricular beats, and wandering pacemakers have been found in copper-deficient hearts (Viestenz and Klevay 1982; Kopp et al. 1983; Medeiros et al. 1991). Lastly, copper deficiency can also induce cardiac failure by altering the myocardial gene expression including contractile proteins, Ca\(^{2+}\)-cycling proteins, extracellular matrix collagens and others (Elsherif et al. 2004). However, the molecular mechanisms involved in cardiomyopathy induced by copper deficiency remain largely unknown.

Beside chronic copper deficiency, impaired intracellular copper transport also plays an important role in the development of cardiomyopathy. Studies have shown that missense mutations in the second cytochrome oxidase assembly gene Sco2 can result in mitochondrial dysfunction, leading to cytochrome c oxidase deficiency and cardiac hypertrophy (Jaksch et al. 2000; Jaksch et al. 2001; Vesela et al. 2003). As described in the previous section, Sco2
protein is involved in the process of Cu$^+$ transfer to the Cu$_A$ site on the Cox2 subunit of CCO in mitochondria, in order to allow CCO to perform its catalytic function in ATP production. In patients with fatal infantile cardioencephalomyopathy, mutations in Sco2 cause severe CCO deficiency in cardiac and skeletal muscle and lead to the development of hypertrophic cardiomyopathy and encephalopathy (Papadopoulou et al. 1999; Mobley et al. 2009; Joost et al. 2010).

In conclusion, these studies demonstrate the essential roles of copper in the normal heart and mitochondrial function. Disturbances in copper balance in diabetes mellitus may lead to impaired mitochondrial function and thus reduced cardiac function and cardiomyopathy.

1.4 Triethylenetetramine as a potential treatment for diabetic cardiomyopathy

Several studies have found that diabetes patient have a higher copper level in serum, especially in those with cardiovascular disease (Zargar et al. 1998; Viktorinova et al. 2009). However, it is believed that the causative molecular mechanisms involved in copper overload in diabetes mellitus are different from those in Wilson's disease. Copper overload in diabetes occurs in different tissues. It has been suggested that elevated serum copper levels in diabetic patients might be due to an imbalance in distribution between the intracellular and extracellular copper concentration (Walter et al. 1991). In a rat model of diabetes, the abnormality in copper homeostasis leads to accumulation of chelatable copper in the heart, and this phenomenon has been proposed as the mechanism of cardiovascular damage in diabetes (Cooper et al. 2004; Cooper et al. 2005). Current studies performed by our group have shown that chronic treatment with a divalent Cu(II)-selective chelator (triethylenetetramine) can ameliorate diabetes-induced disturbances in the regulation of copper homeostasis and improve the structure and function of the heart in diabetic rats and humans (Cooper et al. 2004; Cooper et al. 2009).

1.4.1 History of medical use

Triethylenetetramine or trientine (TETA) administered as the dihydrochloride is a divalent Cu(II)-selective chelator that was first introduced by Walshe in 1969 as an alternative treatment for patients with Wilson’s disease who are intolerant to D-penicillamine (DPA) treatment (Walshe 1969). It was found that TETA could be a better copper chelator with
fewer side effects than DPA (Walshe 1973), although at present DPA is still regarded as the first-line treatment in Wilson’s disease. However, the clinical use of trientine as a second line treatment of Wilson’s disease was not available as a therapy until it was first registered by the US FDA in 1985. Due to its potent copper-binding ability, TETA has also been used as a drug to treat acute copper intoxication secondary to the use of copper utensils that can lead to extensive overexposure to dietary copper (Flora and Pachauri 2010). Recent studies in our group have identified potential new uses for TETA as the first in a new class of anti-diabetic molecules. Our studies have shown that TETA can prevent or reverse systemic copper overload, thereby suppressing oxidative stress and preventing tissue damage in diabetes mellitus.

1.4.2 TETA pharmacology

TETA is a tetradeutate ligand which consists of six CH$_2$ groups arranged as three ethylene segments, which structurally is very similar to the physiologically-occurring polyamines, spermine and spermidine (Fig 1-5a). The four nitrogen groups in TETA can bind to free Cu$^{2+}$ ions to form a TETA-Cu$^{2+}$ complex with square planar coordination geometry (Fig 1-5b). This structural arrangement allows TETA to be tightly bound to Cu$^{2+}$ ions with a dissociation constant for Cu$^{2+}$ of 10$^{-15}$ mol/l at pH 7.0 (Qureshi 1987; Campos-Castello 2001). It has been shown that TETA can reduce excess body copper storage by binding to the excess free copper in the blood, and enhancing urinary copper excretion (Kodama et al. 1997; Walshe 2011). Some studies have suggested that TETA might also be able to block intestinal copper absorption but this remains to be further investigated (Keegan et al. 1999). In terms of medical use, TETA is usually administered in the form of the dihydrochloride salt with a molecular weight of 219.2 Dalton (Taylor et al. 2009).
Figure 1-5 Structure of TETA. A: The linear structure of TETA. B: The square planar coordination of the TETA-Cu$^{2+}$ complex.

Normally, TETA capsules are given to patients for oral administration, or it may be dissolved in aqueous solution and presented as the free-base, TETA, for absorption. In humans, the absorption rate of TETA in the gut through oral administration is relatively poor (Kodama et al. 1997; Keegan et al. 1999). Studies in rats using C$^{14}$-labelled TETA showed that only 6 to 18% of orally administrated drug is systemically absorbed. Current human studies from our group have shown that the recovery observed in urine after administration of oral dose of TETA is around 0.03 to 13.4% in healthy subjects and between 3.7 to 14.6% in diabetic subjects (Lu et al. 2007). Once absorbed into the body, TETA is extensively metabolised, with the majority of absorbed TETA excreted in urine as metabolites (Lu et al. 2010). Using LC-MS, our group has identified two major metabolites of TETA from urine, N1-acetyltriethylenetetramine (MAT) and N$^{1}$,N$^{10}$-diacetyltriethylenetetramine (DAT), both of which are acetylation products of TETA (Lu et al. 2007). However, the enzyme responsible for TETA metabolism is currently under investigation in our group and yet to be established with certainty. As mentioned, the excretion of TETA in humans is mainly through the urine, with the majority excreted as metabolites, and only 0.71 to 4.1% excreted as unaltered parent compound (Kodama et al. 1993; Lu et al. 2007; Lu 2010). Interestingly, it has been reported that patients with diabetes excrete greater amounts of metabolites in the urine than matched healthy subjects, but the implication of this phenomenon remains unknown (Lu et al. 2007).
1.4.3 TETA treatment of STZ-induced diabetic cardiomyopathy in the rat

In order to determine the response to TETA treatment in diabetic cardiomyopathy, extensive experiments were performed using an animal model with streptozotocin (STZ)-induced diabetes (Cooper et al. 2004). In nonclinical studies, we have found that in STZ-induced diabetic rats, TETA treatment increased urinary Cu excretion compared with matched controls (figures 1-6a to c) (Cooper et al. 2004). Furthermore, infusion of a TETA solution into the coronary arteries of ex vivo isolated perfused rat hearts revealed a ~3-fold increase in chelatable myocardial Cu(II) in the diabetic heart (figure 1-6d) (Cooper et al. 2004). These results have provided evidence that an abnormality in copper homeostasis can lead to or cause accumulation of chelatable copper in the heart, which can be ameliorated by TETA treatment.

Figure 1-6 Trientine elicits increased Cu(II) excretion in rats with diabetes. A: Urinary Cu after intravenous TETA (0.1, 1.0, 10, and 100 mg/kg; arrows) in control (●; n = 7) and diabetic (□; n = 7) rats. B: Total urinary copper (mean ± SE; ■ control; □ diabetes). C: Urinary [Cu] (▵) and EPR signal from Cu(II)-trientine complex (▲) after 10 mg/kg (A) and 100 mg/kg (B) TETA. D: Total copper extracted after perfusion of the coronary circulation with TETA.. (mean ± SE; ■ control; □ diabetes). Reproduced from (Cooper et al. 2004).
Our group has also demonstrated the treatment effect of TETA on cardiac structure and function. Our studies have shown that 7-weeks’ oral TETA treatment significantly alleviated heart failure without lowering blood glucose or blood pressure in STZ-induced diabetic rats with established LVH and heart failure (Cooper et al. 2004). Improved cardiomyocyte structure (figure 1-7), and reversed elevations in left ventricular and aortic collagen and beta-1-integrin were found in the TETA-treated diabetic rat.

In addition, ex vivo studies on isolated perfused rat hearts have shown that TETA treatment in our diabetic rat model restored cardiac function with improved cardiac output, as well as restoring both systolic and diastolic function, and repairing the contraction-relaxation mechanism (figure 1-8) (Cooper et al. 2004). In another study, Baynes et al have shown that oral treatment with TETA using an animal model of T2DM, the Zucker Diabetic Fatty (ZDF) rat, can also inhibit the development of cardiomyopathy (Baynes and Murray 2009). Their study has showed that TETA treatment prevented cardiac dilatation, and also improved ejection fraction and myocardial relaxation in these rats (Baynes and Murray 2009). In conclusion, these studies have provided evidence to support the beneficial effect of TETA in restoring cardiac function and structure in diabetes-induced cardiovascular disease.
Figure 1-8 Chronic TETA treatment improves cardiac function in rats with STZ-induced diabetes and heart failure. Trientine was administered for 7 weeks after 6 weeks of established diabetes. ○, untreated diabetes; •, untreated control; □, trientine-treated diabetes; ▪, trientine-treated control; means ± SE, n = 8–11/group. A: Blood glucose; arrow indicates time from which trientine (8–11 mg/day) was administered. B–E were derived from isolated perfused working hearts excised after 7 weeks of trientine treatment. B: CO with increasing atrial filling pressures (preload). C: +dPLV/dt with increasing preload. D: −dPLV/dt with increasing preload. E: Percentage of hearts pumping at each afterload pressure (P < 0.05, Wilcoxon test). *P < 0.05, trientine-treated diabetes vs. untreated diabetes. F: Total copper in 2-min trientine perfusate (n = 10/group, mean ± SE) normalized to heart weights from diabetic (▪) or control (□) animals (*P < 0.04). gHW, grams of heart weight. Reproduced from (Cooper et al. 2004).

TETA treatment in diabetic rats can prevent or ameliorate diabetes-mediated LV and arterial damage by modifying the expression of molecular targets such as transforming growth factor (TGF)-1, Smad4, extracellular matrix (ECM) proteins, extracellular superoxide dismutase (EC-SOD), and heparan sulphate (HS) (Cooper et al. 2005; Gong et al. 2006; Lu et al. 2010). It has been found that diabetic animals have elevated mRNA levels of TGF-β1, Smad4, collagen types I, III, and IV, and fibronectin-1, and plasminogen activator inhibitor-1 in left ventricle and aorta, which can be normalised by TETA treatment. As well, treatment with TETA can also normalise the elevated levels of circulating EC-SOD in T2DM patients.
(Cooper et al. 2005), and restore the arterial HS levels, which were significantly decreased in diabetic rats (Lu et al. 2010). Based on these results, our group has identified potential molecular targets through which TETA could ameliorate diabetic cardiac and arteriovascular disease, which include the suppression of an activated TGF-β1/Smad signalling pathway that mediates increased ECM gene expression and restoration of normal EC-SOD and HS regulation (Cooper 2011).

As mentioned in the previous section, impaired mitochondrial function and structure can lead to abnormal cardiac function in diabetes. Therefore, it is interesting to examine whether TETA can ameliorate the abnormalities in mitochondria. Data from our group’s proteomic analysis of the cardiac left ventricle in diabetic animals suggested that TETA treatment could repair the myocardial mitochondrial structure compared to untreated diabetic controls, and restore the mitochondrial ability to generate ATP through oxidative phosphorylation, thus the metabolic balance between the citric acid cycle and fatty-acid β-oxidation pathways (Jullig et al. 2007).
1.5 Hypothesis and the aims of the proposed research

Diabetes mellitus causes cardiovascular disease, but the mechanisms are incompletely understood. It was found that copper balance tends to be elevated in diabetes compared with non-diabetic control subjects and is not accompanied by systemic copper deficiency, but rather by an imbalance in distribution between intracellular and extracellular copper concentrations. Our group has recently shown that diabetes causes increased systemic levels of chelatable Cu(II) in rodents and humans, probably through hyperglycaemia-evoked increases in Cu(II)-binding to ECM proteins such as collagen. These changes are accompanied by divergent copper-mediated increases in ECM-protein expression and a concomitant copper deficiency inside cardiac myocytes, where most copper is monovalent. Both of these aspects are ameliorated following chronic treatment with TETA, a linear tetra-amine that functions as a divalent Cu(II)-selective chelator. We hypothesise that diabetes-evoked perturbations in intracellular copper regulation might cause the associated cardiovascular disease and can be reversed by chronic treatment with TETA.

The specific aims of this programme research are as follows, namely to:

1. Identify aspects of copper metabolism that are impaired in the left ventricle of the heart in diabetes and their reversibility by TETA treatment.
2. Provide an informative cardiomyocyte model in which to study the molecular and cellular effects of hyperglycaemia and elevated copper levels, and their responses to TETA.
3. Investigate the effects of acute and chronic copper toxicity in the ex vivo isolated perfused rat heart and their interplay with glucose levels.
4. Determine the effects of diabetes on the copper-responsiveness of the ex vivo isolated perfused working heart in an animal model of diabetes, the rat with streptozotocin-induced diabetes.
5. Test the hypothesis that TETA could act as a potential treatment for acute copper toxicity in the heart.
Chapter 2 General Methods: Development and Validation

2.1 Structure of experimental programme

Both cellular and STZ-induced animal models of diabetes were used in this thesis to examine the interplay of hyperglycaemia and copper levels on the structure and function of cardiomyocytes, and to investigate the molecular basis of the dysregulation of copper homeostasis that can lead to or cause cardiac tissue damage. Furthermore, the mechanism of action of a novel therapeutic agent, the Cu(II)-selective chelator TETA, in the prevention and treatment of these cardiac complications, has also been examined in this thesis.

![Diagram](image)

**Figure 2-1** Overall design of experiments employed in this thesis.
2.2 *In vitro* model: continuously cultured P19CL6 cardiomyocytes

P19 cells were originally derived from embryonic carcinoma cell lines obtained from a teratocarcinoma induced in the C3H/He strain of mice (Martin 1981; McBurney and Rogers 1982). Under the influence of different chemical inducers, these undifferentiated pluripotent cells can differentiate into a range of cell types (McBurney *et al.* 1982; Edwards *et al.* 1983). Studies have demonstrated that P19 cells exposed to high concentrations of retinoic acid can differentiate into cells with neuronal and/or glial phenotypes, whereas exposure to a low concentration of dimethyl sulphoxide (DMSO) can promote their differentiation into endodermal and mesodermal phenotypes, including cardiac and skeletal myocytes (McBurney *et al.* 1982; Edwards *et al.* 1983).

The P19 cell line is widely used as an *in vitro* model for cardiogenesis due to the similarity of its differentiation processes to those of *in vivo* cardiomyocytes during embryonic maturation (McBurney *et al.* 1982). Initially, because of their multipotent properties and low differentiation rate into cardiomyocytes, its utility was somewhat limited. However, these limitations were overcome in the later studies as several clonal sub-lines with improved properties were successfully isolated from the original P19 cell line (Rossant and McBurney 1982; Edwards *et al.* 1983; Jones-Villeneuve *et al.* 1983).

One of the clonal sub-lines derived from P19 cells, clone 6 (CL6) or P19CL6, was developed following long-term culture under conditions promoting mesodermal differentiation (Habara-Ohkubo 1996). Compared to original P19 cells, this sub-line has a similar morphology but differentiates into beating cardiomyocytes with far higher efficiency. It was found that 70-80% of P19CL6 cells can differentiated into cardiomyocytes when cultured with 1% (v/v) DMSO, whereas the differentiation frequency of the original P19 cells was only 10-15% under equivalent conditions (Habara-Ohkubo 1996; Peng *et al.* 2002). Furthermore, studies showed that differentiated P19CL6 cells express properties equivalent to those of normal adult cardiac muscle in both physiological and molecular aspects (Peng *et al.* 2002). Spontaneous rhythmic contraction and cardiac markers such as α- and β-cardiac myosin heavy chain are expressed in P19CL6 cells after culture with DMSO for 10 days. Taken together, these results indicate that P19CL6 cells are suitable for studying cardiogenesis *in vitro*.

Compared to primary cultures, there are several advantages in using P19CL6 cells as an *in vitro* model for study of cardiogenesis. Based on published reports, P19CL6 cells are easy to
grow and maintain in the undifferentiated state, and can be differentiated to cardiomyocytes efficiently under simple manipulation of culture conditions (McBurney 1993; Nury et al. 2009). Furthermore, yields from primary myocardial cell culture are relatively low and frequently comprise mixtures of cell types from which pure cardiomyocytes are difficult to isolate (Thum and Borlak 2000). As well, the genetic composition of P19CL6 cells is easier to manipulate than that of primary cells in culture. For these reasons, differentiated P19CL6 cells have been used in this thesis as a cellular model to investigate the effects of diabetes and TETA on cardiomyocyte function.

2.2.1 P19CL6 cardiomyocytes culture and differentiation

P19CL6 cells (obtained from Professor Issei Komuro, Chiba University, Japan) were cultured in growth medium consisting of α-minimum essential medium (α-MEM, GIBCO) supplemented with fetal bovine serum (10% (v/v), GIBCO), sodium bicarbonate (2.2 g/l; Sigma) with 100 U/ml penicillin (Sigma) and 100 µg/ml streptomycin (Sigma). Cells were maintained at 37°C in a cell culture incubator (Esco, USA) with air:CO₂ 95:5 (v/v). Cells were seeded in T25 flasks (Sigma) with 8 ml of growth medium at a suitable density (0.176 x 10⁵ cells/cm²), and passaged every second day.

To induce differentiation, P19CL6 cells were seeded at the same density as above in 12-well plates (Sigma) and α-MEM with ribonucleosides and deoxyribonucleosides (GIBCO) was replaced with α-MEM without ribonucleosides and deoxyribonucleosides, plus DMSO (1% (v/v); BDH, UK) (Differentiation medium). Cells were then continually cultured for the next 14-20 days and medium was changed every second day.

2.2.1.1 Confirmation of differentiation into cardiomyocytes

By monitoring under light microscopy, it was found that P19CL6 cells cultured in differentiation medium proliferated rapidly, reaching confluence on day 3. Thereafter, confluent cultures proliferated continuously over the next 4-6 days to form monolayers. At around days 10 to 12, multilayers had formed at the bottom of the wells. In our experiments, cell contraction was first observed as early as day 12, and was typically present between days 14-18, depending on the size of the culture wells employed for a particular experiment. It was also found that the smaller the well size, the longer the time for the first contraction to be observed. After the first observation of a contraction focus, more foci were typically observed.
to emerge and beat synchronously during the next 4-6 days. Thereafter, individual foci typically remained active for a period of about 2 weeks.

To confirm that differentiated P19CL6 cells were cardiomyocytes, experiments were undertaken to examine their structure, and physiological and molecular properties P19CL6.

Cultured cardiomyocytes are known to contract in response to elevation of extracellular calcium (Nabauer et al. 1989) and alterations in calcium concentration of the medium may affect their contraction rates. Here, calcium chloride (BDH) was added to the medium to determine whether the contraction rates of individual foci would increase. The results calculated from 6 different plates of differentiated P19CL6 cells all showed increased contraction rates in response to elevation of [calcium] (Fig 2-2). This result is consistent with the physiological response of normal cardiomyocytes.

![Figure 2-2](image)

**Figure 2-2** Effect of elevating calcium concentration in the medium on the contraction rates of P19CL6 cardiomyocytes. Calcium chloride was added to elevate the calcium concentration in the culture medium by 20 mM.

For immunocytochemical analysis, paraformaldehyde-fixed cells from both control and differentiated cultures were stained with Phalloidin-Alexafluor-488 4% (w/v) for F-actin, the main component of the thin filaments in cardiomyocytes (Fig 2-3). Contrasting of photomicrographs between normal and differentiated P19CL6 cells indicated that F-actin was highly expressed after differentiation, when their striated structure was clearly observable. In addition, photomicrographs also demonstrated an elongation in cellular shape, a further structural characteristic of differentiated cardiomyocytes (Habara-Ohkubo 1996).
Figure 2-3 Structural changes in differentiated P19CL6 cells. Both non-differentiated and differentiated cells were stained with Phalloidin-Alexafluor-488 for F-actin, the main constituent of the thin filaments in cardiomyocytes, to determine changes in cell structure. This study is representative of n = 3 observations.

<table>
<thead>
<tr>
<th>Target/control gene</th>
<th>Primer sequence (5’ - 3’)</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-myosin heavy chain</td>
<td>For: AGGCCAACACCAACCTGTCC</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>Rev: TTCACGGGCACCCCTTAGAGC</td>
<td></td>
</tr>
<tr>
<td>troponin-T</td>
<td>For: GTGGTGAGGAGGATCGAGGA</td>
<td>215</td>
</tr>
<tr>
<td></td>
<td>Rev: ACCAAGTTGGGCATGAAGA</td>
<td></td>
</tr>
<tr>
<td>β-2-microglobulin</td>
<td>For: ATTTTCAGTGCTGCTACTC</td>
<td>450</td>
</tr>
<tr>
<td></td>
<td>Rev: AGCAGGTTCAAATGAATCTTC</td>
<td></td>
</tr>
</tbody>
</table>

Table 2-1 Primers used in RT-PCR assays for molecular constituents of differentiated cardiomyocytes. Abbreviations: bp, base-pairs; For, forward; Rev, reverse.

**PCR sample preparation**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer</td>
<td>5.0µl</td>
</tr>
<tr>
<td>Primer – For (10µM)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>Primer – Rev (10µM)</td>
<td>0.5µl</td>
</tr>
<tr>
<td>dNTP (10mM)</td>
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</tr>
<tr>
<td>Ampli Tag Gold</td>
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<tr>
<td>MgCl2 (25mM)</td>
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</tr>
<tr>
<td>cDNA sample (1µg)</td>
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</tr>
<tr>
<td>H2O</td>
<td>9.0µl</td>
</tr>
<tr>
<td>Total volume:</td>
<td>50µl</td>
</tr>
</tbody>
</table>

**Standard PCR protocol**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pre-incubation</td>
<td>1 cycle</td>
</tr>
<tr>
<td>2</td>
<td>Amplification</td>
<td>35 cycles</td>
</tr>
<tr>
<td>3</td>
<td>Final elongation</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

Table 2-2 Standard RT-PCR protocol used in for detection of mRNAs corresponding to cardiac markers. For, forward; Rev, reverse.
In our molecular study, the expression of specific cardiac markers was examined in P19CL6 cells at different differentiation states. The cardiac markers troponin-T and β-myosin heavy chain (β-MHC), together with the ‘housekeeping’ gene β-2-microglobulin, were amplified using an established RT-PCR protocol (Table 2-2). Based on the results of this analysis, the expression of troponin-T was found in the cells after 4 days’ culture in differentiation medium whereas the expression of β-myosin heavy chain was first observed only at day 8 (Fig 2-4). In contrast, cells harvested at day 0 (undifferentiated) did not express either cardiac marker. This result suggested that cells cultured in differentiation medium differentiate into cardiomyocytes.

In summary, these studies have shown that P19CL6 cells cultured in differentiation medium can differentiate into cardiomyocytes, which express physiological and molecular properties consistent with those of primary cardiac muscle cells.
2.2.2 Monitoring physical properties of cells by using the xCELLigence system

Cell index is a parameter that can be used to monitor integrated aspects of cell function and physiology. Here, the xCELLigence system (Roche, Germany) was used to monitor the cell index to measure the effects of culturing with different concentrations of glucose and Cu$^{2+}$, in the presence or absence of concomitant TETA treatment.

Compared to more ‘traditional’ viability assays, the xCELLigence system allows non-invasive, continuous measurement of cell index via a purpose-designed micro-titre plate (designated as an ‘E-plate’), which contains interdigitated gold microelectrode-sensors on the bottom of each well, attached to a measuring apparatus (named an ‘SP instrument’). Based on the manufacturer’s description, the local ionic environment at the electrode/solution interface can be affected by the presence of the cell on top of the electrodes, leading to an increase in the electrode impedance. The more cells attached on the electrodes, the larger the increases in impedance. In addition, the impedance can also be affected by the quality of the cells’ interaction with the electrodes. In the xCELLigence system, the electrode impedance is displayed as the ‘cell-index value’ and can be used to monitor integrated changes in cell growth, viability, number and morphology (xCELLigence, Roche).

Before commencing an experiment, 50 µl of differentiation medium was added to each well of an E-plate (96-well) and transferred to an SP instrument for measurement of background values. 100 µl of a suspension of P19CL6 cells was then added to each well of an E-plate (except 3 wells designated for control measurements) and cultured until differentiation (typically 16-20 days). During this period, cells were maintained at 37 ºC in an incubator with 95:5 (v/v) air:CO$_2$, and medium changed every second day. On day 20, cells were examined by light microscopy to determine whether they were differentiated into cardiomyocytes.

After cells had differentiated into cardiomyocytes, medium was replaced with 150 µl of fresh pre-warmed culture medium and they were thereafter monitored (SP instrument, 24 hours) to obtain baseline measurements. When these were completed, medium with different concentrations of glucose, Cu$^{2+}$ and TETA was added to each well and activity monitoring commenced. In this study, the cell index in each well was continuously recorded (24-hour cycle, 15-minute intervals) for the first two hours and thereafter at 1-hour intervals for the next 20 hours) and medium was changed daily. At the end of each experiment, the values of the cell index obtained during the compound activity-monitoring step were normalised to corresponding baseline values before subsequent statistical analysis.
2.2.3 Contractility measurements

P19CL6 cells cultured in differentiation medium developed into cardiomyocytes with multiple foci displaying synchronous contractions by day 16. Contraction rate was measured by counting the mass pulsation (of foci) per minute, in representative fields of an inverted microscope (DM IL LED; Leica, Germany) with temperature control (Tempcontrol-37, Leica). The positions of selected foci were recorded by placing a transparent plastic sheet printed with grids under each cell culture plate and marking (Fig 2-5). After the baseline measurement, reagents (glucose, Cu\textsuperscript{2+} and/or TETA disuccinate as required) were added as required directly into the culture medium. Contraction rates of selected foci were re-recorded on days 2 and 4 and results presented as percentages of corresponding control values without addition of glucose, Cu\textsuperscript{2+} or drug.

![Figure 2-5 System for recording the position of individual foci in the contractility study. The yellow circle represents a field observed under the microscope. For example, the focus in red was recorded as ‘grid 2-1 top-left corner’, and focus in blue was recorded as ‘grid 4-3 bottom-right corner’. The contraction rate of each selected contracting focus was recorded at different times.](image)
2.2.4 Determination of cellular copper concentration by GF-AAS

In this thesis, the cellular copper concentrations in P19CL6 cardiomyocytes cultured under different conditions, were determined by graphite-furnace atomic-absorption spectrometry (GF-AAS; GBS 933AA, Australia). For sample preparation, differentiated cells cultured in 12-well plates were first washed three times with PBS to remove excess culture medium and dead cells. 250 µl of saponin 0.1% (w/v) solution in PBS was then added to each well and incubated (room temperature, 15 minutes) to permit complete cell lysis. The 0.1% (w/v) saponin solution was prepared by dissolving 0.1 g of saponin (Sigma) in 100 ml of water (Milli-Q; Millipore, USA). Cell lysate was transferred into 1.5-ml eppendorf tubes and centrifuged (12,000×g, 15 minutes, 37°C) and supernatants then transferred to new tubes and stored (-20°C) until analysis.

Measurement of cell-copper content was performed as follows. Before the start of each experiment, the GF-AAS was turned on for 2 hours to allow it stabilise. In parallel, the operation conditions for the heating programmes (Table 2-3) were established by using the computer software (v1.33; Avanta, Australia). Measurements were initiated with repeated readings of MilliQ water to clean the reaction tube. Thereafter, a standard curve was constructed to determine the concentration for each sample. Samples were diluted 1:10 (v/v) with pure water, automatically pipetted into the transversely-heated graphite atomiser, and results recorded. For statistical analysis, data were normalised to the total protein concentration of each sample, as determined by the bicinchoninic acid method (section 2.2.6).

<table>
<thead>
<tr>
<th>Step No.</th>
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<th>Ramp time (sec)</th>
<th>Hold time (sec)</th>
<th>Gas Type</th>
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</tr>
<tr>
<td>2</td>
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<tr>
<td>7</td>
<td>2200</td>
<td>1</td>
<td>1</td>
<td>Nitrogen</td>
</tr>
</tbody>
</table>

Table 2-3 Operating conditions employed for measurement of copper by GF-AAS
2.2.5 Measurement of apoptosis

Analysis of apoptosis in P19CL6 cardiomyocytes was performed by using a cell-death detection ELISA PLUS kit (Roche, Germany) as per the manufacturer’s instructions. Briefly, differentiated cells were cultured in 12-well plates and then washed three times with PBS to remove excess culture medium. They were then lysed (lysis buffer, 30 minutes, room temperature), centrifuged (200×g, 10 minutes, room temperature), and supernatants transferred into a new tube. Samples were then diluted 1:10 (lysis buffer) before transfer to a streptavidin-coated micro-plate. Immunoreagent mixtures containing anti-histone/-anti-DNA-antibodies were added to each well and incubated (2 hours, room temperature). Wells were then carefully washed (incubation buffer, three times) to remove non-immunoreactive cell components. Finally, samples were incubated with peroxidase substrate for 10 minutes and the absorbance measured at 405 nm using a micro-plate reader (SpectraMax 340; Molecular Devices, USA) according to the manufacturer’s instructions. For statistical analysis, each value was normalised to the corresponding total protein concentration.

2.2.6 Determination of protein concentration by bicinchoninic acid

Analysis of protein concentrations using the bicinchoninic acid (BCA) assay (Sigma) was performed according to the manufacturer’s instructions. In brief, a standard curve was produced using bovine serum albumin (BSA; Sigma) as the standard for the concentration range of 0 to 1250 µg/ml. 25 µl each of blank, standard and unknown samples were loaded into micro-plate wells in duplicate. 200 µl of the BCA working solution consisting of 50 parts BCA solution and 1 part of CuSO₄ (4%, w/v) was added to each well and mixed thoroughly by using a plate shaker. After incubation (37 °C, 30 minutes), micro-plates were cooled to room temperature before the absorbance was measured (562nm) (SoftMax Pro v4.1.7, Spectra Max 340; Molecular Devices). The protein concentration in each unknown sample was determined from the standard curve.
2.2.7 Measurement of gene expression

2.2.7.1 RNA isolation

In order to obtain RNA samples, P19CL6 cardiomyocytes were cultured in 12 well plates and washed three-times with DEPC-treated PBS to remove excess culture medium and dead cells. An appropriate volume (500 µl) of Trizol reagent (Invitrogen-Life Technologies, Grand Island, USA) was then added to each sample and incubated (room temperature, 5 minutes) to permit complete dissociation of the nucleoprotein complex, followed by transfer of the aliquots into 1.5-ml eppendorf tubes (Eppendorf AG, Germany) and mixing with 100-µl chloroform (Merck, Germany). Samples were mixed by shaking tubes vigorously by hand (15 seconds) and incubating at room temperature (2-3 minutes). They were then centrifuged (12,000 × g, 15 minutes, 4 °C) to separate the mixture into the lower, red phenol-chloroform phase, the inter-phase, and the colourless, upper aqueous phase. The aqueous phase was then carefully transferred to a new tube for RNA precipitation. Next, 250 µl of 100 % isopropanol (Sigma) was added to the aqueous phase and incubated (room temperature, 10 minutes). Samples were then centrifuged (12,000×g, 10 minutes, 4 °C) whereupon RNA formed a gelatinous pellet on the sides and bottom of the tube. Supernatants were then removed from tubes and RNA-pellets washed (500 µl aqueous ethanol 75% v/v; Merck). Samples were briefly vortexed and re-centrifuged (7,500×g, 5 minutes, 4 °C) and the RNA pellet washed and air dried (5-10 minutes). Finally, the RNA pellet was re-suspended in 20 µl nuclease-free water (Ambion/Invitrogen) and stored at -80°C until analysis.

2.2.7.1.1 Assessment of RNA quality and quantity

Properly-extracted RNA must be free from contaminants, including protein, genomic DNA, salts and solvents. Poor RNA quality can cause problems when performing the reverse transcription reaction, which could impair data quality (Barbas et al. 2007). In order to prevent these problems, RNA quality and quantity were determined. In this study, RNA concentration was measured using a NanoDrop® ND-1000 UV-Vis spectrophotometer (NanoDrop Technologies). RNA samples with an OD_{260/280} ratio of 1.8 to 2 and an OD_{260/230} ratio > 1.8 were accepted as “pure”. The integrity of RNA was checked by gel electrophoresis using 1% agarose gels run at 100 V (constant) for 20 minutes. Clear bands for 18S and 28S rRNA (with the 28S band being stronger), were considered to indicate acceptable RNA quality. Any RNA samples that did not reach this standard was either treated with RNase-free...
recombinant DNase I and the extraction then repeated with Trizol reagent, or, in the worst cases, samples were discarded.

2.2.7.1.2 DNase treatment of extracted RNA samples

Recombinant RNase-free DNase I (Roche) is a DNA-specific endonuclease that can randomly degrade both double- and single-stranded DNA to form a mixture of oligo- and mononucleotides in extracted samples without damaging RNA. It is frequently used to eliminate the DNA contamination in RNA preparations. In our experiments, a mixture of 2.5 µl of 10× incubation buffer, 1 µl of recombinant DNase I (10 units) and 1.5 µl of RNase-free water, was combined with 20 µl of an RNA sample and incubated (37 °C, 20 minutes). The reaction mixture was then inactivated by heating and DNase I was then removed by Trizol extraction according to the protocols described in section 2.2.6.1.

2.2.7.2 cDNA synthesis

First-strand cDNA synthesis kits (Roche) were used according to the manufacturer’s instructions. Briefly, a template-primer mixture comprising 0.5 µg of total RNA, 1 µl of anchored-oligo(dT)18 primer (50 pmol/µL), and variable volumes of RNase-free water to constitute a total volume of 13 µl, was prepared in a thin-walled, nuclease-free 0.5 ml tube. This RNA template-primer mixture was then heated in a thermal-block cycler at 65 °C for 10 minutes to ensure the denaturation of RNA secondary structure. Samples were then immediately cooled on ice and mixed with the remaining components, including 4 µL of Transcriptor Reverse Transcriptase Reaction Buffer (5 x concentration), 0.5 µL of Protector RNase Inhibitor (40 U/µl), 2 µL of Deoxynucleotide Mix (10 mM), and 2 µL of Transcriptor Reverse Transcriptase (20 U/µl). Samples were mixed carefully and centrifuged briefly before incubation (50 °C, 60 minutes) in a thermal-block cycler with a heated lid to avoid air condensation on the lip. After incubation, samples were heated (85°C, 5 minutes) before cooling on ice and then stored at -20 °C until analysis.
2.2.7.2.1 Measurement of cDNA concentration

Measurement of cDNA concentrations using Quant-iT Oli-Green ssDNA Reagent and Kits (Invitrogen) was performed according to the manufacturer’s instructions. In brief, 1 µL of each cDNA sample was diluted 200-fold with 1 x TE buffer and a standard curve (with concentrations of 0-500 ng/ml) was prepared using a standard oligonucleotide preparation (Invitrogen). 100 µL each of the diluted sample, standards or blank samples were loaded into a 96-well black plate (Nunc A/S) in duplicate and mixed with 100 µL of working solution containing different concentrations of oligonucleotide in Quant-iT OligoGreen ssDNA Reagent. Fluorescence was measured using a Multi-label Plate Reader (2104 EnVision®; Perkin-Elmer Wallac) at an excitation wavelength of 480 nm and emission wavelength of 520 nm. Based on the results, all cDNA samples were diluted in water to the final concentration (20 ng/µl), and then stored at -80°C for future analysis of gene expression by RT-qPCR.

2.2.7.3 RT-qPCR

Analysis of mRNA expression, using LightCycler 480 DNA SYBR Green I MasterMix kit (Roche), was conducted according to the manufacturer’s instructions. Briefly, one half of each cDNA sample (20 ng/µl) was taken and initially diluted with PCR-grade water to a working concentration of 1ng/µl for RT-qPCR analysis; the remainder of each was pooled to create a standard curve ranging downwards from 20 to 0.015625 ng/µl. Thereafter, a PCR mix solution was prepared that consisted of diluted cDNA sample/standard(blank, in volumes that ranged from 2.5 to 16.5 µl (the final amount depended on the endogenous level of the target gene in the sample preparation), with 3 µl of each of the forward and reverse primers (10 µM primer stock solution), with 22.5 µl of SYBR Green Master Mix and a variable amount of PCR-grade water to make up the total volume of 45 µL. Solutions were mixed well and 15 µl of PCR mix pipetted into each well of a LightCycler 480 multi-well plate, with analysis performed in triplicate. The plate was then sealed with foil, centrifuged (1,500 x g, 2 minutes) and transferred to the plate holder of a LightCycler 480 to commence a qPCR run, as according to the below programme (Table 2-4). Raw data, for example quantification and melting curves, were recorded during the experiment by using LC 480 Software v1.5 (Roche). Relative expression levels of genes under analysis were determined by normalization to the geometric mean of three optimal reference genes (section 2.2.7.3.1).
Table 2-4 Operating programme for qPCR in the LC480 system employed in this thesis.

<table>
<thead>
<tr>
<th>Program</th>
<th>Cycles</th>
<th>Target temperature</th>
<th>Hold (Time)</th>
<th>Analysis mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>1</td>
<td>95°C</td>
<td>5 min</td>
<td>None</td>
</tr>
<tr>
<td>Amplification</td>
<td>45</td>
<td>95°C, 60°C, 72°C</td>
<td>10 sec, 15 sec, 20 sec</td>
<td>Quantification</td>
</tr>
<tr>
<td>Melting</td>
<td>1</td>
<td>95°C, 65°C, 97°C</td>
<td>5 sec, 1 min</td>
<td>Melting Curve</td>
</tr>
<tr>
<td>Cooling</td>
<td>1</td>
<td>37°C</td>
<td>10 sec</td>
<td>None</td>
</tr>
</tbody>
</table>

2.2.7.3.1 Evaluation of reference genes for RT-qPCR analysis in P19CL6 cardiomyocytes

Evaluation and selection of reference genes is a crucial step for qPCR analysis, since using a single, suboptimal or unstable reference gene as normalizer can often give false-positive or false-negative results. It is important to demonstrate that individual genes are robust normalizers under the exact conditions to be used in an experimental protocol. In order to select suitable reference genes for use in these RT-qPCR protocols, experiments were performed on selected cDNA samples using primers specific for candidate reference genes, and the crossing points in each sample were recorded. The crossing-point values were then analysed by two different approaches that employed different software: geNorm (Biogazelle, Belgium) (Fig 2-6) and NormFinder (Denmark) (Fig 2-7), to evaluate the expression stability and suitability of each candidate normaliser gene. In this study, sequences of all primers used for RT-qPCR were designed by using the NCBI programme, Primer_BLAST, with nucleotide sequences obtained from the NCBI.

For studying the acute effects in P19CL6 cardiomyocytes of glucose, Cu²⁺ and TETA, the characteristics of the six candidate reference genes tabulated below (Table 2-5) were analysed. Based on the results of both geNorm (Fig 2-6) and NormFinder (Fig 2-7), Rpl13a, Ndc and U2af were identified as robust normaliser genes for this RT-qPCR analysis.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein L13A</td>
<td>Rpl13a</td>
<td>For: ACAAGAAAAAGCGGATGGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: TTCTCCTCCAGAGTGGCTGT</td>
</tr>
<tr>
<td>TATA box binding protein</td>
<td>Tbp</td>
<td>For: AGAACAAATCCAGACTAGCAGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: GGGAACTTCACATCACAGCTC</td>
</tr>
<tr>
<td>U2 auxiliary factor 35 kDa subunit</td>
<td>U2af</td>
<td>For: CCATTGCCCTCTTTGAACATT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CCTCCCCGTACTTCTCTTCC</td>
</tr>
<tr>
<td>Nucleoporin</td>
<td>Ndc80</td>
<td>For: TTCCCCAACGATGGATTAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CAGCCAGACGTCGGTGGTAGAGTA</td>
</tr>
<tr>
<td>β-actin (sequence 1)</td>
<td>Actb(1)</td>
<td>For: GCTGTATTCCTCCATCGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CACGGTTGGGCCCTTTAGGTTCCAG</td>
</tr>
<tr>
<td>β-actin (sequence 2)</td>
<td>Actb(2)</td>
<td>For: GGATGCAGAAGGAGATTACTGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CCACCGATCCACACAGAGTA</td>
</tr>
</tbody>
</table>

Table 2-5 Primers employed for assessing candidate reference genes.

**Figure 2-6** Analysis of expression stability for candidate reference genes by geNorm.

**Figure 2-7** Analysis of expression stability for candidate reference genes by NormFinder.
In order to study time-dependent effects of glucose, Cu$^{2+}$ and TETA on gene-expression in PC19CL6 cardiomyocytes, the eight candidate reference genes tabulated below (Table 2-7) were examined. Based on the results of analysis by both geNorm (Fig 2-8) and NormFinder (Fig 2-9), Tbp, Hprt and U2af were identified as individual robust normaliser-genes for the planned RT-qPCR analysis.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ribosomal protein L13A</td>
<td>Rpl13a</td>
<td>For: ACAAGAAAAAGCGGATGGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: TTCTCCTCCAGAGTGGCTGT</td>
</tr>
<tr>
<td>TATA box binding protein</td>
<td>Tbp</td>
<td>For: AGAACAATCCAGACTAGCAGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: GGGAACTTCACATCACAGCCT</td>
</tr>
<tr>
<td>U2 auxiliary factor 35 kDa subunit</td>
<td>U2af2</td>
<td>For: CCATTGCCCTCTTTGAACATT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CCTCCCCGTACTTCTCTC</td>
</tr>
<tr>
<td>Nucleoporin</td>
<td>Ndc80</td>
<td>For: TTCCAAAGCATGGATTAGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CAGCCAGACGTGTTGGTAGAGTA</td>
</tr>
<tr>
<td>β-actin (sequence 1)</td>
<td>Actb(1)</td>
<td>For: GCTGTATTCCCTCCATCGTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CACGGTTGGCCTTTAGGGTCAG</td>
</tr>
<tr>
<td>Hypoxanthine phosphoribosyltransferase 1</td>
<td>Hprt</td>
<td>For: GCTTGCTGGTGAAAAGACCTCTCGAAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: CCCTGAAGTACTTATAGTGAAGCAT</td>
</tr>
<tr>
<td>Peptidylprolyl isomerase A</td>
<td>Ppia</td>
<td>For: CGCGTCTCCCTCAGCTGTITG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: TGTAAGTCACCCCCCTGGCACAT</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>For: AGGCCGTTGCTGAGTATGTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rev: TGCTGTGCTCCACCACCTTTCT</td>
</tr>
</tbody>
</table>

**Table 2-6** Primer information for candidate reference genes.

![Figure 2-8](image-url) Analysis of the expression stability for candidate reference genes by geNorm.
2.2.7.3.2 Primer design and optimisation

Sequences of all primers (Table 2-7) used for RT-qPCR were designed using the NCBI software Primer_BLAST using nucleotide sequences obtained from the NCBI (Dr Shaoping Zhang). Pilot PCR runs were performed to select appropriate primer pairs and optimise the amount of cDNA to be used for PCR to analyse each target gene during the actual qPCR run for all samples. In addition, the specificity of individual PCR reactions was also examined by the melting curves obtained in pilot runs.

Figure 2-9 Analysis of the expression stability for candidate reference genes by NormFinder.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
</table>
| Copper transporter 1              | Ctr1   | For: TCAGAATCGGGACCTTATCG  
                              |        | Rev: GAACCATGCCAAACTGAGGT                                      |
| Copper transporter 2              | Ctr2   | For: TGGCCAGTCCCTATCGCATCTCAAT  
                              |        | Rev: ACCACCCGAGGAAAAATCC                                       |
| Divalent metal transporter 1      | Dmt1   | For: CACCGTCAGTACCCAGGT  
                              |        | Rev: CCAATGATTGCAAATCCTCA                                      |
| Copper chaperone for SOD          | Ccs    | For: GAGGCAGGCTGTACTCAAGG  
                              |        | Rev: GTTCCTCAATCAGAAGCA                                        |
| Superoxide dismutase              | Sod1   | For: CAGGACCTCATTTTAAATCCTAC  
                              |        | Rev: TGCCACAGTCTCCACAT                                          |
| Antioxidant protein 1             | Atoxl  | For: AAAGGCTTCTCCCTACTTGG  
                              |        | Rev: TGGACTGACTGACAGTGG                                      |
| ATPase Cu(II) transporter, alpha   | Atp7A  | For: GCAATAGAGGCCATTTCCACC  
                              |        | Rev: AGAGCTGCCGATCTCCAC                                         |
| ATPase Cu(II) transporter, beta    | Atp7B  | For: GCAGATGCTGTCAAACCCAGA  
                              |        | Rev: TGCCACCTGAGTGAAGA                                          |
| Caeruloplasmin                    | Cp     | For: AGGCAACACACCAGGAAAAC  
                              |        | Rev: AGAGGCTGCTGGAGGAAGA                                       |
| Mouse U2af-rs1 region             | Murr1  | For: AGTGCCACCGGAGGAGTT  
                              |        | Rev: CATGCTGAGATGAATTGACT                                       |
| Cytochrome c oxidase 11           | Cox11  | For: TTGCTGCAAGTCTGCAATG  
                              |        | Rev: CCGAATAAACACGCCCTCAA                                       |
| Cytochrome c oxidase 17           | Cox17  | For: ATTGACTGCAGAACTGGAAGA  
                              |        | Rev: AGGCGAAAGCAACTATGAGC                                       |
| SCO cytochrome oxidase 1           | Sco1   | For: GCTCTTTATGGCTGGAATGA  
                              |        | Rev: GTAAAGGCTTTCCCAATGAGC                                      |
| SCO cytochrome oxidase 2           | Sco2   | For: CTTCCGCTGAACTTGCTCTC  
                              |        | Rev: CCCTGAGCCAGTGCATCG                                         |
| Metallothionein 1                 | Mt1    | For: GGGCTGTGTCTGCAAAGG  
                              |        | Rev: GCTGGGTGTCGCTGACT                                      |
| Metallothionein 2                 | Mt2    | For: CCGATCTCTGTCGATAGCTTTCC  
                              |        | Rev: AGGACGACGCTTTGTTGTTG                                       |
| Metallothionein 3                 | Mt3    | For: TGGATATGACCGAGCAGAAG  
                              |        | Rev: TTCTGAGTTGCTGATG                                           |

Table 2-7 Primer sequences for all the target genes studied in the *in vitro* cell culture study.
2.3 Animal care

All animal experimental protocols were approved by The University of Auckland Animal Ethics Committee.

In this thesis, male Wistar rats were obtained from the University of Auckland, School of Biological Sciences, and maintained on a standard autoclaved diet (Teklad; Harlan, UK) and tap water *ad libitum*. Animals were maintained under the following conditions: room temperature, 19-21 °C; humidity, 50-70 %; and a constant 12:12-hour light:dark cycle.

2.3.1 Model of STZ-induced diabetes in Wistar rats

Male Wistar rats (bodyweights 250-300g) were randomised into two groups, ‘diabetic’ or ‘sham’ prior to injection. For the diabetic group, animals were anaesthetised (isoflurane) and received a single injection of streptozotocin (55 mg/kg, Sigma, USA) into the tail vein to induce insulin-deficient diabetes. Control rats (sham) were injected with the corresponding volume of saline. Blood glucose levels (Advantage II system; Roche Diagnostics, Switzerland) and bodyweights were measured at the time of injection and three days thereafter to confirm the presence of diabetes; both variables were thereafter monitored weekly throughout. Diabetes was diagnosed by the presence of an elevated blood glucose value (> 11 mM) on two consecutive measurements at least three days apart in the non-fasting state. In non-diabetic rats, non-fasting blood glucose levels ranged from 4 mM to 6 mM. Rats which showed a loss of 15% of their initial body weight (that is, bodyweight at the time of injection along with other pre-defined criteria, such as immobility, withdrawal or failure to groom) were euthanised and removed from the study.

2.3.2 Treatment of rats with TETA disuccinate

After 8 weeks of diabetes, the rest were assigned to one of four groups: ‘untreated sham’ (n=8), ‘untreated diabetes’ (n=9), ‘TETA-treated diabetes’ (n=9), or ‘TETA-treated sham’ (n=9). In TETA-treated groups, TETA disuccinate (Sigma, USA) was administered in the drinking water (20 mg/day), regardless of weight or glucose level, and continued for eight weeks until organ collection.
2.3.3 Isolated perfused ex-vivo working heart model

After eight weeks’ diabetes, rats from sham and diabetic groups were selected for the cardiac perfusion study. The cardiac perfusion system and procedures used in this study were as developed by Dr Bernard Choong in our group (Cooper et al, 2004). Before the start of an experiment, Krebs-Henseleit bicarbonate buffer (KHB buffer) was added into the perfusion system and preheated to 37°C. The selected rat was then anaesthetised with isoflurane and heparinised (1,000 IU/kg i.v.). The heart was then rapidly excised and immersed in ice-cold KHB buffer to prevent further spontaneous contraction. After trimming the excess tissue, the heart was cannulated for Langendorff perfusion. Meanwhile, the tap in the tube supplying the left-atrial chamber remained closed. After incising the pulmonary artery to ensure adequate ejection of coronary-sinus effusate, the left atrium was slipped onto the atrial cannula and tied in place (silk suture). To position the left atrium, the heart was rotated on the aortic block. Any air bubble in the tube was eliminated before opening the tap in the tube supplying the left atrial chamber, to begin the working-mode perfusion. Once the heart became stable, perfusion was returned to Langendorff mode and a pressure cannula was passed through the apex into the left ventricle for ventricular pressure measurement. The preparation was then returned to working-mode perfusion for the cardiac functional study. Atrial filling pressure was now fixed at approximately 10 cmH₂O and afterload pressure at 82.8 mmHg. Thereafter, cardiac-function parameters including cardiac flow, aortic flow, heart rate and intra-left ventricular pressure were continuously recorded using a PowerLab system (AD instruments, USA).

KHB buffer comprised as follows (all concentrations are final, in mM): NaCl (119, Merck); KCl (4.7, Merck); CaCl₂ (2.5, BDH); MgSO₄ (1.2, Sigma); KH₂PO₄ (1.2, Merck); NaHCO₃ (25, Sigma); pH 7.4. For cardiac perfusion, 10 mM D-glucose (Sigma) was added to the KHB buffer, which was gassed with O₂:CO₂ 95:5 (v/v) and filtered using a VacuCap 90 Filter unit with a 0.2 µm supor membrane fitted (Pall Corporation, USA). After assembly, buffer was stored at 4 °C, and pH adjusted to the final value immediately before use.
2.3.3.1 Chemical response study

In this study, the isolated heart was perfused in working mode with fixation of atrial filling pressure at approximately 10 cmH₂O and afterload at 82.8 mmHg. The heart was first perfused in working mode for 10-15 minutes to allow it to stabilise. Reagents including saline, glucose, CuCl₂ (BDH) and TETA dihydrochloride (Sigma) were first diluted into PBS and then infused (1 ml/min; NE-1000 single syringe pump, USA) as required into the left atrium via the atrial-cannula side-arm in working mode, with continuous recording of values for cardiac function parameters.
2.3.3.2 Pressure study
In each study, the isolated heart first underwent working-mode perfusion with fixation of atrial filling pressure at approximately 10 cm H₂O and afterload pressure at 82.8 mmHg. Cardiac-function parameters including cardiac flow, aortic flow, heart rate and intra-left ventricular pressure were continuously recorded. Atrial filling pressure was decreased to 5 cmH₂O and then slowly increased to 10, 15, 20 and 22.5 cmH₂O and 1-min averages were extracted. The heart was then returned to the initial working-mode perfusion to re-stabilise it. For afterload function assessment, the atrial-filling pressure was then fixed at 10 cmH₂O, and afterload increased from 54.7 mmHg in 8 × 2-min steps. Maximum afterload attained was either 118.5 mmHg or the pressure at which aortic flow became zero. At the end of the experiment, the isolated heart was again returned to the initial working mode perfusion to determine whether irreversible loss of function had occurred during the study.

2.3.3.3 Data analysis
In order to understand the correlation between different cardiac-function parameters, data obtained in chemical response study and pressure study were analysed using Linear mixed effects modelling (LME) by which models were fitted using restricted maximum likelihood (REML) (S+ v8.2, Spotfire, Tibco, Palo Alto, USA).

2.3.4 Tissue collection for study of gene expression
Cardiac LV tissues from sham, diabetic, TETA-treated sham and TETA-treated diabetes rats were previously collected by Dr Deming Dong (Gong et al, 2006). Briefly, following 16 weeks’ diabetes, rats from each treatment group were killed and cardiac left-ventricular tissue collected for molecular genetic analysis. Hearts were rapidly excised from isofluorane-anaesthetised and heparinised rats, and immersed in ice-cold KHB buffer to prevent further spontaneous contraction. The aortic remnant was then ligated to a metal cannula to allow perfusion using a GENIE 220 infusion pump (Kent Scientific) with PBS (100 mM NaCl, 50 mM NaPO₄, pH 7.45, treated with DEPC) at 4 °C. Once perfusion was complete, the left ventricle was excised onto a sheet of sterile RNA-free glass, and tissue cut into 2 smaller pieces. One of these was snap-frozen in optimum cutting temperature (OCT) compound (Tissue-TEK, USA) for immunohistochemistry while the other half was placed in an RNase-free tube containing 1 ml of RNAlater (Qiagen, Germany) for the RT-qPCR study. Tissue
was stored at -80°C until required. In order to ensure an RNase-free environment, prior to use all surgical equipment was soaked overnight in 0.5% SDS treated with DEPC and then soaked in chloroform for 10 minutes before autoclaving prior to surgery.

2.3.5 Gene expression study

For study of gene expression in cardiac tissue, a procedure the same as that described in section 2.2.7 was used, except a different method was employed to extract the RNA, and the primers used in the RT-qPCR study were different as they were optimised for analysis of RNA derived from left-ventricular cardiac tissue.

2.3.5.1 RNA extraction from heart tissue

All RNA samples extracted from cardiac left-ventricular tissues were previously prepared by Jingshu Xu under the supervision of Dr Shaoping Zhang. Briefly, total RNA was extracted using RNeasy Fibrous Tissue Mini kits (Qiagen) according to the manufacturer’s instructions with minor modifications. Cardiac tissues stabilised in RNALater were cut into smaller pieces on an RNase-free surface. Tissues were homogenised using a TissueLyser II (Qiagen) in 300 µl of RLT buffer (lysis buffer) (2 x 3 minutes, 20 Hz). 10 µl of proteinase-K solution and 590 µl of RNase-free water were then added to the tube and the mixture incubated (55°C, 30 minutes). Samples were then centrifuged (10,000 x g, 3 minutes) and supernatants transferred to new tubes. 0.5 volumes of 100% ethanol were added to the samples and then loaded onto RNeasy columns and placed in 2 ml collection tubes. Samples were centrifuged (8,000 x g, 15 seconds) and the flow-through discarded. The columns were then washed with 350 µl of RW1 buffer, and centrifuged (8,000 x g, 15 seconds) and flow-through discarded. Then, DNase treatment consisting of 10-µl RNase-free DNase stock solution and 70 µl RDD buffer were loaded onto RNeasy columns and incubated (20-30 °C, 25 minutes). Columns were then re-washed in a further series of steps using RW1 buffer (x 1) and buffer RPE. For elution, the RNeasy column was transferred into a new 1.5 ml tube. 40-µl RNase-free water was then added and tubes were let to stand for 1 minute before centrifugation (8,000 x g, 1 minute). Samples were then collected and the elution step repeated once more.
2.3.5.2 Primer design and optimisation

Sequences of all primers (Table 2-8) used for RT-qPCR of cardiac tissues were previously designed and optimised by other members of the research group (Dr Shaoping Zhang et al.). Briefly, pilot PCR runs were performed to select appropriate primer pairs and optimize the amount of cDNA to be used for each target gene in the actual RT-qPCR run for all samples. In addition, the purity of the PCR product was also examined by using the melting curve obtained in the pilot run. In addition, the reference genes used in this study was based on the previous evaluation experiments performed by Sebastian Hogl et al in our group.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Symbol</th>
<th>Primer sequence (5' - 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper transporter 1</td>
<td>Ctr1</td>
<td>For: CAACACACCTGGAGAAATGG&lt;br&gt;Rev: CGGGCTATCTTGGAGTCCCTT</td>
</tr>
<tr>
<td>Copper transporter 2</td>
<td>Ctr2</td>
<td>For: TGGAAACGGAGGCCAGAGA&lt;br&gt;Rev: GCCCATGAGGTACTTGAGAGG</td>
</tr>
<tr>
<td>Divalent metal transporter 1</td>
<td>Dmt1</td>
<td>For: AACGGAATAGGCTGGAGGAGT&lt;br&gt;Rev: TGGTGATGAGGACAGAGA</td>
</tr>
<tr>
<td>Cu chaperone for SOD</td>
<td>Ccs</td>
<td>For: CTGTGCACAAAGACCTGAAA&lt;br&gt;Rev: CCATCTTGGGTCTCAACTGAA</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>Sod1</td>
<td>For: GGTCCACGAGAAACAGATG&lt;br&gt;Rev: CAATCACACCAAGAGCCAA</td>
</tr>
<tr>
<td>Antioxidant protein 1</td>
<td>Atox1</td>
<td>For: AAAGCGGTCTCTACTTTG&lt;br&gt;Rev: AGCTGGACTGAGCGTGGT</td>
</tr>
<tr>
<td>ATPase Cu(II) transporter, alpha</td>
<td>Atp7a</td>
<td>For: AGCTTCAGGAGGAGGAGG&lt;br&gt;Rev: AGCTGCTTGGAGCTACAT</td>
</tr>
<tr>
<td>ATPase Cu(II) transporter, beta</td>
<td>Atp7b</td>
<td>For: AACGCGTGCTCTAACTCAAGG&lt;br&gt;Rev: TGCCCGTTTGCTCAACATA</td>
</tr>
<tr>
<td>Caeruloplasmin</td>
<td>Cp</td>
<td>For: CTTCACAAACCGGAAGAAG&lt;br&gt;Rev: ATTGGCTGATGCTAGG</td>
</tr>
<tr>
<td>Mouse U2af-rs1 region</td>
<td>Murr1</td>
<td>For: GTACACGAGCTCAACTC&lt;br&gt;Rev: TCCGTCACTTCCCAAANSG</td>
</tr>
<tr>
<td>Cytochrome c oxidase 11</td>
<td>Cox11</td>
<td>For: GTGGTCCAGAGTTCTCAGAG&lt;br&gt;Rev: CATGATATACAGGAGCA</td>
</tr>
<tr>
<td>Cytochrome c oxidase 17</td>
<td>Cox17</td>
<td>For: TCGGAGAGCTCAGAG&lt;br&gt;Rev: TTTCGCTTCTCAGGAGC</td>
</tr>
<tr>
<td>SCO cytochrome oxidase 1</td>
<td>Sco1</td>
<td>For: GGATCTTATGCTGGAATG&lt;br&gt;Rev: GGAAGGCTTCCCAAATGCTG</td>
</tr>
<tr>
<td>SCO cytochrome oxidase 2</td>
<td>Sco2</td>
<td>For: CAAACACAGGAGAG&lt;br&gt;Rev: CAGGAGCTGAGTAAACCAA</td>
</tr>
<tr>
<td>Metallothionein 1</td>
<td>Mt1</td>
<td>For: AGTGGCTGATGAGCTG&lt;br&gt;Rev: CCGGATCATGTTGAGGAT</td>
</tr>
<tr>
<td>Metallothionein 2</td>
<td>Mt2</td>
<td>For: GCGATCTTCTGTTGATCTC&lt;br&gt;Rev: GCATTTCATGTTGAGG</td>
</tr>
</tbody>
</table>

Table 2-8 Primer sequences for all the target genes examined in the animal study.
2.3.6 Immunohistochemistry

Immunohistochemistry was used in this study to examine the localization of specific molecules in cardiac tissue. The indirect immunolabelling method was employed, in which there are two layers of antibodies. This method has a high sensitivity due to the fact that two or more labelled secondary antibodies can bind to a primary antibody, hence increasing the detectability of the antigen.

Left-ventricular tissues frozen in OCT were previously prepared by other members of the research group (Dr Shaoping Zhang et al). Selected sections were first incubated with 0.1 % TritonX-100 (PBS, room temperature, 15 minutes). Sections were blocked by using normal serum obtained from the species in which the specific secondary antibody was made. This step is necessary to prevent non-specific binding of the primary antibodies to components in the section. A primary antibody was then applied to bind to the specific antigen of interest. After incubation, a secondary antibody conjugated with a fluorophor was used to bind to the primary antibody to detect the localization of the target antigen. During the immunohistochemical procedure, sections were routinely washed in excess PBS at the end of the incubation period for each immunohistochemical reagent.

2.3.6.1 Immunolabelling of CTR1, SOD1, COX17, MT and CCS

Slides with frozen sections were washed and equilibrated in PBS. Selected sections were first incubated with 0.1% TritonX-100 diluted in PBS (room temperature, 15 minutes). Following blocking of sections with normal donkey serum (5% v/v; NDS at 37 °C, 1 hour), sections were incubated with one of the following antibodies (4 °C overnight); rabbit anti-CTR1 (1:250 dilution, Abnova, Taiwan), rabbit anti-SOD1 (1:100 dilution, Novus Biologicals, USA), rabbit anti-COX17 (1:50 dilution, Novus Biologicals, USA), rabbit anti-MT (1:50 dilution, Abcam, UK) or rabbit anti-CCS (1:50 dilution, Novus Biologicals, USA). Sections were then washed and incubated with donkey anti-rabbit IgG-RRX (1:400) (37°C, 1 hour). After washing, sections were dual labelling with anti-WGA 488 (1:1000 dilution, molecular probes, USA) for 1 hour at 37°C before mounted with Prolong Gold ‘antifade’ reagent (Molecular Probes, USA). Slides were then examined by fluorescence microscopy and photomicrography recorded. In order to prevent false positive results, corresponding control experiments were performed in parallel by substituting all the primary antibodies with the corresponding non-immune IgG or serum at equivalent dilutions, and examination by same methods.
Chapter 3 Acute effect of glucose and copper and the potential treatment effect of TETA

3.1 Introduction

T2DM is a metabolic disorder characterized by chronic hyperglycaemia, which results from abnormalities and disruptions of several glucose-control mechanisms. Although the pathological processes remains poorly understood, current studies have suggested that a variety of cellular and molecular defects may contribute to the development of insulin resistance and result in abnormal glucose homeostasis. In patients with diabetes, it is well established that prolonged hyperglycaemia increases the risk of the cardiovascular complications, which are responsible for up to 80% of deaths and are the leading cause of the morbidity and mortality in T2DM.

Patients with long-standing diabetes have higher copper levels in their serum, and this is especially the case in those with cardiovascular disease (Zargar et al. 1998; Viktorinova et al. 2009). It has been suggested that elevated serum copper levels in diabetic patients could be due to an imbalance in distribution between intracellular and extracellular copper concentrations. Studies have suggested that hyperglycaemia can increase the copper levels in the extracellular matrix by reducing the copper binding properties of caeruloplasmin and albumin in patients with diabetes (Islam et al. 1995; Argirova and Ortwerth 2003). The abundance of copper in the extracellular matrix can enhance the production of ROS through the Fenton reaction, resulting in oxidative stress and causing damage to cardiac function. Studies from our group have shown that chronic treatment with a divalent copper-selective chelator, TETA, can ameliorate diabetes-induced disturbances in the regulation of copper homeostasis and improve the structure and function of the heart in diabetic rats and humans.

It is believed that the impairment of both glucose and copper homeostasis occurs long before the onset of the disease. The aim of this chapter was therefore to evaluate the acute effects of hyperglycaemia and elevated copper and their interplay on the physiological and molecular characteristics of cardiomyocytes. In this study, the in vitro cardiomyocyte model and ex vivo perfused heart model were used to examine the acute effects of glucose and copper on cardiac function, intracellular copper levels, and expression levels of mRNAs corresponding to genes involved in intracellular copper transport pathways, as well as their protein distribution and localization. Furthermore, this study also examined the potential treatment effects of TETA in the cardiac model of acute copper toxicity.
3.2 Results

3.2.1 Acute hyperglycaemia

3.2.1.1 Acute effects of glucose on cell viability

To examine the acute effects of hyperglycaemia on cardiomyocyte viability, P19CL6 cardiomyocytes were cultured in specially-designed micro-titre plates (Roche Cat No 05232368001) with different concentrations of glucose. In this study, P19CL6 cardiomyocytes were either cultured at 37°C in medium containing normal (5.5 mM), elevated (16.7 mM) or high (33.3 mM) glucose concentrations. The cell index in each group was continuously monitored using the xCELLigence system, at 15 minutes intervals for 2 hours (Fig 3-1a).

![Figure 3-1](image)

**Figure 3-1** Acute effects of hyperglycaemia on P19CL6 cardiomyocytes viability. (a) Cell index recorded by the xCELLigence system following the addition of glucose at indicated concentrations to the culture medium. Data are mean ± SEM, n = 8/group. (b) Percentage change in cell index after 2 hours incubation with different concentrations of glucose. Data are median with interquartile range, n = 8/group.
The results (Fig 3-1a) showed a slight apparent increase in cell index for all 3 groups over the 2 hour period. However, there was no significant difference \((P = 0.96)\) between the control group and the high-glucose group after 2 hours’ incubation (Fig 3-1b). This result suggests that acute hyperglycaemia did not cause glucotoxicity on the cell number, viability, and morphology of P19CL6 cardiomyocytes under the culture conditions employed.

3.2.1.2 Acute effects of hyperglycaemia on copper uptake in cardiomyocytes

As discussed in the previous chapter, elevated serum copper levels in diabetic patients could be due to an imbalance in distribution between the intracellular and extracellular copper concentrations. In order to examine the acute effects of hyperglycaemia on cell copper levels, P19CL6 cardiomyocytes were cultured under different concentrations of glucose for 2 hours and cells were then collected for GF-AAS analysis to determine the intracellular copper concentrations. It was found that increased glucose concentrations in culture medium may interfere slightly with copper levels in P19CL6 cardiomyocytes (Fig 3-2). However, results from one-way-ANOVA showed that there was no statistically-significant difference in cell copper concentration between the control and high-glucose groups \((P = 0.82)\). These results indicate that acute hyperglycaemia did not affect the copper levels in P19CL6 cardiomyocytes.

**Figure 3-2** Effects of acute hyperglycaemia on cell copper levels in P19CL6 cardiomyocytes cultured with different glucose concentrations for 2 hours. Relative cell copper levels were analysed by GF-AAS. Data are mean ± SEM, \(n = 3\)/group.
3.2.1.3 Acute effect of hyperglycaemia on myocardial gene expression

RT-qPCR was used to examine the acute hyperglycaemic effects on mRNA levels of target genes involved in control of intracellular copper transport in P19CL6 cardiomyocytes. For each target gene, mRNA levels were measured in each individual RNA sample prepared from differentiated cells cultured with different glucose concentrations for 2 hours. Results are shown in Tables 3-1 a-e according to their functions in the intracellular copper transport pathways.

<table>
<thead>
<tr>
<th>Genes involved in cellular copper uptake</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr1</td>
<td>0.93 ± 0.06</td>
<td>1.03 ± 0.09</td>
<td>0.99 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Ctr2</td>
<td>0.93 ± 0.03</td>
<td>0.97 ± 0.08</td>
<td>1.05 ± 0.14</td>
<td></td>
</tr>
<tr>
<td>Dmt1</td>
<td>0.96 ± 0.07</td>
<td>0.94 ± 0.11</td>
<td>0.88 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-1a Acute effects of hyperglycaemia on the mRNA levels of genes involved in cell copper uptake. Data are normalised to the control (5.5 mM glucose) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

<table>
<thead>
<tr>
<th>Genes involved in delivery of copper to components of the secretory pathway</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atox1</td>
<td>1.00 ± 0.02</td>
<td>0.95 ± 0.06</td>
<td>0.94 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>Atp7a</td>
<td>0.88 ± 0.04</td>
<td>0.98 ± 0.06</td>
<td>0.79 ± 0.11</td>
<td></td>
</tr>
<tr>
<td>Atp7b</td>
<td>0.99 ± 0.06</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Cp</td>
<td>0.87 ± 0.07</td>
<td>0.79 ± 0.08</td>
<td>0.72 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>Murr1</td>
<td>0.99 ± 0.05</td>
<td>0.91 ± 0.08</td>
<td>0.95 ± 0.13</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-1b Acute effects of hyperglycaemia on the mRNA levels of genes involved in the secretory pathway. Data are normalised to the control (5.5 mM glucose) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.
<table>
<thead>
<tr>
<th>Genes involved in cytosolic copper transport</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ccs</em></td>
<td>0.95 ± 0.07</td>
<td>0.80 ± 0.05</td>
<td>0.89 ± 0.07</td>
<td></td>
</tr>
<tr>
<td><em>Sod1</em></td>
<td>0.96 ± 0.02</td>
<td>0.95 ± 0.08</td>
<td>0.97 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3-1c** Acute effects of hyperglycaemia on the mRNA levels of genes involved in delivery of cytosolic copper to the copper-requiring enzymes. Data are normalised to the control (5.5 mM glucose) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

<table>
<thead>
<tr>
<th>Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cox11</em></td>
<td>0.90 ± 0.05</td>
<td>1.00 ± 0.16</td>
<td>0.88 ± 0.20</td>
<td></td>
</tr>
<tr>
<td><em>Cox17</em></td>
<td>0.97 ± 0.03</td>
<td>0.86 ± 0.10</td>
<td>0.87 ± 0.13</td>
<td></td>
</tr>
<tr>
<td><em>Sco1</em></td>
<td>0.99 ± 0.06</td>
<td>0.94 ± 0.03</td>
<td>0.99 ± 0.14</td>
<td></td>
</tr>
<tr>
<td><em>Sco2</em></td>
<td>0.99 ± 0.06</td>
<td>0.83 ± 0.15</td>
<td>0.99 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3-1d** Acute effects of hyperglycaemia on the mRNA levels of genes involved in the delivery of copper to (mitochondrial) cytochrome c oxidase. Data are normalised to the control (5.5mM glucose) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

<table>
<thead>
<tr>
<th>Metallothionein gene family</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mt1</em></td>
<td>0.85 ± 0.09</td>
<td>0.68 ± 0.21</td>
<td>0.90 ± 0.23</td>
<td></td>
</tr>
<tr>
<td><em>Mt2</em></td>
<td>0.99 ± 0.05</td>
<td>1.11 ± 0.17</td>
<td>1.55 ± 0.42</td>
<td></td>
</tr>
<tr>
<td><em>Mt3</em></td>
<td>0.81 ± 0.06</td>
<td>0.81 ± 0.09</td>
<td>0.90 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3-1e** Acute effects of hyperglycaemia on the mRNA levels of genes encoding the metallothionein protein family. Data are normalised to the control (5.5mM glucose) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

These RT-qPCR data showed that there was no significant difference in mRNA levels between control and high-glucose groups in any of the target genes examined (Table 3-1). The results provide evidence that short-term hyperglycaemic exposure did not affect the mRNA levels corresponding to genes involved in control of the intracellular copper transport pathways.
3.2.1.4 Effects of short-term glucose infusion on cardiac function of the isolated perfused rat heart

Male Wistar rats with body weights between 250 to 300 g were randomly assigned to control and glucose-treated groups. Following their *a priori* assignment to the treatment group, rats were kept in the animal unit for another 8 weeks. Thereafter, their hearts were isolated for working-model perfusion as described in chapter 2.

In order to examine the acute effects of hyperglycaemia on cardiac function, glucose (final concentration ~25 mM) was infused into isolated rat hearts for 2 minutes via the main coronary arteries and the changes in cardiac function were measured at 30 seconds intervals for 7 minutes (Fig. 3-3).

The results showed that short-term infusion with saline (control) had no effect on measured cardiac functions. Fig 3-3 (a-g) shows that the heart rate, cardiac output, aortic flow, systolic pressure, left ventricular pressure development and relaxation of the perfused heart remained constant after saline infusion. By contrast, for the glucose infusion group, our results showed that short-term glucose infusion (1 M) tended to induce a small increase in some indices of cardiac function. Both cardiac output and aortic flow increased after infusion with glucose, reaching the maximum effect 30 seconds after initiation of infusion followed by a slow decline back to normal. By comparison, measurement of cardiac function between the control and treatment groups at specific time points (Fig. 3-4) showed that cardiac output and aortic flow were slightly increased after glucose infusion, although the result of ANOVA analysis suggested that there is no significant difference between the control and treatment group in terms of cardiac output ($P=0.059$) and aortic flow ($P=0.447$). These results suggest that acute glucose infusion did not cause toxic effect on the *ex vivo* perfused heart model.
Figure 3.3 Acute glucose infusion in the isolated perfused hearts of normal rats. Changes in (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure were recorded and compared between the control (saline) and glucose-treated groups. Data are mean ± SEM, n = 7/group.
Figure 3-4 Acute effects of hyperglycaemia on cardiac function at different time points. Measurements of (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure at different time points were shown and compared between the control and glucose-treated groups. Data are median with interquartile range, n = 7/group.
3.2.2 Acute copper toxicity

3.2.2.1 Acute effect of excess copper on cell viability

To examine the acute effects of copper on cardiomyocyte viability, P19CL6 cardiomyocytes were cultured in a specially-designed micro-titre plate with different concentrations of copper. In this study, P19CL6 cardiomyocytes were either cultured in control medium (no additional copper) or medium with additional copper of 20, 40 and 120 µM added as Cu(II). The cell viability in each group was continuously recorded using the xCELLigence system at 15 minutes intervals for 2 hours (Fig 3-5a).

![Figure 3-5](image)

**Figure 3-5** Acute effects of copper on P19CL6 cardiomyocyte viability. (a) Cell index as recorded by xCELLigence system following the addition of CuCl₂ in culture medium. Data are mean ± SEM, n = 8/group. (b) Percentage of cell index changed after 2 hours incubation with different concentrations of Cu. Data are median with interquartile range, n = 8/group.
The results of the normalised cell index for all 4 groups showed a slight increase over the 2 hour period. However, there was no statistically significant difference between the control and the copper-treated groups over this time (Fig 3-5b). These results suggest that incubation with high levels of copper did not cause toxic effects on the cell number, viability or morphology of P19CL6 cardiomyocytes.

### 3.2.2.2 Acute effects of excessive copper exposure on intracellular copper concentration

In order to examine the acute effects of added copper on cell copper levels, P19CL6 cardiomyocytes were cultured under different concentrations of copper for 2 hours and cells were then collected for GF-AAS analysis to determine the intracellular copper concentrations. It was found that increased copper concentrations in the culture medium increase copper levels in P19CL6 cardiomyocytes (Fig 3-6). The relative intracellular copper concentrations in P19CL6 cardiomyocytes seemed to increase following the addition of Cu(II) to the culture medium. Compared to the control group, the relative intracellular copper concentrations in cardiomyocytes cultured with 120 µM copper showed a nearly two-fold increase. However, results from one-way-ANOVA have shown that there was no statistically significant difference on intracellular Cu concentration between control and all other copper-treated groups ($P=0.107$).

![Graph](image)

**Figure 3-6** Acute effects of copper on cell copper levels. The relative intracellular copper concentrations in P19CL6 cardiomyocytes cultured with different copper concentrations for 2 hours were analysed by GF-AAS. Data are mean ± SEM, n = 3/group
3.2.2.3 Acute effects of excess copper on myocardial gene expression

RT-qPCR was used to examine the acute effects of addition of Cu²⁺ to the medium on mRNA levels of target genes involved in control of intracellular copper transport in P19CL6 cardiomyocytes. For each target gene, mRNA levels were measured in each individual RNA sample prepared from differentiated cells cultured with different copper concentrations for two hours. Results are shown in Tables 3-2 a-e according to their functions in the intracellular copper regulation pathways.

### Table 3-2a

<table>
<thead>
<tr>
<th>Genes involved in cell copper uptake</th>
<th>[Copper] μM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr1</td>
<td>0.93±0.06</td>
<td>1.00±0.09</td>
<td>1.00±0.08</td>
<td>1.00±0.081</td>
<td>1.01±0.14</td>
<td>0.96±0.14</td>
<td></td>
</tr>
<tr>
<td>Ctr2</td>
<td>0.93±0.03</td>
<td>1.10±0.13</td>
<td>1.05±0.10</td>
<td>1.07±0.075</td>
<td>0.92±0.08</td>
<td>0.82±0.08</td>
<td></td>
</tr>
<tr>
<td>Dmt1</td>
<td>0.95±0.07</td>
<td>0.90±0.11</td>
<td>0.93±0.12</td>
<td>0.86±0.094</td>
<td>0.78±0.15</td>
<td>0.77±0.09</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2a Acute effects of elevated copper on the mRNA levels of genes involved in cell copper uptake. Data are normalised to the respective control value (without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

### Table 3-2b

<table>
<thead>
<tr>
<th>Genes involved in delivery of copper to components of the secretory pathways</th>
<th>[Copper] μM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atox1</td>
<td>1.00±0.02</td>
<td>1.09±0.10</td>
<td>1.00±0.06</td>
<td>1.10±0.12</td>
<td>0.97±0.04</td>
<td>0.92±0.04</td>
<td></td>
</tr>
<tr>
<td>Atp7a</td>
<td>0.88±0.04</td>
<td>0.98±0.07</td>
<td>0.98±0.10</td>
<td>0.93±0.05</td>
<td>0.91±0.11</td>
<td>0.86±0.12</td>
<td></td>
</tr>
<tr>
<td>Atp7b</td>
<td>0.99±0.06</td>
<td>0.93±0.17</td>
<td>0.92±0.10</td>
<td>0.87±0.09</td>
<td>0.83±0.13</td>
<td>0.88±0.13</td>
<td></td>
</tr>
<tr>
<td>Cp</td>
<td>0.87±0.07</td>
<td>0.87±0.13</td>
<td>0.90±0.11</td>
<td>0.80±0.14</td>
<td>0.68±0.12</td>
<td>0.69±0.10</td>
<td></td>
</tr>
<tr>
<td>Murr1</td>
<td>0.99±0.05</td>
<td>0.99±0.06</td>
<td>1.02±0.04</td>
<td>0.98±0.06</td>
<td>0.96±0.11</td>
<td>0.92±0.14</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2b Acute effects of elevated copper on the mRNA levels of genes involved in secretory pathway. Data are normalised to the control (without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

### Table 3-2c

<table>
<thead>
<tr>
<th>Genes involved in cytosolic copper transport</th>
<th>[Copper] μM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccs</td>
<td>0.95±0.07</td>
<td>0.96±0.06</td>
<td>0.96±0.11</td>
<td>0.90±0.06</td>
<td>0.81±0.02</td>
<td>0.78±0.04</td>
<td></td>
</tr>
<tr>
<td>Sod1</td>
<td>0.96±0.02</td>
<td>1.03±0.04</td>
<td>1.01±0.05</td>
<td>1.00±0.05</td>
<td>0.98±0.07</td>
<td>0.98±0.06</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2c Acute effects of elevated copper on the mRNA levels of genes involved in cytosolic copper regulation. Data are normalised to the control (without additional Cu) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.
Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway

<table>
<thead>
<tr>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox11</td>
<td>0.90±0.05</td>
<td>0.93±0.12</td>
<td>0.93±0.14</td>
<td>0.87±0.12</td>
<td>0.94±0.22</td>
<td>0.88±0.16</td>
</tr>
<tr>
<td>Cox17</td>
<td>0.97±0.03</td>
<td>0.97±0.11</td>
<td>0.98±0.08</td>
<td>0.96±0.13</td>
<td>0.91±0.13</td>
<td>0.92±0.15</td>
</tr>
<tr>
<td>Sco1</td>
<td>0.99±0.06</td>
<td>0.94±0.04</td>
<td>1.00±0.07</td>
<td>0.83±0.07</td>
<td>0.77±0.06</td>
<td>0.74±0.11</td>
</tr>
<tr>
<td>Sco2</td>
<td>0.99±0.06</td>
<td>0.97±0.12</td>
<td>1.02±0.09</td>
<td>0.97±0.11</td>
<td>0.79±0.13</td>
<td>0.86±0.10</td>
</tr>
</tbody>
</table>

Table 3-2d Acute effects of elevated copper on the mRNA levels of genes involved in the delivery of copper to (mitochondrial) cytochrome c oxidase. Data are normalised to the corresponding control (without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

<table>
<thead>
<tr>
<th>Metallothionein gene family</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT1</td>
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<td>0.96±0.20</td>
<td>1.90±0.46</td>
<td>2.20±0.98</td>
<td></td>
</tr>
<tr>
<td>MT2</td>
<td>0.99±0.05</td>
<td>1.05±0.20</td>
<td>1.10±0.20</td>
<td>1.65±0.48</td>
<td>2.99±0.20</td>
<td>4.58±1.83</td>
<td></td>
</tr>
<tr>
<td>MT3</td>
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<td>0.92±0.14</td>
<td>0.85±0.13</td>
<td>0.81±0.075</td>
<td>0.87±0.14</td>
<td>0.84±0.11</td>
<td></td>
</tr>
</tbody>
</table>

Table 3-2e Acute effects of elevated copper on the mRNA levels of genes encoding members of the metallothionein protein family. Data are normalised to corresponding control values (without additional Cu) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group. Data with significant difference to control group are shown in red (P = 0.017).

Figure 3-7 The relative mRNA levels of Mt2 following addition of Cu(II) to the culture medium. Results are means ± SEM. *P=0.017 vs control (without additional Cu), n=5/group.
The data obtained from RT-qPCR showed that there was no significant difference in mRNA levels between control and copper-treated groups in the majority of the target genes examined (Table 3-2 (a-e)). However, it was found that additional copper did affect the gene expression of one member of the metallothionein gene family. It was found that the mRNA levels of Mt2 were significantly increased ($P = 0.017$) following addition of Cu(II) to the culture medium. Compared to the control group, there was a 4-fold increase in Mt2 expression in cardiomyocytes cultured with 120 µM of added Cu(II) (Fig 3-7). Additional to the effect on Mt2, the mRNA levels of Mt1 trended upwards following the addition of Cu(II), although the difference in Mt1 mRNA was not statistically significant ($P = 0.15$). These results show that short-term exposure of cardiomyocytes to copper did not significantly affect the mRNA levels corresponding to genes involved in the intracellular copper transport pathways, but did however elevated Mt2 mRNA, which mediates copper detoxification.

### 3.2.2.4 Effects of short-term copper infusion on cardiac function in isolated perfused rat hearts

Male Wistar rats with body weights between 250 to 300 g were randomly assigned to control and copper-treated groups. Following selection, rats were kept in the animal unit for another 8 weeks. Thereafter, their hearts were isolated and studied by using working-mode perfusion. In order to examine the acute effects of acute copper elevation on cardiac function, different amounts of cupric chloride (CuCl$_2$) were infused into isolated rat hearts via the main coronary arteries and the changes in cardiac function measured at one-minute intervals for the next 25 minutes (Fig 3-8).

The results show that short-term saline infusion had no measurable effects on cardiac function, since heart rate, cardiac output, aortic flow, systolic pressure, and rates of left ventricular pressure development and relaxation of the perfused heart remained constant after saline infusion. By contrast, in the copper-treated group, the results indicate that short-term perfusion with CuCl$_2$ into *ex vivo* isolated hearts immediately impaired cardiac function in a dose-dependent manner (Fig. 3-9a). At lower dosages of CuCl$_2$ (5-10 µM copper), it was found that the isolated perfused rat heart can recover from the acute impairment induced by short-term perfusion with increased copper and restore their cardiac function to baseline values.
Figure 3-8 Short-term effects of bolus infusion of CuCl₂ solutions on the performance of *ex-vivo* perfused rat hearts studied at two-minute intervals. Effects of elevated copper concentration on stroke volume (a), heart rate (b), cardiac output (c), aortic flow (d), ventricular pressure development (e), relaxation (f) and systolic pressure (g) were recorded for 25 minutes after Cu²⁺ boluses were administered. Values of final [Cu] are given in ranges because the final concentration achieved in any experiment depended on the total volume of the perfusion circuit including the coronary vasculature, which varies between animals within limits. Data are mean ± SEM, n = 7/group.
However, the impairment in cardiac function became irreversible as the copper concentrations increased. As shown in figure 3-8c, cardiac output decreased immediately following copper infusion, and progressively declined still further in the next 25 minutes. It was found that cardiac output dropped within 10 minutes by more than 35% in the isolated heart perfused with 10-15 μM CuCl₂, and by 50% with 15-20 μM CuCl₂ (Fig 3-9a). Besides the changes in cardiac output, all other measures except systolic pressure also declined dose-dependently following the infusion of CuCl₂.

Besides the impairment of cardiac function, high levels of copper (> 20 μM) infused into isolated perfused hearts led to myocardial contracture. It was found that six out of seven isolated hearts perfused with the highest level of copper had failed functionally during the experiment (Fig 3-9b). Based on our observations, isolated perfused hearts treated with high levels of infused copper showed increased stiffness of the cardiac muscle. At the end of the experiments, the isolated heart was severely contracted, firm and felt like a ‘stone’ (‘stone heart’).

Figure 3-9 Short-term infusion of copper impaired cardiac function in isolated perfused working rat hearts. (a) The correlation between Cu-infusion level and cardiac output at the 10-minute time-point. (b) Percentage of hearts that remained pumping at each time point.
Results from 2-way ANOVA provide further supporting evidence concerning the effects of acute copper infusion on the functions of isolated-perfused rat hearts from normal animals. It was found that the cardiac function in the copper-infused group was significantly different from that in the control group (Table 3-3). The heart rate \((P=0.037)\), cardiac output \((P=0.0001)\), aortic flow \((P=0.0022)\) and minimum dP/dt \((P=0.014)\) decreased significantly after copper infusion.

Copper-mediated effects to lower cardiac output appear to have exceeded the effect to lower heart rate, indicating that acute copper infusion may have at least two effects: one resulting in progressive lowering of the heart rate (figure 3-8b), and a second impairing the pump function independent of the lowering of heart rate. These results suggested that short-term infusion of copper into the coronary vasculature of the isolated perfused heart can cause copper toxicity and impaired cardiac function.

<table>
<thead>
<tr>
<th></th>
<th>(p)-value (acute copper effect)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke volume</td>
<td>0.0598</td>
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<tr>
<td>Heart rate</td>
<td>0.0373</td>
</tr>
<tr>
<td>Cardiac output</td>
<td>\textbf{0.0001}</td>
</tr>
<tr>
<td>Aortic flow</td>
<td>\textbf{0.0022}</td>
</tr>
<tr>
<td>Max +dP/dt</td>
<td>0.2130</td>
</tr>
<tr>
<td>Min -dP/dt</td>
<td>\textbf{0.0143}</td>
</tr>
<tr>
<td>Systolic pressure</td>
<td>0.0785</td>
</tr>
</tbody>
</table>

| Table 3-3 Two-way-ANOVA results for acute effects of infused copper on cardiac function as compared to saline-perfused control hearts. Due to the large number of severely failed hearts, data from 20+ µM copper groups has been excluded from this analysis. Data with significant difference to the control group are shown in red, \(n = 7\)/group |
3.2.3 Interactions between acute hyperglycaemia and copper toxicity

3.2.3.1 Interaction between the acute effects of glucose and copper on cell viability
To examine the interplay between acute elevation in glucose and copper values on cardiomyocyte viability, P19CL6 cardiomyocytes were cultured in the micro-titre plate, purpose-designed for measurement of cell index values, with different concentrations of glucose and copper. In this study, cells were cultured under three different concentrations of glucose, 5.5, 16.7 and 33.3 mM, with or without the additional copper at 20, 40 and 120 µM in the medium. The cell index in each group was continuously recorded using the xCELLigence system at 15-minute intervals for 2 hours (Fig 3-10).

Figure 3-10 Acute interplay between elevations of glucose and copper on the viability of P19CL6 cardiomyocytes. Normalised cell index was recorded by applying the xCELLigence system following the addition of CuCl₂ to culture medium along with (a) 5.5 mM glucose, (b) 16.7 mM glucose and (c) 33.3 mM glucose. (d) Summary of relative cell index values after 2-hours’ incubation with different concentrations of glucose and copper. Data are mean ± SEM, n = 8/group.
The results show that the normalised cell index values for all experimental groups slightly increased over the 2 hour period. However, there was no statistically significant difference between the control and the copper-treated groups under different glucose concentrations (Fig 3-10d). Based on the experimental results, there was no evidence to support that suggestion that the interplay between acute elevations in glucose and copper might affect cell number, viability, and morphology of P19CL6 cardiomyocytes ($P=0.80$).

### 3.2.3.2 Effects of acute glucose-copper interaction on intracellular copper levels

In order to examine the interplay between acute elevations in glucose and copper on intracellular copper levels, P19CL6 cardiomyocytes were cultured with three different concentrations of glucose, 5.5, 16.7 and 33.3 mM, and with or without additional copper concentrations of 20, 40 and 120 µM. Samples were collected after 2 hours for GF-AAS analysis to determine the intracellular copper levels.

![Figure 3-11](image)

**Figure 3-11** Interplay between acute elevations in glucose and copper on intracellular copper levels. The relative intracellular copper levels of P19CL6 cardiomyocytes cultured with different treatment conditions for 2 hours were analysed by GF-AAS. Data are mean ± SEM, $n=3$. *** $P<0.001$ for 120 µM vs 0 µM copper.

Relative copper levels in cardiomyocytes increased following the addition of CuCl$_2$ to the culture medium (Fig 3-11), but this effect was not influenced by the different concentrations of glucose in the culture medium. Compared to the control group (without additional copper), cardiomyocytes cultured with 120 µM copper showed more than 2-fold increases in
intracellular copper concentrations (Fig 3-11). Results from two-way-ANOVA show a significant effect of additional copper on intercellular copper levels between control and copper-treated groups ($P=0.0005$). However, no evidence was found in this experiment to support the existence of an interaction between acute elevation in medium glucose concentration and copper on cell copper levels ($P=0.93$).

### 3.2.3.3 Interaction effects between acute elevations in medium glucose and copper on myocardial gene expression in P19CL6 cardiomyocytes

RT-qPCR was used to examine the interaction between acute elevations of glucose and copper on mRNA levels of target genes involved in control of intracellular copper uptake pathways in P19CL6 cardiomyocytes. For each target gene, mRNA levels were measured in each individual RNA sample prepared from P19CL6 cardiomyocytes cultured with different concentrations of glucose and copper in the medium for 2 hours. Results were shown in Table 3-4 (a-e) with genes grouped according to their presumed functions in intracellular copper regulation.

<table>
<thead>
<tr>
<th>Genes involved in cellular copper uptake</th>
<th>[Glucose] mM</th>
<th>[Copper] μM</th>
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<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>5.5</td>
<td>10.0±0.09</td>
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<tr>
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<td>16.7</td>
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<td>0.99±0.11</td>
<td>1.06±0.10</td>
<td>0.93±0.11</td>
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<tr>
<td></td>
<td>33.3</td>
<td>0.99±0.09</td>
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<td>0.95±0.13</td>
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<td>0.95±0.10</td>
<td>0.90±0.11</td>
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</tr>
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<tr>
<td></td>
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<td>1.00±0.11</td>
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</tr>
<tr>
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<td>33.3</td>
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<td>0.98±0.17</td>
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<td></td>
</tr>
<tr>
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<tr>
<td></td>
<td>16.7</td>
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<td>0.91±0.11</td>
<td>0.86±0.10</td>
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</tr>
</tbody>
</table>

**Table 3-4a** Acute interaction between elevated glucose and copper on mRNA levels of genes involved in cell copper uptake. Data are normalised to the control (5.5mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.
### Genes involved in delivery of copper in the secretory pathway

<table>
<thead>
<tr>
<th>Genes involved in cytosolic copper transport</th>
<th>[Glucose] mM</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
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<td></td>
</tr>
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<td>0.92±0.04</td>
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<tr>
<td></td>
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</tr>
<tr>
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</tbody>
</table>

| Table 3-4b | Acute effects of the interaction between elevations of glucose and copper on mRNA levels of genes involved in the intracellular copper secretory pathways. Data are normalised to the corresponding control values (5.5 mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group. |

### Genes involved in cytosolic copper transport

<table>
<thead>
<tr>
<th>Genes involved in cytosolic copper transport</th>
<th>[Glucose] mM</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
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</tr>
<tr>
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</tbody>
</table>

| Table 3-4c | Acute interaction effects between elevated glucose and copper on mRNA levels of genes involved in the control of cytosolic copper transport. Data are normalised to corresponding control values (5.5 mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group. |
### Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway

<table>
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<tr>
<th></th>
<th>[Glucose] mM</th>
<th>[Copper] µM</th>
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<th>10</th>
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<th>80</th>
<th>120</th>
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</thead>
<tbody>
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<tr>
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**Table 3-4d** Acute effects of interaction between elevated glucose and copper on mRNA levels of genes involved in delivery of copper to (mitochondrial) cytochrome c oxidase. Data are normalised to corresponding control values (5.5 mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

### Metallothionein gene family

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**Table 3-4e** Acute effects of interaction between elevated glucose and copper on mRNA levels of genes encoding members of the metallothionein protein family. Data are normalised to the corresponding control values (5.5mM glucose without additional Cu) and presented as relative mRNA level. Data are mean ± SEM, n = 5/group.
Figure 3-12 The relative mRNA levels of Mt1 and Mt2 in P19CL6 cardiomyocytes cultured with different concentrations of glucose and copper. (a) Relative mRNA levels for Mt1, **$P=0.009$ 120 µM added copper vs control (no added copper) (b) Relative mRNA levels for Mt2, ***$P<0.0001$ for 120 µM added copper vs control (no added copper). Results are means ± SEM, n=5/group.

The data obtained from RT-qPCR shows that there was no significant difference in mRNA levels between control and the treatment groups for the majority of the target genes examined (Table 3-4 (a-e)). None of the target genes examined in this experiment have shown any interaction effects between acute elevation of glucose and copper. However, addition of copper had significant effects on the expression of two members of the metallothionein gene family. It was found that the mRNA levels of both Mt1 and Mt2 were significantly increased ($P = 0.009$ and $P < 0.0001$, respectively) following addition of 120 µM Cu$^{2+}$ to the culture medium. Compared to the control group, there was a 2-fold and 4-fold increase in Mt1 and Mt2 mRNA levels, respectively, in cardiomyocytes cultured with 120 µM of additional copper (Fig 3-12) and these effects were not modified by differences in glucose concentration in the culture medium. These results provide evidence that short-term exposure to elevated copper can affect the mRNA levels corresponding to genes involved in the defence against elevated cytoplasmic copper. However, no interaction effects between glucose and copper was identified in this experimental system.
3.2.4 TETA as a potential treatment for acute copper toxicity

3.2.4.1 The effects of acute TETA treatment in P19CL6 cardiomyocytes

In order to determine the effects of acute TETA treatment in P19CL6 cardiomyocytes, cells were cultured in medium containing different concentrations of glucose, in the presence or absence of TETA at concentrations greater than or equal to those occurring in TETA-treated patients. The viability of cells in each group was continuously tracked using the xCELLigence system at 15 minutes intervals for 2 hours (Fig 3-13).

![Figure 3-13](image-url) Acute effects TETA treatment on the viability of P19CL6 cardiomyocytes. Normalised cell index was recorded using the xCELLigence system following the addition of TETA to the culture medium with glucose (mM) of (a) 5.5, (c) 16.7, (e) 33.3. Data are mean ± SEM, n=4/group. Shown are values for the normalised cell index at the 2-hour time point with glucose (mM) of (b) 5.5, (d) 16.7, and (f) 33.3. Data are median with interquartile range, n=4/group.
The results showed that the normalised cell index for both control and TETA-treated groups increased slightly over the 2-hour period (Fig 3-13a, c and e). By comparison, the percentage change in the cell index after the 2-hours period (120-min time-point) showed no significant difference between the control group and the TETA-treated groups at the three different glucose concentrations (Fig 3-13b, d and f). Based on these results, there was no evidence to show that TETA treatment had any adverse effects on cell number, viability, and morphology of P19CL6 cardiomyocytes in the short term.

3.2.4.2 The effects of acute TETA treatment on isolated ex vivo perfused rat heart

To determine the effects of acute TETA treatment in the perfused rat heart model, male Wistar rats with body-weights between 250 to 300 g were selected and kept for another 8 weeks in the animal unit. Their hearts were then isolated for working-model perfusion and were randomly assigned to control or TETA-treated groups. In order to examine the adverse effects of TETA on cardiac function, TETA solution (1 mM) was bolus-infused into isolated rat hearts for 2 minutes via the main coronary arteries and the changes in cardiac function were measured at one-minute intervals for 25 minutes thereafter (Fig 3-14).

The results showed that short-term infusion with saline had no effect on the indices of cardiac function measured. The heart rate, cardiac output, aortic flow, systolic pressure, left ventricular pressure development and relaxation of the perfused heart remained consistent before and after saline infusion (Fig 3-14). For the TETA-treatment group, the results were similar to the control group. Perfusion with 1 mM TETA for 2 minutes had no measurable effect on cardiac function. ANOVA analysis showed that there was no statistically significant difference between responses in the control group and the treatment group. These results indicate that short-term TETA infusion caused no measurable adverse effects on cardiac function in the ex vivo perfused rat heart model.
Figure 3-14 Short-term bolus infusion of TETA (1 mM) in isolated perfused rat hearts. Changes in (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure were recorded and compared between the control and TETA-treated group. Data are mean ± SEM, n = 7/group.
To further determine the possible effects of TETA, cardiac function was measured in the isolated perfused working heart in response to different atrial filling pressures (Fig 3-15) and afterload pressures (Fig 3-16). During these experiments, baseline readings recorded as atrial filling pressure (preload pressure) were increased from 5 cmH\textsubscript{2}O to 22.5 cmH\textsubscript{2}O. Filling pressure was then fixed at 10 cmH\textsubscript{2}O, and afterload pressure increased from 72 mmHg to 157 mmHg. After recording the baseline reading, isolated perfused rat hearts were randomly assigned to either the control or TETA-treated groups. Saline or 1 mM of TETA solution was then bolus-infused into isolated rat heart for 2 minutes via the main coronary arteries, and the changes in cardiac function in response to different preload and afterload pressures were recorded and compared with the baseline readings.

Results from these experiments show that increased atrial filling pressure in isolated perfused rat hearts results in major increases in stroke volume, cardiac output and aortic flow (Fig 3-15 a, c and d). However, no differences were detected in heart rate, systolic pressure, and left ventricular pressure development and relaxation (Fig 3-15b, e, f and g). On the other hand, increased afterload pressure reduced the stroke volume, cardiac output and aortic flow (Fig 3-16 a, c and d). In addition, it was found that the left ventricular pressure development and relaxation in the isolated perfused heart was slightly increased in response to increased afterload pressure (Fig 3-16 e and f). Furthermore, the systolic pressure was also found to increase as the afterload pressure increased (Fig 3-16g). But once again, no effects were found on heart rate (Fig 3-16b).

Compared to the baseline values, there were no significant differences in cardiac function after short-term infusion of saline and TETA in both preload and afterload experiments. These results indicate that short-term infusion of TETA had no measurable adverse effects on cardiac function in the \textit{ex vivo} perfused heart model.
Figure 3.15 Effect of bolus-infusion of TETA (1 mM) on cardiac function in response to increased atrial filling pressure. Changes in (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure were recorded before and after TETA infusion. Data are mean ± SEM, n = 7/group.
Figure 3-16 Effect of bolus infusion of TETA (1 mM) on cardiac function in response to increased afterload pressure. Changes in (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure were recorded before and after TETA infusion. Data are mean ± SEM, n = 7/group.
3.2.4.3 Effects of TETA treatment on P19CL6 cardiomyocytes exposed to elevated copper levels

In order to examine the potential treatment effects of TETA on acute effects of elevated copper in cardiomyocytes, P19CL6 cardiomyocytes were cultured in medium containing different concentrations of glucose and copper, as in previous experiments, with or without the addition of TETA (at a 1:1 molar ratio to copper). In this study, the treatment effect of TETA on P19CL6 myocytes cultured with addition of 40 µM (Fig 3-17) and 120 µM (Fig 3-18) Cu were examined. The cell indexes in each treatment group were continuously recorded using the xCELLigence system at 15-minute intervals for 2 hours.

Figure 3-17 Effects of TETA treatment on P19CL6 cardiomyocytes cultured with 40 µM of added copper. Normalised cell indexes were recorded using the xCELLigence system following addition of TETA to the culture medium with glucose (mM) at (a) 5.5, (c) 16.7, and (e) 33.3. Data are mean ± SEM, n=4/group. Percentage changes in cell index after a 2-hour experimental period (120-min time point) with glucose (mM) (b) 5.5, (d) 16.7, and (f) 33.3. Data are median with interquartile range, n=4/group.
Figure 3-18 Effects of TETA treatment on P19CL6 myocytes cultured with 120 µM of added copper. Normalised cell indexes were recorded using the xCELLigence system following the addition of TETA to the culture medium with glucose (mM) (a) 5.5, (c) 16.7, and (e) 33.3. Data are mean ± SEM, n=4/group. Percentage changes in cell index after the 2-hour treatment period (120-min time point) with glucose (mM) (b) 5.5, (d) 16.7, and (f) 33.3. Data are median with interquartile range, n=4/group.

The normalised cell index values for all sample groups increased slightly over the 2-hour experimental period. When compared with the percentage changes in the cell index after the 2-hour period, no significant difference was present between the control group, the copper-treatment group and the TETA-treatment group across the different glucose concentrations. Based on these experimental results, there was no evidence to indicate that TETA had any measurable effect on P19CL6 cardiomyocytes exposed to elevated added copper.
It was found in the previous experiments that the mRNA levels of both Mt1 and Mt2 were significantly increased in P19CL6 cardiomyocytes cells following the addition of Cu to the culture medium. Therefore, we were interested to examine the treatment effect of TETA on the mRNA levels of those two target genes. In this experiment, P19CL6 myocytes were cultured with different concentrations of glucose and copper in the medium for 4 hours, with or without TETA treatment during the latter 2 hours (Fig 3-19). For the TETA-treatment group, TETA was added into the medium at a 1:1 molar ratio to added copper. The mRNA levels were measured in each individual RNA sample and results are shown in Tables 3-5 and 3-7.

![Figure 3-19](image)

**Figure 3-19** Experimental design for RT-qPCR analysis to examine the treatment-effects of TETA on target genes.

Results from this experiment were consistent with the previous results as the mRNA levels of both Mt1 and Mt2 were significantly increased in P19 cardiomyocytes following the addition of CuCl2 to the culture medium. Comparing the data between the copper-treated group and TETA-treatment group, there was no evidence to support that the increased mRNA levels in Mt1 and Mt2 can be ameliorated following acute treatment with TETA (Fig 3-20 and Fig 3-21). No significant difference was found in the mRNA levels between the Cu-treated group and TETA-treated group in either Mt1 (Table 3-6) or Mt2 (Table 3-8) genes. These results indicate that TETA did not directly reverse the molecular changes elicited by acute addition of elevated copper in P19CL6 cardiomyocytes.
Table 3-5 Treatment effects of TETA on Mt1 mRNA levels. Data are normalised to corresponding control values (5.5 mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 6/group.

Table 3-6 Newman-Keuls multiple comparisons test for the effects of TETA treatment on Mt1 mRNA levels. Results with significant differences are shown in red.

Figure 3-20 Comparison of Mt1 mRNA levels in P19CL6 cardiomyocytes treated with 120 µM added copper and with or without equimolar TETA treatment. The relative mRNA levels of Mt1 were compared among control, copper-treated and copper-TETA-treated groups in P19CL6 cardiomyocytes cultured in medium with glucose (mM) (a) 5.5, (b) 33.3. Data are medians with interquartile ranges, n = 6/group. Data with **, P<0.01 and ***, P<0.001 are significantly different from corresponding controls.
Relative mRNA levels for $Mt2$

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**Table 3-7** Effects of TETA treatment on Mt2 mRNA levels in P19CL6 cardiomyocytes treated with added copper for 2 hours. Data are normalised to control values (5.5 mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 6/group.

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**Table 3-8** Newman-Keuls multiple comparisons test for TETA-treatment on Mt1 mRNA levels. Results indicative of significant differences are shown in red.

**Figure 3-21** Comparisons of Mt2 mRNA levels in cultured P19CL6 cells treated with 120 µM added copper with or without equimolar TETA treatment. Relative Mt2 mRNA levels were compared among control, copper-treated and TETA/copper-treated groups in P19CL6 cardiomyocytes cultured in medium with glucose (mM) (a) 5.5, or (b) 33.3. Data are median with interquartile range, n = 6/group. Data with ***, $P<0.001$ are significantly different from corresponding controls.
3.2.4.4 Effects of TETA treatment in isolated perfused rat hearts treated with acutely toxic levels of copper

Our previous studies have demonstrated the acute effects of toxic copper levels in isolated perfused rat hearts. Short-term perfusion of CuCl$_2$ into *ex vivo* isolated rat hearts immediately impairs cardiac function in a dose-dependent manner (for example, Fig. 3-9a). It was found that the cardiac output fell by more than 50% within 10 minutes in isolated hearts bolus-perfused with 15-20 μM Cu$^{2+}$. The aim of this study was therefore to evaluate the treatment effects of TETA on the function of isolated perfused hearts with cardiac dysfunction caused by acute copper toxicity. In addition, the possible mechanisms involved in TETA treatment would be examined by comparing the effects of SOD1 treatment.

In this study, hearts isolated from weight-matched male Wistar rats were first infused with 15-20 μM CuCl$_2$ for 2 minutes to induce acute impairment of cardiac function. Ten minutes after initiation of the copper infusion, 1mM TETA or 1000 units of SOD1 were infused over two minutes into the functionally-impaired hearts via the main coronary arteries, and subsequent changes in cardiac function measured at 1 minute intervals for the next 15 minutes (Fig 3-22).

Results from this experiment showed that isolated hearts perfused with 15-20 μM CuCl$_2$ for 2 minutes displayed cardiac dysfunction associated with 40% and 60% loss in cardiac output respectively, in the TETA-treatment and SOD1-treatment groups at the 10-minute time point. The results obtained from this experiment were consistent with our previous data, which demonstrated a 50% loss in cardiac output following the two-minute infusion of 15-20 μM CuCl$_2$. After the infusion of two treatments, it was found that cardiac function in the TETA-treated group increased immediately and remained constant throughout the remainder of the experiment. The heart rate, cardiac output, aortic flow, systolic pressure, and rates of left-ventricular pressure development and relaxation were all increased dramatically after the infusion of TETA (Fig 3-22). Compared to the continuous loss of cardiac function caused by acute copper infusion in the isolated heart, TETA treatment not only prevented functional impairment but also restored function in the copper-impaired heart (Fig 3-23).

Based on observations during these experiments, the myocardial condition of the isolated heart improved promptly following the infusion of TETA, as no irreversible myocardial contraction was present on examination and the tissue remained ‘soft’ at the end of the experiment. Thus, TETA prevented the development of the stone heart.
By contrast, there was no evidence to suggest that infusion of SOD1 has any effect on the functional impairment caused by acute copper treatment, as all the measurements of cardiac function remained consistently low after the infusion of SOD1.

Results of statistical analysis further supported the treatment effects of TETA on isolated rat heart with acute copper impairment. Significant differences in cardiac output were found in the copper-treated group between the baseline and end-point values. However, no significant difference ($P > 0.05$) was found in cardiac output between the baseline and end-point values in the TETA-treatment group (Table 3-9). In order to show that the treatment effect of TETA was not due to differences between animals, comparisons were made between the different treatment groups at different time-points (Table 3-10). Comparisons between different treatment groups showed that the treatment effects of TETA were not due to underlying differences in animals as no significant difference was found between the copper-treated TETA/copper-treatment groups in cardiac function at baseline and before the infusion of TETA.

These results provide strong evidence that short-term infusion of TETA can at least partially restore cardiac function of the copper-perfused heart.
Figure 3.22 Treatment effects of TETA and SOD1 administered at time = 20 minutes on functional parameters of isolated perfused rat hearts following induction of acute copper-induced impairment at time = 10 minutes. Changes in (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure were recorded for different treatment groups. Data are mean ± SEM, n = 7/group.
Figure 3-23 Shown are values of cardiac output, a measure of integrated cardiac function, from isolated perfused rat hearts at the three indicated time-points in the four experimental groups of Fig 3-22. The three treatment groups were all bolus-infused with an acutely-toxic level of copper (16.7 mM) at time = 10 minutes and rescue treatments (TETA or SOD1) were administered by bolus infusion beginning at time = 20 minutes. Data are mean ± SEM, n = 7/group.

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</tbody>
</table>

Table 3-9 Values calculated by Tukey’s multiple-comparisons test comparing cardiac function measured in isolated rat hearts of different treatment groups at the indicated time points. Results with significant differences are shown in red.

<table>
<thead>
<tr>
<th>Bonferroni post-hoc tests</th>
<th>Baseline</th>
<th>10 minutes after first infusion</th>
<th>End of experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline vs Cu-treated</td>
<td>$P &gt; 0.05$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Saline vs TETA-treatment</td>
<td>$P &gt; 0.05$</td>
<td>$P &lt; 0.001$</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Saline vs SOD1-treatment</td>
<td>$P &gt; 0.05$</td>
<td>$P &lt; 0.001$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Copper-treated vs TETA-treatment</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Copper-treated vs SOD1 -treatment</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>TETA-treatment vs SOD1-treatment</td>
<td>$P &gt; 0.05$</td>
<td>$P &gt; 0.05$</td>
<td>$P &lt; 0.01$</td>
</tr>
</tbody>
</table>

Table 3-10 Comparison between different treatment groups of cardiac function in isolated heart at different time points. Results with significant differences are shown in red.
3.3 Discussion

3.3.1 Effects of transient acute hyperglycaemia on indices of myocardial function

Alteration in glucose homeostasis has been linked to the pathogenesis of diabetes-induced cardiovascular disease. As described in the previous section, chronic hyperglycaemia in diabetic patients can impair the function of ventricular myocytes via different mechanisms. It has been suggested that the effect of hyperglycaemia occurs long before the onset of overt disease. Therefore, studies using an in vitro cardiomyocyte model and the ex-vivo perfused heart were conducted to examine the acute effect of hyperglycaemia.

Acute hyperglycaemia did not impair indexes of growth in P19CL6 cardiomyocytes. The percentage changes in cell index for these cells cultured in normal glucose (5.5 mM) medium were not significantly different from those of cells cultured in high glucose (16.7 mM or 33.3 mM). These results indicate that elevated extracellular glucose concentrations did not affect the cell number, viability or morphology of these cells. In addition, studies in ex-vivo isolated perfused rat hearts showed similar results for the acute effects of hyperglycaemia. Infusion of elevated glucose into isolated perfused rat hearts showed a temporary increase in indices of cardiac function but no significant changes in cardiac function occurred in the longer term. When taken together, these studies provide robust evidence to support the suggestion that acute hyperglycaemia may not induce toxic effects in the myocardium under the conditions studied here.

According to results from published reports, it has been proposed that acute hyperglycaemia can affect cardiomyocyte function in vitro through increased oxidative stress (Ha and Lee 2000). However, there is little evidence to show that acute hyperglycaemia could affect the physiological characteristics of the myocardium. In addition, it is worth mentioning that most in vitro studies have only investigated longer-term effects of hyperglycaemia (several days to 2 weeks). By contrast, available data on acute hyperglycaemic effects in the 0 to 2-hour time frame are limited. For ex-vivo isolated perfused heart experiments, the results obtained from this study are contrary to previous published reports which indicated that acute perfusion of high glucose in normal subjects may induce vasoconstriction and prolong the re-polarization time (QT interval) (Marfella et al. 2000; Ceriello et al. 2002). A possible explanation for this discrepancy is the difference in perfusion time. In our study, isolated hearts were only perfused with glucose solution for 2 minutes, compared to two to six hours in other studies (Marfella et al. 2000; Ceriello et al. 2002). It was believed that myocardium could tolerate
temporary changes in glucose concentration without causing any damage to cardiac structure and function. It is likely that 2 minutes infusion of high glucose in our study is not long enough to induce glucotoxicity in the isolated perfused heart. In addition, it is worth mentioning that the temporary increase in cardiac function in isolated hearts perfused with acute hyperglycemia could be due to its osmotic effect.

In the second part of this study, the effects of acute hyperglycaemia on intracellular copper uptake and transport in cardiomyocytes were examined in both cellular and molecular levels. Results from GF-AAS have shown that the intracellular copper levels for P19CL6 cardiomyocytes cultured under normal glucose (5.5 mM) medium were not significantly different from those cultured in high glucose (16.7 mM or 33.3 mM). Further results from RT-qPCR also showed no significant difference in mRNA levels for genes involved in intracellular copper uptake and transport, this study provided strong evidence that acute hyperglycaemia did not affect the copper uptake in myocardium.

Taken together, these data indicate that acute hyperglycaemia did not induce glucotoxic effects on either cellular or molecular characteristics of the myocardium. Further studies on myocardium with more prolonged hyperglycaemia are recommended in future work.

3.3.2 Acute effects of copper toxicity on the myocardium

Here we showed that addition of copper into the culture medium did not induce cytotoxic effects in P19CL6 cardiomyocytes. The percentage change in cell index for cells cultured at normal copper (no added CuCl₂) were not significantly different from those cultured at high copper (20-120 µM). However, studies on ex-vivo isolated perfused hearts have shown the opposite with the acute effects of copper toxicity. Short-term perfusion of CuCl₂ into the ex-vivo isolated heart can immediately impair cardiac function in a dose-dependent manner. Isolated hearts perfused with high copper values showed increased stiffness and contraction, resulting in a state similar to “stone” heart. A possible explanation for this finding could be the antioxidant effect of vitamin C in the medium, which neutralised the toxic effects of excess free copper on P19CL6 cardiomyocytes.

Data from the studies using ex-vivo isolated hearts suggested that perfusion of additional copper could have adverse effects on cardiac function. By comparing the results with previous published reports, it is suggested that additional copper can induce a toxic effect on
myocardium through at least two different potential mechanisms. The first of these is the promotion of oxidative stress. As described in the previous chapter, 1.3.3, ionic copper can damage cellular components by generating ROS such as superoxide anion, hydrogen peroxide and hydroxyl radical. Hence, infusion of CuCl$_2$ into perfused rat hearts increases the production of ROS, which may severely compromise cardiomyocyte health and viability, resulting in reduction of cardiac function and heart failure.

In addition to the production of oxidative stress, disturbance of the Na$^+$/K$^+$–ATPase pump and the Na$^+$/Ca$^{2+}$ exchanger could be the other major mechanism involved in copper toxicity in the isolated heart (Benders et al. 1994; McDonough et al. 1996; Levine et al. 2011). Studies in cultured human skeletal muscle cells have shown that copper overload can inhibit the Na$^+$/K$^+$–ATPase pump, resulting in rapid increase of cytoplasmic free sodium. Increased cytoplasmic sodium can activate the Na$^+$/Ca$^{2+}$ exchanger, leading to increased calcium concentration and enhanced myocardial contractility (Benders et al. 1994). Based on the observations made during experiments that the isolated perfused heart, infusion with high copper levels showed increases stiffness and contraction in cardiac muscle, thus providing evidence that elevated ionic copper could impair myocardial calcium homeostasis.

In the second part of this study, the effects of excess copper on intracellular copper transport in cardiomyocytes were examined at both organ/cellular and molecular levels. Differentiated P19CL6 cardiomyocytes cultured with additional copper in the medium showed a non-significant trend towards increasing copper concentration. By RT-qPCR, it was found that the mRNA levels of metallothionein gene-family members were affected by the additional copper. The mRNA levels of both $Mtl$ and $Mt2$ were significantly increased in relation to the intracellular copper concentration in P19CL6 cardiomyocytes. This result is consistent with others which have demonstrated that increased intracellular copper can lead to the induction of $Mt$ mRNA expression in mammals (Furst et al. 1988; McArdle et al. 1990; Bremner 1998). In vitro studies have also revealed that MT protects against oxidative injury by reacting directly with ROS, including superoxide and hydroxyl radicals and hydrogen peroxide (Kang 1999; Tapia et al. 2004). This further supports the suggestion that the production of oxidative stress is one of the potential mechanisms in copper-induced cardiac damage. Taken together, these results indicate that additional copper in the culture medium may increase intracellular copper and induce oxidative stress in P19CL6 cardiomyocytes. In response to this toxic effect, these cells increase $Mtl$ and $Mt2$ mRNAs, presumably to
detoxify ROS, regulate intracellular copper homeostasis and thus to prevent cellular damage caused by additional free copper.

In summary, this study has demonstrated the effects of acute copper toxicity in the myocardium. It is suggested here that free copper ions can induce cardiac damage through the production of ROS and disruption of the Na⁺/K⁺–ATPase pump and the Na⁺/Ca²⁺ exchanger. In response to the increase in extracellular copper concentration, the myocardium can activate cell-protective antioxidant defences, which result in increased mRNA expression of \( Mt1 \) and \( Mt2 \), through which they can maintain intracellular copper homeostasis and prevent cellular damage. Further increases in intracellular copper overwhelm the defence mechanisms, leading to impairment of cardiac function and heart failure.

### 3.3.3 Interaction between acute hyperglycaemia and copper toxicity on myocardium

Based on the results obtained in this study, no evidence was found to show that acute hyperglycaemia can interact with excess copper to affect the cellular and molecular characteristics of the myocardium. In previous studies, a trend towards increased intracellular copper was found in P19CL6 cells with added copper in medium. Here, the effects of additional copper on intracellular copper concentrations were further confirmed. P19CL6 cardiomyocytes cultured with additional copper in the medium showed significant increases in cellular copper that was dose-dependent. A two-fold increase in cellular copper concentration was found in P19Cl6 cells cultured with 120 \( \mu \)M Cu compared over control values. Since there are no previous published studies known to us which have examined the effects of excessive copper on myocardial copper concentrations, results from this study were compared to similar studies based on other organs. The results from this study are in concordance with the previous published reports that showed a linear relationship between cell copper and extracellular copper concentration (Stuart and Johnson 1986; Arredondo et al. 2000). Here, results from RT-qPCR show that copper treatment increases mRNA levels of \( Mt1/Mt2 \). Since MT acts as an indicator/marker for intracellular copper concentrations, these findings provide strong evidence that increased extracellular copper can alter myocardial copper status.
3.3.4 Effects of TETA treatment on acute myocardial copper toxicity

TETA is a linear tetra-amine that functions as a divalent Cu(II)-selective chelator. In patients with diabetes, it has been shown that TETA can lower excessive copper storage by binding to excess free Cu(II) and enhancing urinary copper excretion without lowering plasma copper levels (Cooper et al. 2004; Cooper et al. 2009). Based on the copper-binding properties of TETA, it was hypothesised here that TETA can be used as a treatment for the myocardial impairment caused by acute copper toxicity.

The results from this study have shown that short-term perfusion of TETA can partially restore cardiac function in ex-vivo isolated rat hearts exposed to acutely toxic copper levels. Compared to the continuous loss of cardiac function in isolated hearts without such TETA treatment, it not only prevented further impairment from acute copper toxicity but also restored cardiac function in copper-impaired hearts. Furthermore, the effects of TETA treatment were copper-specific as no improvement or adverse effect of cardiac function were observed when TETA was perfused alone. Lastly, no treatment effect of SOD1 was found in isolated hearts with acute copper damage, even though further loss in cardiac function was apparently prevented following the infusion of SOD1.

The results from this study have indicated that a certain amount of catalytically-active copper may accumulate in the myocardium following the short-term infusion of CuCl₂. This catalytically-active myocardial copper may induce toxic effects that impair cardiac function through the production of ROS, causing oxidative stress and disruption of the Na⁺/K⁺–ATPase pump and the Na⁺/Ca²⁺ exchanger. In addition, the excess free myocardial copper is chelatable and can be removed by short-term infusion of TETA into the coronary circulation.

In addition, this study has provided further insight into the mechanism involved in myocardial copper toxicity. It is believed that SOD1 can protect cells from the toxic effects of superoxide by converting it into oxygen and hydrogen peroxide. Infusion of SOD1 into isolated hearts exposed to acutely toxic copper levels tended towards preventing further damage in cardiac function, consistent with the idea that production of oxidative stress did indeed play a role in copper-mediated cardiac dysfunction. However, cardiac function was not restored following the infusion of SOD1, indicating that cardiac dysfunction in the copper-perfused heart may not be entirely due to oxidative impairment of cardiac function, but to a disruption of the intracellular balance of copper. Alternatively, SOD1 may not have gained access to the intracellular compartment.
In summary, this study has shown that TETA treatment can restore intracellular copper levels and prevent oxidative damage by removing excess chelatable copper accumulated in the myocardium and thereby restores cardiac function in the isolated perfused heart.

### 3.4 Conclusion

The results presented in this study have demonstrated the effects of excess chelatable copper on the myocardium and of its treatment with TETA. Short-term increase in extracellular copper disrupts myocardial copper homeostasis, and results in increased intracellular copper. In response to this increase, cardiomyocytes can activate the cell-protective antioxidant defence system by increasing the expression of MT1 and MT2, which can bind to chelatable copper ions to form MT-Cu complexes, thereby detoxifying the copper excess, regulating intracellular copper homeostasis, and preventing cellular damage from copper-induced oxidative processes. However, as more copper is transported into the cells, the amount of chelatable copper will eventually overwhelm the defence mechanisms, resulting in production of ROS and probable disruption of the Na\(^+\)/K\(^+\)–ATPase pump and the Na\(^+\)/Ca\(^{2+}\) exchanger. The mechanisms induced by elevated chelatable copper ions will thus impair myocardial function and may lead to heart failure.

In addition, the results from this study have demonstrated the treatment effect of TETA in myocardium subject to acute copper toxicity. Results showed that short-term treatment with TETA was able to restore cardiac function impaired by acute copper toxicity in the heart. Although the mechanism is not fully understood, it is believed that TETA can restore intracellular copper homeostasis and prevent oxidative damage by removing excess chelatable copper accumulated in the myocardium, thereby restoring the cardiac function.
Chapter 4 Effects of prolonged exposure to different glucose and copper concentrations on the structure and function of cultured cardiomyocytes and isolated-perfused working hearts

4.1 Introduction
Copper is an essential nutrient required by all living organisms to support a substantive number of critical enzyme-catalysed reactions. Cardiac tissue needs a substantial amount of copper in order to sustain mitochondrial oxidative phosphorylation to generate the large amounts of ATP required for muscle contraction, peptide hormone biogenesis, oxidative stress protection, and other critical functions (Cederbaum and Wainio 1972; Medeiros et al. 1993). Defects in copper metabolism, such as chronic deficiency and impaired intracellular copper transport, can modify the copper supply to mitochondria and lead to cardiac dysfunction. On the other hand, excessive amounts of copper in the cardiac tissues can cause toxicity due to unbound copper ions which react readily with hydrogen peroxide and superoxide anion to catalyse the production of highly-toxic ROS such as the hydroxyl radical that can cause damage to lipid, protein and DNA (Kalyanaraman 1982). Therefore, copper homeostasis in cardiac muscle is tightly regulated to ensure adequate intracellular supply whilst minimising any potential toxic effects.

In patients with diabetes, studies have shown that chronic hyperglycaemia can disrupt the copper balance in the myocardium and cause elevated serum copper levels, particularly in those patients with the complications. It has been shown that copper imbalance in the myocardium can be directly or indirectly involved in the pathogenesis of diabetes-induced cardiovascular disease. Recent studies from our group have shown that chronic treatment with a divalent Cu(II)-selective chelator, TETA, can ameliorate diabetes-induced disturbances in the regulation of copper homeostasis and improve the structure and function of the heart in diabetic rats and humans.

It has been described in previous chapters that short-term exposure to excessive amounts of free Cu(II) can impair the physiological and molecular characteristics of the myocardium. However, the molecular effects of hyperglycaemia in the myocardium were not detected in our acute studies. The aim of this chapter was therefore to evaluate the chronic effects of elevations in glucose and copper on the two models of cardiac structure and function that have been described in chapter two, and thereby to elucidate the effects of glucose-copper interactions on the physiological and molecular characteristics of the myocardium.
These models are interpreted to provide evidence concerning the molecular and pathophysiological mechanisms that contribute to the effects of hyperglycaemia and copper toxicity in the heart.

In these studies, an *in vitro* cardiomyocyte culture model and an *ex vivo* isolated perfused heart model were designed and applied to measure the following:

- chronic effects of glucose and copper on cardiac function;
- cardiomyocyte copper levels (as possible indicators of cellular copper uptake); and
- mRNA levels of genes involved in the intracellular copper-regulatory pathways;

In addition, this study also examined the potential treatment effects of TETA on cardiac tissues undergoing exposure to chronic elevations in copper levels.
4.2 Results

4.2.1 Chronic glucose elevation
To model the ‘chronic’ effects of hyperglycaemia on cardiomyocyte properties, P19CL6 cardiomyocytes were cultured as described in chapter two, in medium containing different concentrations of glucose, and medium was changed every second day.

In these studies, P19CL6 cardiomyocytes were cultured in medium with glucose concentrations that were ‘physiological’ (5.5 mM), ‘moderately elevated’ (16.7 mM) or ‘seriously elevated’ (33.3 mM). These three glucose levels were chosen to reflect the clinical interpretation of the relative severity of hyperglycaemia in diabetic patients (Fig 4-1).

![Figure 4-1](image)

**Figure 4-1** Experimental design for *in vitro* cardiomyocyte culture experiments to examine the effects of prolonged exposure to elevated glucose.

It is noted at the outset that in these studies, four-days’ culture with elevated copper and/or glucose concentrations has been interpreted to model ‘chronic’ exposure, although it is understood that some scientists may consider that longer exposures may be required to merit the description of chronic. Consequently, individual experiments probing effects of ‘chronic exposure’ have been labelled clearly to indicate that culture in each case was for 4-day periods.
4.2.1.1 Physiological effects of prolonged elevations in medium glucose on cultured P19CL6 cardiomyocytes

Values of the cell index for each experimental group were continuously recorded using the xCELLigence system at six-hour intervals for four days, as described in chapter two (Fig 4-2a). To recapitulate, the cell index value is considered to comprise an integrated signal reflecting cell number, viability and morphology.

![Image](image-url)

**Figure 4-2** Effects of chronic elevations in medium glucose on the mean cell index values of cultured P19CL6 cardiomyocytes. (a) Cell index was recorded over the shown intervals by using the xCELLigence system following the addition of glucose to the culture medium to the final concentrations shown. (b) Average normalised cell index value during different days after initiation of culture in the indicated concentrations of glucose. Data are mean ± SEM, n = 8/group

The results (Fig 4-2a) showed that the normalised cell index for P19CL6 cardiomyocytes cultured in physiological glucose medium essentially remained constant over the four-day period. However, a marked increase ($P = 0.0498$) in normalised cell index values occurred in both high-glucose groups compared with control over the four-day period. There was a three-
fold increase in cell index values in each high glucose group compared to control after four day’s culture (Fig 4-2b). These results indicate that prolonged hyperglycaemia had marked effects in P19CL6 cardiomyocytes on those aspects of cellular physiology measured by the cell index value.

Further experiments were designed to examine the effects of chronic glucose elevation in the culture medium on the contractile function of P19CL6 cardiomyocytes. The contraction rates of randomly-selected foci (5 per well) were recorded at different time points using a microscope with temperature control (Fig 4-3).

**Figure 4-3** Effect of prolonged hyperglycaemia on foci contraction rate. (a) Observation data on foci contraction rate following the addition of glucose in the culture medium. Data are mean ± SEM, n = 31 per group in quintuplicate. (b-c) Percentage changed in contraction rate after (b) 2 days and (c) 4 days cultured with addition glucose in the medium. Data are medium with interquartile range.
Figure 4-4 Effects of four-days’ culture at stated glucose concentrations on the longevity of contracting foci in cultured P19CL6 cardiomyocytes. (a) Percentage of foci which remained contracting at indicated days. (b) Percentage of foci which remain contracting after 4 day’s culture with addition of glucose in the culture medium. Data are mean ± SEM, n = 31. **P < 0.001 control vs 33.3 mM glucose (Chi-square test).

Results from this study showed that the contraction rate of foci increased moderately over the 4-day period in cells cultured at 5.5 and 16.7 mM glucose. Although there was a decline in the contraction rate between days 2 and 4 in cells cultured at 33.3 mM glucose, this difference was not significant (P = 0.73) between the control and high-glucose groups (Fig 4-3c). Besides the effect on contraction rate, ~15% of foci had completely ceased contracting after 4 days’ culture with 33.3 mM glucose (Fig 4-4a). Results from Chi square test have shown that there was a significant difference (P < 0.001) in the number of contracting foci between the control and high-glucose (33.3 mM) groups at day 4 (Fig 4-4b). Taken together, these results indicate that 4-day’s culture at markedly-elevated extracellular glucose can affect the properties of contraction in cultured P19CL6 cardiomyocytes, affecting more prominently the numbers of contracting foci than the rate of contraction.
The next study was designed to determine whether chronic extracellular glucose elevation could induce apoptosis in P19CL6 cardiomyocytes. Cells were cultured in medium for four days at physiological (5.5 mM) or elevated (16.7 mM and 33.3 mM) glucose concentrations. At the end of the 4-day period, cells were lysed (using lysis buffer) and nucleosome quantity measured by using a cell-death detection assay (ELISA<sup>plus</sup>) to determine the intensity of apoptosis in each sample (Fig 4-5).

Results from this study showed that the relative nucleosomes values in the high-glucose group were lower than in the normal-glucose group. Glucose levels caused a significant difference in relative nucleosome values between study groups ($P = 0.023$, one-way ANOVA). These results suggest that four days’ culture in the high glucose medium did not induce apoptosis in P19CL6 cardiomyocytes.

**Figure 4-5** Apoptosis in P19CL6 cardiomyocytes cultured with different glucose concentration. Data are median with interquartile range, n=6 per group. *$P < 0.05$* control vs 16.7 mM glucose (Dunnett's Multiple Comparison Test)

Besides the effects on cell contraction and apoptosis, it was also noted that there was an increase in cell number in both the high-glucose groups. The apparent sizes of the contracting foci were also observed by inspection to be reduced after prolonged culture with 33.3 mM glucose.

In summary, these results provide evidence that chronic elevations in extracellular glucose affected the characteristics of cultured P19CL6 cardiomyocytes, including modification of aspects of their contractile behaviour.
4.2.1.2 Effects of prolonged elevation of extracellular glucose levels on copper levels in cultured P19CL6 cardiomyocytes

Based on our group’s findings in the hearts of animals with 12 to 16 weeks’ diabetes, it was hypothesised that culturing with elevated glucose might lower copper uptake and therefore cell copper levels in cultured P19CL6 cells. In order to examine the chronic effects of elevated glucose on cell copper levels, P19CL6 cardiomyocytes were cultured with different glucose concentrations for four days, and samples were then analysed by GF-AAS to determine cellular copper concentrations (Fig 4-6).

Fig 4-6 Effects of glucose elevation on cellular copper concentration. Shown are the effects of stated glucose concentrations on copper levels in cultured P19CL6 cardiomyocytes, where copper values were determined by GF-AAS. (a) Relative intracellular copper concentrations after four days culturing. (b) Ratios of cell copper levels in cells cultured in 16.7 and 33.3 mM glucose for two hours or four days normalised to control values (corresponding cultures in 5.5 mM glucose). Data from two different studies have been expressed relative to control values and combined. Data are mean ± SEM, n = 3/group.

Results from GF-AAS demonstrated that elevated glucose concentration in culture medium may slightly interfere with copper uptake in P19CL6 cardiomyocytes (Fig 4-6a). However, the differences in cellular copper concentrations between normal and high-glucose groups under the conditions employed were found to be non-significant using one-way ANOVA (P = 0.41). In addition, comparison between results from acute (2-hour) and chronic (4-day) studies (Fig 4-6b) further indicated that prolonged glucose elevations did not modify copper levels in P19CL6 cardiomyocytes.
4.2.1.3 Effects of prolonged elevated glucose on gene expression in cardiomyocytes

Real-time qPCR (RT-qPCR) was used to measure the effects of four days’ culture at elevated glucose on mRNA levels of genes involved in intracellular copper-regulatory pathways in cultured P19CL6 cardiomyocytes. For each target gene, mRNA levels were measured in each individual RNA sample prepared from P19CL6 cardiomyocytes cultured with different glucose concentrations for four days. Results are shown in the following tables according to the allocated functions of each in the intracellular copper pathways (Table 4-1a-e).

<table>
<thead>
<tr>
<th>Genes involved in cellular copper uptake</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctr1</td>
<td></td>
<td>1.09 ± 0.06</td>
<td>1.58 ± 0.27</td>
<td>1.35 ± 0.17</td>
</tr>
<tr>
<td>Ctr2</td>
<td></td>
<td>1.01 ± 0.02</td>
<td>0.95 ± 0.24</td>
<td>0.89 ± 0.28</td>
</tr>
<tr>
<td>Dmt1</td>
<td></td>
<td>1.02 ± 0.05</td>
<td>1.17 ± 0.12</td>
<td>1.15 ± 0.11</td>
</tr>
</tbody>
</table>

Table 4-1a Prolonged effect of elevated glucose on the mRNA levels of genes involved in cellular copper uptake. Data have been normalised to control values (5.5 mM glucose) and presented as relative mRNA levels. Data are mean ± SEM, n = 8/group in duplicate.

Figure 4-7 Prolonged effect of glucose on the mRNA expression of gene involved copper uptake. The mRNA levels corresponding to Ctr1, a membrane copper transporter, in P19CL6 cardiomyocytes after 4 days’ culture at indicated glucose concentrations. Data are individual values expressed as mean ± 95% CI and have been presented relative to corresponding values in the 5.5 mM control, n = 8/group.
Genes involved in delivery of copper to components of the secretory pathway

<table>
<thead>
<tr>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atox1</td>
<td>1.02 ± 0.03</td>
<td>0.85 ± 0.15</td>
<td>0.76 ± 0.13</td>
</tr>
<tr>
<td>Atp7a</td>
<td>1.01 ± 0.06</td>
<td>0.75 ± 0.11</td>
<td>0.74 ± 0.13</td>
</tr>
<tr>
<td>Atp7b</td>
<td>1.06 ± 0.08</td>
<td>0.57 ± 0.17</td>
<td>0.59 ± 0.07</td>
</tr>
<tr>
<td>Cp</td>
<td>1.00 ± 0.04</td>
<td>0.48 ± 0.06</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>Murr1</td>
<td>1.00 ± 0.01</td>
<td>0.77 ± 0.06</td>
<td>0.71 ± 0.09</td>
</tr>
</tbody>
</table>

Table 4-1b Prolonged effect of glucose on the mRNA expression of gene involved in secretory pathway. Data have been normalised to corresponding control values (5.5 mM glucose) and are presented as relative mRNA levels. Data are mean ± SEM, n = 8/group. Results that are significantly different from controls are shown in red and corresponding P-values are as shown in Fig 4-8.

Figure 4-8 Prolonged effect of glucose on the mRNA expression of gene involved in secretory pathways. The mRNA levels corresponding to (a) Atp7a, (b) Atp7b, (c) Cp and (d) Murr1 in P19CL6 cardiomyocytes after 4 days’ culture at stated glucose concentrations. Results are individual values with mean ± 95% CI and have been presented relative to control values (5.5 mM), n = 8/group. *, P<0.05 and **, P<0.01 vs control
Table 4-1c Prolonged effect of glucose on the mRNA expression of genes involved in cytosolic copper control. Data are normalised to corresponding control values (5.5 mM glucose) and have been presented as relative mRNA levels. Data are mean ± SEM, n = 8/group.

<table>
<thead>
<tr>
<th>Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ccs</em></td>
<td>1.03 ± 0.03</td>
<td>0.96 ± 0.20</td>
<td>1.07 ± 0.20</td>
<td></td>
</tr>
<tr>
<td><em>Sod1</em></td>
<td>0.98 ± 0.05</td>
<td>1.04 ± 0.11</td>
<td>0.89 ± 0.14</td>
<td></td>
</tr>
</tbody>
</table>

Table 4-1d Prolonged effect of glucose on the mRNA expression of genes involved in mitochondria pathway. Data have been normalised to control values (5.5 mM glucose) and presented as relative mRNA levels, mean ± SEM, n = 8. Results significantly different from corresponding control values are shown in red and corresponding P-values are as stated in Fig 4-9.

<table>
<thead>
<tr>
<th>Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway</th>
<th>[Glucose] mM</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cox11</em></td>
<td>1.00 ± 0.02</td>
<td>0.71 ± 0.07</td>
<td>0.62 ± 0.05</td>
<td></td>
</tr>
<tr>
<td><em>Cox17</em></td>
<td>1.10 ± 0.06</td>
<td>0.89 ± 0.10</td>
<td>0.77 ± 0.09</td>
<td></td>
</tr>
<tr>
<td><em>Sco1</em></td>
<td>0.99 ± 0.02</td>
<td>0.99 ± 0.12</td>
<td>1.21 ± 0.26</td>
<td></td>
</tr>
<tr>
<td><em>Sco2</em></td>
<td>1.03 ± 0.03</td>
<td>1.01 ± 0.15</td>
<td>1.30 ± 0.22</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4-9 Prolonged effect of glucose on the mRNA expression of gene involved in mitochondria pathway. The mRNA levels corresponding to (a) *Cox11* and (b) *Cox17* in P19CL6 cardiomyocytes after 4 days’ culture at stated glucose concentrations. Results are individual values with mean ± 95% CI and have been presented relative to control (5.5 mM glucose), n = 8/group. *, P<0.05 and **, P<0.01 vs control
<table>
<thead>
<tr>
<th>Glucose [mM]</th>
<th>5.5</th>
<th>16.7</th>
<th>33.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt1</td>
<td>1.05 ± 0.07</td>
<td>0.56 ± 0.16</td>
<td>0.44 ± 0.13</td>
</tr>
<tr>
<td>Mt2</td>
<td>1.17 ± 0.10</td>
<td>0.75 ± 0.26</td>
<td>0.58 ± 0.26</td>
</tr>
<tr>
<td>Mt3</td>
<td>0.98 ± 0.05</td>
<td>2.31 ± 0.63</td>
<td>2.62 ± 0.82</td>
</tr>
</tbody>
</table>

**Table 4-1e** Prolonged effect of glucose on the mRNA expression in metallothionein gene family. Data normalised to corresponding control values (5.5 mM glucose) are presented as relative mRNA levels, mean ± SEM, n = 8/group. Results with significant differences are shown in red and P-values are as stated in Fig 4-10.

**Figure 4-10** Prolonged effect of glucose on the mRNA expression in metallothionein gene family. The mRNA levels corresponding to three metallothionein genes, (a) *Mt1*, (b) *Mt2*, and (c) *Mt3* in P19CL6 cardiomyocytes after 4 days’ culture at stated glucose concentrations. Results are individual values with means ± 95% CI presented relative to control (5.5 mM glucose), n = 8/group. *P*<0.05 vs control.
Four days’ culture at elevated glucose concentrations did not affect the mRNA levels in P19CL6 cardiomyocytes of genes involved in cell-membrane copper uptake or the transport of copper to SOD1. Although the mRNA levels of copper transporter 1 (Cttr1) trended towards increase in both high-glucose groups (Fig 4-7), the prolonged effect of glucose was not significant ($P=0.22$) compared to control values using one-way AVONA.

By contrast, the mRNA levels of genes involved in the transport of copper via the intracellular secretory pathways and the mitochondrial pathway were affected by prolonged culture at elevated glucose concentration. In the secretory pathway, it was found that prolonged exposure to elevated glucose can significantly reduce the mRNA levels of Atp7b ($P=0.013$), Cp ($P<0.0001$) and Murr1 ($P=0.0088$) in P19CL6 cardiomyocytes (Fig 4-8). A trend towards decrease in the mRNA levels of Atp7a and Atox1 were also noted, although the differences were not significant ($P=0.11$ and $P=0.16$, respectively). In the mitochondrial pathway, similar effects of elevated glucose were observed for the mRNA levels of Cox11 and Cox17 (Fig 4-9). Elevated glucose also decreased the mRNA levels of Cox11 ($P=0.0036$) and Cox17 ($P=0.039$) in P19CL6 cardiomyocytes whereas by contrast, mRNA levels of Sco1 and Sco2 were not affected.

Furthermore, it was found that elevated glucose also modified mRNA levels of metallothionein genes. The mRNA levels of Mt3 were significantly increased ($P=0.049$) in P19CL6 cardiomyocytes cultured with high glucose. There was a statistically-significant two-fold increase in Mt3 expression in the high glucose group compared to the control. On the other hand, the mRNA levels of both Mt1 and Mt2 trended toward decrease, differences compared to control were not statistically significant (Fig 4-10).

In summary, the data obtained from these RT-qPCR experiments have demonstrated that 4-day’s culture in high glucose can affect the mRNA levels of genes implicated in some but not others of the intracellular copper-regulatory pathways. Although there was no significant differences found for genes involved in transmembrane copper uptake and copper transport to Sod1, significant differences in the mRNA levels between normal and high glucose groups were found in genes involved in the transport of copper in some of the intracellular secretory pathways and the mitochondrial pathway. In addition, the mRNA levels corresponding to Mt3 were also altered by culture at elevated glucose concentrations.
4.2.2 Chronic copper elevation

4.2.2.1 Effects on physiological variables of prolonged culture at elevated copper concentrations of P19CL6 cardiomyocytes

To examine the effects of prolonged culture at elevated copper concentrations on cardiomyocyte activity, P19CL6 cardiomyocytes were cultured with different concentrations of copper as described in chapter two. In this study, P19CL6 cardiomyocytes were cultured either in control medium (no added copper) or medium with additional copper at 20 µM, 40 µM or 120 µM (final concentrations). Cell indexes in each group were continuously recorded using the xCELLigence system at 6-hour intervals for 4 days (Fig 4-11a).

![Figure 4-11](image)

Figure 4-11 Prolonged effect of elevated copper concentration on cell index. (a) Cell index recorded using the XCELLigence system initiated after the addition of copper to the culture medium. (b) Normalised cell index after 4 days’ culture at indicated copper concentrations. (c) Percentage change in cell index between control and 120 µM-copper group at day 4. Data in (a) and (b) are mean ± SEM, n=8/group. Data in (c) are median with interquartile range, n=8/group. **P<0.01 vs 0 µM control
Results from these experiments indicate that four days’ culture at elevated copper concentrations could affect the normalised cell index of P19CL6 cardiomyocytes in a dose-dependent manner. It was found that the cell index of P19CL6 cardiomyocytes cultured with additional copper in the medium fell during the four-day period (Fig 4-11b). Instead of the 20% increase in cell index observed in control group at day 4, there was a 30% decrease in the cell index of cells cultured at 120 µM copper. By using paired t-test for the pre-planned pairwise comparisons, it was found that the difference in percentage change in cell index after four days between the control and 120 µM Cu group was significant ($P=0.0099$) (Fig 4-11c). These results indicate that prolonged culture at high copper concentrations can affect aspects of the physiological structure and function of P19CL6 cardiomyocytes.

Further *in vitro* experiments using P19CL6 cardiomyocytes were designed to examine the effects of culture at elevated copper concentrations on myocyte contractility. To achieve this objective, the contraction rates of randomly-selected foci were analysed at different time points, as described in chapter two, and results were as shown in Fig 4-12.

It has thus been shown that culture at elevated copper concentrations altered the focal contraction rate in a dose-dependent manner over the four-day period (Fig 4-12a). Contraction rates of selected foci were decreased by between 20% and 100% in the high-copper concentration groups (Fig 4-12b). Besides the contraction rate, it was found that foci cultured with additional copper lost their contraction ability (Fig 4-12c). These results indicated that 30% of foci lost their contraction ability in culture medium with 40 µM copper, and none of the foci cultured with additional 120 µM were able to contract at the end of day 4 (Fig 4-12d). However, foci cultured with control and 20-µM copper retained their contractile ability throughout the four-day period. Analysis using chi-square test indicates that prolonged exposure to excessive copper had significant ($P<0.001$) effects on the number of contracting foci. Taken together, the results from this study suggest that prolonged exposure to elevated copper can impair the contractile function of cultured cardiomyocytes.
Figure 4-12 Effects of culturing at elevated copper concentrations on contraction rates of foci in cultured PC19CL6 cardiomyocytes. (a) Focal contraction rates were measured following the addition of CuCl$_2$ to the culture medium to stated final concentrations. (b) Percentage change in contraction rates after 4 days’ culture in medium with additional CuCl$_2$. (c) Percentage of foci which remained contracting following the addition of CuCl$_2$ to the medium. (d) Percentage of foci which remained contracting after 4-days’ culture at elevated copper concentrations. Data in (a), (c) and (d) are mean ± SEM, n = 16. Data in (b) are mean ± 95% CI, n = 16. ***, P<0.001; **, P<0.01; and *, P<0.05 vs 0 µM control.
The final part of this study was to determine whether four days’ culture at elevated copper concentrations could induce apoptosis in P19CL6 cardiomyocytes. P19CL6 cardiomyocytes were cultured with or without addition of copper (0, 20, 40 or 120 µM CuCl₂). At the end of the four-day period, cells were lysed and relative nucleosome levels measured (cell death-detection ELISA plus) to determine the amount of apoptotic cells in each sample (Fig 4-13).

Results from these experiments showed that the relative nucleosome values in P19CL6 cardiomyocytes cultured with additional copper were significantly higher than control values. ($P=0.0077$ by one-way ANOVA). This finding indicates that prolonged cultured with elevated copper can induce apoptosis in P19CL6 cardiomyocytes.

![Figure 4-13](image)

**Figure 4-13** Relative nucleosome values, a measure of apoptosis, in P19CL6 cardiomyocytes cultured at different copper concentrations. Data are median with interquartile range presented relative to values at 0 µM (control), n=6 per group. **$P<0.01$, *$P<0.05$ vs control.**

In summary, these results provide evidence that four days’ culture at elevated copper concentrations can modify certain physiological characteristics of cardiomyocytes, associated with lowering of the numbers and contraction rates of contractile foci, and elevated apoptosis.
4.2.2.2 Effect of elevated extracellular copper concentrations on copper levels in cultured cardiomyocytes

In order to examine the chronic effects of elevated copper on cell copper levels, P19CL6 cardiomyocytes were cultured for 4 days at different concentrations of added copper, and concentrations then determined by GF-AAS.

Increased copper in the medium was found to increase cell copper concentrations in P19CL6 cardiomyocytes (Fig 4-14a). By using one-way ANOVA, this effect of extracellular copper was found significant ($P=0.0091$). In addition, by comparing the results between acute (two-hour) and chronic (four-day) studies (Fig 4-14b), it was found that prolonged culture with control medium only (no added copper) did not change the cell copper concentration in P19CL6 cardiomyocytes. However, a significant difference ($P<0.0001$) in intracellular copper concentrations in P19CL6 cardiomyocytes was found between short- and long-term culture with elevated copper. There was a more than six-fold increase in cells cultured for 4 days. These results suggest that exposure to elevated copper for 4 days can alter cellular copper balance or uptake in P19CL6 cardiomyocytes.

**Figure 4-14** Effects of elevated extracellular copper concentrations on the cellular copper levels of P19CL6 cardiomyocytes. (a) Relative intracellular copper concentrations in myocytes cultured at different extracellular copper concentrations were measured by GF-AAS. (b) Comparison between the effects of two hours’ and four days’ culture at elevated copper concentrations on cardiomyocyte copper levels. Data from two independent studies have been expressed relative to the corresponding control to minimize variability and aggregated values are presented. Data are mean ± SEM, n = 3/group: **, $P<0.01$; *, $P<0.05$ vs 0-µM control; ***, $P<0.001$ four days’ vs two hours’ exposure.
4.2.2.3 Effects in cultured cardiomyocytes of elevated extracellular copper concentrations on the expression of intracellular copper-regulatory genes

RT-qPCR was used to measure the effects of four days’ culture at elevated copper concentrations on mRNA levels of target genes involved in pathways of intracellular copper regulation in P19CL6 cardiomyocytes. For each gene, mRNA levels were measured in each individual RNA sample prepared from P19CL6 cells that had previously been cultured for four days at indicated copper concentrations. Results are shown in the following tables with genes grouped according to their assigned function in the intracellular copper-regulatory pathways (Table 4-2 a-e).

<table>
<thead>
<tr>
<th>Gene involved in cellular copper uptake</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl</td>
<td></td>
<td>1.13±0.09</td>
<td>1.12±0.14</td>
<td>1.18±0.10</td>
<td>1.31±0.29</td>
<td>1.23±0.37</td>
<td>0.93±0.07</td>
</tr>
<tr>
<td>Ctrl2</td>
<td></td>
<td>1.01±0.04</td>
<td>0.90±0.10</td>
<td>0.95±0.08</td>
<td>1.11±0.07</td>
<td>1.20±0.12</td>
<td>1.06±0.16</td>
</tr>
<tr>
<td>Dmt1</td>
<td></td>
<td>1.04±0.09</td>
<td>1.22±0.14</td>
<td>1.05±0.05</td>
<td>1.10±0.09</td>
<td>1.07±0.09</td>
<td>1.07±0.09</td>
</tr>
</tbody>
</table>

Table 4-2a Effect of prolonged exposure to elevated copper on the mRNA expression of gene involved in copper uptake. Data are normalised to the control (0µM copper) and presented here as relative mRNA level. Data are mean ± SEM, n = 5 per group

<table>
<thead>
<tr>
<th>Genes involved in delivery of copper to components of the secretory pathway</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atox1</td>
<td></td>
<td>1.03±0.04</td>
<td>1.25±0.17</td>
<td>1.16±0.13</td>
<td>1.04±0.23</td>
<td>0.96±0.14</td>
<td>1.21±0.21</td>
</tr>
<tr>
<td>Atp7a</td>
<td></td>
<td>0.95±0.05</td>
<td>0.92±0.06</td>
<td>0.90±0.04</td>
<td>0.85±0.10</td>
<td>0.85±0.05</td>
<td>0.79±0.04</td>
</tr>
<tr>
<td>Atp7b</td>
<td></td>
<td>1.07±0.13</td>
<td>1.32±0.23</td>
<td>1.12±0.21</td>
<td>1.02±0.18</td>
<td>0.89±0.28</td>
<td>0.66±0.15</td>
</tr>
<tr>
<td>Cp</td>
<td></td>
<td>1.02±0.06</td>
<td>0.75±0.11</td>
<td>1.51±0.36</td>
<td>1.62±0.56</td>
<td>1.40±0.43</td>
<td>0.66±0.14</td>
</tr>
<tr>
<td>Murr1</td>
<td></td>
<td>1.00±0.02</td>
<td>1.17±0.11</td>
<td>1.14±0.09</td>
<td>1.17±0.07</td>
<td>0.99±0.04</td>
<td>1.18±0.11</td>
</tr>
</tbody>
</table>

Table 4-2b Effect of prolonged exposure to elevated copper on the mRNA expression of gene involved in secretory pathway. Data are normalised to the control (0µM copper) and presented here as relative mRNA level. Data are mean ± SEM, n = 5 per group with duplicate.
### Table 4-2c

Effect of prolonged exposure to elevated copper on the mRNA expression of gene involved in cytosolic copper control system. Data are normalised to the control (0µM copper) and presented here as relative mRNA level. Data are mean ± SEM, n = 5 per group with duplicate.

<table>
<thead>
<tr>
<th>Gene</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ccs</td>
<td>1.05±0.04</td>
<td>0.99±0.11</td>
<td>1.06±0.09</td>
<td>0.97±0.14</td>
<td>1.05±0.10</td>
<td>0.98±0.08</td>
<td></td>
</tr>
<tr>
<td>Sod1</td>
<td>0.94±0.07</td>
<td>1.09±0.17</td>
<td>1.12±0.21</td>
<td>1.19±0.17</td>
<td>1.23±0.17</td>
<td>1.19±0.17</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4-2d

Effect of prolonged exposure to elevated copper on the mRNA expression of gene involved in mitochondria pathway. Data are normalised to the control (0µM copper) and presented here as relative mRNA level. Data are mean ± SEM, n = 5 per group with duplicate.

<table>
<thead>
<tr>
<th>Gene</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cox11</td>
<td>1.00±0.04</td>
<td>0.93±0.07</td>
<td>0.99±0.05</td>
<td>0.83±0.14</td>
<td>0.85±0.20</td>
<td>0.73±0.05</td>
<td></td>
</tr>
<tr>
<td>Cox17</td>
<td>1.20±0.09</td>
<td>1.23±0.13</td>
<td>1.45±0.19</td>
<td>1.43±0.16</td>
<td>1.33±0.10</td>
<td>1.16±0.06</td>
<td></td>
</tr>
<tr>
<td>Sco1</td>
<td>0.99±0.04</td>
<td>1.26±0.14</td>
<td>0.97±0.14</td>
<td>1.09±0.19</td>
<td>1.01±0.09</td>
<td>0.99±0.16</td>
<td></td>
</tr>
<tr>
<td>Sco2</td>
<td>1.04±0.04</td>
<td>1.30±0.20</td>
<td>1.37±0.18</td>
<td>1.48±0.32</td>
<td>1.09±0.07</td>
<td>1.23±0.16</td>
<td></td>
</tr>
</tbody>
</table>

### Table 4-2e

Effect of prolonged exposure to elevated copper on the mRNA expression of metallothionein gene family. Data are normalised to the control (0µM copper) and presented here as relative mRNA level. Data are mean ± SEM, n = 5 per group with duplicate. Results with significant differences from control are shown in red and P-values are as shown in Fig 4-14.

<table>
<thead>
<tr>
<th>Gene</th>
<th>[Copper] µM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt1</td>
<td>1.07±0.11</td>
<td>2.06±0.58</td>
<td>2.20±0.43</td>
<td>2.62±0.57</td>
<td>4.69±0.28</td>
<td>5.58±0.62</td>
<td></td>
</tr>
<tr>
<td>Mt2</td>
<td>1.24±0.15</td>
<td>2.18±0.14</td>
<td>3.52±0.82</td>
<td>4.22±0.74</td>
<td>8.94±1.21</td>
<td>13.7±2.36</td>
<td></td>
</tr>
<tr>
<td>Mt3</td>
<td>0.93±0.06</td>
<td>1.55±0.37</td>
<td>1.92±0.69</td>
<td>6.25±3.91</td>
<td>9.36±3.45</td>
<td>11.7±4.47</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-15 Effects of prolonged exposure to elevated copper on the mRNA levels of members of the metallothionein gene family. The mRNA levels of (a) Mt1, (b) Mt2, and (c) Mt3 in P19CL6 cardiomyocytes after 4 days’ culture at different copper concentrations. Results are individual values with means ± 95% CI and are presented here relative to control (0-µM group), n = 5/group: **, P<0.01, *, P<0.05 vs 0-µM control values.

The data obtained from RT-qPCR have shown that there were no significant differences in mRNA levels between control and copper-treated groups in the majority of the target genes examined (Table 4-2 a-e). However, additional copper evoked the gene expression of the metallothionein gene-family members in a dose-dependent manner. The mRNA levels of Mt1, Mt2 and Mt3 were all significantly increased following addition of CuCl₂ to the culture medium. Compared to the control group, there was a more than 10-fold increase in the mRNA levels of Mt1 (P<0.0001) and Mt2 (P<0.0001), and a five-fold increase in the mRNA levels of Mt3 (P<0.0001) in cardiomyocytes cultured with 120 µM of added copper (Fig 4-15). These results provide evidence that prolonged exposure to excessive copper can significantly elevate the mRNA levels corresponding to genes for metallothionein protein family members.
4.2.2.4 Effect of prolonged copper infusion on cardiac function of isolated-perfused hearts from normal rats

Male Wistar rats, with body-weights of between 250 to 300g, were randomly assigned to control and copper-treated groups. Following their selection, rats were kept in the animal unit for another 8 weeks. Animals hearts were then isolated for working-model perfusion. In order to examine the effects of toxicity caused by elevated copper on cardiac function, different concentrations of cupric chloride (CuCl₂) solution were infused into isolated rat hearts via the main coronary arteries for 10 minutes and changes in cardiac function were measured at one-minute intervals for a further 25 minutes (Fig 4-16).

The results showed that prolonged saline infusion had no effect on cardiac function. Measurement of heart rate, cardiac output, aortic flow, systolic pressure, left-ventricular pressure development and relaxation of the perfused heart remained constant after saline infusion. By contrast, for the copper-treated group, the results indicated that 10-min infusion of even low dosages of CuCl₂ to the ex vivo isolated heart could immediately impair cardiac function in a dose-dependent manner (Fig 4-16). At very low dosages of copper (1-1.5 μM copper), heart rates were affected by the prolonged infusion but most indexes of cardiac function were unaltered.

However, irreversible loss of cardiac function ensued as the concentration of infused copper increased. As shown in figure 4-16c, although there was a trend to recovery in cardiac function after copper infusion, isolated perfused hearts were unable to fully recover their function. The loss in cardiac output was greater than 15% in the isolated hearts perfused with 1.5-2 μM copper, and 25% with 2-2.5 μM Cu after 10 minutes’ infusion (Fig 4-17a). In isolated perfused heart infused with 2.5-3 μM Cu, more than 50% of the cardiac function was lost.

In comparing these results to those with the acute copper infusion, it was observed that prolonged infusion with a much lower copper dosage could induce relatively greater impairment in the isolated perfused heart (Fig 4-17b). All other measurements except systolic pressure were shown to decline following the copper infusion. Furthermore, stone heart or ischaemic myocardial contracture was also found in isolated hearts with prolonged copper infusion: more than 80% of isolated hearts infused with copper concentrations higher than 2.5 μM underwent functional failure during the time-period studied.
Figure 4-16 Effect of 10-min copper infusion on cardiac function of ex-vivo-perfused hearts isolated from normal rats. Effects of different copper concentrations on stroke volume (a), heart rate (b), cardiac output (c), aortic flow (d), ventricular pressure development (e), relaxation (f) and systolic pressure (g) were recorded each minute for 25 minutes. Data are mean ± SEM, n = 5/group.
Figure 4-17 Ten minutes’ infusion of elevated copper impaired cardiac function in isolated-perfused working rat hearts. (a) The correlation between the concentration of copper infused and cardiac output measured at the 10-minute time-point after initiation of the copper infusion. (b) Comparison of the percentage of cardiac output remaining at the 10-minute time-point after initiation of the copper infusion between acute and prolonged copper infusion.

Results from two-way ANOVA provide further evidence concerning the effects of 10-minutes’ copper infusion on cardiac function of isolated-perfused working rat hearts. It was found that the cardiac function in the copper-treated groups was significantly different from that of the control group (Table 4-3). Cardiac output ($P=0.0002$) and aortic flow ($P=0.0009$) decreased significantly after 10-minutes’ copper infusion. These results suggest that prolonged infusion of copper to isolated perfused hearts can cause acute copper toxicity, which causes substantive impairment of cardiac function.

<table>
<thead>
<tr>
<th>Two-way ANOVA P-values (effects of 10-min copper infusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke volume</td>
</tr>
<tr>
<td>Heart rate</td>
</tr>
<tr>
<td>Cardiac output</td>
</tr>
<tr>
<td>Aortic flow</td>
</tr>
<tr>
<td>Max +dP/dt</td>
</tr>
<tr>
<td>Min -dP/dt</td>
</tr>
<tr>
<td>Systolic pressure</td>
</tr>
</tbody>
</table>

Table 4-3 Shown are the results of two-way ANOVA for the effects of 10-minutes’ copper infusion on indexes of cardiac function. Results for hearts infused with >2.5-µM copper have been excluded from this calculation due to the large number that failed in this group. Data with significant differences to the control group are shown in red. * P-values were determined by comparison with the saline-infused group.
4.2.3 Interaction between chronic elevated glucose and copper

4.2.3.1 Physiological effect of chronic elevated glucose and copper interplay on P19CL6 cardiomyocytes

To examine the interplay between the effects of elevations in glucose and copper on the activity of cardiomyocytes, P19CL6 cardiomyocytes were cultured as described in chapter two with different concentrations of glucose and copper added to the medium. Here, P19CL6 cardiomyocytes were cultured under three different concentrations of glucose (5.5 mM, 16.7 mM and 33.3 mM) with or without added copper (20 µM, 40 µM and 120 µM) in the medium. The cell index in each group was continuously recorded by using the xCELLigence system at six-hour intervals for four days (Fig 4-18).

Similar to the results obtained in the previous studies, it was found that both four days’ elevated glucose and prolonged exposure to elevated copper can alter the normalised cell index for P19CL6 cardiomyocytes (Fig 4-18). P19CL6 cardiomyocytes cultured with high glucose had increased cell index values compared to those cultured with normal glucose (Fig 4-18a, c, e). As well, the effects of four days’ elevated glucose were not affected by the addition of copper to the medium. On the other hand, the effects of four days’ elevated copper could also be seen in each of the glucose-treatment groups. It was found that elevated copper could affect the normalised cell index of P19CL6 cardiomyocytes in a dose-dependent manner. The cell index of P19CL6 cardiomyocytes cultured with additional copper in the medium decreased over the four-day period. However, the term for the interaction between elevated glucose and elevated copper was not statistically significant in this study. Analysis by two-way ANOVA further supports the effects of chronic hyperglycaemia and excessive copper on the cell index of P19CL6 cardiomyocytes. These results show that four-days’ elevated glucose ($P=0.0025$) can lead to a significant increase in cell index, whereas prolonged copper elevation can significantly decrease ($P=0.029$) the cell index in P19CL6 cardiomyocytes. However, there was no interaction ($P=0.75$) between chronic hyperglycaemia and copper toxicity detected in this study.
**Figure 4-18** Interplay between 4-day’s elevated glucose and copper in the culture medium on the cell index of the P19CL6 cardiomyocytes. Cell index values were recorded using the xCELLigence system following the addition of CuCl$_2$ to culture medium with glucose (mM) of (a) 5.5, (c) 16.7 and (e) 33.3. Percentage changes in cell index after 96 hours culture with glucose (mM) of (b) 5.5, (d) 16.7 or (f) 33.3 at indicated copper concentrations. Data in (a), (c) and (e) are mean ± SEM, and those in (b), (d) and (f) are mean ± 95% CI, n = 8/group.
In the second part of this study, the *in vitro* experiments using P19CL6 cardiomyocytes were designed to examine the interactions between chronic elevations of glucose and copper in the medium and their effects on indexes of contractile function in cardiomyocytes. The contraction rate of randomly selected foci were recorded at different time points as described in chapter two (Fig 4-19).

Chronic glucose elevation and excessive Cu were each able to modify the contraction rates and/or number of contracting foci of P19CL6 cardiomyocyte cultures. Elevated glucose lowered the average rate at which foci contracted and reduced the number of contracting foci after prolonged incubation (Fig 4-19 & 4-20). On the other hand, the effects of elevated copper on both the contraction rate and number of foci were much greater. For foci cultured with high copper (120 µM), numbers of foci were significantly lower at day 2 and all had ceased contracting by day 4.

Beside the individual effects of elevations in glucose and copper, results from this study have also demonstrated the interaction effects between chronic glucose elevation and copper toxicity on both contraction rate and numbers of contracting foci. It was found that cardiomyocytes cultured with both elevated glucose and copper together showed a greater decrease in contraction rate. Foci of cells cultured in high glucose were more sensitive to excessive copper. By comparison of foci cultured with additional 20 µM copper, the percentage of contracting foci at day 4 dropped from 100% in medium with 5.5 mM glucose to around 25% in medium with 33.3 mM glucose (Fig 4-20). A similar pattern was also observed for foci cultured with additional 40 µM copper.

Results from two-way ANVOA provided further information concerning the interaction between chronic glucose elevation and excess copper on the contraction rate and numbers of foci in P19CL6 cardiomyocytes (Table 4-4). The interactions between chronic elevations in glucose and copper on the contraction rate (*P*=0.047) and the percentage of contracting foci (*P*=0.043) at day 4 were both significant.
Figure 4-19 Interaction effects of four days' culture with elevated glucose and copper on the contraction rates and numbers contracting of foci in P19CL6 cells. Shown are contraction rates after the addition of CuCl$_2$ to the culture medium with glucose (mM) of (a) 5.5, (c) 16.7 and (e) 33.3. Percentage changes in contraction rate after 4 days' culture with glucose (mM) of (b) 5.5, (d) 16.7 and (f) 33.3 glucose with additional copper as indicated. (g) Summary of percentage changes in contraction rates. Data in (a), (c), (e) and (g) are mean ± SEM. Data in (b), (d) and (f) are mean ± 95% CI, n=16/group. ***, $P<0.001$; **, $P<0.01$; and *, $P<0.05$ vs 0-µM control values.
Figure 4-20 Time-dependent effects of elevations of glucose and copper in the medium on the percentage of contracting foci remaining in P19CL6 cardiomyocytes at the end of (a) 2 and (b) 4 days’ culture.

<table>
<thead>
<tr>
<th></th>
<th>Contraction rate</th>
<th>Numbers contracting</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 2</td>
<td>Day 4</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.017</td>
<td>0.018</td>
</tr>
<tr>
<td>Copper</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Interaction</td>
<td>0.26</td>
<td>0.047</td>
</tr>
</tbody>
</table>

Table 4-4 Two-way ANOVA of data in Fig 4-19 pertaining to the effects of chronic elevations in glucose and copper on properties of contraction foci in P19CL6 cardiomyocytes. P-values for significant effects are shown in red.
The last part of this study was to examine the interaction effects of four days’ elevations in glucose and copper on apoptosis in P19CL6 cardiomyocytes. Cells were cultured in medium for 4 days with different concentrations of glucose and copper. After this, cells were lysed and their relative nucleosome value was measured (cell-death detection by ELISA\textsuperscript{plus}) to determine the proportion of apoptosis in each sample (Fig 4-21).

![Figure 4-21](image)

**Figure 4-21** Apoptosis in P19CL6 cardiomyocytes cultured at indicated glucose and copper concentrations. Data are mean ± SEM, n=6/group.

Results from these experiments have shown that the relative nucleosome values in high-glucose groups were lower than in physiological-glucose group. On the other hand, chronic copper toxicity in can induce apoptosis in P19CL6 cardiomyocytes in a dose-dependent manner. Additionally, the effects of four days elevated copper was not affected by the glucose concentrations. Furthermore, no interaction between elevated glucose and copper values in the culture medium were detected in this study.

Results from two-way ANOVA provide further evidence concerning the effects of four days’ culture with elevated glucose and copper on apoptosis in P19CL6 cardiomyocytes. Elevated glucose significantly suppressed ($P=0.021$) whereas copper induced ($P<0.0001$) apoptosis in these cells. However, there was no interaction ($P=0.38$) between elevated copper and glucose values detected in this study.
4.2.3.2 Interactions between elevations in glucose and copper in the culture medium on copper levels in P19CL6 cardiomyocytes

In order to examine the interaction between elevations in glucose and copper content of the medium on copper uptake in cardiomyocytes, P19CL6 cardiomyocytes were cultured under different concentration of glucose and copper for 4 days, and cellular copper concentration measured by GF-AAS as described in chapter two.

![Graph showing the interaction between glucose and copper](image)

**Figure 4-22** Interplay between concentrations of glucose and copper on cellular copper in cultured P19CL6 cardiomyocytes. Copper concentrations of cells cultured under different treatment conditions for 4 days were analysed by GF-AAS and expressed as relative [Cu] values. Data are mean ± SEM, n = 3/group.

Based on the results from this study, it was determined that increased copper in culture medium increased the cellular copper concentrations in P19CL6 cardiomyocytes (Fig 4-22), consistent with increased cellular copper uptake. In addition, this study showed that elevated glucose can affect the copper levels in these cells. In each copper-treated group, there were clear trends indicating that P19CL6 cardiomyocytes cultured in high glucose have lower levels of cellular copper than those cultured with physiological glucose, consistent with decreased copper uptake. However, the interaction terms in the two-way ANOVA between different concentrations of glucose and copper and uptake were not significant in this study.
Table 4-5 Two-way ANOVA results for effects of four days’ culture with elevated glucose and copper on cardiomyocyte-copper levels in data of Fig 4-21. Significant values are shown in red.

Results from two-way ANOVA showed that both 4-days’ culture in elevated glucose and copper concentrations had significant effects on copper levels in P19CL6 cardiomyocytes (Table 4-5). These data indicate that chronic hyperglycaemia significantly decreased ($P=0.042$) the cellular copper levels whereas elevated copper caused the opposite effect in significantly increasing ($P<0.001$) intracellular copper. However, there was no significant interaction ($P=0.43$) between the effects of glucose and copper in this study.

4.2.3.3 Interactions of chronic elevated glucose and copper on myocardial gene expression

RT-qPCR was used to examine the interplay between elevated glucose and copper in the culture medium on mRNA levels of target genes involved in intracellular copper-regulatory pathways in P19CL6 cardiomyocytes. For each target gene, mRNA levels were measured in each individual RNA sample prepared from cells cultured with different additional glucose and copper concentrations in the medium for 4 days. Results have been shown in the following tables according to the genes’ assigned functions in intracellular copper-regulatory pathways (Table 4-6).
### Genes involved in cellular copper uptake

<table>
<thead>
<tr>
<th>Gene</th>
<th>[Glucose] mM</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ctr1</strong></td>
<td>5.5</td>
<td>1.13±0.09</td>
<td>1.12±0.14</td>
<td>1.18±0.10</td>
<td>1.31±0.29</td>
<td>1.23±0.37</td>
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<tr>
<td></td>
<td>16.7</td>
<td>1.98±0.31</td>
<td>1.78±0.19</td>
<td>1.52±0.04</td>
<td>1.33±0.17</td>
<td>1.11±0.12</td>
<td>1.18±0.12</td>
</tr>
<tr>
<td></td>
<td>33.3</td>
<td>1.64±0.15</td>
<td>1.93±0.26</td>
<td>1.50±0.12</td>
<td>1.18±0.18</td>
<td>1.11±0.16</td>
<td>1.05±0.19</td>
</tr>
<tr>
<td><strong>Ctr2</strong></td>
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<td>1.01±0.04</td>
<td>0.90±0.10</td>
<td>0.95±0.08</td>
<td>1.11±0.07</td>
<td>1.20±0.12</td>
<td>1.06±0.16</td>
</tr>
<tr>
<td></td>
<td>16.7</td>
<td>1.27±0.30</td>
<td>1.11±0.34</td>
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<td>1.24±0.24</td>
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<td>1.36±0.22</td>
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<td></td>
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<tr>
<td><strong>Dmt1</strong></td>
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<tr>
<td></td>
<td>16.7</td>
<td>1.33±0.13</td>
<td>1.06±0.04</td>
<td>1.12±0.13</td>
<td>1.12±0.19</td>
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<td>0.94±0.12</td>
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<td>33.3</td>
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<td>1.09±0.16</td>
<td>1.02±0.07</td>
<td>0.94±0.13</td>
</tr>
</tbody>
</table>

**Table 4-6a** Effects in P19CL6 cardiomyocytes of four-days’ culture with indicated glucose and copper levels on mRNA levels of genes involved in cellular copper uptake. Data are normalised to corresponding control values (5.5 mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

---

### Genes involved in cytosolic copper transport

<table>
<thead>
<tr>
<th>Gene</th>
<th>[Glucose] mM</th>
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<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>120</th>
</tr>
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<tbody>
<tr>
<td><strong>Ccs</strong></td>
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<td>16.7</td>
<td>1.17±0.28</td>
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<td>1.19±0.17</td>
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<td>1.09±0.29</td>
<td>1.20±0.31</td>
<td>1.09±0.28</td>
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**Table 4-6b** Effects in P19CL6 cardiomyocytes of four-days’ culture at indicated concentrations of glucose and copper on the mRNA levels of genes involved in transport of copper across the cell membrane. Data are normalised to control values (5.5-mM glucose without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

---

**Figure 4-23** The effects in P19CL6 cardiomyocytes of 4-day’s culture at different concentrations of glucose and copper, as indicated, on the expression of mRNA levels for genes involved in cell-membrane copper uptake. Relative mRNA levels for (a) *Ctr1* and (b) *Dmt1* in response to different treatment conditions. Results are mean ± SEM, n=5/group.
Gene involved in delivery of copper to components of the secretory pathways

<table>
<thead>
<tr>
<th>Gene</th>
<th>[Glucose] mM</th>
<th>[Copper] µM</th>
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<th>20</th>
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<th>80</th>
<th>120</th>
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<tr>
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</tr>
<tr>
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<tr>
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<tr>
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<td>1.18±0.11</td>
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<tr>
<td></td>
<td>16.7</td>
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</tr>
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</table>

Table 4-6c Effects in P19CL6 cardiomyocytes of four-days’ culture at indicated glucose and copper concentrations on mRNA levels of genes involved in the transport of copper in the intracellular secretory pathways. Data are normalised to corresponding control values (5.5 mM-glucose group without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

Figure 4-24 Effects in P19CL6 cardiomyocytes of four days’ culture at indicated glucose and copper concentrations on mRNA levels of genes involved in the transport of copper in the intracellular secretory pathways. Relative mRNA levels for (a) Atp7b, (b) Cp and (c) Murr1 in response to different treatment conditions. Results are mean ± SEM, n=5/group.
| Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway |
|-----------------------------------------------|---------------------|----------------|-----------------|----------------|----------------|----------------|
| [Glucose] mM | [Copper] µM          |
|               | 0          | 10         | 20         | 40         | 80         | 120          |
| **Cox11**    |            |            |            |            |            |               |
| 5.5          | 1.00±0.04  | 0.93±0.07  | 0.99±0.05  | 0.83±0.14  | 0.85±0.20  | 0.73±0.05    |
| 16.7         | 0.75±0.01  | 0.63±0.02  | 0.62±0.11  | 0.61±0.15  | 0.63±0.14  | 0.66±0.13    |
| 33.3         | 0.66±0.05  | 0.73±0.16  | 0.55±0.10  | 0.64±0.23  | 0.57±0.19  | 0.66±0.23    |
| **Cox17**    |            |            |            |            |            |               |
| 5.5          | 1.20±0.09  | 1.24±0.13  | 1.45±0.19  | 1.43±0.16  | 1.33±0.10  | 1.16±0.06    |
| 16.7         | 1.24±0.09  | 1.14±0.11  | 1.01±0.13  | 1.05±0.10  | 1.06±0.16  | 1.04±0.09    |
| 33.3         | 1.00±0.15  | 1.04±0.11  | 0.98±0.13  | 0.98±0.13  | 1.10±0.13  | 1.05±0.17    |
| **Sco1**     |            |            |            |            |            |               |
| 5.5          | 0.99±0.04  | 1.26±0.14  | 0.97±0.14  | 1.09±0.19  | 1.01±0.09  | 0.99±0.16    |
| 16.7         | 1.15±0.16  | 1.19±0.18  | 1.36±0.36  | 1.13±0.35  | 1.23±0.36  | 1.22±0.36    |
| 33.3         | 1.40±0.39  | 1.83±0.76  | 1.62±0.50  | 1.31±0.42  | 1.29±0.36  | 1.33±0.69    |
| **Sco2**     |            |            |            |            |            |               |
| 5.5          | 1.04±0.04  | 1.30±0.20  | 1.37±0.18  | 1.48±0.32  | 1.09±0.07  | 1.23±0.16    |
| 16.7         | 0.86±0.16  | 1.00±0.20  | 0.82±0.22  | 0.90±0.20  | 0.85±0.20  | 0.85±0.20    |
| 33.3         | 1.22±0.31  | 0.96±0.36  | 0.81±0.23  | 1.03±0.32  | 0.95±0.26  | 0.79±0.29    |

**Table 4-6d** Effects in P19CL6 cardiomyocytes of four days’ culture at indicated glucose and copper concentrations on mRNA levels of genes involved in the pathway of transport of copper to the mitochondrion and its insertion into cytochrome c oxidase. Data are normalised to control values (5.5-mM glucose group without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

**Figure 4-25** Effects in P19CL6 cardiomyocytes of four days’ culture at indicated glucose and copper concentrations on mRNA levels of genes involved in the pathway of transport of copper to the mitochondrion and its insertion into cytochrome c oxidase. Relative mRNA levels for (a) Cox11, (b) Cox17 and (c) Sco2 in response to different treatment conditions. Results are means ± SEM, n=5/group.
<table>
<thead>
<tr>
<th>Metallothionein gene family</th>
<th>[Glucose] mM</th>
<th>[Copper] μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td><em>Mt</em>1</td>
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<td>1.07±0.11</td>
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<tr>
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<td></td>
<td>33.3</td>
<td>0.35±0.17</td>
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<tr>
<td><em>Mt</em>2</td>
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<td></td>
<td>33.3</td>
<td>0.29±0.19</td>
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<tr>
<td><em>Mt</em>3</td>
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<td>0.93±0.06</td>
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<tr>
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<td>33.3</td>
<td>3.25±1.24</td>
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**Table 4-6e** Effects in P19CL6 cardiomyocytes of four days’ culture at indicated glucose and copper concentrations on mRNA levels of members of the metallothionein gene family which guard against elevated intracellular levels of copper and zinc. Data have been normalised to controls (5.5-mM glucose group without additional copper) and presented as relative mRNA levels. Data are mean ± SEM, n = 5/group.

**Figure 4-26** Effects in P19CL6 cardiomyocytes of four days’ culture at indicated glucose and copper concentrations on mRNA levels of members of the metallothionein gene family, which guard against elevated intracellular levels of copper and zinc. Relative mRNA levels for (a) *Mt*1, (b) *Mt*2 and (c) *Mt*3 in response to different treatment conditions. Results are means ± SEM, n=5/group.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Glucose effect</th>
<th>Copper effect</th>
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</tr>
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<tr>
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<tr>
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Table 4-7 Results of two-way ANOVA test for the changes in mRNA levels in P19CL6 cardiomyocytes cultured for four-days at different media concentrations of glucose and copper. Statistically-significant effects are shown in red.

These results have shown, in P19CL6 cardiomyocytes, that mRNAs corresponding to a number of genes which play significant roles in the intracellular copper-regulatory pathways, exhibited significant concentration-dependent responses to changes in medium concentrations of glucose and/or copper after four days’ culture (Table 4-6 a-e).

For example, four days’ culture at elevated glucose concentrations evoked increases in mRNAs corresponding to Ctr1 and Mt3, but suppression of those for Atp7b, Cp, Murr1, Cox11, Cox17 and Sco2. These findings are generally consistent with our previous RT-qPCR study of the effects of chronic glucose elevation on mRNA expression and point to a mechanism by which glucose can modulate the pathways of intracellular copper regulation.

The effects of elevated copper on mRNA levels of Mt1, Mt2 and Mt3 in this study were consistent with those we obtained previously. Thus, the mRNA levels corresponding to each of the three metallothionein genes increased in relation to rising extracellular copper (Fig 4-26).
In addition, four other copper-regulating genes were found to be affected by prolonged exposure to elevated copper which, for example, elicited modest but significant, dose-dependent suppression of Atp7b mRNA after four day’s culture.

Copper and glucose interacted to modify the levels of mRNAs corresponding to both Ctrl and Dmt1. Culture of cardiomyocytes at elevated copper but physiological glucose concentrations did not affect either Ctrl or Dmt1 mRNAs. However, by contrast, both mRNAs became progressively lower as extracellular glucose was increased in the presence of elevated copper concentrations (Fig 4-23). Therefore, glucose and copper interacted significantly to down-regulate these two mRNAs, each of which encodes an important cell-membrane copper transporter. These findings are consistent with the lowering of copper uptake into cardiomyocytes in the presence of combined elevations in extracellular concentrations of copper and glucose, such as have been reported to occur in diabetic patients and rats.

By contrast, the effects of altered copper concentrations on Cp mRNA levels generated an inverse-U-shaped curve (Fig 4-24b). This finding indicates that low levels of additional copper in the medium induced Cp mRNA levels but that as copper concentrations increased still further into the (presumably) toxic range, mRNA levels of Cp became suppressed.

In addition to the independent effects of culture with elevated glucose or copper concentrations, several mRNAs demonstrated significant interaction effects, some of which have already been discussed above. Further examples are provided by the effects of combined elevations in glucose and copper to elicit elevation of the mRNAs corresponding to Mtl1 and Mtl2. Levels of both mRNAs demonstrated increased sensitive to elevated medium-copper concentrations in the presence of elevated glucose and two-way ANOVA confirmed the presence of statistically-significant interaction effects.
4.2.4 TETA as a potential treatment for cells damaged by chronic hyperglycaemia and copper toxicity

4.2.4.1 Potential adverse effects of TETA and its protective effect against cell damage caused by chronic hyperglycaemia in P19CL6 cardiomyocytes

In order to examine the potential beneficial or adverse effects of TETA on myocardium damaged by chronic hyperglycaemia, P19CL6 cardiomyocytes were cultured in medium containing different concentrations of glucose, with or without TETA. In this study, TETA at concentrations of 20 µM, 40 µM or 120 µM was added into the medium with different glucose concentrations, and the cell index for each group was continuously recorded using the xCELLigence system at six-hour intervals for four days (Fig 4-27).

The first part of this study showed that prolonged culturing with TETA had no measurable adverse effects on P19CL6 cardiomyocytes. No significant difference ($P=0.95$) was found in cell index between control (5.5 mM glucose) groups and the TETA-treated groups during the 4-day period (Fig 4-27a). TETA had no effect on the cell index in P19CL6 cardiomyocytes after 4-days’ treatment (Fig 4-27e). There was thus no evidence to show that TETA treatment had any measurable adverse effects on cell number, viability, or morphology of P19CL6 cardiomyocytes under long-term treatment.

In the second part of this study, different concentrations of TETA were applied to P19CL6 cardiomyocytes cultured in high-glucose medium. Results showed that the differentiated cells cultured in high glucose demonstrated a significant increase in cell index. However, this was not reversible by addition of TETA to the medium. There was no significant difference in the cell index ($P=0.55$) after the 4-day period between the non-treated group and the TETA-treated groups for either 16.7 mM or 33.3 mM glucose groups. These results indicate that TETA had no measurable effect on the changes in cardiomyocytes caused by culture at elevated glucose levels.
Figure 4-27 Shown are effects of TETA on cell index of P19CL6 cardiomyocytes cultured under conditions of chronic hyperglycaemia. Normalised cell index was recorded by using the xCELLigence system following the addition of TETA to the culture medium with (a) 5.5 mM glucose, (b) 16.7 mM glucose and (c) 33.3 mM glucose. (d) Normalised cell index at day 4. (e) Change in cell index after 4 days. Data are mean ± SEM, n=3/group.
In order to further examine the effects of TETA on P19CL6 cardiomyocytes cultured at elevated glucose concentrations, cells were cultured under different concentrations of glucose, with and without addition of TETA. The contraction rates of randomly selected foci were recorded at different time points as described.

Results from this study found that prolonged culture with TETA had no measurable adverse effects in P19CL6 cardiomyocytes. No significant differences were found during the 4-day period (Fig 4-28&29) between the 5.5-mM glucose control group and TETA-treated groups in contraction rates of foci ($P=0.94$) of the ability of foci to contract ($P=0.94$). These results provided further confirmation that TETA did not affect the contractile function of cultured P19CL6 cardiomyocytes.

In order to measure the effects of TETA on cardiomyocytes cultured for 4 days in high glucose, different concentrations of TETA were applied to differentiated P19 cells cultured in high-glucose medium. The results showed that the contraction rate of cell foci was slightly reduced after four days. As well, a decrease in the number of contracting foci was also found. However, TETA treatment did not reverse the changes in contraction induced by high glucose. In comparing the changes in contraction rate ($P=0.78$) and the contracting foci ($P=0.45$) after the 4-day period, no significant difference was found between the control group and the TETA-treated groups for either 16.7 mM- or 33.3 mM-glucose groups. These results suggest that four-day’s with TETA did not modify the effects of chronic glucose elevation on cardiomyocytes.
Figure 4-28 Adverse and treatment effect of TETA on contraction rate in foci with chronic hyperglycaemia. The rate of foci contraction following the addition of TETA in the culture medium with (a) 5.5mM, (c) 16.7mM and (e) 33.3mM glucose. Percentage changed in contraction rate after 4 days cultured in the medium with (b) 5.5mM, (d) 16.7mM and (f) 33.3mM glucose plus addition CuCl₂. (g) Summary on percentage changed in contraction rate. Data are mean ± SEM, n=3 per group in quintuplicate.
Figure 4-29 Effects of TETA on the percentages of foci that continued to contract in P19CL6 cardiomyocytes cultured with the indicated concentrations of glucose and copper. Shown are changes in the number of contracting foci remaining after 4 days’ culture with (a) 5.5 mM, (b) 16.7 mM or (c) 33.3 mM glucose, in the presence or absence of TETA concentrations. (d) Shows a summary of changes in the number of foci under different treatment conditions. Data are mean ± SEM, n=3/group.
The third part of this study was designed to examine whether prolonged exposure to TETA could induce apoptosis in P19CL6 cardiomyocytes. Cells were cultured in medium containing different concentrations of glucose, with or without equivalent concentrations of TETA for four days. At the end of the 4-day period, cells were lysed as described and their relative nucleosome values measured by the cell-death-detection, ELISA plus to determine the apoptosis rate in each sample (Fig 4-30).

![Figure 4-30 Apoptosis in P19CL6 cardiomyocytes cultured with different concentration of glucose and TETA. Data are mean ± SEM, n=6 per group.](image)

Our results indicate that culture with TETA over 4 days did not induce apoptosis in P19CL6 cardiomyocytes. No significant difference ($P=0.41$) was found between the non-TETA-treated, 5.5-mM glucose group and TETA-treated groups in relative nucleosome values after the 4-day period (Fig 4-30). Furthermore, it was also found that addition of TETA into the high glucose medium did not affect the nucleosome value ($P=0.28$). These results provide strong evidence to show that TETA treatment elicited neither adverse effects in inducing apoptosis nor neutralising effects against chronic glucose elevations in cultured P19CL6 cardiomyocytes.
The last part of this study was designed to determine whether cellular copper levels were affected by the presence of TETA in the medium. Cells were cultured under different concentrations of glucose and TETA for four days and cellular copper concentrations determined by GF-AAS as described.

![Figure 4-31](image)

**Figure 4-31** Effects of TETA on cellular copper levels in cultured P19CL6 cardiomyocytes. Relative cellular copper concentrations of cells cultured at indicated treatment conditions for 4 days were determined by GF-AAS. Data are mean ± SEM, n = 3/group.

Thus different concentrations of TETA in the culture medium did not affect the cellular copper concentrations in P19CL6 cardiomyocytes cultured for 4 days (Fig 4-31). No significant differences were found in relative intracellular copper concentrations between the untreated-control group and TETA-treated groups ($P=0.95$: one-way ANOVA). These results indicate that TETA in the medium did not affect the intracellular Cu balance or impair cellular Cu uptake in P19CL6 cardiomyocytes.
4.2.4.2 Using TETA to modify the effects of elevated copper in cultured cardiomyocytes

As described in the previous chapter, TETA is a Cu$^{II}$-selective chelator that can bind to excess free divalent copper ions. Because of this copper-binding property, it was suggested that TETA would bind to excess free copper and prevent copper toxicity in the myocardium. In order to examine this possibility, *in vitro* experiments were designed using P19CL6 cardiomyocytes, which were cultured for four days with different concentrations of glucose and copper, with or without the addition of TETA to the medium (Fig 4-32). Changes in both physiological and molecular characteristics of cultured cardiomyocytes were recorded in this study.

![Experimental design for in vitro cardiomyocyte culture experiments](image)

**Figure 4-32** Experimental design for *in vitro* cardiomyocyte culture experiments to examine the effects of TETA on chronic copper toxicity.
In order to examine the ability of TETA to prevent copper toxicity in cardiomyocytes, P19CL6 cardiomyocytes were cultured as described with different concentrations of glucose and copper, with the addition of TETA (1:1 ratio to copper) for four days. The cell index in each group was continuously recorded by using the xCELLigence system at 6-hour intervals for four days and the results are presented in Fig 4-33, 34 & 35.

Consistent with the previous studies, the normalised cell index was significantly increased when cells were cultured with high-glucose medium, but on prolonged exposure to copper, the cell index was reduced.

In P19CL6 cardiomyocytes cultured under low-copper conditions (20 µM), it was found that the copper had no effect on the cell index irrespective of the glucose levels in the medium (Fig 4-32 a-d). No significant differences in the cell index were found between control, copper-treated and copper/TETA-treated groups after four days’ culture (Fig 4-33e). Therefore, TETA had no demonstrated effects at this concentration (Fig 4-33). On the other hand, the cell index of cardiomyocytes cultured with high levels of additional copper (40 µM and 120 µM) was slightly decreased after four days’ culture (Fig 34-35). Compared to the cells cultured with TETA, the cell index was restored towards normal. However, there were no significant differences in cell index between control copper-treated groups and those treated with copper and equimolar TETA. Taken together, these results indicated that TETA did not modify the effects of copper treatment in P19CL6 cardiomyocytes.
Figure 4-33 Shown are the prevention effects of TETA on the responses to elevated copper (20 µM) in P19CL6 cardiomyocytes cultured for 4 days under the indicated conditions. Normalised cell-index values were measured by using the xCELLigence system following the addition of copper and TETA to the culture medium with glucose at (a) 5.5 mM, (b) 16.7 mM or (c) 33.3 mM. (d) Normalised cell index at day 4. (e) Percentage changes in cell index after 4 days’ culture. Data are mean ± SEM, n=3/group.
Figure 4-34 Shown are the prevention effects of TETA on the response to elevated copper (40 µM) in P19CL6 cardiomyocytes cultured for 4 days under the indicated conditions. Normalised cell-index values were recorded by using the xCELLigence system following the addition of both Cu and TETA to the culture medium with glucose concentrations of (a) 5.5 mM, (b) 16.7 mM or (c) 33.3 mM. (d) Normalised cell index at day 4. (e) Changes in cell index after 4 days. Data are mean ± SEM, n=3/group.
Figure 4-35 Shown are the prevention effects of TETA on the response to elevated copper (120 µM) in P19CL6 cardiomyocytes cultured for 4 days under the indicated conditions. Normalised cell-index values were recorded by using the xCELLigence system following the addition of both Cu and TETA to the culture medium with glucose concentrations of (a) 5.5 mM, (b) 16.7 mM or (c) 33.3 mM. (d) Normalised cell index at day 4. (e) Changes in cell index after 4 days. Data are mean ± SEM, n=3/group.
To further examine possible effects of TETA on responses to copper in cultured cardiomyocytes, cells were cultured for four days with different concentrations of glucose and copper with TETA (at a 1:1 molar ratio to added copper). The contraction rates of numbers of randomly selected foci were recorded at different time points as described and have been presented here according to the specific copper concentrations (Fig 4-35).

Results from these studies were consistent with the previous results, namely that the contraction rates of foci and their ability to contract were affected by both chronic hyperglycaemia and copper. In addition, this study also identified apparent interaction effects between chronic hyperglycaemia and the response to copper, by which foci of cardiomyocytes cultured in high-glucose medium were more sensitive to elevated copper in the medium. Besides these effects, we also demonstrated effects of TETA in preventing copper-mediated effects in cultured cardiomyocytes. By comparing the results in each individual copper-treatment group, it was found that differentiated foci cultured with combined copper and TETA retained their ability to contract and to maintain their contraction rates throughout the study period. At the end of day 4, almost 100% of foci had lost their contraction ability in the high-copper treatment group (120 µM), compared to more than 75% of foci that retained their contracting ability in the group treated with TETA and copper. Taking these results together, more than 80% of foci cultured with both copper and TETA retained their ability to contract at the end of day four, which was similar to the results obtained in the corresponding control group. However, TETA was unable to prevent the impairment in contraction of foci that was induced by chronic hyperglycaemia in the cultured cardiomyocytes.

Taken together, results from this study have suggested that the presence of TETA in the medium can neutralise some of the toxic effects of excessive copper in P19CL6 cardiomyocytes.
Figure 4-36 Shown is evidence that TETA can prevent the damage to contractile properties of P19CL6 cardiomyocytes cultured with elevated copper added to the medium. Presented in (a), (c) and (e) are changes in the contraction rates of foci after 4 days’ culture at indicated glucose concentrations with added copper of (a) 20, (c) 40 or (e) 120 µM, with or without equimolar TETA. Shown in (b), (d) and (f) are changes in the numbers of contracting foci remaining after 4 days’ culture with additional copper at (b) 20, (d) 40 or (f) 120 µM, with or without equimolar TETA. Data indicated by *, P<0.05, **, P<0.01 and ***, P<0.001 are significantly different from the corresponding control groups, and those by +, P<0.05, and ++, P<0.001 from the corresponding copper-treated group. Data are mean ± SEM, n=3/group.
In order to determine whether TETA could prevent copper toxicity-induced apoptosis in P19CL6 cardiomyocytes, cells were cultured for four days with different concentrations of glucose and copper, in the presence of TETA (at a 1:1 molar ratio to copper). At the end of the 4-day period, P19CL6 cardiomyocytes were lysed as described, and their relative nucleosome values were measured by using the cell death detection, ELISA plus to determine the amount of apoptosis rate at each culture condition (Fig 4-37).

Results from the apoptosis study showed that chronic exposure to excessive copper induced apoptosis in P19CL6 cardiomyocytes. Cells cultured under high-copper conditions had higher relative nucleosome values than matched controls. Significant differences were present in relative nucleosome values between control and copper-treated groups. When TETA was added to the medium, relative nucleosome values were similar to those in matched control groups. Indeed, no significant differences were present in relative nucleosome values between most of the control and TETA-treated groups. Furthermore, in cells cultured with 120 µM Cu, relative nucleosome values were significantly reduced ($P < 0.01$) by the presence of equimolar TETA. These results provide strong evidence that the presence of TETA in the medium neutralised the toxic effects of excessive copper by preventing apoptosis in P19CL6 cardiomyocytes. However, TETA did not prevent the changes in nucleosome values induced by chronic hyperglycaemia in these cells.
Figure 4-37 Evidence that TETA prevents apoptosis in P19CL6 cardiomyocytes cultured with elevated copper concentrations in the medium. Relative nucleosome values were measured after 4 days’ culture with added copper of (a) 20, (b) 40 or (c) 120 µM, with or without addition of equimolar TETA. Data indicated by *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$ are significantly different from values in the corresponding control groups; and those indicated by **, $P<0.01$ from the corresponding copper-treated groups. Data are mean ± SEM, n=6/group.
In the previous studies, it was found that addition of TETA to the medium could prevent copper-mediated damage in P19CL6 cardiomyocytes. However, the mechanisms of TETA in preventing such copper toxicity are unknown. It is possible that TETA could act either through lowering the intracellular copper concentration or neutralising its toxic effects by binding to the excess free copper. In order to learn more about this mechanism, the latter part of this study was designed to determine whether addition of TETA to the medium could lower the levels of copper in cells cultured under high-copper conditions. In this study, cardiomyocytes were cultured with different concentrations of glucose and copper, with or without concomitant addition of TETA (at a 1:1 molar ratio to copper), for four days. Samples were then collected and analysed by GF-AAS to determine cell copper concentrations (Fig 4-38).

In this study, copper levels in myocytes correlated with the concentrations of copper added to the culture medium. Furthermore, cells grown in the presence of TETA had lower copper levels than corresponding, non-TETA-treated groups. Cell copper levels differed significantly between copper- and copper/TETA-treated groups. These results suggest that TETA may act by binding to excess free copper, thereby lowering the amount of copper being taken up into the cells. However, measured copper values also showed that the TETA did not fully prevent the uptake of excess copper, as the intracellular copper levels in the TETA-treated group were significantly higher than those in matched control groups. This finding indicates that the copper uptake function of these cells was not completely suppressed by the presence of TETA, which was also unable to prevent changes in cell copper levels induced by chronic glucose elevation in the culture medium. Taken together, this study has shown that TETA partially decreases the uptake of copper into P19CL6 cardiomyocytes at the same time as it prevents copper-mediated damage. Lowering of cardiomyocyte copper levels may well contribute to its ability to protect them against damage in this system.
Figure 4-38 Shown is evidence that TETA treatment lowers cell copper levels in P19CL6 cardiomyocytes cultured with elevated copper in the culture medium. The relative cell copper values measured in P19CL6 cardiomyocytes after 4 days’ culture with added copper of (a) 20, (b) 40 or (c) 120 µM, with or without addition of equimolar TETA. Data indicated as: *, P<0.05; **, P<0.01 and ***, P<0.001 are significantly different from matched control groups; and those indicated as +, P<0.05 and ++, P<0.01 from corresponding copper-treated groups. Data are mean ± SEM, n=3/group.
Previous studies, which probed the chronic effects of copper toxicity in the isolated perfused heart, have shown that perfusion of peri-physiological levels of CuCl\(_2\) into \textit{ex vivo} isolated hearts for a period of about 10 minutes, can impair cardiac function in a dose-dependent manner (Fig 4-15). Isolated hearts infused with greater than 3 \(\mu\)M CuCl\(_2\) underwent failure within 10 minutes. The aim of this study was therefore to evaluate the preventive effects of TETA treatment against copper toxicity in isolated perfused hearts. Here, isolated hearts from weight-matched male Wistar rats were infused either with CuCl\(_2\) solution (0.5 mM/ml stock solution) alone or co-infused with both CuCl\(_2\) and TETA solutions (1:1 molar ratio) for 10 minutes; and changes in cardiac function measured at 1-minute intervals for 25 minutes after treatment onset (Fig 4-39).

Ten minutes’ saline infusion into \textit{ex vivo} isolated hearts had no adverse effects on cardiac function. In the group treated with copper alone, infusion of 0.5 mM CuCl\(_2\) immediately impairs cardiac function, causing heart failure within a few minutes. All measurements of cardiac function were dramatically impaired after this treatment. However, when TETA was infused simultaneously, the toxic effects of the copper were neutralised. The measured values of heart rate, cardiac output, aortic flow, systolic pressure, left ventricular pressure development and relaxation of the isolated perfused heart were all found to be constant and unchanged after the simultaneous infusion of TETA and CuCl\(_2\).

These results indicate that TETA infused in 1:1 ratio with CuCl\(_2\) was able to completely prevent cardiac dysfunction induced by the acute toxic effects of copper.
Figure 4-39 Shown are the effects of TETA treatment to prevent acute copper toxicity in perfused working hearts isolated from Wistar rats. Presented are the preventive effects of TETA against toxic effects of copper on stroke volume (a), heart rate (b), cardiac output (c), aortic flow (d), ventricular pressure development (e), cardiac relaxation (f) and systolic pressure (g) that were measured for 25 minutes. Data are mean ± SEM; n = 5, saline-treated group; n=3, 0.5-mM copper group; n=4 in the copper-/TETA-treated group. P = 0.0000 for each comparison of TETA vs TETA + CuCl₂.
4.2.4.3 Using TETA to reverse cardiac damage induced by copper toxicity

The studies presented above have demonstrated that TETA treatment can prevent copper toxicity in the ex-vivo perfused working hearts of normal rats. In order to model the effects by which TETA ameliorates myocardial copper toxicity, P19CL6 cardiomyocytes were first cultured with different concentrations of glucose and copper in the medium for two days. At the end of day 2, medium was replaced with fresh medium containing either copper with equimolar TETA, or TETA alone, and cells then cultured for a further two days (Fig 4-40). Changes in both physiological and molecular characteristics of P19CL6 cardiomyocytes were recorded.

![Diagram](image)

**Figure 4-40** Experimental design for *in vitro* cardiomyocyte culture model for the effects of TETA on myocardium subject to copper toxicity.
In order to examine the effects of TETA on the activity of cardiomyocytes cultured at high glucose and copper concentrations, cells were cultured as described for two days. On day 2, either copper plus TETA, or TETA at a 1:1 ratio with the initial copper concentration, were added into the medium and cultured for a further two days. Cell index values were continuously recorded by using the xCELLigence system at 6-hour intervals for four days and results have been presented in Fig 4-41.

**Figure 4-41** Shown are the effects of TETA on cell index values of P19CL6 cardiomyocytes exposed to indicated values of copper, glucose and TETA according to the experimental design of Fig 4-40. Graphs show responses to: (a) 20 µm Cu with copper and TETA (Cu+TETA); (b) 20 µm Cu with TETA only; (c) 40 µm Cu with copper and TETA; (d) 40 µm Cu with TETA only; (e) 120 µm Cu with copper and TETA; and (f) 120 µm Cu with TETA only. Data are mean ± SEM, n = 3/group. No significant difference was found among these groups.
Here it was found that the normalised cell index was modified when they were cultured with elevated glucose and copper levels over the study period. The cell index was significantly increased in cells cultured with high-glucose medium only but 120 μM copper lowered the cell index. These results were consistent with the previous results.

Moderately elevated copper levels (20 μM and 40 μM) had no measurable effect on cell-index values in cultured cardiomyocytes. There was also no significant difference in cell-index values between control, copper-treated and copper-/TETA-treatment groups. High copper concentrations (120 μM) slightly lowered cell-index values. Cell-index values trended slightly higher in cells treated with Cu+TETA compared with copper alone Cu-treated group but lower than the control group, but differences failed to reach statistical significance.

Cell-index values for cells grown in the presence of TETA alone were similar to those treated with Cu+TETA. In cells cultured with the lower copper concentrations (20 and 40 μM), there were no significant differences between control, copper-treated and TETA-treated groups. In cells cultured with 120-μM copper, there was an apparent trend towards partial recovery of cell index, but changes were not significant.

Taken together, results obtained from the xCELLigence system were unable to demonstrate significant treatment effects of TETA on cell damage caused by elevated copper in P19CL6 cardiomyocytes during the 4-day study period.
To further examine the effects of TETA on copper toxicity in P19CL6 cardiomyocytes, cells were first cultured for two days with different concentrations of glucose and copper. At the end of day 2, medium was replaced with fresh medium containing either copper-TETA (1:1 molar ratio to initially-added copper) or TETA alone, and cells were then cultured for two more days. Contraction rates of randomly-selected foci were recorded at different time points as described and data are presented in Fig 4-42.

Results from this study were consistent with the previous findings: contraction rates and persistence of foci were affected by both elevated glucose and copper concentrations. In addition, this study also demonstrated the presence of interaction effects between elevated glucose and copper concentrations, since contracting cardiomyocyte foci were more sensitive to the effects of elevated copper when cultured in high glucose.

Furthermore, this study also identified effects of TETA on differentiated P19CL6 cardiomyocytes cultured at high copper concentrations. Here, in cells exposed to lesser elevations in medium copper (20 and 40 µM), both copper-TETA (1:1) and TETA tended to modestly improve both persistence and contraction rate of foci (Fig 4-42). By using chi-square test, it was found that TETA treatment can significantly reverse the effect of excess copper on cardiomyocytes. In addition, neither Cu-TETA (1:1) nor TETA alone reversed the alterations in focal contraction evoked by 120-mM copper. Almost 100% of foci had lost their ability to contract by the end of day four, irrespective of TETA treatment (Fig 4-42f). In addition, TETA had no effect on alterations in focal contraction induced by chronic glucose elevation.

Taken together, these results indicate that treatment with copper-TETA or TETA alone was insufficient to reverse the damage induced by elevated copper in P19CL6 cardiomyocytes.
Figure 4-42 Shown are the effects of TETA on aspects of contractile function in P19CL6 cardiomyocytes exposed to indicate values of copper, glucose and TETA according to the experimental design of Fig 4-40. Change in focal contraction rates after 4-days’ culture with additional copper at (a) 20 µM, (c) 40 µM and (e) 120 µM, with or without added TETA. Foci contracting after 4-days culture with additional copper at (b) 20 µM, (d) 40 µM and (f) 120 µM, with or without TETA. Data indicated by ***, P<0.001 are significantly different from corresponding control groups, and by *, P<0.05, compared to the copper-treated group. Data are mean ± SEM, n=3/group.
In order to determine whether TETA could decrease apoptosis in cardiomyocytes cultured at high glucose, cells were first cultured with different concentrations of glucose and copper for two days. At the end of day 2, medium was replaced with fresh medium containing either copper-TETA (1:1 molar ratio to initially-added copper) or TETA alone, and cells were then cultured for two more days. At the end of the 4-day period, cells were lysed and relative nucleosome values measured by cell-death detection ELISA\textsuperscript{plus} as described, to determine the amount of apoptosis (Fig 4-43).

Here, exposure to excess copper induced apoptosis in cardiomyocytes. Cells cultured in 40- and 120-µM copper had high relative nucleosome values compared to corresponding controls (Fig 4-43 b-c). Relative nucleosome values differed significantly between control and copper-treated groups. However, there was no increase in cells cultured with 20 µM additional copper.

Cardiomyocytes grown for the last two days in copper-TETA (1:1) had slightly lower relative nucleosome values compared to the corresponding groups treated with copper alone. Thus, treatment with copper-TETA significantly decreased relative nucleosome values at 120 µM copper, whereas values did not differ between control and copper-TETA groups.

Effects on relative nucleosome values were similar in cells grown with TETA or Cu-TETA (1:1) (Fig 4-43a). Effects of culture with 20 µM and 40 µM added copper were not significantly different. However, the effects of TETA were significant in cells cultured with medium at 120 µM added copper. In addition, it was found that TETA was more effective than Cu-TETA (1:1 molar ratio) in comparing the relative nucleosome values. Cells which received TETA had lower relative nucleosome values than those with Cu+TETA. However, the difference was not significant. In summary, these results provided strong evidence that both Cu+TETA and TETA treatment may be able to reduce chronic Cu toxicity induced apoptosis in P19CL6 cardiomyocytes.
Figure 4-43 Evidence that TETA decreases apoptosis in P19CL6 cardiomyocytes cultured at elevated copper concentrations. Cells were treated according to the design shown in Fig 4-40. They were cultured for four days at the shown glucose concentrations with the three treatment groups also having the indicated medium-copper concentration: (a) 20, (b) 40 or (c) 120 µM. At end-of-day 2, medium was replaced with that containing the indicated treatments: copper, copper-TETA (1:1 molar ratio), or TETA alone. Presented are relative nucleosome values after day 4. Data indicated by **, \( P<0.01 \) and ***, \( P<0.001 \) are significantly different from corresponding control groups; and by *, \( P<0.05 \) significantly different from the copper-treated group. Data are mean ± SEM, n=6/group.
In the previous studies, we found that treatment with either Cu-TETA (1:1 molar ratio) or TETA alone could reverse copper-mediated damage in P19CL6 cardiomyocytes. In order to examine whether the treatment outcome is related to the cell-copper levels, cardiomyocytes were first cultured with different concentrations of glucose and copper for two days (as in Fig 4-40). At the end of day 2, medium was replaced with fresh medium containing copper, copper-TETA (1:1 molar ratio) or TETA alone (1:1 ratio with the initial added copper concentration), and cultured for a further two days. Cells were then analysed for copper by GF-AAS (Fig 4-44).

The results show that treatment with Cu-TETA (1:1 molar ratio) lowered the copper concentrations in cardiomyocytes cultured in elevated glucose at 20- and 40-µM added copper, compared to those treated with equivalent copper concentrations only. Their cell copper concentrations were elevated compared to control (0 added copper) and were significantly lower than those of groups treated with copper alone. However, in cells cultured with 120 µM copper-containing medium, copper values in the Cu-TETA (1:1 molar ratio) group did not differ significantly from the copper-only-treated group. Taken together, these results suggest that treatment with copper-TETA (1:1 molar ratio) may have a minor effect on lowering cellular copper concentrations in P19CL6 cardiomyocytes.

Effects in cells treated with TETA alone were similar to those treated with copper-TETA (1:1 molar ratio). The effect of TETA was significant in cells cultured with 120-µM copper. TETA may have been marginally more effective than copper-TETA (1:1) in lowering cell copper but the difference was not statistically significant.

In summary, these results provide strong evidence that treatment with either Cu:TETA (1:1 molar ratio) or TETA alone can lower cell copper in P19CL6 cardiomyocytes.
Figure 4.4 Evidence that TETA lowers cell copper in P19CL6 cardiomyocytes cultured at elevated copper concentrations. Cells were cultured for four days at the indicated glucose concentrations and the three treatment-groups also had the indicated medium-copper concentrations: (a) 20, (b) 40 or (c) 120 µM (as in Fig 4-39). At end-day 2, medium was replaced with that containing the indicated treatments: copper, copper-TETA (1:1 molar ratio), or TETA alone (equimolar with prior medium copper), and cells cultured for 2 more days. Presented are relative copper concentrations after day 4. Data indicated as *, P<0.05; **, P<0.01; and ***, P<0.001 are significantly different from matched control groups; and as †*, P<0.05; ††*, P<0.01 and †††*, P<0.001 significantly different from the corresponding copper-treated group. Data are mean ± SEM, n=3/group.
4.3 Discussion

4.3.1 Effects of chronic elevations in glucose on myocardial models

Alterations in glucose homeostasis have been linked to the pathogenesis of diabetes-induced cardiovascular disease (Voors and van der Horst 2011). It is believed that chronic hyperglycaemia can affect the physiological and molecular characteristics of the myocardium via different molecular pathways (Aneja et al. 2008; Voors and van der Horst 2011). Here, experiments were performed to investigate the effects of 4-days’ culture in medium with elevated glucose concentrations on the properties of cultured cardiomyocytes.

In terms of physiological variables, the chronic effects of hyperglycaemia on cultured cardiomyocytes were found to be inconclusive in this study. The results obtained from the xCELLigence system and the apoptosis study showed that culture with elevated glucose stimulated some variables in cardiomyocytes. In particular, significant increases in the normalised cell index concomitant with decreased relative nucleosome values were identified in cells cultured in high-glucose medium. However, other data from an observational study indicated impairment of some contractility-associated functions: in the high-glucose group, the number of contracting foci was decreased significantly and their size was found to be decreased after prolonged culture. These results indicate that elevations in the extracellular glucose can impair these aspects of myocardial function. However, it is difficult to determine what these findings might mean for physiological cardiomyocytes in the heart.

According to results from published reports, it has been proposed that chronic hyperglycaemia can affect the physiological characteristic of cardiomyocytes in vitro. It was reported that chronic hyperglycaemia could cause cardiac contractile dysfunction as a result of oxidative stress and disruption of the calcium balance in cardiac tissues (Bracken et al. 2006). Several studies have demonstrated that hyperglycaemia-induced oxidative stress reduces the activity of gap junctions in cardiomyocytes (Kuroki et al. 1998; Sato et al. 2002). Intercellular communication between cardiomyocytes is primarily through the gap junctions and is important for the maintenance of cellular homeostasis and normal electrical activity (van Veen et al. 2001). Reduced gap junction activity can impair intercellular communication between cardiomyocytes resulting in contractile dysfunction. Besides the effects on gap junction, hyperglycaemia can also affect the function of the sarcoplasmic reticulum resulting in cellular calcium imbalance (Belke et al. 2004; Tang et al. 2010). A study by Gupta et al provided further evidence to show that elevation of extracellular glucose concentrations could
increase the intracellular calcium concentration in cardiomyocytes (Gupta and Wittenberg 1993). Since cardiac contraction is partially regulated by calcium concentration, calcium imbalance will lead to cardiac contractile dysfunction.

Several published reports have also demonstrated that chronic hyperglycaemia can induce apoptosis in cardiomyocytes through increased oxidative stress (Cai et al. 2002). It has been shown that hyperglycaemia can activate the mitochondrial cytochrome c-mediated caspase-9 activation pathway and the local renin-angiotensin system (RAS) to initiate apoptosis (Cai et al. 2002; Jiang and Wang 2004; Lim et al. 2004).

The results of our current studies are consistent with some of these findings. Discrepancies between cell activity and viability may reflect the nature of continuously-cultured P19CL6 cardiomyocytes. As described in chapter 2, the differentiation rate for P19CL6 was about 70-80% and the undifferentiated cells proliferated at lower rates in the medium. It was noted that P19CL6 cardiomyocytes proliferated at a faster rate when cultured in high-glucose medium (results not shown). It is possible that the some of the effects of elevated glucose on P19CL6 cardiomyocytes may be driven by an increase in undifferentiated cell numbers. In addition, medium glucose concentrations when cells are cultured in physiological-glucose medium may decline during the course of the experiment to levels that no longer support cell growth and induce apoptosis in the cells, leading to a false conclusion that high glucose could induce cell activity and viability.

In the second part of this study, the effect of chronic hyperglycaemia on intracellular copper transport in cardiomyocytes was examined in both physiological and molecular level. Results from GF-AAS have showed that differentiated P19 cells cultured under high glucose (16.7mM and 33.3mM) medium have a slightly lower intracellular copper concentration than those cultured in normal glucose (5.5 mM). However, the difference was not significant mainly due to the low copper content in the control medium and small sample size. In medium with additional copper, the difference in intracellular copper concentration between different glucose group become more obvious and it clearly showed that chronic hyperglycaemia can disturb the cellular copper uptake in differentiated P19 cells.

Chronic hyperglycaemia affected mRNA levels of several genes involved in pathways of intracellular copper regulation. Messenger RNA of the cell-membrane copper transporter, Ctr1, was significantly increased, whereas the expression of genes involved in the secretory pathways (Atp7a, Atp7b, Cp and Murr1) were all significantly decreased. These changes are
consistent with insufficient intracellular copper supply and indicate that P19CL6 cardiomyocytes attempted to restore intracellular copper supply to organelles by increasing the levels of cellular copper uptake and lowering the export of intracellular copper. The results from this study are consistent with the results obtained in our GF-ASS study, which showed a lower intracellular copper concentration in P19CL6 cardiomyocytes cultured in high-glucose medium. In addition, the decrease in mRNA expression of $Mt1$ and $Mt2$, which can act as indicators for intracellular copper levels provided further evidence that cell-copper uptake became impaired in cardiomyocytes cultured in high glucose.

RT-qPCR studies were also consistent with the idea that chronic hyperglycaemia can affect the copper supply to mitochondria in cardiomyocytes. Messenger RNA levels corresponding to the copper chaperones, $Cox11$ and $Cox17$, both of which are involved in transporting copper to the mitochondria, were significantly decreased in P19 cardiomyocytes cultured in high-glucose medium. Based on these results, it can be hypothesized that the amount of copper being transported to the mitochondria was decreased. These results are consistent with previous conclusions from this thesis that chronic hyperglycaemia can cause copper deficiency in P19CL6 cardiomyocytes. Since the intracellular distribution of copper is regulated in response to metabolic demand and changes in the cell environment, intracellular copper deficiency will lower the bioavailability of copper and less copper chaperones will be required for transporting copper to the mitochondria. Furthermore, this result is also consistent with the results obtained from the contraction study, which showed that the cardiac contractile function was affected by chronic hyperglycaemia. Since copper ions are needed for cellular respiration to generate ATP, insufficient copper ion supply to the cytochrome c oxidase (CCO) in mitochondria will affect the production of ATP, resulting in cardiac dysfunction.

In summary, this study has demonstrated the effects of chronic hyperglycaemia on the myocardium. It is suggested that chronic hyperglycaemia can induce myocardial dysfunction through the disruption of cellular copper uptake and distribution to organelles. In order to reverse the trend towards intracellular copper deficiency, cardiomyocytes will increase the expression of $CTR1$ to increase the copper uptake and suppress the expression of $ATP7A$, $ATP7B$, CP and MURR1 to lower intracellular copper excretion. Furthermore, intracellular copper deficiency will also affect the intracellular distribution of copper ions, which impairs the supply of copper to the mitochondria for ATP production, and resulting in cardiac dysfunction.
4.3.2 Effects of prolonged culture in medium with elevated copper

In this study, the effects of elevated copper were investigated in both in-vitro and ex-vivo models. It was found that exposure to excess free copper ions can affect myocardial cell activity, contractility and viability. As discussed in the previous chapter, free copper ions can induce cardiac damage through the production of ROS and the disruption of the Na⁺/K⁺-ATPase pump and the Na⁺/Ca²⁺ exchanger (Benders et al. 1994; McDonough et al. 1996; Levine et al. 2011). It is believed that chronic copper toxicity may induce damage in the myocardium through the same mechanisms. Besides the toxicity of excessive free copper ions, results from this study have indicated that the exposure time may also play an important role in determining the effects of copper toxicity in the myocardium.

From in vitro studies, it was found that the effects of copper on cardiomyocytes are correlated with the exposure time. From the acute study, we found that despite the increase in intracellular copper concentrations and mRNA levels of MT, acute copper toxicity did not affect the physiological characteristics of P19CL6 cardiomyocytes measured here. However, results from the study of longer exposure have shown that both physiological and molecular characteristics of P19CL6 cardiomyocytes were affected after more prolonged culture in medium with additional copper. The longer exposure to excess free copper ions affected myocardial cell activity, contractility and viability. This discrepancy between acute and more prolonged effects suggests that copper-mediated damage in P19CL6 cardiomyocytes is time-dependent.

Results from our studies of copper levels have provided further insights into the role of exposure-time on copper-mediated cardiac damage. Compared to the results obtained from the acute study, it was found that P19CL6 cardiomyocytes cultured with additional copper for four days had significantly higher levels of cellular copper. These results suggest that cellular copper uptake is time-dependent, and that increased exposure-time will allow more extracellular copper ions to be taken up into the cells. In addition, since the levels of copper added to the medium were the same in both short- and longer-term studies, these findings indicate that copper-mediated damage was probably due mainly to the elevation in intracellular copper concentrations. Combining these results with previous findings, it is proposed that longer exposure times allows more extracellular free copper to be transported into the cells, accumulating to toxic levels which overwhelm the intracellular defence mechanisms, thereby causing damage to the cells. Further evidence to support this hypothesis was found in the RT-qPCR study, in which the mRNA levels of MT1, MT2 and MT3 were
increased dramatically in P19CL6 cardiomyocytes cultured with high-copper medium in order to lower copper-mediated oxidative damage.

In addition, results from the ex vivo study also demonstrated the importance of exposure time in copper-mediated cardiac damage. It was found that both short- and longer-term infusion of copper into ex vivo isolated hearts could impair cardiac function in a dose-dependent manner. However, longer copper-infusion times can induce cardiac dysfunction at significantly lower concentrations of infused copper. Based on these results, it is considered likely that the amount of copper required to induce cardiac damage will be lower if the exposure time is longer. This indicates that exposure time is an important factor in determining the effects of copper on cardiac function of the isolated rat heart. In addition, this study has demonstrated that small changes in cell-copper balance can result in severe damage to the myocardium, with copper concentrations as low as 1.5 μM able to impair cardiac function in isolated-perfused rat hearts with longer infusion times. The level of 1.5 μM copper is significant since non-protein bound copper levels in mammals are considered to comprise 1-5% of total copper, and would thus be similar. Therefore, the results of these studies raises the question of the relevance of these findings to states where myocardial copper regulation is disturbed, such as in diabetes mellitus.

Taken together, the evidence derived from these studies indicates that the effects of copper on the myocardium are both time-dependent and dose-dependent. It is concluded that prolonged copper exposure could allow extracellular copper ions to be transported across the cell membrane and accumulate inside the cell, resulting in increased intracellular copper concentrations that overwhelm intracellular defence mechanisms causing severe damage to the myocardium.

4.3.3 The interaction between elevated glucose and copper toxicity

Results from this study indicate that P19CL6 cardiomyocytes exposed to high glucose are more sensitive to the effects of elevated copper. In physiological aspects of this work, it was found that addition of extra copper to the culture medium of cells grown under hyperglycaemic conditions can cause a greater loss of contracting foci, as well as an increase in apoptosis. In the studies of cell copper responses, however, levels in the 120 μM glucose-treated group were lower than in the controls (5.5 μM glucose-treated). Further evidence was provided by the RT-qPCR study in which an interaction effect of high glucose and copper
toxicity on the mRNA levels of *MTs* was evident, as demonstrated by a significantly greater increase in response to additional copper. Since *MTs* have antioxidant functions that are thought to be mediated by intracellular sequestration of metal ions, the greater increase in *MT* suggests that the cells grown in high glucose for prolonged periods were more sensitive to the additional copper. Although the mechanisms are not fully understood, it is possible that chronic glucose elevation in the medium can increase copper sensitivity in P19CL6 cardiomyocytes through the induction of oxidative stress. It is likely that this oxidative stress can either cause direct damage to cardiomyocytes or suppress the protective mechanisms against oxidative stress. In return, cardiomyocytes become less tolerant to copper toxicity leading to an increase in copper sensitivity.

In our previous studies, it was found that P19CL6 cardiomyocytes grown under chronic elevation of glucoses have lower cell copper values (Fig 4-22). In response to low cell copper levels, the mRNA levels for certain genes changed in a manner consistent with restoration of copper balance. In this study, results from RT-qPCR showed that additional copper in the medium could reverse some of the changes caused by 4-days’ culture in elevated glucose, for example by lowering the elevated mRNA levels of *Ctr1* (Table 4-6a). Increased extracellular copper concentrations lowered *Ctr1* expression, which indirectly provides evidence that copper is deficient in the cells cultured under high-glucose conditions.

Taken together, results from this study suggest that chronic hyperglycaemia can affect the copper sensitivity of the myocardium, resulting in lowering of intracellular copper content, which could lead to myocardial copper deficiency in the longer term.

**4.3.4 Treatment effects of TETA**

The first part of this study has shown that prolonged cultured with additional TETA did not produce any measurable adverse effects on cardiomyocytes. No significant difference was found between control and TETA-treated groups in both physiological and molecular characteristics of P19CL6 cardiomyocytes. These results were consistent with several other studies that have demonstrated the safety of TETA in animal models and human clinical trials.

In the second part of this study, the results showed that the presence of TETA can prevent copper toxicity-induced damage in the myocardium. From the *in vitro* study, it was found that
the presence of TETA in the medium could neutralise the effects of excessive free copper on P19CL6 cardiomyocytes. No significant difference was found in terms of physiological characteristics between control- and TETA-treated groups. From the ex vivo study, parallel infusion of copper and TETA (at 1:1 molar ratio) into isolated-perfused hearts from normal rats was able to prevent copper-mediated impairment of myocardial function. All measurements of cardiac function remained constant throughout the experimental period. In addition, during the optimisation of the experiments, it was found that TETA, given at the molar 1:1 ratio to copper, was required to prevent copper toxicity, since a lower ratio of TETA to copper resulted in cardiac dysfunction (results not shown). These results suggest that TETA can prevent copper toxicity-induced damage in the myocardium.

Compared to the results in a published report, it has been suggested that TETA can prevent Cu toxicity by binding to free extracellular Cu. However, it is worth mentioning that despite a lack of physiological changes, the intracellular copper concentration was significantly increased in P19CL6 cardiomyocytes, even in the presence of TETA. Furthermore, elevation of copper levels in these cells was not completely suppressed by the presence of TETA. As described in the previous section, increased cell copper concentrations can also induce cardiac dysfunction in a dose-dependent manner. A possible explanation for this discrepancy is that TETA may be transported across the cell membrane and bind to intracellular free copper, thus preventing or minimising the induction of oxidative stress in P19CL6 cardiomyocytes. Although the mechanism is currently unknown, it is suggested that TETA can be transported across the cell membrane through two different mechanisms. Since TETA’s structure is very similar to the principal endogenous polyamines, spermine and spermidine, it is possible that it can directly move across the membrane through the polyamine uptake pathway. Another possibility is that the formation of a TETA-copper complex may enable TETA to be transported into the cell along with copper through the copper transport pathway. However, the measurement of copper by GF-AAS cannot distinguish between copper ions and a TETA-copper complex, and further research is required to confirm the exact mechanisms involved in TETA treatment responses in cardiomyocytes.

In the last part of the study, an in vitro model was used to investigate the treatment effects of TETA in reversing copper-induced cardiomyocyte damage. When compared to the copper-treated group, results suggested that both copper-TETA and TETA treatments might improve the end-points in physiological measurements of P19CL6 cardiomyocytes, but these trends
were not statistically significant. It was also found that both Cu+TETA and TETA treatments could prevent further damage induced by additional copper but were unable to reverse the damage already incurred by cardiomyocytes. However, it is worth mentioning that some differentiated foci recovered their contractility after TETA treatment (results not shown), even though the numbers were not significant.

In examining the GF-AAS results, it was found that both Cu-TETA and TETA treatments could significantly lower the intracellular copper concentrations in P19CL6 cardiomyocytes. These results are consistent with previous studies which suggested that increased extracellular copper concentrations can lead to increased intracellular copper, which is chelatable and can be removed from cardiomyocytes by TETA treatment. It is believed that TETA treatment can improve indexes of cardiomyocyte structure and function by removing excessive free intracellular copper from cardiomyocytes, thereby lessening copper-mediated damage. This in turn may allow cardiomyocytes to undergo an as-yet unknown regeneration process to restore both cardiac function and structure.

In summary, this study demonstrated the treatment effects of TETA in preventing copper toxicity in cardiomyocytes. Although the mechanisms are not fully understood, these results suggest that TETA may be able to bind to extracellular and intracellular free copper to neutralise the toxic effects of free copper in the myocardium and restore copper homeostasis. Removal of free copper can apparently enable cardiomyocytes to regenerate and recover functionally and structurally.
4.4 Conclusion

The results presented in this chapter demonstrated the effects of elevated glucose and copper toxicity on myocardial structure and function. It was found that states of both chronic glucose elevation and copper excess could disrupt the copper balance in cardiomyocytes, resulting in cellular dysfunction. However, it was shown that these states could induce cardiac dysfunction through different mechanisms. In P19CL6 cardiomyocytes, prolonged exposure to elevated glucose could result in relative intracellular copper deficiency whereas prolonged exposure to additional copper can lead to copper overload. As described in the previous chapter, both copper deficiency and copper overload can lead to cardiac dysfunction. However, the effects of copper imbalance on cardiomyocytes were dependent on the copper dosage and exposure times. In response to these changes, it was found that cardiomyocytes will alter the mRNA levels of genes involved in the copper transport pathway consistent with restoration of copper balance and prevention of oxidative damage. Besides the individual effects of hyperglycaemia and copper toxicity, it was also found that these two factors can interact with each other to produce major damage in the myocardium. It was found that chronic glucose elevation could reduce the myocardial tolerance to copper excess, resulting in greater induction of myocardial damage. Based on these results, it is suggested that chronic hyperglycaemia-induced intracellular myocardial copper deficiency could be due to the changes in copper sensitivity, leading to lowering of the capacity of cardiomyocytes to resist copper-mediated toxicity.

In addition, the results described here also demonstrated the treatment effects of TETA in the myocardium with respect to chronic copper toxicity. It was found that TETA was able to protect cardiomyocytes against extracellular copper toxicity. However, it had minimal effects on reversal of cardiac damage already induced by excess copper. Although the mechanisms are not fully understood, the results from this study indicate that, in the myocardium, TETA may be able to bind to both extracellular and intracellular free copper to neutralise the toxic effects of free copper and restore copper homeostasis. As a result, cardiomyocytes may able to regenerate and restore cardiac function and structure.
Chapter 5 Effects of copper and TETA treatment on the structure and function of the diabetic rat heart

5.1 Introduction

As described in the previous chapter, intracellular copper homeostasis is tightly regulated to ensure adequate supply to copper-requiring enzymes and processes without concomitant effects of toxicity or deficiency. Impaired regulation of copper transport pathways can lead to tissue copper deficiency or overload states, which in turn can cause organ damage. In patients with diabetes, it has been found that chronic hyperglycaemia can cause defective copper regulation that can result in higher plasma copper levels, especially in those patients with cardiovascular disease (Zargar et al. 1998; Viktorinova et al. 2009). It has been suggested that elevated plasma copper levels in diabetic patients can be due to an imbalance in distribution between intracellular and extracellular copper concentrations (Walter et al. 1991). In a rat model of diabetes, the abnormality in copper homeostasis leads to accumulation of trientine-chelatable Cu\textsuperscript{II} in the coronary arteries, and this phenomenon has been proposed as a mechanism of cardiovascular damage in diabetes (Cooper et al. 2004; Cooper et al. 2005).

Triethylenetetramine or trientine (TETA) is a divalent Cu\textsuperscript{II}-selective chelator that was first introduced by Walshe in 1969 as an alternative treatment for patients with Wilson’s disease (Walshe 1969). Recent studies in our group have identified new uses for TETA as first in a new class of anti-diabetic molecules. Our studies show that TETA can prevent or reverse diabetic Cu\textsuperscript{II} overload, thereby suppressing oxidative stress and preventing or reversing tissue damage in diabetes mellitus. Current studies performed by our group show that chronic treatment with TETA can ameliorate diabetes-induced disturbances in the regulation of copper homeostasis and improve the structure and function of the heart in diabetic rats and humans (Cooper et al. 2004; Cooper et al. 2009).

In the previous chapters, functional and molecular changes have been described that occur in response to elevated glucose and copper in two myocardial models, \textit{in vitro} cultured myocytes and \textit{ex vivo} isolated-perfused working rat heart. The aim of the studies described in chapter 5 was to extend the previous observations by comparing and contrasting functional and molecular changes in sham and diabetic animals, and to elucidate the mechanisms by which TETA treatment could contribute to observed therapeutic effects.
5.2 Results

5.2.1 Functional changes in hearts from rat with STZ-induced diabetes

In order to determine the effects of diabetes on cardiac function, male Wistar rats with body-weights between 250 and 300 g, were randomly assigned to control or diabetic groups. Following selection, they received a single injection of STZ or saline and were thereafter kept in the animal unit for another eight weeks. Their hearts were then isolated for working-model perfusion, and saline was infused into each heart for two minutes via the main coronary arteries. In this study, the stability of the heart perfused under standard pressures of 10-cm H₂O preload and 82.8-mm Hg afterload was first measured at 2-minute intervals for 10 minutes; thereafter saline was infused for 2-minute followed by a 23-minute run-out period; cardiac parameters were measured at one-minute intervals for 25 minutes after initiation of the saline infusion (Fig 5-1).

As in the previous cardiac perfusion studies reported in this thesis, different units for preload and afterload have been used as is usual in such studies: these reflect the different techniques employed to measure preload and afterload pressures in the model, which reflect equivalent pressure measurements in patients. In addition, the term ‘diabetic heart’ has been used here as a contraction of ‘heart from an STZ-diabetic rat’.

As described in the previous experiment, short term infusion of saline into the isolated heart from control did not induce any adverse effect on any cardiac parameters. However, two minutes’ saline infusion was associated with significant lowering of heart rate ($P<0.0001$), cardiac output ($P<0.0001$), aortic flow ($P<0.0001$) and max left ventricular pressure ($P<0.0001$) in isolated diabetic heart (Fig 5-1 b, c, d and e).

Since there were no changes in stroke volume, the observed lowering of cardiac output and aortic flow would appear to be mainly due to the lower heart rate. Furthermore, a similar trend was also found in isolated-perfused hearts from diabetic rats without saline infusion (results not shown), which suggested that the fall in heart rate was due to the attenuation in cardiac condition caused by diabetes rather than any effect of saline infusion. Taken together, these results indicate that isolated diabetic rat hearts were less stable than those from sham-treated normal rats.
To further investigate the functional differences between control and diabetic hearts, indices of cardiac function were measured in the isolated-perfused working heart preparation, in response to different atrial-filling pressures (Fig 5-2) and afterload pressures (Fig 5-3).

Results from these experiments confirmed that increased atrial-filling pressures can lead to major increases in stroke volume ($P<0.0001$), cardiac output ($P<0.0001$), aortic flow ($P<0.0001$) and systolic pressure ($P<0.0001$) (Fig 5-2 a, c, d and g) in both normal and diabetic hearts (Starling effect). Compared with controls, diabetic hearts had a markedly lower heart rate ($P=0.0084$) that trended to fall progressively as filling pressures increased ($P=0.20$). In contrast, however, the attenuation in heart rate did not affect the cardiac output in diabetic hearts as filling pressure increased to compensate. As a consequence, the disparity between control and diabetic hearts in stroke volume trend to increase with filling pressure increased ($P=0.079$). In addition, both left-ventricular development and relaxation pressures and systolic pressure tended to be slightly attenuated in diabetic hearts as filling pressure increased, although differences were not statistically significant.

On the other hand, increased afterload pressure significantly reduced stroke volume ($P<0.0001$), cardiac output ($P<0.0001$) and aortic flow ($P<0.0001$) in both normal and diabetic hearts (Fig 5-3 a, c and d). In contrast, other cardiac parameters such as heart rate, maximum left ventricular pressure, relaxation pressure and systolic pressure were all found to be increased as the afterload pressure increased. In addition, diabetic hearts were less resistant to the effects of increasing afterload than control (sham-treated) hearts. It was also found that diabetic hearts trended to undergo larger falls in cardiac output ($P=0.14$) and stroke volume ($P=0.14$) as the afterload pressures increased (Fig 5-3a and c). Furthermore, the left-ventricular development pressure (Max–dP/dt) was impaired in diabetic compared with control hearts and did not respond to the increasing afterload pressure ($P<0.001$) (Fig 5-3e), whereas there was no difference in aortic flow and systolic pressure in response to increasing afterload pressure between the normal and the diabetic preparations. In summary, the diabetic heart was found to be less tolerant to both increasing preload and afterload pressures.
Figure 5-1 Shown are values for indices of cardiac function in isolated working rat-heart preparations during saline perfusion at constant preload (10 cm-H$_2$O) and afterload (82.8 mmHg) pressure levels. Measured indices were: (a), stroke volume; (b), heart rate; (c), cardiac output; (d), aortic flow rate; (e), ventricular pressure development; (f), ventricular pressure relaxation; and (g), systolic pressure measured at 1 minute intervals for 25 minutes. Data are mean ± SEM, n = 7/group.
Figure 5-2 Shown are functional responses of isolated-perfused working rat hearts from diabetic and control rats during step-wise elevation in preload at a constant afterload pressure of 82.8 mmHg. Presented are measurements of: stroke volume, (a); heart rate, (b); cardiac output, (c); aortic flow rate, (d); rate of ventricular pressure development, (e); rate of ventricular pressure relaxation, (f); and systolic pressure, (g). These were measured at progressively increasing filling pressures as shown. Data are mean ± SEM, n = 7/group.
Figure 5-3 Shown are the responses of indices of cardiac function to stepwise elevation in afterload pressures of isolated hearts from diabetic and matched control rats perfused at a constant preload pressure of 10 mmHg. Presented are measurements of: stroke volume, (a); heart rate, (b); cardiac output, (c); aortic flow, (d); rate of ventricular pressure development, (e); rate of ventricular pressure relaxation, (f); and systolic pressure, (g); were measured under different afterload pressures as indicated. Data are mean ± SEM, n = 7/group.
5.2.2 Sensitivity to copper infusion in the diabetic rat heart

In order to determine the copper sensitivity of the diabetic rat heart, male Wistar rats with body-weights between 250 and 300 g were randomly assigned to control or diabetic groups. Following selection, rats received a single injection of streptozotocin or saline as described and were thereafter kept in the animal unit for another eight weeks. Hearts were then isolated for working-model perfusion: different concentrations of CuCl\(_2\) (aq) were infused into isolated rat hearts via the main coronary arteries for two minutes, achieved copper concentrations determined, and changes in the seven indices of cardiac function measured at one-minute intervals for 25 minutes (Fig 5-4).

Consistent with our previous study, the results obtained here also showed that cardiac function in saline-infused diabetic hearts declined slightly during this experiment. Both heart rate and cardiac output declined by about 10% during the study-period.

Two minutes’ infusion of CuCl\(_2\) (aq) into isolated-perfused diabetic hearts, elicited immediate dose-dependent impairment in indices of cardiac function (Fig 5-4). Similar to non-diabetic rat heart, it was found that the diabetic heart was also able to tolerate the infusion of a low copper dosage (5-10μM). Impairment in cardiac function became irreversible as the copper concentration of the perfusate increased. As shown in figure 5-4c, although there was a trend towards cardiac recovery after copper infusion, isolated-perfused hearts did not recover fully. On average, the fall in cardiac output was greater than 50% in diabetic hearts infused with copper at concentrations above 10 μM. By comparison of these results to those of controls, it was found that diabetic hearts were more sensitive to elevated copper in the perfusate \((P=0.0001)\). The percentage loss in cardiac output after copper infusion was higher in the diabetic hearts. Besides the differences in cardiac output, LME analysis also showed that copper infusion have a greater impact on all other cardiac parameters \((P<0.0001)\) in isolated diabetic rat hearts.

Cessation of pump function was also more common in diabetic than control hearts. For example, when 50% of the diabetic hearts perfused with 15-20 μM copper (final perfusate concentrations) had functionally failed (cardiac output = 0), more than 85% of control hearts were still pumping. These results indicate that diabetic hearts are less tolerant of acute copper-mediated impairment than non-diabetic hearts.
Figure 5-4 Shown are the effects of 2-minutes’ infusion of CuCl$_2$ solutions on functional indices of isolated diabetic rat hearts perfused at constant preload (10 cmH$_2$O) and afterload (82.8 mmHg) pressure values. Copper was infused between $t = 10$ and 12 min. Measured indices of cardiac function were: stroke volume, (a); heart rate, (b); cardiac output, (c); aortic flow rate, (d); maximum left-ventricular $dP/dt$, (e); minimum left-ventricular $dP/dt$, (f); and systolic pressure, (g). All measurements of cardiac function were recorded for 25 minutes. Data are mean ± SEM, $n = 7$/group.
5.2.3 TETA treatment of copper-infused diabetic rat hearts

In the previous sections, cardiac function was shown to be impaired in diabetic hearts. Isolated-perfused diabetic rat hearts were unable to maintain cardiac function during perfusion and were less tolerant to increases in preload and afterload pressures. Therefore, the aim of the study reported in chapter 5.2.3 was to test whether short-term infusion of TETA could prevent or ameliorate the functional impairment in isolated-perfused diabetic rat hearts. Furthermore, this study also examined the potential treatment effects of TETA on diabetic hearts undergoing acute copper toxicity.

In the prevention study, diabetic rat hearts were isolated for working-model perfusion and randomly assigned to control- or TETA-treated groups. Saline (control) or 1 mM TETA solutions were infused into isolated hearts via the main coronary arteries for two minutes, and the changes in cardiac function measured at one-minute intervals for the next 25 minutes (Fig 5-5). As described in the previous experiment, cardiac function in diabetic hearts slowly declined after short-term saline infusion. Heart rate, cardiac output, aortic flow, maximum left ventricular pressure and systolic pressure were all found to decline during this experiment. By comparison with controls, it was found that short-term infusion of TETA could stabilise the isolated heart and partially prevent cardiac dysfunction. Although TETA infusion did not prevent the decrease in heart rate \( (P=0.97) \), both cardiac output \( (P<0.0001) \), aortic flow \( (P<0.0001) \), maximum left ventricular pressure \( (P<0.0001) \) and systolic pressure \( (P=0.0004) \) were better able to be maintained after the infusion of TETA. As well, the stroke volume of diabetic hearts were found to be slightly increased with short-term infusion of TETA.

In the treatment study, STZ-induced diabetic rat hearts were first perfused for 20 minutes (baseline + 10 minutes) to allow functional loss before the infusion of TETA. TETA solutions at 1 mM and 10 mM respectively were infused into isolated rat hearts for two minutes via the main coronary arteries and the changes in cardiac functions were measured at one minutes intervals for 25 minutes (Fig 5-6). The results showed that short-term infusion of TETA was able to ameliorate the functional loss in isolated diabetic heart. Cardiac output, aortic flow, left ventricle development pressure, relaxation pressure and systolic pressure were found to be increased after TETA infusion. However, there was no significant difference between the treatment effect of 1 mM and 10 mM TETA.
Figure 5-5 Effects of TETA in isolated working hearts from control and diabetic rats perfused at constant preload (10 cm-H$_2$O) and afterload (82.8 mmHg) pressures. TETA was infused from t = 10 to 12 minutes and perfusion conditions thereafter were equivalent to those during the 10-min run-in period. Functional indices were: stroke volume, (a); heart rate, (b); cardiac output, (c); aortic flow, (d); maximum left-ventricular dP/dt, (e) minimum left-ventricular dP/dt, (f); and systolic pressure, (g). All measurements of cardiac function were recorded for 25 minutes. Data are mean ± SEM, n = 7/group.
Figure 5-6 Effects of two doses of TETA (infusion-fluid concentrations of 1 and 10 mM; actual concentrations in perfusate of 15-25 μM and 180-400 μM) and control (0 μM TETA) on indices of cardiac function in isolated working heart preparations from diabetic rats perfused at constant preload (10 cm-H₂O) and afterload (82.8 mmHg) pressures. Saline was infused from t = 10 until 12 minutes, and TETA from t = 20 until 22 minutes. Run-out conditions after t = 22 min were equivalent to those during the run-in and t = 12 to 20 periods. Indices of cardiac function measured: stroke volume, (a); heart rate, (b); cardiac output, (c); aortic flow, (d); maximum left-ventricular dP/dt, (e) minimum left-ventricular dP/dt, (f); and systolic pressure (g). Data are mean ± SEM, n = 7/group.
To further investigate the treatment effects of TETA on the diabetic heart, indices of cardiac function were measured in the isolated-perfused working heart at different preload (Fig 5-7) and afterload pressures (Fig 5-8). During this study, baseline readings were recorded as atrial filling pressure (preload pressure) was increased stepwise from 5 cm H$_2$O to 22.5 cm H$_2$O. Filling pressure was then fixed at 10 cm H$_2$O, and afterload pressure increased stepwise from 54.7 mm Hg to 118.5 mm Hg. After taking the baseline reading, hearts were randomly assigned to either non-treated diabetes (control) or TETA-treated diabetes groups. Saline or 1-mM TETA solution was then infused for two minutes via the main coronary arteries and the changes in indices of cardiac function in response to the different preload and afterload pressures were recorded and compared with the baseline reading to examine the effects of TETA. These repeated-measures data have been analysed by linear mixed-effects modelling.

Results from these experiments showed that short term infusion of saline did not affect the cardiac function of diabetic hearts in response to increased atrial filling pressure (Table 5-1). There was no difference between before and after saline infusion in all cardiac parameter in responses to increased atrial filling pressure. In contrast, the heart rate of diabetic hearts was found to be significantly lower after 2 minutes infusion of 1 mM TETA ($P<0.0001$) (Table 5-2). However, this effect did not alter the heart rate in response to increased atrial filling pressure ($P=0.72$).

In response to increased afterload pressure, results from the experiments showed saline infusion did not have effect on diabetic hearts. No significant difference was found between the periods before and after saline infusion in all cardiac parameter in responses to increased afterload pressure (Table 5-3). However, several cardiac parameters such as stroke volume, maximum left ventricular pressure, left ventricular relaxation pressure and systolic pressure were found to be altered following TETA infusion (Table 5-4). These cardiac parameters were found to be less able to cope with the increased afterload pressure after TETA infusion. Similar responses were also found in cardiac output and aortic flow, although differences were not statistically significant.
Figure 5-7 Response of isolated-perfused diabetic hearts to increasing preload pressures before and after TETA infusion. (a), heart rate (b), cardiac output (c), aortic flow (d), ventricular pressure development (e), relaxation (f) and systolic pressure (g) were measured under different filling pressures before and after TETA infusion. Data are mean ± SEM, n = 7.
Figure 5-8 Response of isolated diabetic hearts perfused at the constant preload pressure of 10 cmH$_2$O to stepwise increments in afterload pressures following two minutes’ infusion of 1 mM TETA. (a), heart rate (b), cardiac output (c), aortic flow (d), ventricular pressure development (e), relaxation (f) and systolic pressure (g) were measured under different afterload pressures before and after TETA infusion. Data are mean ± SEM, n = 7/group.
### Table 5-1 Effects of saline infusion on diabetic Wistar rat in response to increase preload pressure.
Statistically-significant effects are shown in red. Preload (Effect of increase preload pressure), Saline (Effect of saline infusion) and Preload:Saline (Interaction effect between preload and saline).

<table>
<thead>
<tr>
<th>Linear Mixed effects model</th>
<th>Stroke volume</th>
<th>Heart rate</th>
<th>Cardiac output</th>
<th>Aortic flow</th>
<th>Max dP/dt</th>
<th>Min dP/dt</th>
<th>Systolic pressure</th>
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<tbody>
<tr>
<td>Preload</td>
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<td>0.0001</td>
<td>0.0001</td>
<td>0.34</td>
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<tr>
<td>Saline</td>
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<td>0.43</td>
<td>0.78</td>
<td>0.94</td>
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<tr>
<td>Preload:Saline</td>
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<td>0.51</td>
<td>0.42</td>
<td>0.88</td>
<td>0.54</td>
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</table>

### Table 5-2 Effects of TETA infusion on diabetic Wistar rat in response to increase preload pressure.
Statistically-significant effects are shown in red. Preload (Effect of increase preload pressure), TETA (Effect of TETA infusion) and Preload:TETA (Interaction effect between preload and TETA).

<table>
<thead>
<tr>
<th>Linear Mixed effects model</th>
<th>Stroke volume</th>
<th>Heart rate</th>
<th>Cardiac output</th>
<th>Aortic flow</th>
<th>Max dP/dt</th>
<th>Min dP/dt</th>
<th>Systolic pressure</th>
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<td>TETA</td>
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<td>Preload:TETA</td>
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<td>0.65</td>
<td>0.6</td>
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### Table 5-3 Effects of saline infusion on diabetic Wistar rat in response to increase afterload pressure.
Statistically-significant effects are shown in red. Afterload (Effect of increase afterload pressure), Saline (Effect of saline infusion) and Afterload:Saline (Interaction effect between afterload and saline).

<table>
<thead>
<tr>
<th>Linear Mixed effects model</th>
<th>Stroke volume</th>
<th>Heart rate</th>
<th>Cardiac output</th>
<th>Aortic flow</th>
<th>Max dP/dt</th>
<th>Min dP/dt</th>
<th>Systolic pressure</th>
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</thead>
<tbody>
<tr>
<td>Afterload</td>
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<td>0.0001</td>
</tr>
<tr>
<td>Saline</td>
<td>0.23</td>
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<td>0.018</td>
<td>0.29</td>
<td>0.58</td>
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<tr>
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<td>0.56</td>
<td>0.33</td>
<td>0.26</td>
<td>0.29</td>
<td>0.69</td>
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</table>

### Table 5-4 Effects of TETA infusion on diabetic Wistar rat in response to increase afterload pressure.
Statistically-significant effects are shown in red. Afterload (Effect of increase afterload pressure), TETA (Effect of TETA infusion) and Afterload:TETA (Interaction effect between afterload and TETA).

<table>
<thead>
<tr>
<th>Linear Mixed effects model</th>
<th>Stroke volume</th>
<th>Heart rate</th>
<th>Cardiac output</th>
<th>Aortic flow</th>
<th>Max dP/dt</th>
<th>Min dP/dt</th>
<th>Systolic pressure</th>
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<tbody>
<tr>
<td>Afterload</td>
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<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.013</td>
<td>0.0001</td>
</tr>
<tr>
<td>TETA</td>
<td>0.013</td>
<td>0.0034</td>
<td>0.33</td>
<td>0.37</td>
<td>0.35</td>
<td>0.056</td>
<td>0.024</td>
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<tr>
<td>Afterload:TETA</td>
<td>0.037</td>
<td>0.6</td>
<td>0.14</td>
<td>0.13</td>
<td>0.04</td>
<td>0.017</td>
<td>0.026</td>
</tr>
</tbody>
</table>
The previous study has demonstrated the acute effects of copper toxicity in the isolated-perfused diabetic heart, and the results showed that short-term (two minutes) perfusion of CuCl₂ to ex vivo isolated hearts can immediately impair cardiac function in a dose-dependent manner (Fig. 5-4). It was found that in isolated hearts perfused with 15-20 μM copper, more than 60% of the cardiac output was lost within 10 minutes. The aim of this chapter was therefore to evaluate the treatment effects of TETA on isolated-perfused diabetic hearts with cardiac dysfunction caused by acute copper toxicity. In this study, isolated hearts from diabetic rats were first infused with 15-20 μM CuCl₂ for two minutes to impair cardiac function. Ten minutes after the infusion of copper had begun, 1 mM TETA (stock solution) was infused into the copper-treated hearts via the main coronary arteries for two minutes, and changes in cardiac function were measured at one-minute intervals for a further 15 minutes (Fig 5-9).

Results from this study showed that two minutes’ infusion of 15-20 μM CuCl₂ into diabetic hearts caused severe cardiac dysfunction, with a more than 50% fall in cardiac output at the 10-minute time point in TETA-treatment group. After short-term infusion of TETA, the results in this study showed that the cardiac function of isolated diabetic hearts was immediately restored, which was consistent with the previous experiment on the normal sham heart. Measurements of heart rate, cardiac output, aortic flow, systolic pressure, left ventricular development pressure and relaxation were all increased dramatically after the infusion of TETA (Fig 5-9). However, it was found that the recovery rate in the diabetic heart was slower than the control. In the normal heart with acute toxicity, TETA infusion restored cardiac output immediately and reached maximum effect within two minutes. On the other hand, diabetic hearts were less sensitive to TETA treatment and cardiac output was still recovering after 15 minutes. However, there was no significant difference in the treatment outcomes between control and diabetic hearts.
Figure 5-9 Treatment effects of TETA on isolated-perfused diabetic hearts with acute Cu toxicity. Isolated heart from STZ induced diabetic rats was first infused with 15-20μM CuCl₂ for 2 minutes to induce damage in cardiac function. 10 minutes after the infusion of copper, an acute treatment of 1mM TETA was infused into the damaged heart via the main coronary arteries for 2 minutes. Changes in (a) stroke volume, (b) heart rate, (c) cardiac output, (d) aortic flow, (e) ventricular pressure development, (f) relaxation and (g) systolic pressure were recorded for different treatment groups. Data are mean ± SEM, n = 7.
5.2.4 Molecular changes in diabetic rat hearts and treatment effects of TETA

In this study, male Wistar rats, whose body-weights ranged between 250 and 300 g, were randomly assigned to one of four groups: “untreated control” (n=8), “TETA-treated control” (n=9), “untreated diabetes” (n=9) or “TETA-treated diabetes” (n=9) for 16 weeks. For TETA-treated groups, trientine (20 mg/day) was added to the drinking water from the beginning of week 9 to the end of week 16. Thereafter, hearts were excised, and cardiac left-ventricular tissue was collected and divided immediately into two halves, one of which was used for RNA extraction while the other was snap-frozen for subsequent immunohistochemistry. The design of this study was as outlined below in Fig. 5-10.

![Diagram](image)

**Figure 5-10** General experimental design for molecular studies in diabetic and matched-control rats. Male Wistar rats were assigned to one of four groups: “untreated control” (n=8), “TETA-treated control” (n=9), “untreated diabetes” (n=9) or “TETA-treated diabetes” (n=9) for 16 weeks. For TETA-treated groups, trientine (20 mg/day) was added into the drinking water during weeks 9 to 16. At the end of week 16, cardiac left-ventricular tissue was collected for RNA extraction and immunohistochemistry.
RT-qPCR was used to examine mRNA levels corresponding to target genes involved in the intracellular copper-regulatory pathways in cardiac left ventricular tissue. For each gene, mRNA levels were measured in each RNA sample prepared from individual rats. Results have been listed in the following tables, in which genes have been grouped according to their functional involvement in intracellular copper homeostasis.

<table>
<thead>
<tr>
<th>Genes involved in cellular copper uptake</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Gene name</td>
<td>Sham</td>
<td>Sham-TETA</td>
<td>STZ</td>
<td>STZ-TETA</td>
</tr>
<tr>
<td><strong>Ctr1</strong></td>
<td>0.93±0.13</td>
<td>0.89±0.10</td>
<td>0.83±0.13</td>
<td>0.92±0.10</td>
</tr>
<tr>
<td><strong>Ctr2</strong></td>
<td>1.04±0.05</td>
<td>1.11±0.04</td>
<td>1.30±0.05</td>
<td>1.49±0.09</td>
</tr>
<tr>
<td><strong>Dmt1</strong></td>
<td>1.06±0.06</td>
<td>1.08±0.07</td>
<td>0.75±0.03</td>
<td>0.78±0.05</td>
</tr>
</tbody>
</table>

**Table 5-5a** Effects of diabetes and TETA treatment on levels of mRNAs corresponding to target genes involved in cellular copper-uptake pathways. Data are normalised to the corresponding control (sham) values and have been presented as relative mRNA levels. Data are mean ± SEM, n ≥ 8/group. Data significantly different from corresponding control values are shown in red.

**Figure 5-11** Genes involved in cellular copper uptake whose mRNA levels in left-ventricular myocardium were significantly altered by diabetes. Relative mRNA levels corresponding to (a) **Ctr2** and (b) **Dmt1** for each treatment group are shown here. Results are individual values with mean (95% CI), n ≥ 8/group. Data with **, P<0.01 and ***P, <0.001 are significantly different from corresponding sham-control groups.
Figure 5-12 Immunohistochemical analysis of CTR1 expression in cardiac left-ventricular tissue. Photomicrographs of cardiac tissue that has been triple-labelled (with anti-CTR1 antibodies, WGA and DAPI) and compared between Sham-, STZ-, and TETA-treated STZ groups: a-c: CTR1 (red); d-f: WGA (green, surface membrane stain); g-i: DAPI (blue, nucleic acid stain) j-l: merged photomicrographs of CTR1 (red), WGA (green, surface membrane stain) and DAPI (blue, nucleic acid stain). Representative scale bar shown in (l) is 50 µm. Data shown are representative of four independent replicates in each group. Abbreviations: DAPI, 4', 6-diamidine-2-phenylindole; STZ, streptozotocin; and WGA, wheat-germ agglutinin.
The results from these studies showed that mRNA levels corresponding to several genes involved in the cellular copper-regulatory pathways were altered in the cardiac left-ventricular tissue of diabetic rats, and that some of these changes were ameliorated by chronic TETA treatment (Table 5-5 a-e).

Messenger RNA levels of *Ctr2* and *Dmt1* were significantly altered in diabetic heart tissue (Table 5-5a). These genes are associated with the movement of copper through membranes. Messenger RNA levels of *Ctr2* were significantly increased in diabetic myocardium (Fig 5-10a), by on average ~ 30% above control values. In contrast, mRNA levels of *Dmt1* were lower in diabetic hearts (Fig 5-10b). However, chronic TETA treatment did not restore levels of either in treated diabetic myocardium. In addition, no evidence was found in this experiment to support the notion that mRNA levels corresponding to the high-affinity cell-membrane copper transporter, *Ctr1*, were affected in the diabetic heart.

In order to identify the potential changes in expression of CTR1, immunohistochemistry was performed in sections of left-ventricular tissue from male Wistar rats in the different treatment groups. Representative photomicrographs of cardiac cells labelled with anti-CTR1 antibody, WAG (a marker for the plasma membrane) and DAPI are shown in Fig 5-12. CTR1 staining in the normal (sham) heart was mainly present in the plasma membrane, as expected, and only a very small amount of CTR1 staining was present in the intracellular space. However, it was found in diabetic hearts that the expression of CTR1 was internalised and the staining was more intense in the intracellular space as compared to the control study. However, there was no significant difference in the localisation of CTR1 between diabetic and TETA-treated diabetic tissue, so chronic TETA treatment was apparently unable to ameliorate the changes in CTR1 distribution induced by diabetes. Similar staining patterns were observed in the other three rats of each treatment group.
Table 5-5b Shown are the effects of diabetes and TETA treatment on mRNA levels of target genes involved in delivery of copper via the intracellular secretory pathways. Data have been normalised to corresponding controls (Sham) and presented as relative mRNA levels. Data are mean ± SEM, n ≥ 8/group. Those with significant differences compared to corresponding Sham values are shown in red and those with significant differences compared to corresponding STZ-treated values in green.

Figure 5-13 Shown are mRNA levels corresponding to genes involved in the delivery of copper via the secretory pathways whose mRNA levels in left-ventricular myocardium were significantly altered by diabetes and/or TETA treatment. Relative mRNA levels of (a) Atp7a, (b) Cp and (c) Murr1 for each treatment group are shown here. Results are individual values, with means (± 95% CI), n ≥ 8/group. Data indicated by *, P < 0.05 are significantly different from the corresponding sham values; and by +, P<0.05 significantly different from the corresponding diabetic (STZ) group. Abbreviations: STZ, streptozotocin; TETA, triethylenetetramine.
In the secretory pathway, the results showed that the mRNA levels of Cp were significantly increased in STZ-induced diabetic hearts (Fig 5-13b). Compared to the control group, there was a 70% increase in the mRNA levels of CP in diabetic hearts. In addition, although the result was not significant, it was found that the mRNA levels of Murr1 were slightly reduced in diabetic heart (Fig 5-13c). With chronic TETA treatment, the results showed that the mRNA level of Murr1 was brought back to normal levels despite persistent hyperglycaemia. However, no effect was found in the mRNA levels of CP. Interestingly, an unexpected result was found in this study, which showed that with chronic TETA treatment, the mRNA levels of ATP7A was significantly increased in the diabetic heart (Fig 5-13a). Lastly, no significant changes in ATOX1 and ATP7B were found between different treatment groups.

In the pathway associated with delivery of copper to cytosolic SOD1, Ccs mRNA levels were significantly reduced, by ~30%, in diabetic heart compared with controls (Fig 5-14). Moreover, TETA treatment restored Ccs mRNA levels to normal, despite persistent hyperglycaemia. By contrast, neither diabetes nor TETA treatment altered Sod1 mRNA levels.

In order to further examine the effects of diabetes on the expression of CCS and SOD1, immunohistochemistry was performed in left ventricular tissue sections from male Wistar rats of each treatment group. Representative photomicrographs of cardiac cells labelled with anti-CCS antibodies, WAG (a marker for the plasma membrane) and DAPI are shown in Fig 5-15. Results showed that CCS staining in normal sham hearts was present in both the plasma membrane and the intracellular space (Fig 5-15a), and CCS staining was more intense in the intracellular space in the diabetic heart (Fig 5-15b) but no significant difference was apparent in the localization of CCS. Alterations in CCS expression in diabetic heart were partially restored by TETA treatment. For example, the intensity of CCS staining was slightly decreased in TETA-treated diabetic hearts but remained stronger compared to control levels (Fig 5-15c). Immunohistochemical analysis for Sod1 showed that it was detectably expressed only in the intracellular space (Fig 5-16). Similar to the results for CCS, diabetes induced myocardial SOD1 expression (Fig 5-16a). Compared to controls, SOD1 staining was more intense in the intracellular space (Fig 5-16b). However, chronic treatment with TETA did not ameliorate the changes in SOD1 expression, since no significant difference was present in the staining intensity between untreated and TETA-treated diabetic hearts (Fig 5-16c). Results shown are representative of the four rats in each treatment group.
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Sham</th>
<th>Sham-TETA</th>
<th>STZ</th>
<th>STZ-TETA</th>
</tr>
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<tr>
<td>Ccs</td>
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<td>0.68±0.07</td>
<td>1.03±0.10</td>
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<td>Sod1</td>
<td>1.02±0.07</td>
<td>1.01±0.06</td>
<td>0.96±0.04</td>
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**Table 5-5c** Effects of diabetes and TETA on mRNA levels of target genes involved in delivery of Cu to SOD1 in the cytosolic location. Data are normalised to the corresponding control (Sham) and presented as relative mRNA levels. Data are mean ± SEM, n ≥ 8/group: groups significantly different from control (Sham) are in red, and those significantly different from diabetic (STZ) in green (t-test).

**Figure 5-14** Shown are effects of diabetes and TETA on mRNA levels of target genes involved in delivery of copper to SOD1 localised in the cytosol. Relative mRNA levels of Ccs for each treatment group are shown. Results are individual values with means (± 95% CI), n ≥ 8/group. Data indicated as **, P<0.01 are significantly different from control (Sham) and ++, P<0.05 from the diabetic group.
Figure 5-15 Immunohistochemical analysis of CCS expression in left ventricular myocardium. Photomicrographs of cardiac tissue triple-labelled with Ccs, WGA and DAPI were compared between control (Sham), diabetic (STZ) and TETA-treated diabetic groups. a-c: Ccs (red); d-f: WGA (green, surface membranes stain); g-i: DAPI (blue, nucleic acid stain); j-l: merged micrographs of Ccs (red), WGA (green, surface membranes stain) and DAPI (blue, nucleic acid stain). Representative scale bar shown in (l) is 50 µm. Data shown are representative of four independent replicates in each group. Abbreviations: DAPI, 4’, 6-diamidine-2-phenylindole; STZ, streptozotocin; WGA, wheat-germ agglutinin.
Figure 5-16 Immunohistochemistry analysis of SOD1 expression in the left ventricle. Photomicrographs of cardiac tissue triple-labelled with Sod1, WGA and DAPI were compared between control (Sham), diabetic (STZ) and TETA-treated diabetic groups. a-c: SOD1 (red); d-f: WGA (green, surface membranes stain); g-i: DAPI (blue, nucleic acid stain); j-l: merged micrograph of Sod1 (red), WGA (green, surface membranes stain) and DAPI (blue, nucleic acid stain). Representative scale bar shown in (l) is 50µm. Data shown are representative of four independent replicates in each group. Abbreviations: DAPI, 4’, 6-diamidino-2-phenylindole; STZ, streptozotocin; WGA, wheat-germ agglutinin.
Several genes that function in the pathway of copper supply to the mitochondrion and its insertion into Cco were found to be modified by diabetes and/or TETA treatment in the left-ventricular myocardium of diabetic hearts (Table 5-5d). Compared to respective control values, the levels of mRNAs corresponding to Cox11, Cox17 and Sco1 were all significantly decreased in left-ventricular tissue from diabetic hearts. Following chronic TETA treatment, Cox17 mRNA levels were restored to normal despite persistent hyperglycaemia (Fig 5-17b). Messenger RNA levels of Cox11 (Fig 5-17a) and Sco1 (Fig 5-17c) also apparently trended towards normal but effects were not statistically significant.

In order to further examine the effects of diabetes COX17 expression, immunohistochemistry was performed on cardiac left-ventricular sections from each treatment group. Representative photomicrographs of tissue labelled with anti-COX17 antibodies, WAG and DAPI are shown in Fig 5-18. COX17 staining in control (Sham) heart was mainly present in the intracellular space of cardiomyocytes (Fig 5-18a) and appeared to be more intense in the diabetic heart (Fig 5-18b). However, no difference was found in the localisation of COX17. With chronic TETA treatment, COX17 staining was partially restored. The intensity of COX17 staining was found to be slightly reduced in the TETA-treated diabetic heart but remained stronger compared to the control (Fig 5-18c). The result was consistent with similar staining patterns observed in the other three rats of each treatment group.
Genes involved in delivery of copper to cytochrome c oxidase in the mitochondrial pathway

<table>
<thead>
<tr>
<th>Gene Name</th>
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<th>STZ</th>
<th>STZ-TETA</th>
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<tr>
<td>Cox17</td>
<td>1.04±0.15</td>
<td>0.83±0.10</td>
<td>0.75±0.05</td>
<td>1.11±0.14</td>
</tr>
<tr>
<td>Sco1</td>
<td>1.00±0.04</td>
<td>0.93±0.04</td>
<td>0.85±0.06</td>
<td>0.96±0.05</td>
</tr>
<tr>
<td>Sco2</td>
<td>0.91±0.12</td>
<td>0.87±0.14</td>
<td>0.93±0.16</td>
<td>1.13±0.11</td>
</tr>
</tbody>
</table>

**Table 5-5d** Shown are effects of diabetes and TETA treatment on mRNA levels of target genes involved in the pathway of copper delivery to the mitochondria. Data are normalised to corresponding controls (Sham) and have been presented as relative mRNA levels. Data are mean ± SEM, n ≥ 8/group; those with significant differences from the corresponding control (Sham) are shown in red, and from the corresponding diabetic (STZ) group in green.

**Figure 5-17** Shown are effects of diabetes and TETA on levels of mRNAs corresponding to target genes that function in the delivery of copper to the mitochondria. Relative mRNA levels of (a) Cox11, (b) Cox17 and (c) Sco1 for each treatment group are shown. Results are individual values with means (± 95% CI), n ≥ 8/group. Data with *, P<0.05 and **, P<0.01 are significantly different compared to corresponding controls (Sham), and with *P<0.05 from diabetic (STZ) groups.
Figure 5-18 Shown is the immunohistochemical analysis of COX17 expression in left-ventricular myocardium. Photomicrographs of tissue triple-labelled with anti-COX17 antibodies, WGA and DAPI were compared among control (Sham), diabetic (STZ) and TETA-treated STZ groups. a-c: COX17 (red); d-f: WGA (green, surface membranes stain); g-i: DAPI (blue, nucleic acid stain); j-l: merged picture of Cox17 (red), WGA (green, surface membranes stain) and DAPI (blue, nucleic acid stain). Representative scale bar shown in (l) is 50 µm. Data shown are representative of four independent replicates in each group. Abbreviations: DAPI, 4’, 6-diamidine-2-phenylindole; STZ, streptozotocin; WGA, wheat-germ agglutinin.
### Table 5-5e

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Sham</th>
<th>Sham-TETA</th>
<th>STZ</th>
<th>STZ-TETA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mt1</td>
<td>0.96±0.09</td>
<td>1.30±0.20</td>
<td>0.45±0.05</td>
<td>0.37±0.03</td>
</tr>
<tr>
<td>Mt2</td>
<td>0.97±0.10</td>
<td>1.20±0.21</td>
<td>0.62±0.06</td>
<td>0.54±0.08</td>
</tr>
</tbody>
</table>

Shown are the effects of diabetes and TETA treatment on the levels of mRNAs corresponding to members of the metallothionein family. Data are normalised to respective control (Sham) values and have been presented as relative mRNA levels: mean ± SEM, n ≥ 8/group. Data significantly different from controls are shown in red.

**Figure 5-19** Shown are the effects of diabetes (STZ) and TETA treatment on the levels in left-ventricular myocardium of mRNAs corresponding to metallothionein genes. Relative mRNA levels of (a) Mt1 and (b) Mt2 for each treatment group are shown here. Results are individual values with means (± 95% CI), n ≥ 8/group. Data with **, P<0.01 and ***, P<0.001 are significantly different from corresponding controls.

Furthermore, this study has provided strong evidence that diabetes also affects the mRNA levels of the metallothionein gene family (Table 5-5e). The mRNA levels of both MT1 (Fig 5-19a) and MT2 (Fig 5-19b) was found to be significantly decreased in diabetic heart tissue. Compared to the control, more than a 50% reduction in mRNA levels was found in diabetic heart for both MT1 and MT2. However, chronic treatment with TETA was unable to reverse these changes in the diabetic heart.
Figure 5-20 Shown are immunohistochemical analyses of MT expression in representative cross-sections of left-ventricular myocardium. Photomicrographs display cardiac tissue triple-labelled with anti-MT antibodies, WGA and DAPI. Comparisons have been made between control (Sham), diabetic (STZ) and TETA-treated diabetic groups. a-c: MT (red); d-f: WGA (green, surface membranes stain); g-i: DAPI (blue, nucleic acid stain); j-l: merged micrography of MT (red), WGA (green, surface membranes stain) and DAPI (blue, nucleic acid stain). Representative scale bar shown in (l) is 50 µm. Data are representative of four independent replicates in each group. Abbreviations: DAPI, 4’, 6-diamidino-2-phenylindole; STZ, streptozotocin; WGA, wheat-germ agglutinin.
Figure 5-21 Immunohistochemical analysis of MT expression in longitudinal sections of left-ventricular myocardium. Photomicrographs display cardiac tissue triple-labelled with anti-MT antibodies, WGA and DAPI. Comparisons have been made between control (Sham), diabetic (STZ) and TETA-treated diabetic groups. a-c: MT (red); d-f: WGA (green, surface membranes stain); g-i: DAPI (blue, nucleic acid stain); j-l: merged micrography of MT (red), WGA (green, surface membranes stain) and DAPI (blue, nucleic acid stain). Representative scale bar shown in (l) is 50 µm. Data are representative of four independent replicates in each group. Abbreviations: DAPI, 4’, 6-diamidine-2-phenylindole; STZ, streptozotocin; WGA, wheat-germ agglutinin.
In the immunohistochemical studies, MT staining in control (sham) hearts was found to be most dense in the nuclei of cardiomyocytes (Fig 5-20a) and intercalated discs (Fig 5-21a) whereas it was not co-localised with nuclei associated with the *perimysium*, most of which are probably vascular. There was also substantive background staining throughout the cardiomyocytes. By comparing photomicrographs between normal and diabetic myocardium, it was observed that MT staining in diabetic heart may be slightly stronger in the intercalated disc (Fig 5-21b). However, the significance of this apparent difference is uncertain. Changes in MT expression in diabetic heart may well have been partially restored by chronic TETA treatment. The intensity of MT staining was found to be slightly reduced in TETA-treated diabetic hearts but remained stronger compared to controls (Fig 5-20c and Fig 5-21c). The results were consistent with similar staining patterns observed in the other three rats in each treatment group.

In summary, the data obtained from RT-qPCR experiments and immunohistochemical studies showed that diabetes affects the expression of genes involved in the intracellular copper-regulatory pathways in left ventricular myocardium. Some of the observed molecular defects in the diabetic heart can be ameliorated by TETA treatment.
5.3 Discussion

5.3.1 Physiological changes in the diabetic heart

Chronic cardiovascular complications are the leading cause of mortality and morbidity in patients with diabetes. Cardiovascular disease is responsible for up to 80% of deaths in patients with T2DM (Winer and Sowers 2004). Although the mechanisms remain uncertain, it has been shown that diabetes may cause marked ultrastructural damage in the heart and impaired cardiac function in rats and humans (Penpargkul et al. 1980; Jackson et al. 1985).

In this study, results showed that cardiac function was impaired in male Wistar rats with 8-weeks’ diabetes. Compared with controls, isolated diabetic hearts were less stable during the time period studied. Their mechanical performance, as reflected for example in measurements of heart rate, cardiac output and aortic flow, was found to decline slowly during the experimental period irrespective of treatment, whereas control hearts tended to be more stable and not to decline. In addition, ex vivo diabetic hearts were less tolerant when challenged by stepwise increments in preload or afterload pressures.

The results obtained in this study are in concordance with previous studies which utilised a similar experimental design to demonstrate impaired cardiac function in male Wistar rat with 16 weeks’ diabetes (Cooper et al. 2004). When comparing result between these two studies, it is particularly worth mentioning that cardiac function in isolated hearts with 16 weeks’ diabetes was worse than in those with 8 weeks’ disease. These findings confirm that the chronic metabolic defects of diabetes can cause progressive myocardial damage. Comparing these results to previously published reports, it is suggested that diabetes can impair cardiac function through two different potential mechanisms. The first is myocardial fibrosis - the increased formation and deposition of collagen in the heart. As described in the previously in chapter 1.2.3.3, myocardial biopsies from diabetic patients have shown significantly increased collagen deposition around the blood vessels and between the myofibers (Regan et al. 1977). It is believed that increased content and altered three-dimensional organization of fibrous connective-tissue structures account for the myocardial stiffness and dysfunction in diabetic cardiomyopathy (Riva et al. 1998). Besides the formation of collagen, it is believed that mitochondrial dysfunction can also contributed to cardiac dysfunction in the diabetic heart. As described in the previous chapter, metabolic balance is disturbed in diabetes mellitus. It has been shown that the diabetic heart exhibits a reduction in glucose utilisation (glycolysis and glucose oxidation) and increased reliance on fatty acid oxidation as an energy
source. Defective mitochondrial metabolism, possibly with uncoupling of electron transport with oxidative phosphorylation, is thought to result in reduced ATP synthesis and impaired energy supply which in turn can lead to contractile dysfunction and ventricular failure (Rodrigues et al. 1995; Belke et al. 2000; Boudina et al. 2005).

In addition to the impairment of cardiac function in the diabetic heart, the results from this study also showed that diabetic hearts were more sensitive to the infusion of free copper than controls. Short-term infusion of CuCl\(_2\) into the ex-vivo isolated-perfused working diabetic heart immediately impaired cardiac function in a dose-dependent manner. However, the percentage loss in cardiac output after copper infusion at effective levels in diabetic hearts was greater than that in matched controls. In addition, contraction was more common in diabetic than control hearts. These results indicate that the heart in diabetic animals is less tolerant to copper-mediated damage. It is possible that increased copper sensitivity in isolated diabetic hearts may be due to oxidative damage induced by the chronic metabolic disturbances of diabetes. Oxidative stress induced by chronic hyperglycaemia has been reported to directly damage cardiomyocytes or alternatively to reduce the capacity of antioxidant defence mechanisms (Valko et al. 2007). As a consequence, cardiomyocytes could become less tolerant to copper toxicity and thus display increased sensitivity to the pro-oxidant effects of free copper.

Lastly, sarcoplasmic reticulum dysfunction in diabetic hearts could also contribute to the increase in copper sensitivity. As described in the previous chapter, infusion of copper into the isolated heart may disturb the Na\(^+\)/K\(^+\)–ATPase pump and the Na\(^+\)/Ca\(^{2+}\) exchanger, leading to the increased intracellular calcium concentration and resulting in myocardial contraction (Horackova and Murphy 1988; Benders et al. 1994). Since the major function of the sarcoplasmic reticulum is to regulate the intracellular calcium mobilisation, sarcoplasmic reticulum dysfunction may reduce the capacity of myocardium to handle excess calcium and become less tolerant to calcium toxicity (Lopaschuk et al. 1983; Bers 1985; Bouchard and Bose 1991). Such a mechanism could contribute to increased myocardial copper sensitivity in the diabetic heart.
5.3.2 Molecular changes in the diabetic heart

The results from RT-qPCR and immunohistochemistry (IHC) analysis showed that diabetes did affect the mRNA levels of genes involved in the intracellular regulation of copper homeostasis. Although no difference was found in the mRNA levels of Ctr1, IHC studies showed that Ctr1 itself was internalized and relocated from cell membrane to intracellular space in diabetic hearts. This result is consistent with previous published reports which indicated that cells regulate the expression of Ctr1 through translocation and posttranslational stability, rather than transcriptional regulation (Lee et al. 2000; Petris et al. 2003; Guo et al. 2004). Based on these previously published reports, it is considered that Ctr1 internalised in the intracellular space will decrease cellular copper uptake by the myocardium (Dancis et al. 1994; Petris et al. 2003). Furthermore, a significant decrease in the mRNA levels of Dmt1 in diabetes was also observed in the RT-qPCR experiments, which could further contribute to decreased intracellular copper uptake by the myocardium. Although, DMT1 is mainly responsible for iron uptake, several studies in yeast have shown that it may also mediate cellular copper uptake (Arredondo et al. 2003; Collins et al. 2005). Taken together, these results are consistent with the previous reports which suggested that copper balance in the diabetic heart is impaired and that copper uptake may be decreased in cardiomyocytes. This idea is further supported by the increased mRNA levels of Ctr2 in the diabetic hearts. Previous published reports on Ctr1-knockout mice have demonstrated the low-affinity copper-uptake ability of Ctr2 under copper-deficient conditions (Lee et al. 2002). It has been proposed that the expression of Ctr2 can increase in response to lowered intracellular copper concentrations (van den Berghe et al. 2007; Blair et al. 2010). Here, it is hypothesized that the expression of Ctr2 is increased in diabetic cardiomyocytes to compensate for the lowered copper uptake by Ctr1 in order to attempt to restore intracellular copper balance in the myocardium.

In our studies of the pathway for copper delivery to cytosolic SOD1, mRNA levels of Ccs were shown to be significantly reduced in the diabetic heart. Ccs is the copper chaperone for copper/zinc superoxide dismutase 1. Some studies have proposed that Ccs expression is related to cell copper levels and can serve as a biomarker of the intracellular copper state (Bertinato et al. 2010). In animal models, it has been shown that Ccs protein expression was increased in the tissues of copper-deficient animals (Prohaska et al. 2003; West and Prohaska 2004). In contrast, decreased Ccs expression was observed in copper-overload animals. Based on these results, depressed mRNA levels of Ccs could signal copper overload in diabetic...
myocardium. This result provides a possible explanation for the alteration in the expression of Ctr1, which was suppressed in diabetic myocardium to minimise the uptake of copper. In addition, although there was no evidence to show that the expression of Ccs is related to cardiac hypertrophy, it has been demonstrated that Ccs-mediated HIF-1 alpha activation and VEGF expression may play a role in cardiac hypertrophy (Jiang et al. 2007). Reduced expression of Ccs may alter the activity of these two downstream pathways resulting in cardiac hypertrophy.

Results from RT-qPCR studies also suggest that diabetes can affect the mRNA levels of Cp in the myocardium. Here, Cp mRNA levels were found to be significantly increased in diabetic rat heart. Serum CP was reportedly increased in diabetic patients and highly correlated with serum copper levels (McMillan 1989; Daimon et al. 1998). Others have shown that plasma ferroxidase activity, which is catalysed almost entirely by CP, is substantively deficient in diabetes and restored by TETA treatment (Lu et al. 2010).

However, the effects of diabetes on the expression of CP in the myocardium have not yet been investigated. As well, it has been suggested that increased serum CP levels could be also due to the increase in oxidative stress in diabetes (Cunningham et al. 1995). As described in the previous chapter 1.3.2.2, CP is a circulating copper-dependent ferroxidase that is involved in the transportation of copper in the plasma. In addition to its function in copper transport, it has been suggested that CP may also be involved in antioxidant defence by acting as a scavenger to inhibit superoxide-induced lipid peroxidation and uncontrolled oxidation of Fe(II) to Fe(III). It is possible that the increased expression of CP in diabetic myocardium in could contribute to the prevention of oxidative damage mediated by chronic hyperglycaemia. Taken together, elevated mRNA expression of Cp could signal abnormally high oxidative stress in myocardium. In addition, it is worth mentioning that the mRNA levels of both Dmt1 and Cp were affected in diabetic hearts, which may indicate that copper imbalance in diabetes may also interfere with the intracellular iron homeostasis in the myocardium (Hellman and Gitlin 2002; Madsen and Gitlin 2007).

In addition, the results in this study have also indicated that the metallothionein gene family is altered in diabetic hearts. Here, it was found that mRNA levels corresponding to both Mt1 and Mt2 were suppressed in diabetic heart. As described in the previous chapter, MTs can regulate intracellular copper homeostasis and prevent cellular damage induced by oxidative stress (Kelly and Palmiter 1996; Palmiter 1998; Sato and Kondoh 2002; Gold et al. 2008).
Studies using MT-knockout mice and cell lines have shown that impaired MT expression can reduce the capacity for copper storage and enhance the sensitivity to excess copper and oxidative stress (Park et al. 2001; Qu et al. 2002; Tapia et al. 2004). Based on these published data, it is suggested that decreased expression of MT in diabetic myocardium could reduce the cardiomyocytes’ tolerance to intracellular copper and increase copper sensitivity. The results obtained in isolated heart perfusion were possibly consistent with this idea, since they have shown that diabetic hearts are more sensitive to copper infusion than controls.

Furthermore, results from RT-qPCR studies also indicated that diabetes can affect the copper supply to mitochondria in the diabetic heart. Messenger RNAs corresponding to the copper chaperones, Cox11 and Cox17, involved in transporting copper ions to the mitochondria, were significantly decreased in the diabetic rat heart. However, the results from the IHC studies were unable to demonstrate the differences in Cox17 expression between normal and diabetic hearts. Based on these results, it is concluded that the amount of copper being transported to the mitochondria was decreased. Since the intracellular distribution of copper is regulated in response to metabolic demand and changes in cell environment, decreased copper uptake by Ctr1 will decrease the bioavailability of copper ions and fewer copper chaperones will be required for transporting copper to the mitochondria. Since copper ions are needed for cellular respiration to generate ATP, inadequate copper supply to cytochrome c oxidase (Cco; complex IV) in mitochondria could impair the production of ATP, resulting in cardiac dysfunction. As well, mRNA levels of Sco1, a Cco-assembly gene was found to be significantly reduced in diabetic rat myocardium. Sco1 is required for the assembly of the Cco complex, and defects in Sco1 may result in complex IV deficiency (Leary et al. 2004). This result provides further evidence that mitochondrial function is impaired in diabetic hearts. Taken together, the results from this study are consistent with prior reports that diabetes causes mitochondrial dysfunction in the myocardium. Furthermore, these results are also consistent with results from the physiological studies which showed that the cardiac contractile function was affected in isolated diabetic hearts.

In summary, it is suggested that oxidative stress induced by chronic hyperglycaemia can modify intracellular copper homeostasis in the diabetic heart by suppressing myocardial metallothionein expression. As a consequence, diabetic myocardium became less tolerant to intracellular copper, which leads to the disruption in intracellular copper balance. For example, normal intracellular copper concentrations found in control hearts may elicit toxic manifestations in diabetic heart. Based on this abnormal intracellular copper balance, diabetic
myocardium becomes excessively sensitive to copper, which leads to alterations in the mRNA levels of Ccs and Cp. Furthermore, the expression of Ctr1 becomes re-localised in diabetic myocardium in order to prevent further copper uptake and protect the myocardium from copper toxicity. In response, the intracellular copper concentration becomes lowered in the diabetic heart which may affect the copper supply in other pathways. For example, it is believed that decreased intracellular copper concentrations in diabetic myocardium will diminish the bioavailability of copper ions undergoing transport to the mitochondria. As a result, the expression of genes involved in the mitochondrial pathway became affected in the diabetic myocardium. Since copper ions are needed for cellular respiration to generate ATP, an inadequate copper ion supply to the CCo in mitochondria could well lower the production of ATP, resulting in cardiac dysfunction. Furthermore, decreased copper supply may also affect Ccs-mediated HIF-1 alpha activation and VEGF expression in diabetic hearts, which may contribute to cardiac hypertrophy.

5.3.3 Effects of TETA treatment in the diabetic heart

In isolated-perfused diabetic hearts, it was found that short-term infusion of TETA stabilised heart function and prevented functional loss during experimentation. Consistent with the function of TETA, it is believed that short-term infusion of TETA can chelate excess copper in myocardium thereby restoring trace-metal balance. In return, the membrane potential of diabetic myocardium could be restored to normal allowing sustained calcium influx into cardiomyocytes to maintain contractile function. In contrast, infusion of TETA did not improve or restore the response to increased preload or afterload pressures. Since the impairment in cardiac function observed in these pressure studies was mainly due to the long-term alteration in cardiac structure and probably to mitochondrial dysfunction, it is perhaps unsurprising that infusion of TETA did not reverse these abnormalities over the short treatment period. These results contrast with previous data from our group, which demonstrate that the abnormalities in myocardial structure and mitochondrial structure/function can be reversed by chronic TETA treatment (Cooper et al 2004, 2009). In those studies, cardiac function in response to increased preload or afterload pressures was found to be improved in a similar experimental methodology in diabetic rats that received TETA treatment for six to eight weeks. Furthermore, treatment effects of TETA on diabetic hearts with acute copper toxicity were also demonstrated. As hypothesized, short-term
infusion of TETA reversed copper-mediated dysfunction in diabetic hearts, and the results were similar to findings in control rat hearts.

Concerning molecular mechanisms, it was found that chronic TETA treatment in diabetic rat could ameliorate some of the alterations in left ventricles of diabetic heart. Decreased mRNA levels of *Murr1* and *Ccs* were both returned to normal in diabetic hearts following chronic TETA treatment. As stated before, *Ccs* could be a biomarker in determining the intracellular copper availability in the myocardium. Restoration of *Ccs* levels could indicate that the Cu balance in the diabetic heart was restored after TETA treatment. Furthermore, the results are consistent with restoration of mitochondrial function in the TETA-treated diabetic hearts. Messenger RNA levels corresponding to *Cox11*, *Cox17* and *Sco1* were normalised in the diabetic heart after chronic TETA treatment. Based on these results, it is believed that the copper supply to the mitochondria was restored in TETA-treated diabetic hearts. This result is consistent with previous unpublished data from our group which shows that the activity of complex IV in the mitochondria is restored in TETA-treated diabetic hearts (Zhang S et al, unpublished).

In contrast, some of the alterations in left ventricles of diabetic hearts were unaffected after chronic TETA treatment. For example, the expression of *Ctr2*, *Dmt1*, *Cp*, and *Mt1* and *Mt2* were altered by TETA but not restored in TETA-treated diabetic hearts. A possible explanation for this is that these alterations in the copper-regulatory pathway members are due to factors other than copper imbalance. As suggested previously, altered *Mt* expression may be caused by the effects of chronic hyperglycaemia rather than copper imbalance *per se*. This idea is further supported by the result obtained in the *in vitro* cardiac model which showed the expression of *Mt* was suppressed by chronic hyperglycaemia. For Dmt1 and Cp, alteration in mRNA expression may be caused by other mechanisms, as these two genes were also involved in intracellular iron homeostasis in myocardium. In addition, the increased mRNA levels of *Cp* could be due to the increased oxidative stress in diabetic hearts. In summary, this study provided strong evidence that chronic TETA treatment was able to ameliorate some of the molecular alterations in the left ventricles of diabetic hearts.
5.4 Conclusion

The results in this study have demonstrated the effects of diabetes on both functional and molecular properties of the myocardium. Compared to control, it was found that cardiac function in diabetic hearts were impaired, and showed increased sensitivity to copper excess. In addition, the expression of many of the genes in the intracellular copper-regulatory pathways was altered in diabetic myocardium, consistent with impaired intracellular copper regulation and providing potential explanations for at least some of the results obtained in the perfused heart studies.

With chronic TETA treatment, some of the alterations seen in diabetic myocardium were reverted to normal, despite persistent hyperglycaemia. Based on the evidence from these studies, it is believed that impairment of the intracellular copper-regulatory pathways plays an important role in the development of diabetic cardiomyopathy, and that TETA can be used as a treatment to restore the intracellular regulation of copper and improve cardiac function in diabetes.
Chapter 6 Final discussion and conclusions

The research reported in this thesis has investigated the molecular basis of the dysregulation of copper homeostasis in diabetes, and its roles in the causation of damage in cardiomyocytes, and of heart disease (diabetic cardiomyopathy) in rats used as a model of diabetes. The animal model used, the STZ-diabetic rat, closely resembles those forms of diabetes characterised by severe insulin deficiency, such as in particular type-1 diabetes (T1DM).

The central theme of this work has been the interplay of the defective regulation of carbohydrate metabolism, ‘hyperglycaemia’, in diabetes with the concomitant defects in the homeostatic regulation of copper that occur in the disease, and how these two pathogenetic processes might combine to impair the structure and function of isolated cardiomyocytes, the myocardium, and the heart.

The mechanism of action of a novel therapeutic agent, the Cu(II)-selective chelator TETA, in the prevention and treatment of these complications has also been studied and reported here.

The format adopted in this thesis document is that each study has been presented with its own detailed discussion. This final chapter has thus been used to bring together the various themes presented in the individual sections and chapters of the thesis, to recall the key findings and provide a context in which to view the results when taken together.

The thesis ends with a brief discussion of how future work might be directed.

6.1 Hyperglycaemia in diabetes mellitus

Changes in glucose homeostasis have been linked to the pathogenesis of diabetes-induced cardiovascular disease (Voors and van der Horst 2011). It is believed that hyperglycaemia can affect the physiological and molecular characteristics of the myocardium via different mechanistic pathways. In patients with diabetes, studies have showed that chronic hyperglycaemia can disrupt the copper balance in the myocardium resulting in elevated serum copper levels in patients with diabetic complications (Zargar et al. 1998; Viktorinova et al. 2009). It has also been proposed that this impairment in copper regulation may be involved, directly or indirectly, in the pathogenesis of diabetes-induced cardiovascular disease. Thus, it could serve as a new target for pharmacological intervention.
6.2 Chronic hyperglycaemia: a driver in diabetes-induced cardiovascular disease

This study provided evidence that hyperglycaemia-induced cardiac dysfunction is mainly due to the long-term downstream effects of hyperglycaemia rather than acute changes in glucose metabolism. It was found that acute hyperglycaemia caused no measurable toxic effects on either physiological or molecular characteristics in the models employed: the *in vitro* continuously-cultured cardiomyocyte model and the *ex vivo* isolated-perfused rat heart. In contrast, cardiac dysfunction and changes in gene expression were identified in myocardium from rats with severe chronic hyperglycaemia.

A possible explanation for this discrepancy may be associated with the diabetes-mediated activation of antioxidant mechanisms in the myocardium. It is widely thought that hyperglycaemia can impair cardiomyocyte function through the induction of oxidative stress (Ha and Lee 2000; Nishikawa *et al.* 2000; Marfella *et al.* 2001). In order to prevent oxidative damage, antioxidant mechanisms such as SOD1 and glutathione reductase will be activated to eliminate reactive oxygen species (ROS) such as superoxide anion and hydroxyl radical that would otherwise be produced by hyperglycaemia. It is possible that the elevated ROS levels that can generate the oxidative stress induced by acute hyperglycaemia (Hunt *et al.* 1988; Wolff *et al.* 1991) can be neutralised or eliminated by these antioxidant processes. However, these mechanisms were found to be impaired in chronic hyperglycaemia-associated diseases such as diabetes mellitus. As a consequence, hyperglycaemia-induced oxidative stress could cause direct damage to the myocardium resulting in cardiac dysfunction.

6.2.1 Increased copper sensitivity in myocardium

One of the major findings in this thesis is that chronic hyperglycaemia can affect copper sensitivity in both our cellular model and the rat model of STZ-induced diabetes employed. It was found that cardiac cells grown under chronic hyperglycaemic conditions and isolated-perfused diabetic hearts were both more sensitive to the addition of free copper than were comparable controls. Although the mechanism is not fully understood, it is possible that chronic hyperglycaemia increases copper sensitivity in the myocardium through the induction of oxidative stress. It is thought that the resulting oxidative stress can either lower the capacity of the myocardial defence mechanisms against oxidative stress or cause direct damage to cellular components (Valko *et al.* 2007), probably through the ultimate generation of hydroxyl radicals (Hunt *et al.* 1988). Besides, several studies have reported that enzymes
such as SOD1 and glutathione reductase, which mediate antioxidant defences, become impaired in chronic hyperglycaemia-associated diseases such as diabetes.

In addition, the results obtained in the gene-expression studies of this thesis have shown that the expression of two metallothionein gene-family members became suppressed in the myocardium of rats with chronic diabetes. It is known that MTs provide an antioxidant defence mechanism in the myocardium, and also play roles in the regulation of the intracellular copper homeostasis, thus preventing cellular damage induced by oxidative stress (Kelly and Palmiter 1996; Palmiter 1998; Sato and Kondoh 2002; Gold et al. 2008). Studies using MT-knockout mice and cell lines have shown that impaired MT expression can reduce the capacity for copper storage, and enhance the sensitivity to excess copper and oxidative stress (Park et al. 2001; Qu et al. 2002; Tapia et al. 2004).

It is suggested here that decreased expression of MTs in diabetic myocardium would lower the cardiomyocytes’ tolerance to elevated intracellular copper and thus result in increased copper sensitivity. Taken together, lowering the capacity of the anti-copper and anti-oxidant defence mechanisms in the myocardium would lead to an increase in copper sensitivity.

As well as the suppression of antioxidant mechanisms, chronic hyperglycaemia induced-oxidative damage to cellular components may also contribute to the increase in copper sensitivity. Recent studies have shown that sarcoplasmic-reticulum function was impaired in the diabetic heart. Since its major function is to regulate intracellular calcium mobilisation, sarcoplasmic reticulum dysfunction may lower the capacity of the myocardium to handle excess calcium, and thus interfere in the distribution of intracellular calcium in the diabetic heart (Lopaschuk et al. 1983; Bers 1985; Bouchard and Bose 1991). As described in the previous chapter, excess free copper in the myocardium can disturb intracellular calcium balance via effects on the Na⁺/K⁺–ATPase pump and the Na⁺/Ca²⁺ exchanger (Horackova and Murphy 1988; Benders et al. 1994). Lowered tolerance to intracellular calcium imbalance in the diabetic heart may indirectly affect myocardial copper sensitivity.

Taken together, these findings provide valuable insights into the mechanisms by which copper imbalance in diabetes might affect anti-oxidant defence mechanisms and consequent organ function. It is possible that increased myocardial copper sensitivity interacts with the alteration in copper homeostasis in diabetes.
6.2.2 Alteration in copper balance leads to intracellular copper deficiency

In order to prevent damage from copper elevation or deficiency, the intracellular copper concentration is tightly regulated by the copper homeostatic system through regulation of copper uptake, distribution, storage and excretion (Balamurugan and Schaffner 2006). In previous sections, it was shown that chronic hyperglycaemia-induced oxidative stress can lower the myocardial capacity for copper storage, for example by lowering myocardial MT levels. As a consequence, some of the intracellular bound Cu(I) may undergo redox cycling to form Cu(II), which may then cause oxidative damage to cellular components, resulting in increased copper sensitivity. Under this abnormal state, physiological intracellular copper concentrations can now become toxic for the diabetic heart. Therefore, the intracellular copper balance needs to be reset to reduce the excess copper activity in diabetic myocardium in order to prevent the copper-mediated damage that would otherwise occur. This hypothesis is consistent with the results obtained in our in vitro studies, which showed that cardiomyocytes grown in a state of chronic hyperglycaemia have a lower intracellular copper concentration compared to control values. Although the mechanisms are not fully understood, changes in gene expression of proteins involved in the intracellular copper-regulatory pathways may contribute to the lowering of intracellular copper in diabetic myocardium.

In myocardium from a rat model of STZ-induced diabetes, CTR1-protein expression was internalised and relocated away from the cell membrane into intracellular space in the left-ventricular myocardium. Taking these observations together with previous reports supports an hypothesis that internalisation of CTR1 would lower myocardial copper uptake, acting as a defence mechanism against copper excess and pro-oxidant stress generation (Dancis et al. 1994; Petris et al. 2003). Furthermore, a significant decrease in Dmt1 mRNA expression was also observed by RT-qPCR: this alteration could further contribute to lowering the myocardial copper uptake. Although Dmt1 is mainly responsible for cell iron uptake, several experiments in yeast have showed that it may also mediate intracellular copper uptake (Gunshin et al. 1997). Taken together, these mechanisms can lower the amount of copper being taken up in the myocardium and thus prevent further elevations in the intracellular activity of copper.

Besides the alteration in cell copper levels, increased Ctr2 mRNA expression in the diabetic heart could further lower cytoplasmic copper in cardiomyocytes. As described earlier in this thesis, Ctr2 is predominantly localised in intracellular organelles that are reminiscent of late endocytic and lysosomal compartments (van den Berghe et al. 2007). Ctr2 can regulate the
accumulation of intracellular copper and by releasing copper ions from intracellular vascular stores to different copper chaperones (Kampfenkel et al. 1995; Rees et al. 2004; Blair et al. 2009). It is possible that cardiomyocytes increase the Ctrl2 expression would accelerate the release of copper from intracellular vascular stores, to compensate the reduction in copper supply in myocardium.

Through such mechanisms, intracellular copper concentrations and consequent catalytic activity can be lowered to levels that the diabetic myocardium can tolerate. However, this lowering of intracellular copper can be overdone, leading to a copper-deficiency state in the diabetic myocardium, thus impairing the supply of copper to organelles that contain key intracellular copper enzymes, such as Cco (in mitochondria) and SOD1 (in cytoplasmic endocytic/lysosomal vesicles). The consequent insufficiency in cellular copper supply would thus impair cardiac function in the diabetic myocardium through functional impairment in, for example, copper enzymes such as SOD1 and Cco, leading to the development of diabetes-associated cardiovascular disease.

6.2.3 Copper mediated damage in the diabetic myocardium

Under normal circumstances, copper-associated cellular damage is either due to copper overload or copper deficiency. However, the mechanisms involved in diabetes may be more complex and share elements of both processes. It has been suggested that elevated serum copper levels in diabetes patients may be due to an imbalance in distribution between the intracellular and extracellular copper concentrations (Walter et al. 1991), although what exactly that might mean and how it might come about was not discussed. As a consequence of our groups’ work, it has become apparent that diabetic myocardium may suffer damage caused by imbalance between elevated extracellular Cu(II) and concomitant intracellular deficiency of copper, which is predominantly Cu(I).

6.2.3.1 Cardiac damage induced by extracellular copper excess

Several studies have found that diabetic patients have higher serum copper levels, especially those with diagnosed cardiovascular disease (Zargar et al. 1998; Viktorinova et al. 2009). However, the difference in serum copper levels between diabetic patients and normal healthy
controls is only about 1-2 µM and there has been ongoing debate about whether such a small change in copper concentrations could lead to major damage in the myocardium.

In this thesis, we have demonstrated that infusion of additional Cu\(^{2+}\) (aq) at concentrations as low as 1.5 µM can cause major functional impairment in ex vivo isolated hearts. Impaired cardiac function and muscle contraction was observed immediately very soon after initiation of Cu\(^{2+}\) infusion. Based on these observations, we hypothesise that elevated serum copper levels in diabetes patients could be a factor in causation of myocardial copper toxicity. However, non-protein-bound copper in the plasma is mainly complexed to histidine and polyhistidine oligopeptides (Sarkar et al. 1993).

There are no available published reports known to us concerning the bioactivity of copper-histidine complexes in the heart, so the catalytic ability of elevated histidine-bound copper remains uncertain and will need to be ascertained by experiment.

Based on our data considered in the context of previously published studies, it is suggested here that excess free copper can induce toxic effects in myocardial tissue through at least two distinct mechanisms. The first is the induction of oxidative stress. Free copper ions can cause damage to cellular components by catalytic interactions with ROS such as superoxide anion and hydrogen peroxide that ultimately form hydroxyl radicals (Simpson et al. 1988; Kadiiska and Mason 2002). Thus increased extracellular copper could contribute to the increased production and/or transformation of ROS in diabetic tissues, thus severely compromising the health and viability of cardiomyocytes, and resulting in cardiac dysfunction and heart failure. In clinical studies, elevated serum levels of extracellular superoxide dismutase (EC-SOD, SOD3) were identified in diabetic patients, and its activity correlated strongly with the degree of oxidative injury found in the vascular system (Fukai et al. 2002; Kimura et al. 2003; Cooper et al. 2005). These findings provide further evidence supporting a potential role by which elevated serum copper levels could contribute to oxidative stress generation. Furthermore, the increased oxidative stress induced by extracellular copper may further reduce the copper tolerance in diabetic myocardium, which could in turn contribute to the impairment of copper distribution between the intracellular and extracellular spaces.

Besides the production of oxidative stress, extracellular copper toxicity can affect cardiac function through disturbance of the Na\(^{+}/K^{+}\)-ATPase pump and the Na\(^{+}/Ca^{2+}\) exchanger (Benders et al. 1994; McDonough et al. 1996; Levine et al. 2011). Studies in cultured human skeletal muscle cells have shown that copper overload can inhibit the Na\(^{+}/K^{+}\)-ATPase pump,
resulting in a rapid increase in cytoplasmic free $\mathrm{Na}^+$, which will therefore activate the $\mathrm{Na}^+$/\mathrm{Ca}^{2+}$ exchanger, leading to an increase in $\mathrm{Ca}^{2+}$ concentration, and resulting in myocardial contraction (Benders et al. 1994). Our experiments showing that isolated-perfused hearts infused with $\mathrm{Cu}^{2+}$ showed increase stiffness and irreversible contraction of the left-ventricular muscle providing evidence that excess copper may well impair myocardial calcium homeostasis.

6.2.3.2 Cardiac dysfunction and deficient copper in cardiomyocytes

In rats with chronic STZ-induced diabetes, myocardial cellular copper levels are deficient by about 50%, as determined by studies using the reference methods particle-induced X-ray emission spectroscopy (PIXE) coupled with Rutherford backscattering spectroscopy (RBS) (Cooper GJ et al, unpubl. data). To our knowledge there are no available reports of myocardial copper levels in patients with diabetes and heart disease. The decrease in myocardial copper concentrations in diabetic rats may lead to symptoms of copper deficiency similar to those that occur in the various copper deficiency syndromes in animals and patients. Results from this thesis provide evidence that defects in myocardial copper regulatory mechanisms in diabetes that impair cellular copper uptake and trafficking to intracellular compartments could lead to defective copper incorporation into enzymes such as SOD1 and Cco (and in particular, its copper-containing subunits, COI and COII).

In the rat model of diabetes employed in this thesis, we also found that intracellular copper deficiency may affect myocardial contractile function. Isolated diabetic hearts were less stable throughout the experimental period than matched controls. Indices of mechanical performance of diabetic hearts, such as heart rate, cardiac output and aortic flow, declined slowly during the experimental period in control animals. In addition, the diabetic heart was less tolerant to increases in either preload or afterload pressures. These observations are consistent with our group’s previous studies, which showed an impairment of cardiac contractile function in rats with 16-weeks’diabetes (Cooper et al. 2004).

Based on previously published reports, our group believes that the impaired contractile function in the diabetic heart is caused in the main by alteration in organ structure and function caused by diabetes-induced copper deficiency. Myocardial copper deficiency in other contexts is accepted as the cause of various cardiovascular disorders, including cardiac hypertrophy, fibrosis and myofibrillar dysfunction (Prohaska and Heller 1982; Elsherif et al.
that mimic those which occur in diabetic heart disease. Alterations in myocardial composition and the three-dimensional molecular organization of fibrillar/contractile myocardial proteins can cause myocardial stiffness and dysfunction in diabetic cardiomyopathy (Riva et al. 1998), which manifests in part as diastolic dysfunction. Lastly, copper deficiency can also induce cardiac failure by altering myocardial gene expression of proteins including contractile proteins, Ca\(^{2+}\)-cycling proteins, extracellular matrix collagens and others (Elsherif et al. 2004).

In addition, copper deficiency also can affect copper supply to mitochondria in the STZ-induced diabetic heart. It was found in this thesis that mRNA expression of the chaperones, COX11 and COX17, both of which are involved in transporting copper to the mitochondria, was significantly decreased in myocardial tissue from diabetic rats. These results are consistent with deficient copper supply to the mitochondria. Since the intracellular distribution of copper is regulated in response to metabolic demand and the changing cell environment, decreased copper uptake by CTR1 will lower the bioavailability of copper ions and fewer copper chaperones will be required for transporting the metal to the mitochondria. Since copper ions are needed for cellular respiration to generate ATP, inadequate supply of copper ions to Cco will impair the production of ATP, which results in elevated production of superoxide anions from inefficient coupling of electron transport to oxidative phosphorylation, and to cardiac dysfunction.

Information concerning aspects of such problems are available from animals receiving diets deficient in copper, which result in decreased levels of cellular ATP and phosphocreatine, and elevated ribose 5-phosphate and phosphocholine levels (Kopp et al. 1983). Also, the mRNA expression of one of the COX assembly genes, Sco1, was found in this thesis to be significantly decreased in the diabetic rat myocardium. Sco1 is known to be required for the assembly of complex IV, and genetic defects in Sco1 cause complex IV deficiency (Leary et al. 2004).

These results, when taken in conjunction with the original findings reported in this thesis, provide important evidence that mitochondrial function is impaired in diabetic hearts. Mitochondria are key organelles that catalyse the metabolism of metabolic fuels via cellular respiration and ATP production via oxidative phosphorylation. Their uncoupling, such as is promoted by copper deficiency, will result in reduced ATP synthesis and impaired energy supply which will lead to contractile dysfunction and ventricular failure (Rodrigues et al. 2003; Mandinov et al. 2003),
1995; Belke et al. 2000; Boudina et al. 2005). The new results reported from this thesis provide important insights concerning the mechanism by which diabetes causes mitochondrial dysfunction in the myocardium.

6.3 Potential mechanisms of TETA in diabetic cardiomyopathy treatment

As noted above, diabetic patients and in particular those with cardiovascular disease have been found to have raised serum copper levels (Zargar et al. 1998; Viktorinova et al. 2009). Here, we have demonstrated that copper imbalance in diabetic myocardium could be related to impairment of antioxidant mechanisms, coupled to lowered intracellular copper stores and decreased myocardial copper. As a consequence, diabetic myocardium may suffer resulting in impaired cardiac function and structure. With chronic TETA treatment, diabetes-induced disturbances in the regulation of copper homeostasis are ameliorated, and both cardiac structure and function are restored in the diabetic heart in rats (Cooper et al. 2004; Gong et al. 2006; Cooper et al. 2009; Lu et al. 2010). Although the molecular mechanisms by which TETA acts are not fully understood, the evidence in this thesis and that of others in our laboratory, suggest that TETA treatment could protect the diabetic myocardium against oxidative damage and restore copper supply to the mitochondria.

In diabetic patients, TETA lowers excess body-copper stores by binding to the excess free, or chelatable copper in blood, enhancing its excretion at least in part through the urine (Cooper et al. 2004; Cooper et al. 2005; Cooper et al. 2009). Whether TETA can also enhance excretion of copper via the bile remains to be determined (Cooper 2011; Cooper 2012).

Furthermore, the ex vivo studies performed in this thesis have also demonstrated that TETA can bind to excess chelatable copper in a 1:1 ratio, thereby shielding the perfused heart from copper-mediated damage. As described in the previous section, excess chelatable copper can induce toxic effects in the myocardium through the induction of oxidative stress. The binding of excess copper could lower free radical production and protect the myocardium against oxidative stress, which would then allow the damaged heart to regenerate as has been observed with chronic TETA treatment (Cooper et al. 2004).

In clinical studies, elevated levels of EC-SOD (SOD3) were demonstrated in diabetic patients (Cooper et al. 2005), and its activity correlated well with evidence of oxidative injury in the vascular system (Fukai et al. 2002; Kimura et al. 2003; Cooper et al. 2005)). Following
TETA treatment in type-2 diabetic patients, elevated EC-SOD levels fell to values similar to those of non-diabetic controls, consistent with suppression of oxidative stress mediated with elevated superoxide anion. This result provides further substantive evidence that TETA-binding of excess chelatable copper can lower the production of radicals in diabetic tissues.

Besides the probable lowering of radical production, TETA can also improve cardiac function by restoring the intracellular trace-metal balance of the myocardium. As described in the previous section, excess chelatable copper may disturb the Na⁺/K⁺–ATPase pump and Na⁺/Ca²⁺ exchanger which modulate cardiac contractile function (Benders et al. 1994; McDonough et al. 1996; Levine et al. 2011). Studies in cultured human skeletal muscle cells have shown that copper overload can inhibit the Na⁺/K⁺–ATPase pump, resulting in defective Na⁺ regulation that can in turn activate the Na⁺/Ca²⁺ exchanger and increase cytoplasmic [Ca²⁺] causing myocardial contraction (Benders et al. 1994). TETA therapy restores myocardial copper homeostasis, which could thus in turn restore the balance of other intracellular trace metals in the myocardium (Cooper et al. unpubl results). In return, the membrane potential of diabetic myocardium may be restored, which repair of Ca²⁺ homeostasis and cardiac contractile function in treated diabetic animals. Further experiments are required to confirm or refute this hypothesis.

Moreover, TETA may also improve mitochondrial function in diabetic myocardium by restoring the copper supply. In this thesis, mRNAs corresponding to Cox11, Cox17 and Sco1 were normalised in diabetic left-ventricular myocardium following TETA treatment. Since Cox11 and Cox17 both mediate intracellular copper transport to the mitochondria, increased expression of these two genes may well signal TETA-evoked restoration of copper supply to the mitochondria. Similarly, proteomic analysis showed that chronic treatment with TETA in diabetic rats can restore the expression of several mitochondrial proteins in myocardium (Jüllig et al 2007). These findings are consistent with recent as-yet unpublished data from our group which have shown that mitochondrial complex IV activity is restored in TETA-treated diabetic hearts (Zhang S et al unpubl data). Furthermore, repair of mitochondrial function may also improve the myocardial structure in diabetic hearts, as elevated left-ventricular mass in diabetes is due in significant part to the enlargement in mitochondria volume (Cooper et al. 2004; Cooper 2012).

Although the mechanism by which TETA restores intracellular copper is not fully understood, the results presented here are consistent with the idea that TETA can cross the
cell membrane and bind to intracellular chelatable copper, thereby beneficially modifying its intracellular regulation. In further recent and as-yet unpublished studies, our group has shown by *in vivo* administration of [14C]-labelled TETA, that the active moiety is taken up into myocardial tissue following its administration in rats and dogs (Cooper GJ et al, unpubl nonclinical data). As described in the previous section, antioxidant mechanisms in the myocardium such as those mediated by metallothionein and SOD1 become impaired by chronic hyperglycaemia. As a consequence, the copper-homeostatic system may act to lower intracellular copper levels, to prevent elevation of free copper from causing oxidative damage in myocardium, leading to copper deficiency in cardiac cells. This it may do by modifying the bioactivity of Ctr1 and related mechanisms. Since TETA can bind to free copper ions and suppress residual catalytic/toxic activity of copper atoms in the myocardium, formation of the TETA-copper complex (Cooper 2011) could allow copper atoms to remain in the myocardium, contributing to the restoration of intracellular copper levels.

In our *in vivo* studies, we demonstrated that when cardiomyocytes are cultured with TETA in the medium, they can tolerate higher amounts of cellular copper without any measurable negative effects. However, studies on the molecular basis of TETA transportation in the myocardium have not yet been performed. Based on the current understanding of TETA biology, it is thought that TETA can be transported across the cell membrane through two different mechanisms. Since its structure closely resembles those of the physiological polyamines spermine and spermidine, it is possible that TETA could be directly transported across the membrane through polyamine transporters. In addition, the formation of the TETA-copper complex may enable TETA to be transported into the cell, bound together with copper via another transport pathway. However, the copper concentrations measured in this thesis were not able to inform concerning to this mechanism, and further research is required to ascertain which of these mechanisms might be involved in its cellular uptake.

In addition, although the structure and function of diabetic rat heart was found to be improved after chronic TETA treatment, this thesis was not designed to determine whether TETA can directly stimulate the regenerative processes in the myocardium. It is believed that cardiac cells can regenerate through different mechanisms. TETA-mediated prevention of myocardial damage could allow innate regenerative processes to take place in myocardium, restoring cardiac structure and function in diabetic hearts (Cooper et al. 2004; Cooper 2012).
6.4 P19CL6 cardiomyocytes as a cellular model to study diabetic cardiomyopathy

P19CL6 cardiomyocytes are multipotent stem cells that can differentiate into different cell lineages under different stimuli. In the presence of dimethyl sulfoxide, P19CL6 cells can differentiate into cardiomyocytes. Indeed, the cells are widely used to study cardiomyocyte development. However, to my knowledge, we are the first group to use P19CL6 cells as a cellular model to study diabetic cardiomyopathy. We were interested to determine whether P19CL6 cells cultured under conditions of chronic hyperglycaemia would behave similarly to cardiomyocytes from diabetic myocardium. Based on the results of this research, we have demonstrated that P19CL6 cardiomyocytes cultured under conditions of chronic hyperglycaemia did indeed show changes in both physiological and molecular aspects that were similar to diabetic myocardium.

In our functional studies, P19CL6 cardiomyocytes showed contractile dysfunction, impaired copper balance and increased copper sensitivity, consistent with findings in diabetic hearts. However, there were important differences: for example, both activity and viability of these cells were increased, which is inconsistent with previous publications concerning diabetic hearts.

A possible explanation for this discrepancy is found in measurements of cell activity and viability is the presence of undifferentiated P19CL6 cells. As described previously in this thesis, the differentiation rate for P19CL6 cells is ~70-80% and undifferentiated cells were able to proliferate at low rate in the differentiation medium. Undifferentiated cells appeared to proliferate at a faster rate when cultured in high glucose medium (results not shown). It is thus possible that the toxic effects of chronic hyperglycaemia on differentiated P19CL6 cells may be overshadowed by increased numbers of undifferentiated P19CL6 cells in high-glucose cultures. As well, glucose in the low-glucose medium (physiological glucose) may decline naturally through consumption by growing cells during the course of the experiments, to levels that no longer support continuing cell growth, increasing apoptosis in differentiated cells, and leading to the apparent conclusion that high glucose can induce cell activity and viability.

At the cellular level, the mRNA expression of genes involved in the copper-regulatory pathways was found to be affected in P19CL6 cardiomyocytes cultured under conditions of chronic hyperglycaemia, which can impair the copper supply to the mitochondria, with suppression of expression of both Cox11 and Cox17 in these cells. This result is consistent
with the alterations found in diabetic hearts in this thesis. However, even though the copper balance in cardiac myocytes in both cellular and animal models tended towards copper deficiency, different responses were found in genes involved in copper uptake. In diabetic rat hearts, expression of certain of these genes was consistent with lowered copper uptake and transport. On the other hand, in P19CL6 cardiomyocytes, alterations in gene expression were consistent with increased copper uptake. A possible explanation for this discrepancy is the difference in extracellular copper concentrations. As stated previously, the serum copper concentration in diabetic subjects is around 14-16 µM (Cooper et al. 2005) and we hypothesise that the lowering of intracellular copper in diabetic hearts is related to the toxic effect of copper in the myocardium. In the P19CL6 cellular model, the copper content in the medium was much lower and this may not have been sufficient to induce a toxic effect on P19CL6 cardiomyocytes, resulting in the different response.

Taken together, the results indicate that P19CL6 cells can be used as a cellular model to characterise the molecular basis of myocardial disease in diabetes and related conditions. However, the limitations of this model need to be considered before drawing conclusions from the data and it requires further development and refinement.

6.5 Limitations and future directions

In this research, the potential mechanisms involved in copper imbalance in diabetic myocardium and its treatment with TETA have been dissected using two models. However, there are a number of limitations and matters that remain unresolved and further experiments are required to better characterise the role of copper in diabetic cardiomyopathy, and the possible mechanisms by which TETA acts to treat this condition.

6.5.1 Regulation of gene expression

Here, the alterations in mRNA expression of genes involved in the intracellular copper regulatory pathways were examined in both cellular and animal models. However, regulation of gene expression occurs not only at the level of transcription, but also at the translational and post-translational levels. In mammals, studies have shown that numerous genes associated with copper metabolism are regulated by post-translational mechanisms. Therefore, quantification of mRNA and protein levels is complementary, and necessary for a
more complete understanding of the mechanisms involved in copper imbalance in diabetic myocardium. Experiments such as western blotting and mass-spectrometric methods should be able to provide supporting data to determine the changes in protein expression and further support the data missing from these thesis studies.

6.5.2 Interactions of trace metals in diabetic myocardium

It is well known that alterations in copper balance can affect the homeostasis of other trace metals. For example, elevated copper levels can lower the levels of manganese, zinc and potassium in the body. In this thesis research, we have investigated the changes in the copper transport pathways in diabetes that may lead to copper imbalance in the myocardium. In molecular studies, we found that both Dmt1 and Cp expression in the myocardium were altered in diabetic rats. Since both Dmt1 and Cp also play roles in iron homeostasis, it is possible that iron balance in diabetic myocardium could also be affected by these processes. As well, copper imbalance can also affect myocardial Ca\(^{2+}\) homeostasis through disturbance of the Na\(^+/K^+\)-ATPase pump and the Na\(^+/Ca^{2+}\) exchanger. However, the molecular aspects of other trace-metal transport pathways and their interaction with copper were not investigated in this thesis. Understanding the intracellular trace-metal regulatory networks in diabetic myocardium may provide new insights into the mechanisms implicated in copper imbalance.

6.5.3 The role of metallothionein proteins in the diabetic heart

Metallothionein proteins (MTs) are major intracellular metal-binding proteins that localise to the membrane of the Golgi apparatus. Several studies have shown that MTs can regulate intracellular copper homeostasis and prevent cellular damage by scavenging radicals, protecting against oxidative stress, and are also involved in cell copper storage, distribution and detoxification (Kelly and Palmiter 1996; Palmiter 1998; Sato and Kondoh 2002; Gold et al. 2008). Several studies have also shown that overexpression of MTs can prevent diabetes-induced deficits in the myocardium by suppressing the induction of oxidative stress (Liang et al. 2002; Ye et al. 2003). However, the mechanisms associated with the copper transport pathways have not been investigated in this thesis. In this research, the expression of MTs was found to be suppressed in the left ventricles of diabetic hearts and this phenomenon has been proposed as an initiating event in the copper imbalance in diabetic myocardium.
Examin ing the copper transport pathways in transgenic animals which overexpress the MTs may improve our understanding of these mechanisms.

6.5.4 TETA transport in the myocardium

In this thesis research, we hypothesise that TETA may able to cross the cell membrane either through the polyamine pathway or by being taken up by a copper-transporting mechanism. The first of these proposed mechanisms is based on the structural similarity between TETA and the biologically-occurring polyamines, spermine and spermidine, which can be transported across the cell membrane through the Na\(^+\)/spermine antiporter (Tanabe 1996; Kobayashi et al. 1999). It is possible that TETA or its main metabolites, N\(^1\)-monoacetyl-TETA and N\(^1\), N\(^{10}\)-diacetyl-TETA (Lu et al. 2010) can be transported across the cell membrane via the same mechanisms as polyamines. The second proposed mechanism is based on the results of the \textit{in vitro} study, which found increased intracellular copper concentrations in P19CL6 cardiomyocytes cultured in medium containing both copper and TETA. It is possible that TETA binds to free copper atoms to form a TETA-copper complex which is then taken up into cells through a copper-transporting mechanism. However, further studies are required to confirm the involvement of such mechanisms in TETA action. If confirmed, this finding would provide a new insight on the mechanism of TETA in restoring the copper imbalance found in diabetic myocardium.

6.5.5 Combination treatment with TETA and insulin for diabetes cardiomyopathy

As demonstrated in this work, chronic TETA treatment can restore cardiac function and structure in the hearts of STZ-diabetic rats. However, some of the changes in the left ventricles of diabetic hearts remained after chronic TETA treatment. For example, the expression of \textit{Ct2}, \textit{Dmt1}, \textit{Cp}, as well as \textit{Mt1} and \textit{Mt2}, all remained unrestored in TETA-treated diabetic hearts. It is possible that these changes in copper-regulatory pathway members are due to factors in diabetes other than copper imbalance. For example, it is believed that altered MT expression might be due to the effects of chronic hyperglycaemia rather than copper imbalance. Since insulin is widely used to control blood glucose in patients with diabetes, combination treatment with insulin and TETA in diabetic subjects may be more effective in ameliorating the abnormalities in diabetic myocardium.
6.6 Conclusions

In conclusion, this thesis has examined the interplay of hyperglycaemia and copper levels on cardiomyocytes structure and function in both a cellular and an animal model. It was found that the copper balance in cardiomyocytes was altered by chronic hyperglycaemia, which elicited decreased cellular copper levels and increased copper sensitivity compared to controls. Based on these data, chronic hyperglycaemia-induced copper imbalance could be due to changes in the copper-transport pathways, with genes involved in antioxidant mechanisms and the mitochondrial pathway found to be suppressed in cardiomyocytes cultured with high glucose. In the STZ-induced diabetic rat model, some of these abnormalities were also found in diabetic myocardium.

Taken together, we propose that copper imbalance in the diabetic myocardium is due to the lowering of intracellular copper capacity caused by chronic hyperglycaemia-induced changes that promote oxidative damage and lower copper storage capacity. As a consequence, some bound Cu (I) ions may become unbound and undergo a redox cycling reaction to form Cu (II) atoms, which can cause oxidative damage to cellular components, resulting in increased copper sensitivity. Therefore, intracellular copper balance needs to be adjusted in order to lower the excessive amounts of intracellular copper in the diabetic myocardium, thereby preventing copper-mediated damage. However, the lowering of intracellular copper may in time lead to symptomatic copper deficiency in the diabetic myocardium, impairing the copper supply to intracellular compartments and copper enzymes such as Sod1 and Cco. As a result, diabetic myocardium may suffer damage from both copper toxicity (external) and copper deficiency (internal), which together impair cardiac structure and function in diabetic subjects.

With chronic TETA treatment, several of the abnormalities found in diabetic myocardium have been shown in this thesis to be ameliorated. Although the mechanisms of drug action are not fully understood, we hypothesise that TETA treatment can improve cardiac function in the diabetic heart through the binding of excess free copper in the myocardium, which can lower copper-mediated oxidative stress, thus restoring copper balance in the diabetic heart. These mechanisms can prevent further damage occurring and allow innate regenerative processes to take place in the myocardium to restore the structure and function of the diabetic heart.
Figure 6-1 Summary diagram. Hyperglycemia induces copper imbalance in diabetic myocardium resulted in both copper toxicity (external) and copper deficiency (internal), which together impair cardiac structure and function in diabetic subjects. TETA treatment can improve cardiac function in the diabetic heart through the binding of excess free copper in the myocardium, thus restoring the intracellular copper balance in the diabetic heart. These mechanisms can prevent further damage occurring and allow innate regenerative processes to take place in the myocardium to restore the structure and function of the diabetic heart.
References


