Endocrine Disruptors: Passage and Actions in Human Placenta

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ABSTRACT

Endocrine disruptors (EDs) are ubiquitous in nature. Bisphenol A (BPA, from polycarbonated plastics), genistein (from soy based diets) and 4-nonylphenol (4-NP, degradation product of alkylphenols from detergents) are three major xeno-estrogen contaminants in New Zealand. The estrogenic action of these chemicals has been reported in many in vitro and in vivo studies. The mounting evidence from experimental animals on increased developmental anomalies, reproductive disorders, metabolic diseases and cancer when exposed to these chemicals during development necessitated the examination whether these compounds could be transferred across the human placenta. Hence the aim of this project was to to study the passage and actions of BPA, genistein and 4-NP in human placenta. To achieve this we developed an ex vivo dually perfused single cotyledon human placental perfusion model in our laboratory. The model was validated by measuring physical and biochemical parameters. To imitate the environmentally relevant conditions, we incorporated the compounds in the perfusion medium from the range of serum concentration reported from pregnant mothers. Once the compounds were incorporated in the maternal perfusate, dually re-circulating perfusion experiments were continued for 3 hours with digitally controlled pumps. We collected the perfusates from maternal and fetal reservoirs every 30 minutes for analysing the compounds and their conjugates. We standardized HPLC / LC-MS/MS methods to measure accurately the concentrations of the compounds in the perfusates. Our results conclusively showed that all these compounds could transfer across the human placenta and reach in the fetal reservoir in an active parent compound. However the rate of transfer and the conjugation pattern varied for these three EDs studied. We also incubated villous and choriodecidulal explants from term placentae with environmentally relevant concentrations of BPA. Our results indicate subtle changes in the expression patterns of key genes involved in placental development. The possibility of altering cytokine secretion could also be observed. Taken together our results showed a materno-fetal transfer of EDs in human placenta with potential in vitro actions. When comparing the available data with our results, the concentration reached in fetal compartment would be sufficient to cause an estrogen receptor mediated effect in the fetus.

DEDICATION

THIS THESIS IS DEDICATED TO MY GURU (TEACHERS), MY PARENTS, MY WIFE AND MY SIBLINGS

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ABBREVIATIONS

AhR	arylhydrocarbon receptor
APCI	Atmospheric Pressure Chemical Ionization
ARTN	Artemin
BisGMA	BPA-diglycidyl methacrylate
BPA	bisphenol A
CYP-450	Cytochrome P-450
DCM	Dichloromethane
DDT	dichlorodiphenyltrichloroethane
DEE	Diethylether
DEHP	di-2-ethyl hexyl phthalate
DES	diethylstilbestrol
DHEAS	dihydroepiandrostenidione sulfate
DoHAD	Developmental origin of Health and Diseaseases
EB	estradiol benozoate
EDs	endocrine disruptors
EPA	Environmental Protection Agency
ER	estrogen receptor
ERR-γ	Estrogen Related Receptor - γ
ESI	Electrospin Ionization
Fc	Fetal reservoir concentration
FITC-DX	Flouresceine Isothiocyanate tagged dextran
Fv	Fetal reservoir volume
hCG	human Chorionic Gonadotropin
HPLC	High Performance Liquid Chromatography
IGF-I & II	Insuline like Growth Factors I & II
IL-10	Interleukin – 10

IL-1β	Interleukin – 1beta
LC-MS/MS	Liquid Chromatography – tandem Mass Spectrometry
LD_{50}	Lethal $Dose_{50}$
LOD	Limit of Detection
Mc	Maternal reservoir concentration
MDR	Multi Drug Resistance
mRNA	messenger RNA
MRP-2	Multidrug resistance Protein 2
Mv	Maternal reservoir volume
NOAEL	No Observable Adverse Effective Level
OH-PCBs	Hydroxylated polybrominated diphenyl ethers
PCB	Polychlorinated biphenyls
PCDD	Polychlorinated dibenzodioxins
PCDFs	Polychlorinated dibenzofurans
P-gp	P-glycoprotein
p-NP (4-NP)	p-nonylphenol or 4-nonylphenol
PR	progesterone receptor
Q-RT-PCR	Quantitative Real time PCR
RfD	Reference dose
SHBG	Sex Hormone Binding Globulin
SLUTs	Sulfotransferases
TBP	TATA box Binding Protein
TCDD	2,3,7,8-Tetrachlorodibenzodioxin
UGT	UDP (Uridine 5’ diphosphate)-glucuronosyl transferase

Chapter 1. GENERAL INTRODUCTION

1.1. INTRODUCTION

Chemical or microbial degradation of many common products liberates chemical compounds with hormone-like actions into the environment. These chemicals are everywhere in the environment and come from products which include common household items, agrochemicals, veterinary medicines and biopharmaceutical wastes. In 1991, researchers attending the Wingspread Conference held in Racine, Wisconsin, USA coined the term ‘endocrine disruptor’ (a compound which mimics or disrupt the actions of hormones) to describe chemical compounds which are believed to disrupt the normal metabolic action of hormones in humans and other animals, causing developmental and reproductive abnormalities, especially those chemicals present as an environmental pollutant [1, 2]. A reduction in the population size of wild and marine animals grown in environments contaminated with pesticides and herbicides has been observed for many years[3]. For example; the crocodiles of chemically contaminated Lake Apoka had underdeveloped penises and developmental abnormalities of the gonad when compared to the crocodiles of uncontaminated lakes in Florida. [4]. In addition, results from studies on laboratory animals and cell lines exposed to environmentally relevant doses of these environmental hormones support the notion that these compounds have the potential to disrupt endocrine function during development [5].

The initial thrust of the research was to identify the compounds with estrogenic actions in sensitive screening tests and once identified they were classified as environmental estrogens which included phyto-estrogens (estrogenic chemicals liberated from plants), myco-estrogens (estrogenic chemicals from fungus) and xeno-estrogens (chemicals used in the manufacture of detergents, plastics etc). Compounds present as components of

common household objects have been found to be associated with estrogenic actions in both *in-vitro* and *in-vivo* assays. Examples of these household compounds are bisphenol A (BPA) in plastics and dental sealants, pthalates in plasticizers, alkyl phenols in paints and detergents, genistein and diadzein in plants, organochlorides in insecticides and certain herbicides and mycoestrogens [6, 7]. Most of these compounds are found to have a weak estrogenic action, of the order of 10^3 to 10^5 times less potent than 17-β-estradiol, the most potent of the major estrogens, which is mediated through the classical nuclear receptor pathways[8]. Further studies have shown that the same compound may possess more than one endocrine mimicking or antagonizing action. For example: genistein; a phytoestrogen, can act either as an estrogen agonist or as an estrogen antagonist. As the list of environmental chemicals with hormone modulating actions expanded, the potential for affecting diverse hormone targets is also increased. Now the same or similar compounds are also found to modulate the actions of androgens, progestins, thyroid, hypothalamic and pituitary hormones [9, 10].

The fact that these compounds are ever present in the environment and the fact that these compounds can also act as agonists or antagonists of various hormones present in the fetus during highly vulnerable periods of fetal growth and development suggests that these compounds may influence outcomes suggested by developmental origins of health and disease hypothesis (DoHAD) [9, 11-14]. The DoHAD hypothesis is based upon the findings that the stress (nutritional / environmental) during critical developmental windows of human development (eg. fetal stage) may programme the fetus, which is vulnerable, to develop chronic diseases during adulthood, due to the formation of a permanent mark in the epigenome [15, 16]. Human development is a complex highly integrated process which continuous from fertilization until death. The integral roles of

various steroid / peptide hormones during the developmental process are very important for normal development.[17-19]. Development is also vulnerable to the interaction between genetics and the environment (epigenetics). During human development, the embryo and fetus is most vulnerable to environmental insults. These insults can lead to an accumulation of the mutagenic and epigenetic events, which can predispose the child to develop diseases, such as cancer and metabolic syndrome, later in adult life [20]. In addition, fetal endocrine feedback mechanisms and the immune system are not fully developed, which predisposes them to have adverse reactions in response to exposure to endocrine disruptors (EDs) even at very low doses [21]. Other factors which make the fetus during the perinatal period highly vulnerable to the insults of hormone mimetics include undeveloped DNA repair enzymes, lack of detoxifying enzymes, primitive liver metabolism, lack of development of the blood-brain barrier and increased metabolic rate [14, 20, 22]. Moreover, transplacental transfer and a placental binding of such compounds were reported for most of the xeno-estrogens[23-25], The placental binding effect is more often observed with highly lipophilic compounds such as organochlorides and dioxins [26]. Direct evidence of developmental effects on the human fetus has not been shown for the majority of these compounds, most likely because only a small number of epidemiological researchers have studied the association between in-utero exposure to these compounds and reproductive and behavioural abnormalities in humans [27-31].

Diethylstilbestrol, an estrogen agonist, was widely prescribed for pregnancy loss and other pregnancy related issues (http://www.cdc.gov/DES/). Higher incidence of cervical adenocarcinoma in daughters of mothers treated with diethylstilbestrol (daughters of seven of eight treated mothers v/s none in control) has shown that diethylstilbestrol can cross human placenta and make subsequent generations susceptible to developmental diseases

and cancer [32]. Furthermore, research has shown that the epigenetic regulation of key genes during vulnerable windows of exposure is one of the important factors that contribute to disease susceptibility [14, 33].

Some studies on experimental animals have shown that a very low concentration of estradiol is sufficient to affect fetal development [34, 35]. These experiments have shown that endocrine disruptors can readily transfer across the placenta and cause developmental abnormality [5, 34-37], but studies in other laboratories could not reproduce the results[38]. Various reasons have been given for the inconsistency in reported results, including variations in strain of the species studied (Wistar rats versus CD-1 rats), different phytoestrogen concentrations incorporated into the feed, and exclusion of a positive control (DES for xenoestrogen study). Data from animal experiments have shown that the placenta can metabolize endocrine disruptors (EDs) and make them less toxic to the fetus. The placenta shows considerable anatomical variation over different species, hence the interpolation of results between species is not possible. Thus, the study of transfer and actions of the common endocrine disruptors in the human placenta is necessary.

Unlike typical toxicity studies the interpretation of risk may not be possible with endocrine disrupting actions of these chemicals as they may follow a non-monotonic dose-response relationship. Moreover, the effects of exposure may vary at different gestational ages. The test chemical may have an entirely different effect in the embryo than adult [39]. Such variations have been observed with the embryo, fetus, neonate, adolescence and adult. Moreover, observed differences could vary in relation to the animal species, route of administration or transport, duration of exposure and gender.

1.2. ENDOCRINE DISRUPTORS

1.2.1. Definition and classification

Environmental Protection Agency (EPA, NIH, USA) accepted definition for an Endocrine Disrupting Chemical is

"an exogenous agent that interferes with the production, release, transport, metabolism, binding action or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental process" [40].

The observation of estrogenic and or antiestrogenic actions of some synthetic chemicals (BPA, nonylphenol, organochloride insecticides, dioxins etc) and natural compounds (phytoestrogens and mycoestrogens) on *in-vitro* estrogen screening assays[7], and the correlation of epidemiological data of reproductive and health disorders in various wild and laboratory animals have all contributed to the development of the endocrine disruptor hypothesis. More and more compounds have been progressively added to the list, and the concept of 'endocrine disrupting action' has been extended to cover disruptors of other hormone systems such as androgen mimetics or antiandrogens, and thyroxine antagonists and thyroxine mimetics [9].

Endocrine disruptors have been classified by structural analogy and by other characteristics. Based upon their mode of action endocrine disruptors are classified into xeno-estrogens, antiandrogens, antiestrogens, thyroxine antagonists.

Table 1-1 Classification of endocrine disrupting chemicals

Classification	Endocrine disrupting chemicals	
Pesticides	2,4-D Atrazine Benomyl Carbaryl Cypermethrin Chlordane (γ-HCH) DDT and its metabolites Dicofol Dieldrin/Aldrin Endosulfan Endrin Heptachlor Hexachlorobenzene (HCB) Iprodione	Kepone (Chlordecone) Lindane Malathion Mancozeb Methomyl Methoxychlor Mirex Parathion Pentachlorophenol Permethrin Simazine Toxaphene Trifluralin Vinclozolin
Organohalogens	Dioxins and furans PCBs	PBBs and PBDEs 2,4-Dichlorophenol
Alkylphenols	Nonylphenols Octylphenols Pentaphenols	Nonylphenol ethoxylatesOctylphenol ethoxylates Butylphenols
Heavy metals	Cadmium Lead	Mercury Arsenic
Organotins	Tributyltin (TBT)	Triphenyltin (TPhT)
Phthalates	Di-ethylhexyl phthalate Butyl benzyl phthalate Di-n-butyl phthalate Di-n-pentyl phthalate	Di-hexyl phthalate Di-propyl phthalate Dicyclohexyl phthalate Diethyl phthalate
Natural Hormones	17β-Estradiol Estrone	Estriol Testosterone
Pharmaceuticals	Ethinylestradiol Mestranol	Tamoxifen Diethylstilbestrol (DES)
Phytoestrogens	Isoflavonoids Coumestans Lignans	Zearalenone β-sitosterol
Phenols	Bisphenol A	Bisphenol F
Aromatic hydrocarbons	Benzo(a)pyrene Benz(a)anthracene Benzo(b/h)fluoranthene 6-hydroxy-chrysene	Anthracene Pyrene Phenanthrene n-Butyl benzene

1.2.2. Structure and mode of action of selected endocrine disruptors

One of the most studied endocrine disruptors falls under the xenoestrogen category. A study has shown that the major estrogenicity in a typical New Zealand food comes from BPA, Genistein and Alkylphenols[41] (Table 1.2). Hence we selected BPA, genistein and 4-nonylphenol in our studies. In brief, natural estrogens impart a physiological action by binding to nuclear estrogen receptors (genomic classical pathway) or by binding to membrane estrogen receptors (non-genomic alternate pathway). It is evident from the research data that xeno-estrogens can bind to estrogen receptors (ER) and can activate the classical and / or alternate pathway to impart its action [42]. It is also reported that xeno-estrogens can modulate the activities of the enzymes involved in xenobiotic metabolism [42]. The major endocrine disruptive actions of xeno-estrogens are mediated through the estrogen receptor pathway (Figure 1.1). The 'enzyme modulating' pathway is complex since it includes various xenobiotic metabolic pathways and the magnitude varies based on the nature of the endocrine disruptor and the metabolic products formed. For example some of the xeno-estrogens can inhibit steroid conjugating enzymes, the actions of which normally reduce the estrogenicity to make more estrogenic compounds in circulation.

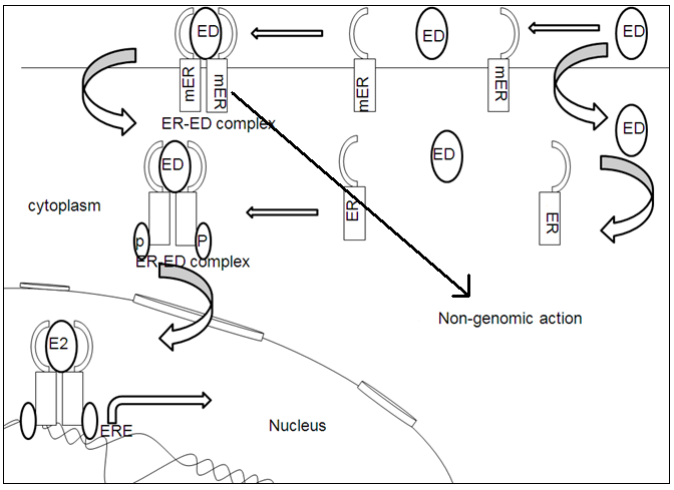

Figure 1-1 Mechanism of action of xeno-estrogens

Endocrine disruptors (ED) can either bind to membrane estrogen receptors (mER, to mediate a non-genomic response) or cytoplasmic estrogen receptors (ER, to mediate a genomic response). Once bound to the ligands the dimerization of estrogen receptors can take place and will activate phosphorylation cascade and enter the nucleus and activates estrogen response elements (ERE) to produce a genomic response (ER = Estrogen receptor, mER = membrane estrogen receptor, ED = endocrine disruptor, ERE = estrogen response element)

Table 1-2 The hierarchy of human exposure to xeno-estrogens (Thomson et al, 2003)

Xenoestrogen	Estimated Blood EQ (mg/L)
BisphenolA	1.6×10^{-3}
Genistein	1.5×10^{-3}
Alkyl phenols	8.4×10^{-4}
Kaempferol	2.8×10^{-4}
Phloretin	2.8×10^{-4}
Diadzein	2.2×10^{-4}
Quercetin	1.9×10^{-5}
Enterolactone	7.8×10^{-6}
Endosulfan	4.5×10^{-6}
PCBs	2.7×10^{-6}
BHA	1.4×10^{-6}
DDT + metabolites	3.2×10^{-7}
Enterodiol	4.0×10^{-8}

PCB = polychlorinated biphenyls, BHA = butylated hydroxyanisol

1.2.2.1. Bisphenol A (BPA)

BPA (Figure 1-2) is a chemical released into food from epoxy resins that coat food packaging (i.e. food and beverage cans)[43]. BPA is also present in polycarbonate plastics, used in common household items like baby bottles, plastic bottles, jugs, CDs, DVDs, electronics, construction glazing, sports safety equipment and medical devices, and is released into the environment upon degradation of these materials. It is also present in

dental sealants as BPA-diglycidyl methacrylate (BisGMA)[44]. Both in-vitro and in-vivo studies have shown that BPA possess a weak estrogenic action [7] and that it can bind to both estrogen receptors α and β; with a stronger affinity for ER β to exert an action [45]. Recently, it has been shown that the endocrine disrupting activity of BPA could be mediated through membrane estrogen receptor pathway [45]. There are reports on developmental abnormalities caused by BPA in rodents at a low exposure level [36]and reviewed in vom Saal FS and Hughes C [37]. Due to its strong interaction, recently Estrogen Related Receptor was also considered as a putative receptor for BPA (Figure 1-3).

Structure of BPA:

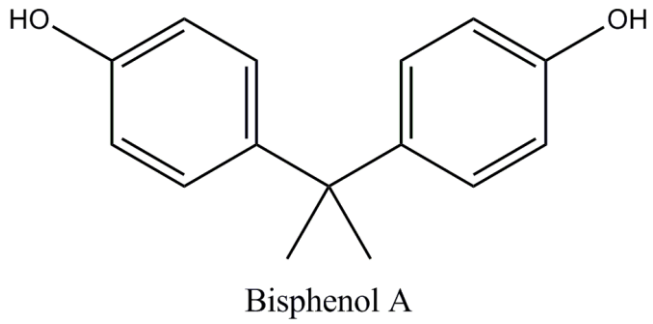

Figure 1-2 Structure of BPA

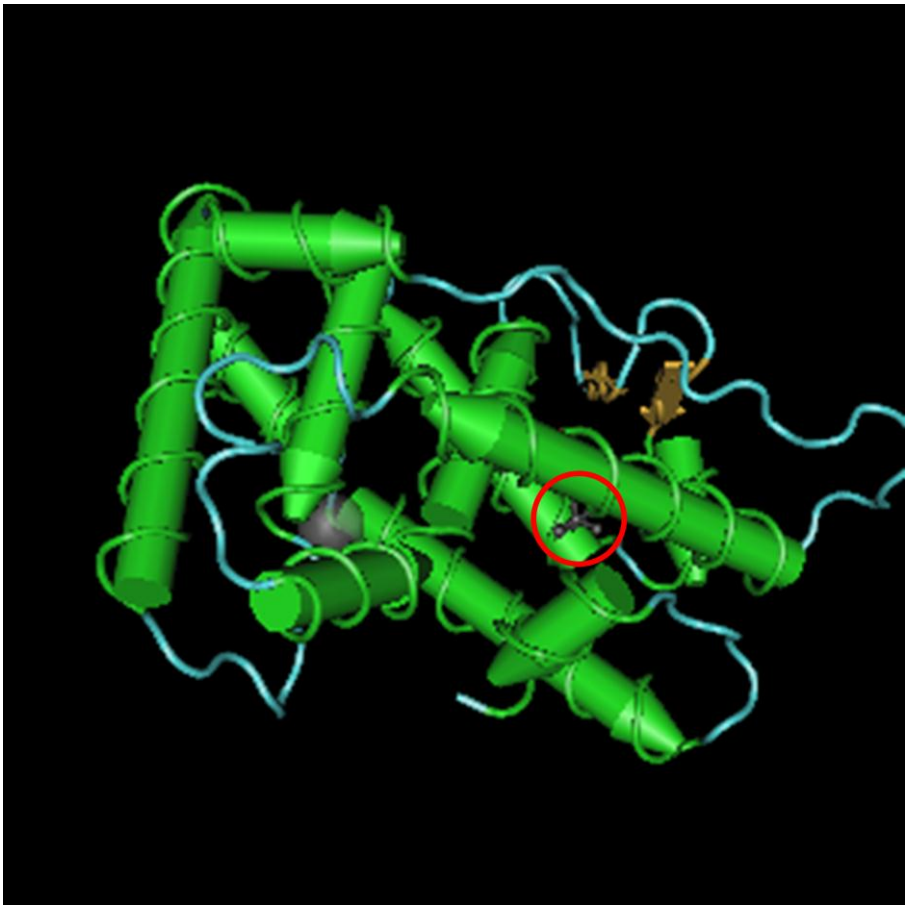

Figure 1-3 X-Ray structure of estrogen related receptor gamma in complex with BPA [46]. BPA was shown in the round insert.

Adapted from http://www.ncbi.nlm.nih.gov/Structure/mmdb/mmdbsrv.cgi?Dopt=s&uid=62662

1.2.2.2. p-nonylphenol (4-nonylphenol, 4-NP)

4-Nonylphenols are biodegradable products of Alkylphenol Ethoxylates (APE) that are used as non-ionic detergents in paints, industrial and household cleaning agents, in pesticide formulation and rubber manufacture (Figure 1-4) [47]. More than 90% of the Alkyl Phenol Ethoxylates produced are nonylphenol ethoxylates [48]. Hence, from the degradation products, 4-nonylphenol (4-NP) forms a significant contributor to environmental contamination. Nonylphenol-9 is used as a contraceptive spermicidal agent and *tris*(nonylphenyl) phosphate is used as an antioxidant in the production of some polystyrene and polyvinyl films, and both were found to be 4-nonylphenol congeners and

contaminate the environment[48]. Human exposure can happen by dermal absorption, ingestion or inhalation. 4-NP and other alkylphenol's weak estrogenic action has been proven by both *in vitro* and *in vivo* methods and their endocrine disruptor action is predominantly mediated through binding to estrogen receptors and modifying physiological function [7].

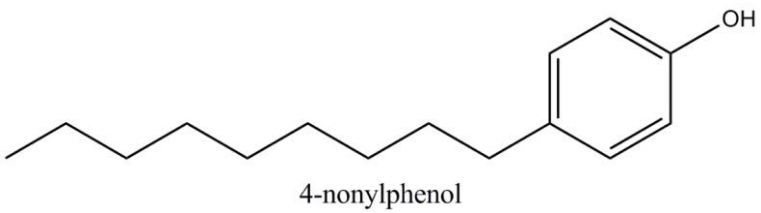

Figure 1-4 Structure of 4-nonylphenol

1.2.2.3. Genistein (Gen)

Genistein is a phytoestrogen present naturally as several β-glucosides which are metabolized by intestinal microflora to genistein (Figure 1-5) [49, 50]. It is present in soya based foods. Genistein can bind to estrogen receptors (ERs) as well as activate a number of estrogen responsive genes *in vitro* including pS2 and *c-fos*. Engel *et al*. reported its presence in the amniotic fluids of human[51] and Todaka *et al*. reported that it can cross the human placenta[52]. Genestein is also an inhibitor of tyrosine kinases that can exert multiple, estrogen-independent, effects on a variety of cellular processes [49, 53].

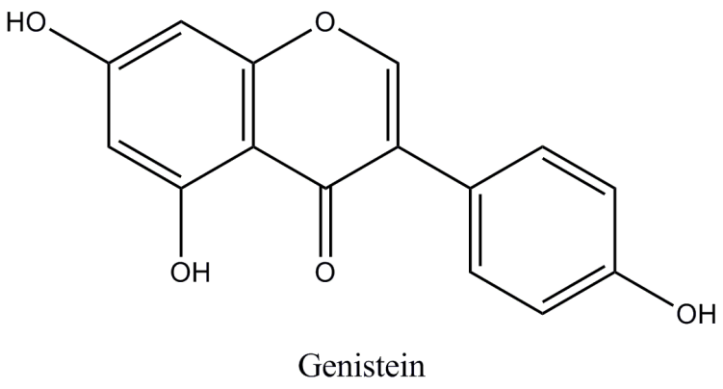

Figure 1-5 Structure of genistein

1.3. EXPOSURE ASSESSMENT AND DOSE EFFECTS OF ENDOCRINE DISRUPTORS

Reports in the literature conflict over the dose of endocrine disruptors that will produce an adverse effect in an adult and/or in a developing fetus. Traditionally toxicologists used a threshold dose, below which the dose was considered to have no significant adverse effects and to be safe for use in non-genomic toxic chemicals [54]. Based upon that a NOAEL (No – Observed – Adverse – Effect – Level) was introduced to depict the highest dose which imparts no adverse effects compared to control (zero) dose [54]. The acceptable daily intake dose (AID) or Reference dose (R_fD) is calculated using NOAEL as standard. It is expressed as mg/kg body weight /day. The concept of threshold theory cannot be applied to endocrine disruptors, as both *in vitro* and *in vivo* studies have shown a non-monotonic response with endocrine disruptors [39]. In a non-monotonic dose response curve, the shape of the curve reverses as the contamination increases as shown in the Figure below (Figure 1-7) [1]. Hence, a variation in effect can be seen well below threshold levels measured using traditional toxicology parameters. Also variations were evident in different developmental stages of organisms at the same dose levels as well as the duration of action of the chemical [39], which implies that a non-toxic low dose can cause an unprecedented adverse reaction in the developing fetus. A similar opinion was putforth recently in an Environmental Protective Agency (EPA), NIH, USA sponsored workshop, which weighed the peer reviewed articles on low dose BPA effects on fetal development [54]. The NOAEL for genistein in rats was found to be 50mg/kg/day and the no observed effective level (NOEL) was found to be 5mg/kg/day [55]. Figure 1.6 shows the common dose response curves observed with xenobiotics and Figure 1.7 shows a typical nonmonotonic response curve on proliferation response of MCF-7 cells (an estrogen responsive breast cancer cell line) to various concentrations of estradiol.

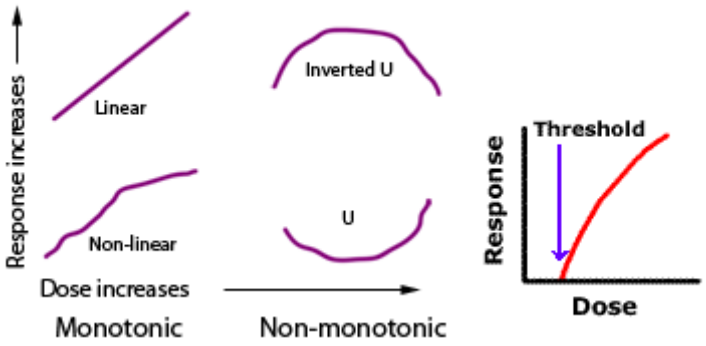

Figure 1-6 Common dose response curves observed with xenobiotics

Adapted from http://www.ourstolenfuture.org/newscience/lowdose/2007/2007-0525nmdrc.html

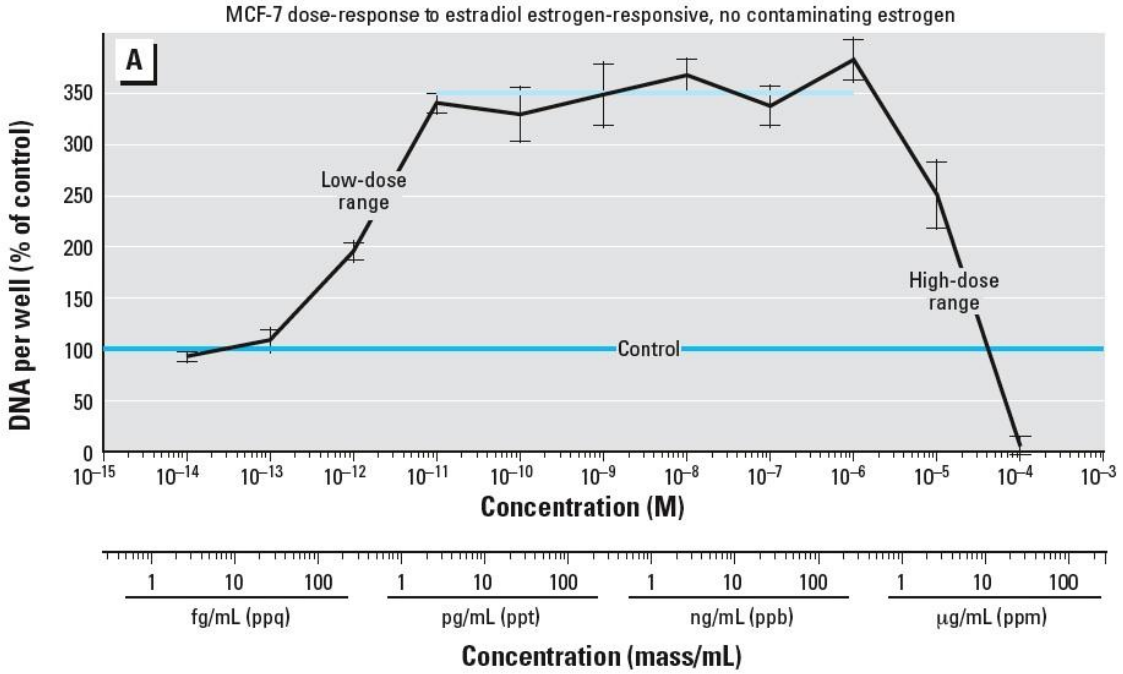

Figure 1-7 Typical non monotonic dose response curve observed after treating a breast cancer cell line (MCF7 cells) with various doses of 17-β-estradiol.

Reproduced with permission from Welshons *et al* (2003) [56].

1.3.1. Low dose effects

Vom Saal has advocated recognising the importance of how much a low dose of an endocrine disruptor can affect development [37]. His research group had reported that a small increment in the estradiol concentration has resulted in a significant increase in the

prostate weight of the male fetuses [5]. The experiment involved surgically implanting silastic estradiol into pregnant mice to mimic 2-8 fold free estradiol in the circulation. However, the higher dose inhibited the prostate development. In another study the same group has shown that the fetal position in the womb affects the prostate weight and other reproductive and developmental parameters due to a small increment in estradiol concentration [57]. Welshon *et al* has argued that low dose effects are mediated through the estrogen receptors and that a different mechanism is involved in toxicological or high dose effects [1]. Studies in prenatal and postnatal exposure to low, environmentally relevant, concentrations of estrogenic chemicals have resulted in a range of developmental effects, such as; sex reversal in turtles [58], reduced testes sizes and sertoli cell numbers in Wistar rats [59], enhanced induction of cytochrome P-450 1A activity in mice [60], increased anogenital distances in CD-1 mice [61], increased preputial gland size in mice [36], and increased reproductive tract organ sizes [5, 62]. Initial studies from vom Saal and others have also shown that it is the low dose of BPA or estrogens which causes the increased prostatic bud number, cell proliferation and adult prostate size in mice [62]. Prins *et al* hypothesized that environmentally relevant doses of BPA increased the sensitivity of the prostate gland to carcinogenesis following adult insults such as elevated circulating estrogens [63]. At low dose of estrogens Putz *et al* observed an inverted manifestation of most of the effects observed at high dose range [63]. In neonates, a low dose of estrogen may alter steroidal gene expression along the developing Wolffian duct and urogenital sinus, which in turn could be responsible for the observed organ-specific responses [63]. The environmentally relevant dose of BPA is considered to be 0.23ng/ml in tissue culture medium [64]. In a recent study by vom Saal *et al*, it was shown that an estrogenic endocrine disruptors (EDs) would be biologically active in the fetus if supplemental estrogenic activity of the free chemical in the blood was equivalent to an

increase of free estradiol of only 0.1 pg/ml (0.37pM estradiol concentration equivalent) [5]. It has been shown that low doses of estrogen can stimulate 2α, and 16α- hydroxylase activity in the liver [65]. Nagel *et al*. (1997) predicted the dose of BPA (fed to pregnant mice) that will be biologically active in mouse fetuses based upon a comparison of BPA and estradiol in terms of both the relative affinity for nuclear ERs and the ability to bind to serum estrogen-binding proteins that effectively restrict estradiol (but not BPA) uptake into cells. This has been referred to as the "physiologic approach" to dose selection [37]. Due to strain and species variability on overt toxicity findings in low dose estrogen exposure, the National Toxicology Programme panel, NIH, USA NTP (2001) advised that further studies should evaluate the effects of EDs on different species and strains in comparison to a positive control [37]. For example the dose of ethinylestradiol in CD-SD rats (50μg/Kg/day) is 100-400 times the dose required to produce an effect in women (0.5μg/Kg/day) [37], while CF-1 mice requires about 25,000 times less dose to produce a similar effect (0.002μg/kg/day [37].

In rats, exposure to low dose BPA in-utero was found to have adverse effects on reproductive function in male offspring. Although researchers have failed to replicate these particular findings, many other researchers have shown low dose effects in mice, rats, fish, snails and frogs [25]. It was found recently that BPA binds to ERR gamma (a nuclear orphan receptor present in brain) with IC_{50} of 13.1 nM, suggesting that it may have a role in toxicity from low dose exposure [66]. At environmentally relevant doses, the only three studies performed and published, to date, have failed to observe any adverse effects on exposure to genistein, BPA, 4-NP [67]. 4-NP was detected in drinking water at 0.7 μg/L [68]. Dietary exposure of 4-NP is estimated to be 7.5 μg/day for an adult man [68].

Infant exposure levels were observed to be about 0.2µg/day in breast fed infants and 1.4 µg/day in infants fed with infant formula [68].

An acute exposure cohort study has found that 2,3,7,8-Tetrachlorodibenzodioxin (TCDD , a type of dioxin) has a high half life ($t_{1/2}$) in humans (4-7 years) [69]. It is detectable at 10 parts per trillion in background plasma and adipose tissue of the general population [70]. The low level considered to be non-toxic to adults may adversely affect fetal growth and development, even increasing the risk of thyroid cancer [70]. Fetuses, newborns and infants are at high risk as they are exposed to higher levels than adults through placental and lactational routes during development [70]. Since dioxins accumulate in fatty tissues, it is unsurprising that $t_{1/2}$ of dioxin has been found to be greater in women than men [70]. TCDD were detected at1.3-2.1 ppt in adipose tissue and 0.76-1.51 ppt in liver of human stillborn babies, suggesting the placental transfer in exposed regions[70]. On a lipid basis comparison dioxin concentration is comparable in maternal serum and cord blood [70]. In Germany breastfed infants have been shown to have 400 pg/g of dioxin levels with a mean of 70 pg/g liver fat [71]. In rodents, dioxin can induce fetotoxicity at a dose equivalent to or less than NOAL when administered to pregnant mothers [72]. Edema and hypoplasia of the lymphoid organs were observed at relatively low doses in rodent fetuses [17]. Dioxin produced reproductive toxicity in offspring in a three generational reproductive function study in rats [71]. Dioxin causes hydronephrosis and cleft palate in mice at doses below which any overt fetal or maternal toxicity is detected [17].

1.3.2. High dose effects

As discussed above, the adverse effects of EDs differ widely depending upon the level of exposure. Recent research has shown that high concentrations of exogenous estrogens, natural or synthetic, administered during early postnatal life can advance the pubertal

process in females, but delay its onset in males [63]. Few groups have observed a significant delay in vaginal opening in rodents at higher concentrations of di-2-ethyl hexyl phthalate (DEHP) [73]. Early post natal exposure to high doses of estradiol benzoate abrogated the puberty marker PPS in males, indicating feminisation [65]. The same study also identified a demasculanization of the hepatic enzyme activity patterns of prepubertal males treated with high concentrations of estradiol benzoate (EB). In contrast, low-dose animals were super-masculinised, suggesting advanced puberty. However the super-masculinization of 35 day old male rats that were neonatally exposed to mid-range concentrations of EB indicated that neonatal imprinting may have occurred in a similar fashion to that of testosterone imprinting [65]. It has been hypothesized that intrauterine high estrogen exposure can predispose the offspring to mammary cancer [74]. In human epidemiological studies, twin pregnancy has been used as an indicator of high estrogen exposure and preeclampsia as an indicator of low estrogen exposure [75]. Potischman N and Troisi R (1999) have reported a high correlation of breast cancer with the former and a low correlation with the latter [75, 76]. A study by Lee *et al*. has shown that BPA disrupts placental function and leads to reproductive disorders [77]. The adverse effects of DES exposure were accompanied by a transient decrease in estrogen receptor (ER) alpha and steroidogenic enzyme mRNA levels in the testis of male mice at 3 weeks of age [78, 79].

The adverse effects observed with 2,3,7,8-Tetrachlorodibenzodioxin (TCDD) exposures were “high-dose effects”. The amounts that can be regarded as high dose vary between species. For example 1µg/kg is the lethal dose-50 (LD50) for guinea pigs, and >1000µg/kg is the LD50 for hamsters [72]. However the levels that induce fetal toxicity in hamsters, rats, mice and guinea pigs were observed to be within a similar range [72]. Multigenerational studies indicate that 1ng/Kg is the Low Observed Adverse Effective

Level (LOAEL) for dioxins (TCDD). TCDD and other Arylhydrocarbon receptor (AhR) ligands are potent developmental toxicants in multiple species [72].

The adverse effects of low and high doses of various endocrine disruptors on various developmental stages are described in the below table (Table 1-3).

Table 1-3 Deleterious effects of EDs on various developmental stages

No.	Chemical	Group	Deletarious effects on exposure at critical windows
1.	Diethylstilbestrol	Estrogen agonist	1. Incidence of clear cell cervical adenocarcinoma in human (in-utero exposure in humans) 2. Incidence of preterm birth and 2nd trimester foetal loss in humans (Kaufman *et al*., 2000) 3. Testicular dysgenesis syndrome? (humans)
2.	Bisphenol A	Xenoestrogen	1. Reproductive abnormalities in experimental animals (vom Saal et al., 1997) 2. Ambiguous genitalia in humans? (Paris *et al*., 2006) 3. Recurrent miscarriages in human (Suigiura-Ogasawara *et al*., 2005) 4. Alters sexual differentiation of brain and behaviour 5. Prostatic interepithelial neoplasia in rodents (Prins *et al*., 2007)

			6. Stimulation of mammary growth and ductal mammary carcinoma in rodents (Murray *et al*., 2007; Durando *et al*., 2007) 7. Alters immune functions in rodents 8. Meiotic aneuploidy in female mice (Hunt *et al*., 2003) 9. Implantation failure in mice (Takai *et al*., 2000; Berger *et al*., 2007) 10. Low birth weight, hypogonadotropism, dampened LH surge in female lambs (Savabieasfahani *et al*., 2006) 11. Modulates drug efflux mechanism of placenta (Jin and Audus 2005)
3.	4-nonylphenol	Xenoestrogen	1. Reproductive abnormalities in rodents (Lee *et al*., 1998) 2. Delayed testes descent in rodents (Lee *et al*., 1998)

4.	Dioxin (TCDD)	Antiestrogen	1. Premature reproductive senescence in female rats (Shi *et al*., 2007) 2. Miscarriage in monkeys (Wilbur, 1984) 3. Incidence of preterm birth in human (Revich *et al*., 2001)
5.	DDT	Xenoestrogen	1. Micropenis in alligators (Guillette *et al.*, 1994) 2. Preterm birth in human (Longnecker *et al*., 2001)
6.	Vinclozolin	Antiandrogen	1. Developmental abnormalities in various systems in rodents (Anway *et al*.,2006a) 2. Transgenerational effects (Anway *et al*., 2006a and b)

1.3.3. Wild and marine animal observations

The first evidence of adverse effects of phytoestrogens in animal reproduction came over 60 years ago from Western Australia where male rams, feeding on clover pasture, became feminised and unable to breed. The cause of this phenomenon was eventually identified as the high levels of the isoflavones genistein and diadzein in [80]. Following a possible dichlorodiphenyltrichloroethane (DDT) contamination of Lake Apopka, Florida, reproductive defects (such as small phallus size, reduced hatchability and poor survivorship) were observed in the alligators. Researchers found that 17-β estradiol levels were doubled in the alligators at 6 months of age and argued that DDT had endocrine disrupting actions. [4]. More recent studies have shown a correlation between residues of dioxin equivalents and reproductive and developmental toxicities in fish-eating bird populations [81]. A report on the decline of bald eagles in a region where the fish are contaminated by DDT also points towards its reproductive toxicity in wildlife populations [82]. There are also reports on industrial pollutants like alkylphenols having estrogenic actions in marine fish [81]. Other reports have shown developmental defects in the oviducts and ovaries in herring gulls of Lake Ontario, thyroid enlargement in 2-3 year old salmon, and reproductive failure and early mortality in fish-eating birds in the Great Lakes. It is hypothesised that these changes may have occurred due to the ED contamination of the environment [83]. However the usefulness of wildlife as a model for human disease is still questionable, due to the exposure of the former to a wide variety of chemicals other than the specific endocrine modulators under investigation.

1.4. BPA CONTROVERSY AND TIME LINE FOR FDA REGULATION

- Large scale use started after 1950 (6 billion pound per year)
- 1988 – Environmental Protective Agency (EPA) – based on Low Observable Adverse Effective Level (LOAEL) = 50mg/Kg/day fixed the Reference dose (RfD) = 50 µg/Kg/day
- 1997 – 2003 – low dose studies (below LOAEL) in rodents showed developmental abnormalities
- 1999 – FDA statement – BPA safe at low levels
- 2003 – 2006 NIH – NTP panel conducted peer-reviewing and reported as safe
- 2007 – NIH – NTP panel re-assessed – some concerns on the potential effects of BPA on the brain, behaviour and prostate gland in human fetus, infants and children
- 2008 FDA – 'no safety concern at current exposure limits'
- 2008 – Canada Banned Polycarbonated (PC) baby bottles
- 2008 – congressional investigation
- 2009 – Some states in USA started some sort of banning BPA use (New York)
- 2010 – FDA – 'some concerns on the potential effects of BPA on the brain, behaviour and prostate gland in human fetus, infants and children'
- 2010 – Canada declared BPA as a toxic chemical

This section is adapted from http://www.ewg.org/reports/bpatimeline (accessed on 08/07/2011)

1.5. MATERNAL EXPOSURE AND METABOLISM

Humans and animals are constantly exposed to many endocrine disruptors. Major exposure occurs through dietary contamination. After ingestion, these chemicals are metabolized by host enzymes to form metabolites could either be inactive or possess potent hormonal activity [84, 85]. Metabolism of these chemicals is dependent upon the physiological conditions of the animal. For example in mammals there are two major pathways for the metabolism of BPA viz. glucuronidation and sulphation [85]. Glucuronide metabolites are generally found to be less hormonally active than the parent compound. While sulphation of BPA reduces its estrogenicity, it can rapidly be acted on by sulphatase to revert back to the parental compound. Hepatic glucuronidation is reduced during pregnancy due to lower levels of multidrug resistant (MDR) proteins [85, 86]. Human fetal liver is also deficient in UDP-glucuronosyltransferases (UGT), and the activity of UGT in rat fetus is non-detectable [85, 87, 88]. These factors can contribute to the accumulation of more toxic parent compounds in the fetus. Cytochrome P-450 (CYP-450) can metabolize BPA into bisphenol-*o*-quinone which has been shown to have enhanced estrogenic [89] or toxic activities including DNA adduct formation with BPA metabolites [84, 85]. Metabolites are excreted in bile; however, greater levels of metabolites (around 3 fold greater) are eliminated via the portal vein in pregnant rats [86]. Earlier studies have shown that BPA is excreted in the faeces, either as free BPA or in hydroxylated and conjugated forms in humans [90], and while the glucuronic acid conjugate, which is the major metabolite of BPA, is excreted through urine [91]. Negishi *et al* (2004) have shown that the concentration-time curve is longer in primates than in rats, [92] This together with other reports on variation in metabolism and disposition of BPA over the species and strains suggests a possible species / strain variability in metabolism and disposition of other endocrine disruptors[93]. Thus variation in

metabolism of BPA has been observed between the species, strain, gender and physiological status of animals [91, 93, 94]. Researchers have also demonstrated the presence of metabolites in the animal fetus such as glucuronides and disaccharide conjugates [91]. Although, the function of these metabolites is unknown, it is hypothesized that they might have lower estrogenic activity than the parent compound. It has been shown that glucuronidation of xeno-estrogens abrogates their estrogenic action in *in-vitro* assays. It has been reported that during pregnancy there is a reduction in the activity of hepatic enzymes involved in glucuronidating xeno-estrogens. [86, 95]. Conversely, there are reports which suggest that the metabolites of 4-NP may impart more endocrine disruptor activity than the parent compound, probably through hydroxylation of the aliphatic side chain [66].

Other researchers have shown that the major source of dioxin intake is through the consumption of contaminated seafood. After ingestion, dioxin is distributed to tissues via blood and accumulates in liver and adipose tissue They also showed that accumulated Polychlorinated dibenzodioxins (PCDD) concentrations in maternal tissues were highest in the placenta and lowest in the cord blood [96]. Different affinities for PCDDs, Polychlorinated dibenzofurans (PCDFs) and Polychlorinated biphenyls (PCBs) were also observed. Transfer of dioxins across the placenta is also related to the concentration of free dioxins in maternal blood. 2,3,7,8-TCDD (a highly toxic environmental dioxin) exists mostly bound to lipoprotein (80%), as well as other plasma proteins (15%) and red blood cells (5%) in the blood [96]. There are wide variations in reports of metabolic activities of different dioxin congeners in rats [96]. Some studies have shown that bisphenols and nonylphenols bioaccumulate in fat (brown fat) and can affect energy balance [97, 98].

Soy isoflavones are cleaved by gut microbial β-glucosidases after ingestion in animals and humans. Aglycones are absorbed and converted to glucuronides and sulphate conjugates in the liver and intestine, and are then released into the circulation [99]. Soy based foods contains estrogenic precursors which, by the action of intestinal microbial glycosidases, are converted to active phytoestrogens such as genistein [52]. Once absorbed, these undergo enterohepatic recirculation and are excreted through urine as glucuronide conjugates [52]. Like other EDs, genistein also undergoes glucuronidation and sulphation. Todaka *et al* observed greater concentrations of genistein in cord blood than in maternal serum [52].

In human volunteers BPA was rapidly eliminated as BPA glucuronide via urine ($t_{1/2}$ = 3.36 hrs) with no gender variation [100]. However, gender differences in serum concentrations of BPA have been reported with a higher concentration in men than women (1.49 ± 0.11 v/s 0.64 ± 0.1) [101]. An interspecies difference was observed between rodents and primates for the metabolism of BPA [92], with primates being found to be the best model to study the toxicokinetics of BPA [102].

Parahydroxybenzoic acid is the primary metabolite of 4-NP. When sulphation and glucuronidation take place metabolites are excreted via urine. No accumulation or placental transfer could be detected at low (environmentally relevant) doses of 4-NP in a rat study [68].

1.5.1. Placental transfer, metabolism and fetal exposure

In humans, most endocrine disruptors are known to transfer across the placenta [51, 96]. TCDD accumulates in the placenta because of the presence of arylhydrocarbon receptors [96, 103]. which slow placental transfer of TCDD due to high octanol/water partition

coefficients [90]. Compounds with a low partition coefficient are transferred quickly across the placenta and their maximal concentrations will be 30-100% of the maximal maternal plasma levels [90]. Dioxins are also transported by binding with proteins; [96] so when dioxin is bound to plasma proteins it cannot cross the placenta and accumulates in maternal blood [96].

Detectable amounts of BPA were found in rat fetuses within 10 minutes of oral administration to their mothers. The levels peaked (0.004 % of the parent dose per g of the fetus) at 20 minutes and then gradually decreased over 10 hours. Initial half life was rapid in the fetus (33 min), but terminal half life was longer (173 hrs) [90]. The researchers also observed that MRT (mean retention time), VRT (variance retention time) and AUC (Area Under the Curve) were also found to vary considerably from the maternal values. BPA can rapidly cross the placenta, as it has a small partition coefficient (P_{ow} 3.32) [90]. BPA remains in fetal tissues longer than in maternal tissues [104]. The pharmacokinetics of BPA at higher doses have been observed to be nonlinear, possibly due to metabolic saturation [104]. Fetal rat microsomes showed sluggish glucuronidase activity [105], suggesting the passage of glucuronidase from placenta [105]. As described in fig 1.4.1, the placental transfer of BPA is thought to follow first order kinetics [104], although in this study there was little evidence of BPA transfer to the fetus. Zalko *et al* (80) recovered 0.4 %, 0.3% 0.6% and 4.1% of total radioactivity from maternal uterus, amniotic fluid, placenta and littermates after a single subcutaneous dose of radioactive BPA during gestation [106]. The kinetics of BPA mimics that of diethylstylbestrol (DES) in all aspects [90].

The capacity of the fetus to conjugate EDs is very much lower than maternal capacity, consequently the parent compound may accumulate and be more toxic to the fetus; this

relationship has been demonstrated with genistein [99]. Thyroid hormone transferred from mother to fetus is critical for brain development [107]. The accumulation of DES in fetal genital organs is one of the reasons for fetal predisposition to reproductive toxicity. The developmental defects observed in the fetus were tabulated in Table. 1.3

1.6. ACTIONS OF ENDOCRINE DISRUPTORS IN THE PLACENTA

In the previous discussion we have seen that EDs can be metabolized and also be transferred through placenta. During these events EDs can cause some alteration in the normal histology of the tissue.

1.6.1. Placental transporters and endocrine disruptors

Many researchers have reported that estrogen and progesterone can influence the function and expression of (Multi Drug Resistance) MDR proteins, p-glycoprotein (p-gp, a prototype of MDR in placenta). It is reported that progesterone at high concentrations inhibits and at low concentration activates the P-gp activity. while progesterone itself is not transferred [108]. Estrogen reverses Breast Cancer Resistant Protein (BCRP, a prototype of ATP Binding Cassette Transporter) mediated drug resistance [109]. Previously it was reported that BPA is a substrate for P-gp in the intestine [110]. In BeWo choriocarcinoma cells BPA has a direct stimulatory action on the drug efflux mechanism, thought to be by direct interaction between BPA and the drug efflux transporter [111]. However, in combination with estradiol, BPA inhibited the efflux mechanism, but did not alter P-gp ATPase activity. Placental transfer of dioxins and furans is thought to be through the fatty acid transport mechanism [26].

1.6.2. Effects on placental hormones

TCDD can decrease the production of progesterone in placental cells [26] and that TCDD administration in CF1 mice increases the amniotic levels of lipid metabolites[112]. The inhibition may be due to reduction in the activity of the mitochondrial enzyme converting cholesterol into pregnenolone ($CYP450_{sec}$) or pregnenolone to progesterone. Inappropriate progesterone secretion leads to adverse pregnancy outcomes. Decreased progesterone levels increase the susceptibility of uterine muscle to contractile stimuli which may result in preterm labour. Halogenated hydrocarbons are associated with intrauterine growth retardation and developmental deficits. Phytoestrogens inhibit progesterone production from choriocarcinoma cell lines [113], but the effect on hCG production varied depending on the phytoestrogen and the dose used [114]. A dose-dependent inhibition of hCG was reported in human term trophoblasts treated with phytoestrogens [115]. These results show that the inhibitory effect of phytoestrogens was mediated by inhibiting hCG production. The phytoestrogen induced the expression of ERα and progesterone receptor (PR) in JEG-3 cells presents another pathway to inhibit proliferation through inducing various hormone receptors [114]. On an equimolar concentration of nanomolar range, 4-NP induced significantly greater quantities of hCG over a prolonged period than did 17-β-estradiol in first trimester placental explants [116]. The enhanced activity of 4-NP may be due to the formation of more stable and potent metabolites [116]. TCDD increases the secretion of immunoactive hCG from primary syncytiotrophoblast-like cultures in a dose-dependent manner, but does not affect the bioactive hormone [117].

1.6.3. Effects of drug and steroid metabolizing enzymes of placenta

CYP-19 (aromatase) catalyzes the aromatization of androgens into estrogens. It was shown in JEG-3 cells that placental aromatase activity was inhibited in a dose-dependent

manner by TCDD treatment [118]. BPA can also inhibit aromatase activity with an IC_{50} of about 300μM, by interacting directly with the aromatase enzymatic complex [119].

1.6.4. Growth and differentiation

When pregnant rats were exposure to TCDD on gestational day 15 (GD15) the levels of eight proteins were significantly increased, of which Hsp27, β-2-macroglobulin (β-2M) and GAPDH are considered to be markers for TCDD toxicity in placenta. the other three proteins whose expression levels were increased were haptoglobulin, C-reactive protein, and apolipoprotein M and are considered to be acute phase proteins [120]. The amount of GAPDH was shown to increase in the placenta after exposure of TCDD, which is the first evidence to suggest that the TCDD exposed placenta was in a hypoxic state. Placental hypoxia, caused by reduction in placental blood flow, is thought to be one of the mechanisms for intrauterine fetal death. On GD20 TCDD exposed placenta is thought to have disrupted blood capillaries and reduced placental blood flow, and researchers have observed that exposure of mice to TCDD resulted in rupture of the blood barrier in the Labyrinthine Zone (LZ) of the placenta [121]. The possibility of oxidative stress in placenta produced by TCDD was related to the expression of Hsp27 and β-2M. [120]. In one study it was shown that TCDD induced the expression of CYP1A1 m-RNA in the placenta of a 1600ng TCDD/kg exposed group. Based on this and previous studies, TCDD-dependent changes in placenta may increase in protein level expression patternsof heat shock protein 27 (Hsp27) and β-2M; and the secondary response is hypoxia characterized by an increased expression levels of GAPDH. Phytoestrogens were found to reduce the proliferation of choriocarcinoma cell lines [113, 114]. 4-NP at equimolar concentrations showed cell differentiation and apoptosis in first trimester human placental explants[116].

1.7. THE ROLE OF THE ENDOCRINE SYSTEM IN FETAL DEVELOPMENT

The endocrine system plays a major role throughout pregnancy, fetal development, growth and parturition. There is interplay of maternal, fetal and placental hormones at all stages of fetal development.

1.7.1. Placental hormones

Initially hormones synthesized from syncytiotrophoblasts are secreted into the maternal compartment and transferred to the fetal compartment. This happens during the 8th week of pregnancy. Syncytiotrophoblasts produce estrogen, progesterone, hCG, placental lactogen, and placental GH (GHv) to maintain the pregnancy [122].

1.7.1.1. Estrogen

Estrone, estradiol and estriol are the three types of estrogens produced by the human placenta near term [123]. The major substrates for estrogen production come from both maternal and fetal adrenal glands [123]. Placental estrogens maintain uteroplacental blood flow [123].

1.7.1.2. Progesterone

At 12 weeks of pregnancy, the placenta produces progesterone. Estrogens, human chorionic gonadotropin (hCG) and IGFs regulate placental progesterone production [124]. Progesterone maintains quiescence of the myometrium and helps to avoid fetal graft rejection, but the real significance of progesterone in the fetus is not yet known. An association between progesterone, estrogen and birth weight or placental weight has been reported [122].

1.7.1.3. Polypeptide hormones

Human Chorionic Gonadotropin (hCG): - Placenta starts producing hCG during the first half of gestation. GnRH acts in a paracrine manner to regulate hCG production [125, 126] and is involved in the maintenance of corpus luteum (CL), fetal testes development, and progesterone production in placenta. Most of the thyrotropic activity in placenta and maternal serum may be due to the TSH-like activities of hCG. It also increases uterine blood flow [122].

Human Prolactin (hPL): - hPL is produced by the placenta during the second half of gestation. It has a weak growth hormone-like activity, which imparts an anti-insulin effect on maternal macronutrient metabolism to enhance the fetal supplementation of glucose and amino acids through maternal intake [125]. Furthermore, it stimulates fetal growth.

Human placental lactogen: - Human placental lactogen promotes early embryonic growth [122], stimulates IGF-1 and insulin production.

Placental Growth Hormone (variant) GHv: - GHv is secreted into the maternal circulation where it inhibits the production of pituitary GH [122]. Intra uterine growth restriction (IUGR) is characterized by a reduced level of GHv in maternal serum and placenta [127].

Renin: - Renin's presence in the chorion is observed throughout pregnancy [128]. It is thought to be involved in the production of fetal angiotensinogen II, which also plays a role in fetal growth [129].

Lutinizing hormone releasing hormone (LHRH), Thyroxine releasing hormone (TRH), Somatostatin, Growth hormone releasing hormone (GHRH): - LHRH may have a paracrine regulation on hCG and steroid production [126]. Placental TRH regulation mimics hypothalamic TRH as the levels of placental TRH changes depending on the

thyroid status of the fetus (increases in case of hypothyroid fetus) [130]. Placental corticotrophin release hormone (CRH) may modulate maternal pituitary and adrenal function. Since the glucocorticoids increase CRH production, fetal glucocorticoids near term can increase both CRH and Pro-opio melano cortin (POMC) to augment prenatal fetal cortisol production [131].

1.7.1.4. Growth factors

Insulin Like Growth Factor I and II (IGF-I and II): - IGF-I regulates placental growth in an autocrine or paracrine manner [132]. IGF II and hCG influence uterine blood flow [122]. They are associated with increased somatic cell proliferation and enhanced transfer of glucose and amino acids across the placenta [122]. IGF-I also influences postnatal growth [122].

1.7.2. Fetal hormones

1.7.2.1. Growth hormone and prolactin

The fetal pituitary produces GH from 8-10 weeks of gestation [133]. GH production increases till mid-gestation and then falls [132]. The pattern of fetal GH production reflects maturation of the hypothalamo-pituitary and forebrain functions of fetus. At mid-gestation the development of the pituitary portal vascular system is indicated by unrestrained secretion of GH. Somatostatin, in late gestation regulation of GH appears. [133]. GH secretion is thought to modulate hypothalamic function in the fetus [132]. Mature responses to GH appear postnatally. Prolactin levels are very low until late gestation and secretion increases near term [134]. The intermediary pituitary produces α-MSH and β-endorphin, both of these hormones play a role in fetal growth. The posterior pituitary develops around 10-12 weeks and secretes antidiuretic hormone (ADH) and

oxytocin. ADH is secreted in response to osmotic stimuli and its antidiuretic effects on fetal kidneys are well documented [135]. Fetal ADH rapidly responds to intrauterine stress conditions such as hypoxia and osmolarity changes [132]. ADH prevents lung fluid production in fetal sheep and goats[132]. It is also thought to have an effect on water transport via amniotic membranes [132].

1.7.2.2. Adrenal hormones

Fetal pituitary ACTH stimulates fetal adrenal glands to produce the (Dehydroepiandrosterone) DHEA, pregnenolone and conjugates which are the substrates for placental estrogen synthesis. The fetal adrenal gland can produce all the steroidogenic apoenzymes produced by the adult adrenal, and also produces cortisol. Fetal cortisol regulates hepatic cholesterol synthesis, and is metabolized to inactive cortisone by dehydrogenase enzymes in fetal tissues. This metabolism protects the anabolic environment of the fetus. In late gestation fetal liver and lung have ketosteroid reductase activity, cortisone is metabolised to cortisol, and a cortisol surge observed in late gestation is the major maturational stimulus for preparing the fetus for extrauterine life. Near term the fetal adrenal gland also secretes aldosterone [132]. Aldosterone in the human fetus is unresponsive to the renin angiotensin system and has been found to be involved in sodium excretion in the preterm human baby and fetal sheep [132].

1.7.2.3. Thyroid and parathyroid hormones

Human fetal thyroid buds are visible from day 16-17 of gestation, and thyroglobulin synthesis can be observed on day 70 [132]. The parathyroid gland develops between 5-12 weeks of gestation. Fetal T3 and T4 levels are very low till mid-gestation, and maternal T4 is the major source during this time. Plasma TSH increases during late gestation, and there is an increased secretion of T4 at 35-40 weeks of gestation, which indicates

maturation of the fetal hypothalamic pituitary control of TSH and of thyroid gland responsiveness to TSH. Fetal T4 activates the enzymes responsible for iodothyronine metabolism. Thyroxine regulates growth, brain development, and basal metabolic rate (BMR)during early postnatal life [132].

1.7.2.4. Gonadal hormones

Testosterone production starts in the human male fetus between weeks 10-20. Production is under the control of circulating hCG and fetal LH, with hCG being the main stimulus. Increased testosterone production stimulates sexual differentiation and the formation of accessory sex glands in male fetuses. Fetal testosterone is also important for prostate development, differentiation of phallus and formation of penile urethra. Testosterone is converted to dihydrotestosteone in the fetus with the help of 5-α reductase, and it is essential in differentiation of the urogenital sinus and external genitalia. Dedifferentiation of the mullerian duct is under the control of AMH (antimullerian hormone), produced in the fetal testicular sertoli cells [136]. AMH also has a role in testicular descent [136]. In the female fetus, the mullerian duct develops in the absence of AMH. Gonadal hormones also play a role in sexual differentiation of brain and hypothalamus. The pulsatile release of LHRH is programmed in the female fetus by day 80, which is important for the maintenance of post pubertal ovarian cyclical activity. The feedback mechanism of gonadal steroids starts at day 150 of gestation. Fetal testosterone-mediated suppression of LHRH predominates after day 150 in the male fetus [132].

1.7.2.5. Insulin and glucagon

The pancreas develops by 4^{th} week of gestation. The pancreas secretes insulin, glucagon, somatostatin and pancreatic polypeptide [132], but fetal insulin levels have been observed to be unresponsive to high glucose concentrations. The glucagon level increases

progressively with fetal growth. Glucose transfer occurs by facilitated diffusion, insulin and glucagon do not assist in glucose metabolism in the fetus. Insulin is thought to act as a signal for nutrient requirement, as insulin deficiency leads to reduced fetal growth [122]. It has been shown that maternal corticosteroid administration can lead to increased insulin production in the fetus. A direct correlation between fetal insulin levels and fetal growth has been reported [137]. Diabetic pregnancy increases fetal insulin production and leads to macrosomia [122].

1.8. EFFECTS OF ENDOCRINE DISRUPTORS ON THE DEVELOPING FETUS

Human development starts from fertilization and events in the first week lead to the beginning of implantation of the blastocyst in the endometrium, which is completed by the end of the second week. Gastrulation proceeds in the third week -when early nervous system and cardiovascular systems develop. The conceptus from 4^{th} week of pregnancy till 8^{th} week is known as the embryonic period. The fetal period begins at the ninth week after fertilization and ends at birth. Major organogenesis takes place at the embryonic / fetal stage. They are the most vulnerable stages during human development because environmental chemicals can affect the fetus and produce congenital anomalies. The teratogenic effects of drugs / environmental chemicals are usually studied in high doses. There is mounting evidence from animal experiments that the EDs may act as teratogens and can even cause early embryonic / fetal loss if exposure to ED's occurs.

Researchers have also shown that these chemicals can transfer across the human placenta [25] but the impact on the human embryo viability and fetal development at low environmentally relevant dose is not clear. Diethylstylbestrol (DES) was shown to induce early embryonic loss in pregnant mice as a result of decidual hypoplasia and placental

haemorrhage [138]. Others have also reported a similar mechanistic reduction in viable embryos on exposure to high doses of phthalate esters [139]. The EDs, BPA and genistein were found to act in a dose dependent manner to cause morphological abnormalities in mice embryos [140] with a synergistic effect. However, the teratogenic potential of BPA alone, at a concentration range reported in pregnant mother's serum concentration, has been ruled out in this study [140]. It has been reported that BPA terminates implantation at higher dose [141]. BPA has been shown to induce apoptosis in placental cells at a level relevant to human exposure [142]. Benachour and Aris (2009) further hypothesized that BPA has the potential to produce early embryonic loss and pre-term birth in human. BPA at environmentally relevant concentrations has been shown to affect the *in vitro* development of mice embryos in a dose dependent manner [64]. Dioxin mediates its action by binding to arylhydrocarbon receptors (AhR). The expression of AhR has been reported in 8 cell embryo stage. Hence the changes in differentiation of 8 cell embryos observed on exposure to dioxin are mediated through binding of the AhR in the embryo. A teratogenic dose of dioxin could be detected in embryonic tissues within 30 minutes of maternal exposure [143, 144]. Mouse embryos express ER-α and ER-β quite early in development and the embryo-toxicity of estrogenic EDs may be mediated through ER mediated pathways [145]. When considering the estrogen mimetic / anti-androgenic actions of the above compounds and a common pathway for embryo loss (through decidualization deficit) it can be concluded that the observed effects can be due to endocrine disruptor actions of these compounds on the developing embryo.

1.8.1. Effects of endocrine disruptors in sexual differentiation and congenital malformations of the developing fetus

In humans the sexual differentiation (morphological development as a male and female) happens from seventh week onwards and in males it is under the strict control of androgens (Figure 1-8). The development of penis, scrotum and accessory sex glands are all under the influence of steroid hormonal phase of testicular development [146]. Testosterone and androgen dihydrotestosterone are the two major hormones involved in the above process [147]. In the penile tissues of human male conceptus the androgen receptor is expressed as early as 12-20 weeks of gestation [148]. Hence it is possible that the accumulation of endocrine disruptors (anti-androgens / xeno-estrogens) in the fetal environment can disrupt the normal sexual differentiation. There are some controversial human data on derangement of male sexual differentiation associated with pre-natal exposure to the synthetic estrogen diethylstilbestrol [149]. Studies with diethylstilbestrol (DES) exposed children have shown the importance of a proper balance between testosterone and estrogen for sexual development of a male fetus [150, 151].

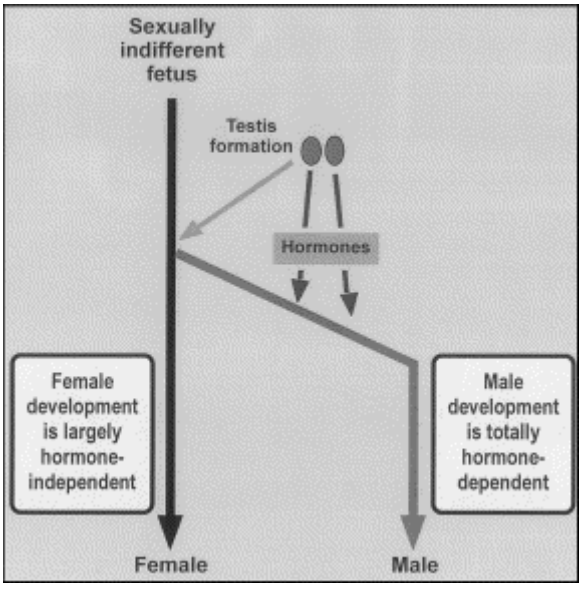

Figure 1-8 Dependancy on sex hormones for the development of male fetus

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Another potential harm observed on exposure to endocrine disruptors was on the development of spermatids and sperm production. The study by West *et al* observed that the exposure to genistein during intra-uterine or neonatal period could interrupt the differentiation of round to elongated spermatids in male rat offspring thus compromising their sperm production [80]. Apoptotic receptors (FasR) in germ cells and their ligands (FasL) in sertoli cells were found to be upregulated causing the apoptosis and hence a reduction in sertoli and germ cell numbers in response to ED exposure in rodents [59, 153] [154]. In rodents BPA caused a reduction in testicular and epididymal sperm counts and a reduction in testicular and seminal vesicular weights [155]. BPA was shown to increase the expression of aromatase in rodent Leydig cells further supporting male reproductive disruption [156]. *In-utero* exposure of male rats to genistein resulted in delayed spermatogenesis and oligospermia [157]. It is reported that the phytoestrogens can upregulate androgen binding globulin [158] as well as Sex Hormone Binding Globulin (SHBG) [49], which reduces serum estradiol levels and facilitates in the binding of the phyto or xeno-estrogens to the estrogen receptors effectively and resulting in an estrogenic or antiestrogenic activity in rodents. Both xeno (e.g. Octylphenol) and phytoestrogens can cross blood testes barrier and come into contact with intratubular germ cells leading to a reduction in the male germ cell proliferation [80]. Taken together these studies have shown the potential developmental reproductive toxicity of endocrine disruptors in experimental animals. Although a parallel study is lacking in humans there is some evidence showing the disruptive actions of EDs on human male sexual differentiation and gonadal function. In a neonatal screening programme of ambiguous genitalia, Paris *et al* (2006) identified three male newborns with male pseudo-hermaphroditism whose mothers had been exposed to EDs during gestation [150]. Based on the findings, it was hypothesised that the pre and neonatal exposure of these children to ED deranged the fetal

sexual differentiation (6-12 weeks PGD) leading to the genital ambiguity [150]. Carlsen *et al*. (1992) reported a decline in semen quantity in normal men during the past 50 years based on published reports [155]. The results reported were controversial and they had regional variation as well [155]. The critics pointed out that there was also a tremendous life style change during this time which could have caused a similar observation. Furthermore, the semen quantity of bulls and other animals has remained stationary for this 50 year period of time [155]. Recently Swan *et al* (2007) reported a correlation between the amount of beef consumed by pregnant women and oligospermia of their sons during adolescence. The reason was speculated to be the intra-uterine exposure to the xeno-estrogens present in the beef [159]. In human seminoma cell lines BPA activates the protein kinase A (PKA) through G-Protein coupled receptor (GPCR) membrane mediated non-genomic action [160].

It is believed that gonadogenesis in the female fetus is under the control of the maternal hormonal environment [147] in that Wolffian ducts regress and Mullerian ducts differentiate in the absence of testosterone . Mullerian duct differentiation is under the influence of maternal hormones [147]. Recent studies have revealed that fetal ovarian development is not a passive event as considered earlier and involves collaboration of several genes [147, 161, 162]. There is an upregulation of many genes like dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (DAX1), wingless-type MMTV integration site family, member 4 (WNT4) and down regulation of those involved in male sexual differentiation like SRY (sex determining region Y)-box 9 (SOX9), Wilms tumour 1(WT1) and splicing factor 1 (SF1) (reviewed in [147]). Cytochrome P 19 (CYP19 or aromatase) expression is detected in somatic cells of fetal ovary during early gestation, which is involved in the conversion of androgens to

estrogens. Estrogen synthesis takes place in fetal ovaries during oogonial phase and is turned off just prior to the initiation of meiosis in female germ cells. Animal experiments have shown the toxicity of endocrine disruptors in the development of female gonadogenesis. For example dioxin congeners were reported to reduce germ cells in the ovaries which may lead into premature menarche [163, 164]. Shi *et al* [165] observed premature reproductive senescence in chronic *in utero* exposure of 2,3,7,8 – TCDD without depleting ovarian follicular reserve. Aneuploidogenic (producing abnormal number of chromosomes) potential of BPA has also been reported [166].

In an endometrial cell line (Ishikawa cell lines), xeno-estrogens [BPA (1μM), 4-NP (5μM), DES (1nM) and 17-β-estradiol (1nM)] were shown to upregulate progesterone receptor. Prenatal exposure to BPA in female rodents produced offspring with an endometriosis like phenotype [167, 168]. Xeno-estrogens were shown to stimulate gonocyte proliferation in a dose-dependent manner, which may disrupt normal signalling events for gonocyte development, and lead to disrupted gonocyte development [169]. There are no conclusive data available on developmental exposure to endocrine disruptors on female gonadal function in human. But the available data on animal experimentation / in-vitro human data and epidemiological studies on diethylstilbestrol (DES) daughters suggests a potential role for developmental exposure to endocrine disruptors towards the development of premature ovarian failure, aneuploidy, polycystic ovarian syndrome and endometriosis in human.

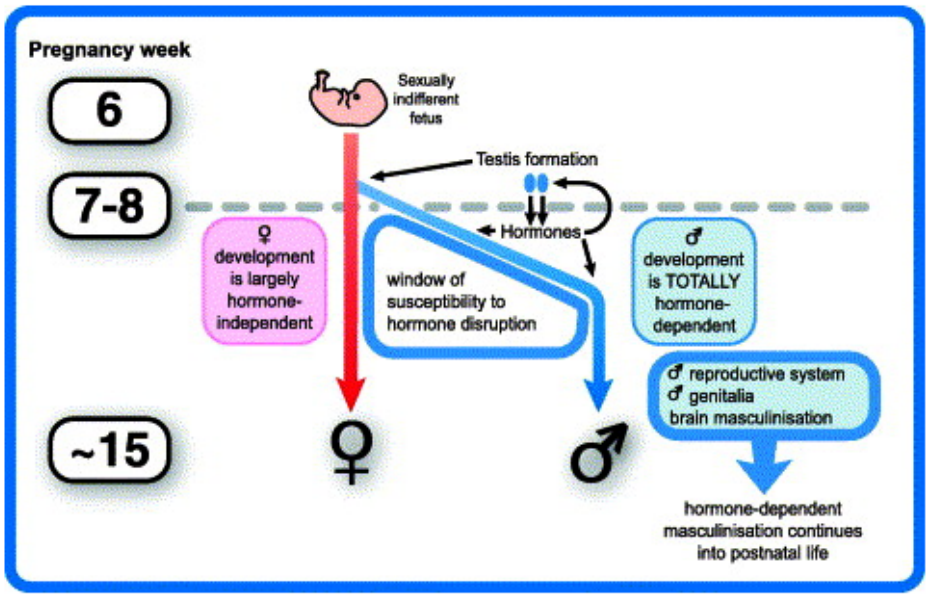

Figure 1-9 Windows of susceptibility to sexual differentiation on exposure to sex hormones

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1.8.2. Congenital malformations of developing fetus

A higher incidence of male reproductive abnormalities like hypospadias, cryptorchidism and testicular germ cell cancer, coupled with a fall in sperm count has been observed globally from the mid 20th century onwards [80]. This condition in humans, called testicular degeneration syndrome, is believed to be due to exposure to EDs. Due to the widespread dissemination of hormonally active chemicals following the industrial revolution, and the use of phytoestrogen in soy-based food, a positive correlation between exposure and the defects described was speculated [80].

It is possible that the oriental population might have developed a resistance to phytoestrogens, as they have consumed a highly estrogenic diet over many generations. The genetic predisposition is another factor explaining western populations being more vulnerable and eastern population more resistant [80]. Beckmann *et al* (2000has cautioned about the importance of monitoring lactose intolerant children receiving soya-based infant formula, as any adverse outcome will manifest only after 20-30 years [80]. The ability of

babies to absorb genistein and diadzein can increase their estrogenic content by more than a thousand-fold over endogenous levels [80, 171, 172]. A study by West *et al* observed that exposure to dietary genistein during the intrauterine or neonatal period interrupts the differentiation of round to elongated spermatids in male offspring, thus compromising their sperm production [80]. Fas receptors in rodent germ cells and Fas ligands in sertoli cells were found to be up-regulated when exposed to phthalate toxicants, possibly leading to apoptosis and reduction in sertoli and germ cell numbers [59, 153]. Nair and Shaha 2003 have shown a similar mechanism with DES [154]. These studies show the deleterious effects of xeno- and phyto-estrogens on spermatogenesis.

1.8.2.1. Cryptorchidism

One to five percent of male neonates suffer from undescended testis into the scrotum (cryptorchidism) [173], which is under hormonal control. The first phase in the process is migration to the groin, and studies in mice have revealed that this is under the control of insulin-like factor (Insl-3) produced by Leydig cells, and that it is inhibited by estrogens. Five to ten percent of cryptorchidism is due to failure to descend during the first phase. The second phase, migration to the scrotal sac, is thought to be influenced by androgens. Failure during this phase will cause the testicles to remain in the inguinal region [174]. A correlation between diethylstilbestrol treatment and cryptorchid children can be traced back to the mid 1970s to early 1980s [175, 176]. Toppari and Skakkebaek (1998) also discussed a positive correlation between cryptorchidism and possible human exposure to xeno-estrogens. Estrogen treatment in pregnant rats also led to similar observations in male offspring [176]. A recent study in mice treated with DES produced a complete suppression of INSL3/RLF mRNA in fetal testes without affecting SF-1, which is a transcription factor involved in expression of INSL-3 genes. None of these promoters

have specific responsive elements for estrogen receptors, and hence the effect of estrogen on these genes is through an uncharacterized mechanism [176]. These studies were followed by studies with aromatase (AROM+) transgenic mice leading to a demonstration of the likelihood of exogenous estrogen actions in testes resulting in cryptorchidism in humans [176]. It is highly probable that the affected pathway involves the hypothalamo-pituitary-gonadotropic axis [176]. Recently, *in utero* exposure of phthalates was also found to suppress (Insuline like -3) Insl 3 in rodents, leading to improper development of adult Leydig cells in certain animals [147, 177].

A 40 year cohort study in East and West Berlin maternity hospitals has shown a strong correlation between cryptorchidism and DDT exposure [176]. Studies of ED exposure coupled with epidemiological observation of increased prevalence of cryptorchidism and hypospadias in ED-contaminated areas lead to the hypothesis that the environmental chemicals influenced testicular descent. Although the experimental results have been criticized, due to the usage of higher than environmentally relevant doses, it is undeniable that in nature it is the combined action of these chemicals that disturbs the dynamics of testicular descent [176]. A 25 year cohort study in Berlin has shown a correlation between the reduction in cryptorchidism and the discontinuance of DDT. Hosie *et al* (2000) has shown increased levels of certain organochloride compounds in patients with cryptorchidism [178]. Cryptorchidism is considered to be a multifactorial disturbance which involves both the hypothalamic pituitary gonadal axis (HPG axis) and specific hormones like Mullerian Inhibitory Substance (MIS) and Inslulin Like 3 / rearranged L-myc Fusion (INSL3/RLF) [176]. Homeobox – 10 a (HOX-10a) mutations were also implicated in some patients [176]. Cryptorchidism is linked to decreased testicular size, decreased sperm counts, infertility and testicular cancer [176].

1.8.2.2. Hypospadias

Hypospadias is a developmental anomaly defined by the displacement of the urethral meatus from the tip of the glans penis to the ventral side of the phallus, scrotum, or perineum, often requiring surgical reconstruction [179]. It is a common condition, reported to occur in 0.02 to 0.4 % of live births [179]. It is thought that the accumulation of anti-androgens in the placental-fetal unit at 6-14 weeks of gestation can significantly reduce the testosterone concentration and cause growth restriction and hypospadias in the human conceptus [147]. ED exposure has been linkedto hypospadias [147]. Since the differentiation of the genital tubercle into male external genitalia has been noted to require signalling factor protein FGF 10 in mice [180], it is possible that estrogen mimics or anti-androgenic compounds in the environment contribute to this malformation by disrupting FGF 10 signalling [147]. Recently dominant expression of estrogen receptors over androgen receptors was reported in the penile tissues of neonates with hypospadias [181]. This dominance may contribute to the endocrine disrupting action of xeno-estrogens in the development of hypospadias. The observation of hypospadias in male children of IVF mothers, and following maternal exposure to endocrine disruptors through environment or diet, emphasizes the role of xeno-estrogens to predispose the condition in male neonates [179].

1.8.3. Other effects of endocrine disruptors on the developing fetus

EDs cause a vast array of developmental defects in the developing fetus. These can vary from fetal loss to developmental abnormalities in different systems in the fetus.

1.8.3.1. Fetal loss

In a large follow-up study on the reproductive performance of daughters prenatally exposed to DES, a high incidence of preterm birth and 2nd trimester fetal loss was shown [182]. A positive correlation has been identified between recurrent spontaneous abortion and serum BPA concentration (2.6ng/ml vs 0.8 ng/ml) in antinuclear antibody-positive individuals [183], but this observation has some shortcomings as the BPA range observed in those who had had a spontaneous abortion was very broad. Hunt *et al* showed an induction of meiotic aneuploidy in mice after exposure to environmentally relevant doses of BPA. This group also found a dose dependent effect on meiotic aneuploidy [184]. Guo *et al* showed early fetal loss (EFL) to single-dose oral exposure of TCDD in pregnant macaques [185]. BPA at environmentally relevant concentrations has been shown to affect the *in vitro* development of mice embryos in a dose-dependent manner [64]. It also affects the postnatal weaning weight, which may contribute to later disease in the adult [186].

1.8.3.2. Preterm birth

Preterm birth has significantly increased in the last decade, reaching about 8 % of total births in USA http://www.cdc.gov/Features/PrematureBirth/[187]. There are a few, inconclusive studies available in the literature regarding endocrine disrupting chemicals and preterm labour (PTL). Few environmental pollutants have been associated with preterm birth. They include lead in the environment and nicotine in smoke. In a large cohort study by Longnecker *et al* (2001) the association between serum Dichlorodiphenyldichloroethylene (DDE) and preterm birth has been established [188]. However no association between serum DDT concentration and PTL were observed in that study [188]. Farhang *et al* (2005) did not find any relationship between first trimester DDE concentration in women and preterm delivery [189]. Saxena *et al* (1981) reported an

association between placental concentrations of organochloride pesticides and preterm birth in the Indian subcontinent [190]. No association between TCDD exposure and PTL was found in a study from Italy [191], while a significant correlation was observed in a highly contaminated area in Russia [192]. There is an increased awareness of the endocrine disrupting activities of phthalates, which are present in plastics, and early fetal loss [193, 194]. Phthalates and their metabolites have been found to increase the PPAR-γ receptor expression in a mouse placental trophoblast cell line [195]. It is hypothesized that phthalates can induce an inflammatory condition through PPAR- γ pathway to decrease gestational length [195]. Sharara (1998) observed a positive correlation between DDT exposure of pregnant women and low birth weight children born to them [82]. Prenatal exposure to BPA also caused a reduction in birth weight [196]. Low birth weight is known to predispose babies to adult onset of disease.

1.8.3.3. Cardiovascular development

There are many studies on the benefits of soy products in cardiovascular protection. It was reported that feeding genistein and daidzein to pregnant rats resulted in offspring with shorter cardiac myocytes producing greater cardiac protection [197]. A recent study has shown the adverse effect of soy-based diets on the cardiac phenotype in a mouse model of human cardiac myopathy (HCM) [198]. HCMis a mutational cardiovascular disease in humans that affects 1 in 500 people [198]. Intrafamilial studies suggest the possibility that epigenetic and environmental factors are involved in modifying the risk of HCM [198]. The influence of sex steroids on cardiac growth has been studied previously. Estrogen and androgen receptors are present in myocardium [199]. By feeding a soy-based diet to male and female HCM mice, the researchers have shown that diadzein and genistein influence cardiac growth in a sex-dependent manner. They further hypothesized that the agonistic

and antagonistic behaviour of phytoestrogens, in the absence or presence of estradiol, might have contributed to the sex-dependent cardiac phenotype. The researchers found augmentation of cardiac growth in male HCM mice and an inhibitory growth in female. An increased caspase-3 activity through the IGF-1/Akt/GSK3β pathway [199] have also been shown, but the effects of dietary estrogens on cardiovascular development remain to be elucidated.

1.8.3.4. Effect on neuroendocrine and brain development and function

There is a positive correlation between the abundance of endocrine disruptors in the environment (BPA, DDT etc) and the prevalence of neurodevelopmental and other developmental defects in humans [107]. Kabuto *et al*. showed that fetuses and neonates exposed to dioxin consistently through the placenta and milk had underdeveloped brains, kidneys and testes [12]. The higher incidence of learning disabilities, ADHD, childhood cancers and juvenile diabetes during mid 1990's may be due to *in utero* exposure of these chemicals [107]. Developmental defects in the neuroendocrine system have been demonstrated in animal models after ED exposure [43]. The AhR antagonists like dioxins can accumulate during the time of neuronal development (i.e. fetal and neonatal periods) and is irreversible [43]. Recent studies have shown a relation between the prenatal exposure of EDs and post natal neurological underdevelopment and poor maternal nursing in rodents[200] and humans [201]. Gestational exposure to BPA caused increased levels of dopamine and serotonin metabolites in the brains of female offspring as well as dams [96]. This phenomenon is hypothesized to be due to the estrogenic action of BPA.

Animal researchers have shown major behavioural changes due to *in utero* exposure of EDs. Two distinct neural circuits appear to mediate sexual behaviours in vertebrates [202]. Parts of anterior hypothalamus and preoptic area (AH-POA) are involved in the control of

mounting and intromission behaviour, where as parts of the ventromedial hypothalamus (VMH) are involved in sexual receptivity [202]. There are numerous structural differences between male and female brain and sex steroid hormones in early development which play a role in the structural differences and sexual differentiation of male and female behaviour [202]. DES administration in rats masculinises and defeminises the female brain and behaviour. The effects also include the enlargement of the sexually dimorphic nuclei of preoptic area and the elimination of cyclic secretion of pituitary LH. Long term DES exposure during development results in the enhanced masculinised juvenile behaviour in Rhesus monkeys (Goy and Deputte 1996). *In utero* exposure of low dose DES in rats increased urine mark deposition in male offspring and increased level of aggressive behaviour [202]. The non reproductive sexually dimorphic behaviour is sensitive to endocrine disruption at environmentally relevant concentrations of EDs [203]. By using BPA and ethinyl estradiol Ryan *et al* have shown an altered anxious behaviour and spatial memory. They also reported that inutero exposure to BPA can disrupt exploration and paired bond formation behaviour in mice [203].

Rubin *et al* (2006) studied the effects of perinatal exposure of BPA on brain development and sexual differentiation in mice [106]. Perinatal BPA exposure obliterated tyrosine hydroxylate nucleus in rostral periventricular preoptic area, which is an important region for estrogen feedback and estrous cycle. The effect was statistically significant in treated mice than control [106]. They also observed alterations in the open-field behaviour in treated mice [106].

Epidemiological correlations between *in utero* exposure of DES and behavioural abnormalities including depression and anxiety were well documented [31]. Thyroid hormones play key role in the fetal brain development. A new study has shown

externalizing behaviour in female children exposed in-utero to BPA [27]. BPA has been shown to increase neuronal differentiation in rodent cells further suggesting a role for neonatal brain disruption [204]. Chronic pre and postnatal exposure to endocrine disruptors in mice induced anxiolytic behaviour, cognitive deficits, and changes in dopaminergic and glutamatergic (NMDA)systems [205].

BPA increases progesterone receptor expression in hypothalamus, which in turn, alters hypothalamic mechanisms and affects the onset of estrus and receptivity of the uterus [206].It has been shown that prenatal exposure to BPA caused a reduction in birth weight, hypergonadotropism and delayed breeding season and dampened LH surge in the female offspring of Suffolk sheep [196]. BPA has also been shown to reduce testosterone production by adversally affecting pituitary function [207].

1.8.3.5. Intergenerational effects

The first observation of intergenerational effects was the finding of clear cell adenocarcinomas in daughters born to DES treated women, and its subsequent occurrence in the granddaughters. The mechanism of intergenerational effects of DES is thought to involve epigenetic regulation [208]. The transgenerational transmission of carcinogenic effects of DES in experimental animals has also been reported [20]. Chapin *et al* reported reduced sperm counts in F2 generations and kidney toxicity in all F0, F1 and F2 generations of p-NP exposed rats [209]. Nagao *et al* (1999) observed slight reductions in implantation sites and live births in a two generation study [210]. In contrast, no intergenerational effects on reproduction or development were found in the few multigenerational studies performed with low doses of BPA [38, 211]. Recently Anway *et al* (2006) reported the transgenerational action of an antiandrogenic ED (vinclozosin) in the four generations studied. The diseases ranged from prostate to kidney and immune

functional disorders. The study has also found testicular effects and mammary carcinoma [212]. Another study from the same group has shown that the epigenetic transgenerational effects of these compounds are the cause for deranged sexual differentiation and multi organ failures [212, 213].

1.8.3.6. Predisposition to metabolic diseases and cancer

Intrauterine exposure to high concentrations of endogenous estrogens has been speculated to predispose offspring to breast cancer [74]. This speculation was based upon a population study where the authors have found the low incidence of breast cancer in pregnancies associated with pre-eclampsia. The authors concluded that the estrogen concentrations in pre-eclamptic pregnancies were low and the low incidence of breast cancer in pre-eclamptic pregnancies implied that it might be because of low intra-uterine estrogen exposure. Elevated levels of natural estrogen during gestation also predispose the children to breast cancer [214]. Epidemiological studies have shown that DES exposure is related to susceptibility to breast cancer and that DES is the only proven transplacental carcinogenic xenoestrogen [214]. Association between intrauterine exposure to DES and the appearance of clear cell adenocarcinoma during puberty of girls has been shown as early as 1971 [20]. Prins *et al* (2006) provided a link between developmental exposure to low-dose of BPA and prostate cancer in adult experimental animals [215]

Wetheril *et al*. showed BPA at environmentally relevant low doses can activate mutant androgen receptor (AR) in androgen dependent and independent cell lines but only had a marginal induction of the wild type AR [166]. The group postulated this to be a one of the possible mechanisms behind the relapses in prostate cancer [166]. In contrast BPA at higher concentration inhibited the proliferation of prostatic growth [166]. There are a few

studies on the correlation between prenatal exposure of EDs and testicular cancers [164, 216], prostate carcinoma (48), brain tumours, childhood leukaemia breast cancer [217, 218] in humans. Gestational exposures to estrogens or antiandrogens can lead to the development of testicular neoplasms in adulthood [20].

Although the epidemiological data for the above hypothesis are limited, there is a large body of data on prenatal exposure and susceptibility to cancer in animal studies [214]. Neonatal exposure to genistein caused uterine adenocarcinoma in adult mice [48]. When comparing the effect of genistein with DES, the hypothesis that it is not the structure of the chemicals that leads to the predisposition to cancer but rather the total estrogenicity is strengthened. On the contrary, Kociba *et al* reported an antitumorigenic action of dioxin (TCDD) in mouse spontaneous mammary carcinoma [219]. Gestational exposure to genistein produced mammary carcinoma in female offspring. Brown *et al* (1998) have reported a single oral prenatal exposure of TCDD increased the susceptibility of offspring to develop mammary tumour when pups are treated with 7,12-Dimethylbenz(a)anthracene (DMBA) during sexual maturity [20]. The AhR is present during organogenesis in most of the tissues. It is also expressed in mammary ducts and developing lobules of pubescent mice [20]. Brown *et al* has also shown an increase in number of terminal buds in the mammary epithelium of TCDD treated mice at sexual maturity They also observed a decrease in the ductal migration of the epithelium into the stromal compartment [20]. These two changes are associated with increased susceptibility to carcinogenesis in rodents [20]. Murray *et al* (2007) have recently shown that fetal exposure to BPA induces preneoplastic lesions in rodent mammary tissues even in the absence of post stimulation [75]. A similar observation was reported in pre-natally exposed rats after chemical challenge at adult stage [11]. BPA also increased the sensitivity of developing mammary

gland to endogenous estrogen [220]. Developmental exposure to BPA also increased susceptibility to develop prostate carcinoma in the presence of stimulating agents in rodents [215, 221].

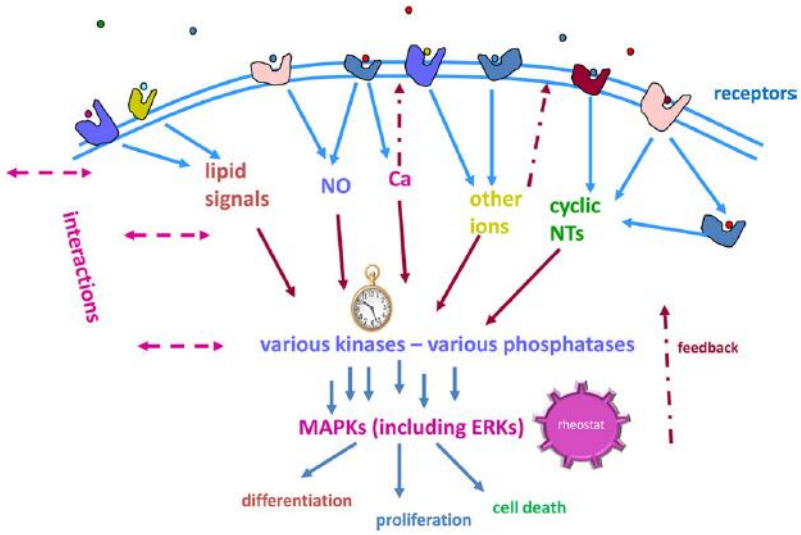

Figure 1-10 Xeno-estrogenic interactions in major signalling pathways to cause cell differentiation, proliferation and death

The figure above shows the proposed mechanism by which xeno-estrogens bind to estrogen receptors to generate second messengers which synergistically activate with other receptors to mediate a signal transduction through Mitogen Activated Protein Kinases (MAPKs) to cause a change in the cellular function. (No = Nitric Oxide, NT = Nucleotides, Receptors = Estrogen Receptors).

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"Obesity, defined as excessive body fat (>25% men, >30% women), is fast becoming a significant human health crisis that is receiving worldwide attention" [9]. Obesity can predispose people to type 2 diabetes, hyperinsulinemia, coronary heart disease, strokes and few forms of cancer [9]. It is caused by a complex interaction between genetic, behavioural and environmental factors [9]. "An emerging hypothesis proposes that *in utero* and early developmental exposure to environmental chemicals may play a role in the development of obesity later in life". Neonatal exposure to DES caused susceptibility to weight gain during adulthood in mice [9]. An increased abdominal fat content was

identified in mice treated with DES, may be due to the alteration of genetic programming of adipocytes by the endocrine active compounds. Also leptin, adiponectin, IL-6 and triglyceride levels of DES treated mice were found to be upregulated, while insulin was reduced. Prenatal exposure to genistein also increased fat deposits in prepubertal mice [9]. The DES treated mice showed islet cell hyperplasia indicating an abnormal glucose metabolism. Many of the organochloride pesticides, growth promoters and pharmaceutical agents were reported to cause obesity in experimental animals [223]. Human adipose tissue expresses both ERα and β. Estrogen decreases the activity of lipoprotein lipase [224]. Masumo *et al* 2002 have shown the ability of BPA to differentiate 3T3L1 (mouse fibroblast cells) to adipocytes. 4-NP can also stimulate proliferation of fully differentiated3T3L1 [224]. Grun *et al* has shown that at high dose, genistein acts as a ligand for PPARγ and stimulates adipogenesis [225] which can lead to obesity. The molecular mechanism of adipogenic stimulation and EDs needs to be further explored.

In utero exposures to estrogenic endocrine disruptors have long been associated with cancers in humans. For example studies have shown that *in utero* DES exposure increased the risk for clear cell adenocarcinoma and melanoma in humans [226]. An NIH funded cohort study examined the association of intra uterine and post natal exposure to estrogenic compounds in the incidence of uterine fibroids in women of age group 35-59 years old. The study has reported an increased risk of uterine fibroid formation in women who was on infant soy based diet during early neonatal periods. The same study also reported a possible association between *in utero* DES exposure and uterine fibroma formation during adulthood [227]. Results from a recent cross-sectional study showed a possible role of endocrine disruptors in human obesity epidemic. The study examined the exposure status of six phthalate metabolites in US population and correlated the metabolic

and physiological changes observed in the exposed individuals. The observed variations in body mass index (BMI) and waste circumference (WC) had a dose-response relationship. The study further showed the age and gender differences on phthalate metabolies in relation to the obesity epidemics [228, 229]. BPA at an environmentally relevant concentration reduced a key adiponectin that is important to prevent human metabolic syndrome. The authors have demonstrated this effect in human adipocyte explant culture and BPA mediated this effect at a concentration of 0.1 and 1 nM [230]. However the mechanism for the inhibition of this adiponectin has not yet been determined. The abilities for EDs to increase adipogenesis through glucocorticoid receptor -1, further speculating the roles for EDs in obesity epidemics [231]. In rodents perinatal exposure to BPA increased adipogenesis in female pups on weaning [232]. This further strengthens the role of BPA in predisposing the fetus to metabolic syndromes.

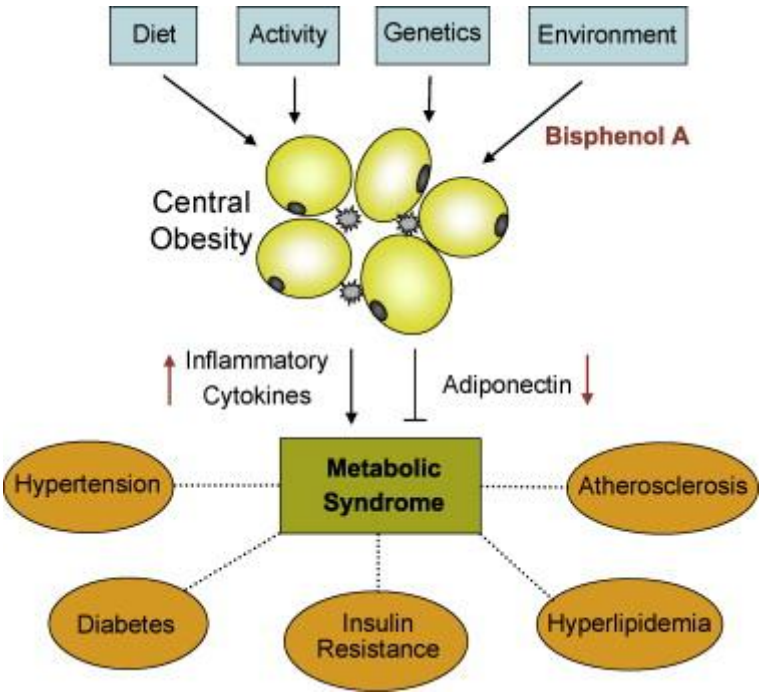

Figure 1-11 A model depicting an integrated view of the various factors that affect the obesity-related metabolic syndrome.

BPA suppresses adiponectin and stimulates inflammatory cytokines by acting on adipocytes and infiltrating macrophages. Adiponectin is an insulin sensitizer whereas cytokines such as IL-6 and TNFα promote insulin resistance. The opposing actions of BPA on these adipokines contribute to the development and manifestation of the metabolic syndrome.

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1.8.3.7. Potential targets of endocrine disruptors

ER is also expressed by various immune system components, especially the thymus, which expresses ER-α and ER-β. Thymus development is mainly dependent on estrogen signalling through ER-α [234, 235]. It has been reported that the neonatal estrogen exposure can cause thymus atrophy in experimental animals. Recently it has been shown that estrogens action on thymus can vary depending upon the stage of exposure ie. whether neonates, pubertal or adulthood and a thymic enlargement was observed in neonates if the exposure occurs after 8 weeks of postnatal life[236]. Genistein's immunosuppression may be due to its protein tyrosine kinase activity at higher concentration [237]. Genistein impaired macrophage functions, inhibited T-cell proliferation *in vitro*, inhibited the production of leukotriene B4 (LTB4) and cytokines [238]. Genistein causes thymic atrophy and consequent reduction in T-cell numbers [239]. Genistein at environmentally relevant dose inhibited IFN-γ production in mice challenged with *Mycobacterium avium* [240]. Some *in vitro* studies have shown that genistein at higher concentrations can mediate immunostimulant activity [47, 241]. The immunosuppressive functions of genistein was found to be useful in preventing cardiac allografts in mice [242] and beneficial in anti-inflammatory effect in guinea pig model of asthma [243]. There is a strong correlation between perinatal exposure of dioxin congeners (PCBs) and consequent T-cell mediated immunosuppression in wild animals [244]. Developmental exposure to endocrine disruptors led to decreased antibody response, hypoplastic thymus, hypoplastic spleen and hypoplastic bone marrow in alligators of Lake Apopka. [245]. Data from

animal studies also supported the hypothesis that exposure to endocrine disruptors during developmental stages has an immunosuppressive action [245]. A nonmonotonic dose dependent immunomodulatory effect was observed on T-cell proliferative effects with BPA treatment [245]. BPA also inhibited macrophage adhesion in plastics which is an indication of inhibited macrophage function. Ahmed has reviewed the few studies in humans relating to immune dysfunction mediated by endocrine disruptors [245]. There are possible correlations between autoimmunity and prenatal DES exposure [246]. Estradiol and BPA were inhibitory to MCP-1 [247], and genistein inhibits NK cell function [248]. Pre-natal exposure to BPA was found to enhance the inflammatory response to allergens in a mouse model of asthma [13].

Data from animal studies have shown a thyrotoxic action of dioxins [70]. There are structural similarities between natural thyroid hormones and TCDD. Data from structure activity relation (SAR) and invitro competitive binding studies have shown that there is significant similarity between T3, T4 and TCDD. However, their affinity for thyroid receptors is less. Dioxin has shown to inhibit signalling events mediated by thyroid hormones after binding to thyroxine receptors. TCDD may displace thyroid hormones from binding the transport proteins and thus increase the elimination process. TCDD induces thyroxin metabolising enzymes and eliminates it rapidly [70]. TCDD can induce the glucuronidation enzymes (UGT) and other metabolizing enzymes through AhR and can increase the excretion of the glucuronidased form of the thyroid hormones through liver [70]. Hyperstimulation of glucuronidase can derange the TSH mediated hypothalamo- pituitary- thyroid feedbacks and precipitate neoplastic conditions of the thyroid follicular cells.

Soy fed human infants show reversible goitre as they are relieved from the symptoms once changed to cow milk or increase the dietary iodine intake [48]. Later genistein and daidzein, the ingredients of a typical soy formula was found to inhibit the thyropoietin (TPO) catalysed iodination and coupling [48]. In the absence of iodine, both genistein and daidzein can bind covalently to the active site of the TPO leading to the concomitant loss of both iodinating and coupling activities. With adequate iodide genistein and daidzein are the alternate substrates and the products are mono, di and tri-iodoisoflavones. Data from invitro research has shown that BPA can bind to thyroid receptors and can suppress the triiodo thyronine mediated transcriptional activity. But a recent assessment of exposure in gestational rats could not establish a thyrotoxic action in the F1 progeny [249]. During fetal and early neonatal periods, disorders of thyroid hormone may lead to development of motor and cognitive disorders. They observed similarities in cognitive and motor behaviour in both the hyt/hyt mouse (congenital hypothyroid model) and the dioxin treated mouse, which indicated that there is a risk of developing similar disorders upon exposure to TCDD [250].

p-NP also posses mild progesterogenic action in-vivo and in-vitro[68]. It can also behave as an antiandrogen [68]. P-NP also affects thyroid and pituitary hormones. Han *et al* showed that prolonged exposure at higher doses can increase serum LH and FSH concentration in adult rats [68]. BPA was also found to cause a dose dependent hyperprolactonemia in rats [251]. Occupational exposure to organophosphates has been shown to increase T4 levels and to inhibit T3 levels in adult human [252].

1.9. ENDOCRINE DISRUPTORS AND NEONATAL MORBIDITY

1.9.1. Reproductive dysfunctions in male

Carlsen *et al*. (1992) reported a decline in semen quantity in normal men during the past 50 years based on published reports [253]. The data reported were controversial and they had regional variation as well [155]. Critics pointed out that there was also a tremendous lifestyle shift during this time which could have caused a similar observation. Furthermore, the semen quantity of bulls and other animals has remained stationary for this 50 year period of time [155].

The effects of 4-NP were studied in experimental animals. In one study, exposure of neonatal rats to 20.8 mg/kg/day of 4-NP resulted in a decrease in reproductive organ weight and delayed testes descent [254]. A similar study performed by Odum and Ashby (2000) could not repeat these results [255], but the strain of rats they used was a different one from the former study. In an adult exposure study by de Jager *et al* (1999), impaired testicular mass and sperm counts, as well as adversely affected seminiferous tubules, were found in 4-NP treated rats [155, 256]. In a multigenerational study, acrosomal integrity was decreased in both treatment (P and F1) groups [257]. Postnatal exposure to BPA in rats on day 21-35 suppressed serum LH, testosterone and estrogen levels, the latter possibly due to inhibition of aromatase activity in Leydig cells [155]. In certain low dose studies, BPA caused a reduction in testicular and epididymal sperm counts in mice and rats, and reduction in weights of testes and seminal vesicles were also reported [155]. Single exposures to TCDD caused impaired spermatogenesis in rats and marmoset monkeys [258, 259]. Histochemically 3-β-hydroxysteroid hydroxygenase activity was reduced in Leydig cells of both species. However, there are also multigenerational studies in the literature which show no adverse effects of the treatment either in parents or in

progeny. Mably *et al* (1992) reported a reduced sperm production without affecting fertility in male rats exposed to TCDD [260]. Rozati *et al* (2002) observed the presence of PCBs in seminal fluids of infertile men, and the absence of PCB's in fertile men (control) [261].

Recently Swan *et al* (2007) reported a positive correlation between the amount of beef consumed by pregnant women and oligospermia of their sons reaching adolescence. The reason was speculated to be intrauterine exposure to the xeno-estrogens present in the beef [159]. *In utero* exposure of male rats to genistein resulted in delayed spermatogenesis and oligospermia [157]. Adult male plasma levels of estradiol are less than 220 pmol/l, while adult female plasma levels are 70-1250 pmol/l. the phytoestrogens can upregulate androgen binding globulin [158] as well as SHBG [49], which reduces serum estradiol levels and facilitates the binding of the phyto- or xeno-estrogens to the estrogen receptors effectively, resulting in an estrogenic or antiestrogenic activity. Both xeno (eg. Octylphenol) and phytoestrogens can cross the blood testes barrier, can come into contact with intratubular germ cells and lead to a reduction in male germ cell proliferation [80].

1.9.2. Reproductive dysfunction in females

An accidental observation by Hunt *et al*. (2003) showed a positive correlation between BPA exposure and aneuploidy in developing mouse oocytes [184]. Recently Sugiura-Ogasawara *et al.* (2005) reported significantly higher concentrations of serum BPA in women (2.6ng/ml) who have had recurrent spontaneous abortions, compared to healthy non pregnant women (0.8ng/ml) from the same city in Japan [183]. Although a correlation between BPA exposure and spontaneous abortion was found, the study had a few shortcomings, thus further research in this area would be advisable [166]. Since there was an observed high correlation between aneuploidy and spontaneous abortion in humans, the

aborted fetuses could be karyotyped to confirm the cause, demanding further exhaustive epidemiological studies [166]. BPA increases progesterone receptor expression in hypothalamus, which in turn, alters hypothalamic mechanisms and affects the onset of estrus and receptivity of the uterus [206]. It has been shown that prenatal exposure to BPA caused a reduction in birth weight, hypergonadotropism and delayed breeding season, and dampened LH surge in the female offspring of Suffolk sheep [196]. 4-NP at higher doses increased uterine weight and advanced the vaginal opening in prepubertal rats [262].

1.10. METHODS FOR MEASURING ENDOCRINE DISRUPTORS IN BIOLOGICAL SAMPLES

As evident from the above discussion, humans are constantly being exposed to endocrine disruptors throughout our development, from embryo through fetus to adolescent stages. It is difficult to assess the amount of exposure to any endocrine disruptor during pregnancy or early childhood development, as there are no monitoring devices to do so.

There is an array of *in vivo* and *in vitro* assays to detect the estrogenic actions of environmental endocrine disruptive chemicals. However, there are only a few methods available to study fetal exposure to these agents, and each of them has their own advantages and disadvantages. Each method is also unique to the detection of a particular class of chemical. Most laboratories depend on chromatographic methods for the detection of EDs in fetal fluids/maternal sera /tissues. Accurate exposure assessment is essential and is fundamental to risk assessment in humans [263]. The detection of EDs in fetal tissue is the gold standard [263]. There are reports from cord blood, placenta and various fluids at the time of pregnancy, but since the fetus starts urinating by the 11^{th} week of gestation and swallows the amniotic fluid, the latter activity represents the major exposure link in fetus [263]. The adverse effects of EDs can also be enhanced by the synergistic actions of many

different chemicals present. Hence, even the dose of one chemical at a sensitivity well below the detection limit can combine with one or more other chemicals to act synergistically to produce an effect [202]. This introduces additional challenges when developing high sensitivity assays for detecting an ED alone or in combination. However, the concept of synergistic action by two or more endocrine disruptors is being questioned. An article published in *Science* regarding the synergistic action of xeno-estrogens [264] has shown that the combination of two or more environmental estrogens were 1000 times more potent in human estrogen receptor-mediated transactivation *in vitro* than used alone. However, due to an inability to reproduce the effects in the same or other labs, the article has since been withdrawn [265]. Detection of various EDs was carried out with Enzyme Linked Immunosorbent Assay (ELISA), Radio Immno Assay (RIA) and Chromatographic methods. The major disadvantage of ELISA and RIA are the specificity of the assay. For some EDs HPLC was found to be sensitive and for combination of EDs in a complex matrix interphases Liquid Chromatography – Tandem Mass spectrometry was a preferred method. The principal methods for detection of EDs in biological samples are tabulated in table 1.4.

Table 1-4 Method to detect selected endocrine disrupting from various biological matrices

SI No.	Compound	Method	Sensitivity	Levels	Endpoint	Mis.
1	Bisphenol	HPLC (ED)[51]	50 pg/ml (LOD)	0.5 (0.5-1.96) ng/ml	Amniotic fluid (before 20 weeks)	
		ELISA[125]	0.5-5000 ng/ml	2.2 ± 1.8 ng/ml 8.3 ± 8.9 ng/ml 1.1 ± 1.0 ng/ml	Fetal serum Amniotic fluid (15-18 weeks) Amniot	Also detected in follicular fluid

					ic fluid (term)	
		ELISA[l 266]	0.2 ng/ml	0.26 ng/ml	Amniotic fluid (11-16 weeks)	Also detected in maternal serum compared with chromosomal abnormality (higher group range 2.8-5.6)
		GC-MS[23]	0.1 ng/ml(plasma LOQ) 0.01 ng/ml (LOD)	2.9 ± 2.5 ng/ml (0.2-9.2) 11.2 ± 9.1 ng/g (1-104.9)	Fetal serum Placental tissue	Also detected maternal plama content
		GC-MS[267]	50pg/ml (LOD) 100pg/ml (LOQ)	ND to 4.05ng/ml	Cord blood	
		Radio-HPLC[91]				
2	Nonylphenol	GC-MS[267]	50pg/ml (LOD) 100pg/ml (LOQ)	ND to 15.17ng/ml	Cord blood	
3	Genistein	HPLC (ED)[51]	0.2 ng/ml (LOD)	1.4 (0.2-7.88)ng/ml	Amniotic fluid (before 20 weeks)	
		GC-MS[263]	0.5 ng/ml	1.69 ± 1.48 ng/ml	Amniotic fluid (15-23 weeks of gestation)	

		Isotope dilution GC-MS[53]	1-3 nM/L	165 nM/L (31.8-417) 64 nM/L (11.4-212)	Cord blood Amniotic fluid	Also detected maternal and conjugated and unconjugated
		Isotope dilution GC-MS [99]	LOD = 2nM	60-80nM 0.25 pM/mg	Rat fetal serum Rat dam's brain	
		TR-FIA[268]	3.2pg/20µl (9.2-1998pg/20µl)	3.2 ± 7.6 nM/L (0-47nM/L)	Maternal serum	Sensitive and specific assay
		LC-MS/MS [52]	500pg/ml (LOD) 0.5 -150 ng/ml (LOQ)	19.4 ± 19.2 ng/ml 7.2 ± 9.1 ng/ml	Cord blood Maternal serum	
		HPLC [52]	1ng/ml (LOD) 1-50ng/ml (LOQ)	5.2 ± 4.7 ng/ml 8.7 ng/ml (n=1)	Cord blood maternal	Sulphated conjugates
		RIA[269]	4.44fmol (1.2 pg) per tube		Human sera	Polyclonal antibodies
		RIA[269]	10.4 fmol (2.8pg)		Human sera	Polyclonal Ab

1.11. SUMMARY AND RESEARCH AIMS

Chemicals with hormone like actions are ubiquitous in environment. The chemicals and their degraded products released from common household materials (BPA in plastics), industrial detergents (4-nonylphenols), veterinary medicines, pesticides, phyto-estrogens and myco-estrogens were found to have estrogenic action and pollute the environment. The observations of reproductive abnormalities in the wild animals in a chemically polluted environment led scientists to hypothesize endocrine disruptive actions of these chemicals on animal development. The estrogenic action of some of these chemicals was identified in both *in vitro* and *in vivo* screening assays. Studies in the experimental animals have shown that most of these chemicals can transfer across the placenta and can cause reproductive and developmental anomalies in the fetus. Studies have also shown that humans are constantly exposed to these chemical. One of the studies has examined the hierarchy of human exposure to xeno-estrogens through dietary consumption and it was reported that BPA, genistein and alkylphenols constitutes major estrogenic chemicals in a typical New Zealand [41]. Based upon this report we selected BPA, genistein and 4-nonylphenol (alkylphenol) in our study. Detectable quantities of these chemicals were reported from both serum of pregnant mothersand umbilical cord blood, the latter showing the possibility of prenatal exposure. The conjugated forms of these compounds are devoid of estrogenic action. Human placenta expresses xeno-biotic conjugating enzymes (glucuronyl transferase and sulphotransferase) and hence if placenta could conjugate these compounds the consequences of prenatal exposure could be minimized. However none of the studies have directly assessed the transfer and biotransformation of these compounds in human placenta. Hence we had taken up this project to measure transplacental transfer and actions of BPA, genistein and 4-nonylphenol in human placenta.

Specific aims of the study

1. To develop an *ex vivo* dual perfusing single cotyledon human placental perfusion model in our laboratory

2. To develop High performance Liquid Chromatography (HPLC) and Liquid Chromatography – tandem Mass spectrometry (LC- MS/MS) to measure BPA, genistein and 4-nonylphenol in perfusates

3. To conduct transfer studies with BPA, genistein and 4-nonylphenol at environmentally relevant concentrations in human placental perfusion model

4. To study *in vitro* actions of BPA in placental explants

Chapter 2. METHODOLOGY

2.1. INTRODUCTION

As described in the previous chapter the embryo and fetal periods of development are the most susceptible period for chemically induced developmental abnormalities. Animal experiments have shown that a majority of the endocrine disruptors (EDs) can transfer across the placenta and produce various teratogenic effects in the fetus [11, 211, 213, 270]. Recent studies have shown that subtle changes taking place in a fetus due to a stressful intra-uterine environment might pre-dispose them to adult onset diseases. This hypothesis known as the Developmental origin of Health and Disease (DoHAD), originally put forth by David Barker relating intra-uterine under nutrition to predisposition to coronary diseases [271] during adulthood. The same theorycould be applied to the long-term effects of environmental endocrine disruptors present during fetal development. The human placenta is a complex organ and functions as a conduit for nutrients and facilitates the gaseous exchange required for the normal development of the fetus. There are many mechanisms by which placenta preferentially transport certain nutrients and drugs against their concentration gradients (ATPase pumps / specific transporters etc) and enzymatically modify endogenous hormones and other chemicals to protect the fetus. To monitor drug / nutrient / chemical transfer across the human placenta development of a well-defined system is required. The potential feto-toxicity prevents direct examination of such changes in pregnant women. Due to the variation in anatomical make up of placentae it is difficult to extrapolate the results from animal experiments. The best alternative was to develop a fully validated *ex vivo* human placental perfusion of a single cotyledon in our laboratory. The first perfusion with single cotyledon was conducted in 1962 by Panigel [272]. The method we validated in our laboratory was a modification of Glance *et al* (1984)[273],

Miller *et al* (1984)[274], Cannell *et al* (1988)[275] and Collier *et al* (2004)[276]. To detect the selected endocrine disruptors and endogenous estrogens in the perfusates we developed High Performance Liquid Chromatography (HPLC) and Liquid Chromatography – Tandem Mass Spectrometry based methods in our laboratory.

2.2. MATERIALS

2.2.1. Chemicals and reagents

The reagents and chemicals used were as tabulated below:-

Table 2-1 Reagents and chemicals used in the study

Name of the chemical / reagent	Catalogue No.	Source
17-beta estradiol	E-8875-5g	Sigma - Aldrich, St. Louise, USA
17-beta estradiol d_3	491187-100mg	Sigma - Aldrich, St. Louise, USA
4-nonylphenol	44-2873, Supelco	Sigma Aldrich, St. Louise, USA
Acetonitrile	1.00003.2500	Merck KGaA, Darmstadt, Germany
Antipyrine	A-5882-100g	Sigma - Aldrich, St. Louise, USA
BD Insyte – IV catheter 20GA, I inch, 1.1 X 25 mm	381433	BD Biosciences, Auckland, New Zealand
Beta estradiol glucuronide	E-1127-5mg	Sigma - Aldrich, St. Louise, USA
Beta estradiol suphate	E-9505-25mg	Sigma - Aldrich, St. Louise, USA
Bisphenol A	239658-50g	Sigma - Aldrich, St. Louise, USA
Bisphenol A d_{16}	DLM1839-1g	Cambridge Isotope Laboratories Ltd, MA, USA

Bovine serum albumin (BSA)	30063572	Sigma - Aldrich, St. Louise, USA
Butyl paraben (*p*-Hydroxybenzoic acid *n*-butyl ester)	54680-50g	Sigma - Aldrich, St. Louise, USA
Calibrated Pharmed tubing 2.0 mm ID	F1825103	John Morris Scientific, Auckland, New Zealand
Charcoal stripped FBS	12676029	Invitrogen corporation, CA, USA
Dichloromethane	1.00044.2500	Merck KGaA, Darmstadt, Germany
Diethyl ether	1.00921.5000	Merck KGaA, Darmstadt, Germany
DMEM-F12 (Phenol Red free)	11039-047	Invitrogen corporation, CA, USA
DMEM-F12 (with Phenol Red)	10565-042	Invitrogen corporation, CA, USA
DNase I – amplification grade	18068-015	Invitrogen corporation, CA, USA
Estriol	E-1253-100g	Sigma - Aldrich, St. Louise, USA
Estrone	46573-10g	Sigma - Aldrich, St. Louise, USA
Ethanol	200-578-6	BDH Lab Supplies, England, UK
Ethyl acetate	1.00868.2500	Merck KGaA, Darmstadt, Germany
Faslodex (ICI 182, 780)	1047-10mg	Tocris Bioscience, Bristol, UK
Fetal bovine serum (FBS)	10099-141	Invitrogen corporation, CA, USA
FITC dextran	FD-4	Sigma - Aldrich, St. Louise, USA
Genistein	G6649-25mg	Sigma - Aldrich, St. Louise, USA
Genistein d_4	DLM-4460-1.2	Cambridge Isotope Laboratories Ltd, MA, USA
Gentamicin	15710-064	Invitrogen corporation, CA, USA
Heparin	Multiparin	CP Pharmaceuticals Ltd, Wexham, UK
L-Glutamine	21051-024	Invitrogen corporation, CA, USA
M-199 media without phenol red	M-3274	Sigma - Aldrich, St. Louise, USA

Methanol	1.06007.4000	Merck KGaA, Darmstadt, Germany
Multisorb Nonwoven swabs (7.5 X 7.5 cm^2)		BioLab Ltd, New Zealand
n-Pentane	1.07177.2500	Merck KGaA, Darmstadt, Germany
Nunc Plates	456537	Nalge Nunc International, NY, USA
Penicillin-Streptomycin	15070063	Invitrogen corporation, CA, USA
Phenacetin	Serva	Serva, Feinbiochemica, Hesperia, CA
Polyvinyl pyrrolidone 40 (PVP-40)	PVP-40-500	Sigma - Aldrich, St. Louise, USA
Single lumen PVC tubes (2mm outer diameter and 1mm inner diameter)		BioLab Ltd, New Zealand
Sodium bi-carbonate	SO129	Scharlau, Barcelonia, Spain
Soft-silk (2-0) (Cutting edge 3/8 - 24mm needle) Silk Suture 2/0 Davis & Geck SS695	BUY124	Helath Support Limited, Auckland, New Zealand
SuperScript® III Platinum® Two-Step qRT-PCR Kit	11735032	Invitrogen corporation, CA, USA
SYBR green reagent	4385612	Invitrogen corporation, CA, USA
Trizol	10296-028	Invitrogen corporation, CA, USA
Trypsin EDTA	15050-065	Invitrogen corporation, CA, USA
Vygon-V-Green IV- Extension Line	7.1100.01	BioLab Ltd, New Zealand

2.3. METHODS

2.3.1. Placental perfusion

To study the transfer and conjugation of selected endocrine disruptors in human placenta a placental perfusion model was standardized in our laboratory.

2.3.1.1. Instruments:

The instruments below were used for conducting perfusion studies

1. Biohazard cabinet: - To manipulate the tissue in an isolated environment and to maintain proper temperature throughout the perfusion period a biohazard cabinet was used. Four electric thermostats (Redring Electrical Ltd., UK) were attached to the biohazard cabinet to maintain a temperature of 37°C inside the cabinet throughout the perfusion period. The temperature was constantly monitored with the help of digital thermometer connected to the thermal probes attached to the biohazard cabinet.
2. Perfusion pumps (Cole Parmer Instrument Co., Chicago, USA) with manual speed control units
3. Carbogen gas (BOC): - This contains a mixture of oxygen and carbon dioxide at 95 and 5 % respectively and the gas delivery was controlled with the regulator attached to the cylinder.
4. Sphygmomanometers (MAC, Japan)
5. Gilson Minipulse-3 digitally controlled pumps (John Morris Scientific, Australia)
6. Heating magnetic Stirrer (VELP Scientifica, Milano, Italy)
7. pH meter
8. Labserve pipettor

2.3.1.2. Preparation prior to collection of the placenta

The Perfusion cabinet and water bath were turned on 2 hours prior to the collection of placenta to maintain a temperature of 37 ^{o}C (heating bars at 75 ^{o}C with the help of a fan in the perfusion cabinet were tuned to maintain this temperature inside the cabinet). The fresh perfusates (Appendix 2) and 1L of PBS were also warmed to 37°C 30 minutes prior to collection of placenta. An autoclaved placental collection tray along with the details of patient consent for collection was given to the reception at birth unit of Auckland City Hospital.

2.3.1.3. Technique standardization using manual pumps:-

Pump calibration and configuration:-

Initial standardization was done with the help of a set of manual pumps as described by Collier *et al* [276] with modifications. A simple experiment was carried out to set the pump head speed to 4 mL / min in the fetal compartment and 10 mL / min in maternal compartment. Distilled water was circulated through the pumps for 30 minutes and collected in a measuring cylinder. The volume collected from the fetal compartment should be exactly 120 mL and from the maternal compartment should be exactly 300 mL. Once these volumes were achieved the pump heads were set and the points were marked on the manual speed controller on both maternal and fetal pumps. This exercise was carried out before each perfusion experiment to ensure that the pumps were properly calibrated for each experiment. One of the problems encountered while using the manual pumps was the uncontrolled flow which inadvertently introduced some bubbles into the fetal compartments and caused early termination of perfusion due to increased pressure and more leaks. Hence perfusions were carried out using digitally controlled pumps.

2.3.1.4. Technique standardization using digital pumps:

The digital pumps used for perfusion experiments were as shown in the Figure 2-8. The standardization of the experiment using these pumps tremendously improved the perfusion success as it imparted a uniform smooth flow and greater control over adjusting its flow rate. Once the 3-way taps were configured appropriately as shown in Figure 2-8 a simple experiment was performed each day before the start of the actual perfusion experiment to adjust the flow rates in both the maternal and fetal compartments. Perfusates were allowed to flow through both compartments using two different pumps adjusted at a flow rate of 4 mL/min (fetal compartment) and 10 mL/min (maternal compartment). The perfusates coming out from the other end of the tube attached to the pump were collected with the help of a measuring cylinder at specific time intervals and the flow rates were calculated. Before the actual experiment was started, the pumps were calibrated and then Milli Q water and absolute ethanol were pumped through the tubes to clean up the system.

2.3.1.5. Placental collection and preparation of placenta

The placenta was transferred to the laboratory immediately after (within 10 minutes) C-section. Once in the laboratory, the tray containing the placenta was placed over a water bath containing distilled water at 37°C to maintain the warmth of the placenta. Gross morphological examination was conducted immediately for the suitability of using the placenta in perfusion experiments. The placentae showing any of the following abnormalities were excluded from the study.

a) Widespread calcified areas

b) Blood clots and haemorrhage

c) Fragile maternal surface, as evidenced by tears and sinusoids in the maternal cotyledons

d) Rigid chorionic vessels

e) Absence of terminal branches to a cotyledon

The time from birth to cannulation affects the viability of placenta and in-turn the success of the perfusion experiment. The examination was completed as quickly as possible (approximately 10 minutes). Once a suitable cotyledon was identified, the entire water bath containing the placenta was transferred to the perfusion cabinet (biohazard cabinet) and placed over a heated magnetic stirrer (maintained at 37°C). The fetal reservoir containing perfusate was also placed in the neighbouring heated magnetic stirrer maintained at 37°C. One end of the arterial catheter tube was immersed in the fetal reservoir (200 mL perfusate in 250 mL glass bottle) and connected to the peristaltic pump. The perfusate was then re-circulated in the fetal reservoir by turning on the pump at a very slow head speed (~1 rpm). A small portion of amniotic membrane lining the chorionic artery was excised with the help of scissors. A nick was then generated in the artery (3^{rd} or 4^{th} order of the artery) with a 20 gauge IV catheter to create the suitable entry-point (Figure 2-1). We carefully inserted the Polyethylene tube (PE tube) (2.0 mm OD and 1.0 mm ID) coming from the other end of tube immersed in fetal perfusate (Figure 2-2). We secured the catheter into the chorionic artery with a surgeon's knot (nylon 2-0) (Figure 2-3). A tertiary vein corresponding to the artery and supplying the same cotyledon was catheterized in the similar manner. A correctly established fetal circuit was demonstrated by the return of perfusate through the venous catheter. At this stage, the returning fluid would be heavily contaminated with RBCs, and would be very slow. Upon successful establishment of fetal circulation the pump speed was increased slowly to reach a speed of 4 mL / min. The pressure fluctuations could be observed in the in-line

sphygmomanometer. As one or more air bubbles could make a drastic change in fetal pressure and could adversely affects the perfusion, extra care was given to avoid any extraneous introduction of air at this stage.

Figure 2-1 Creating an entry-point for the arterial (fetal) catheter tubing

A 20 G intravenous (IV) catheter is used here to produce a nick in the chorionic vessels.

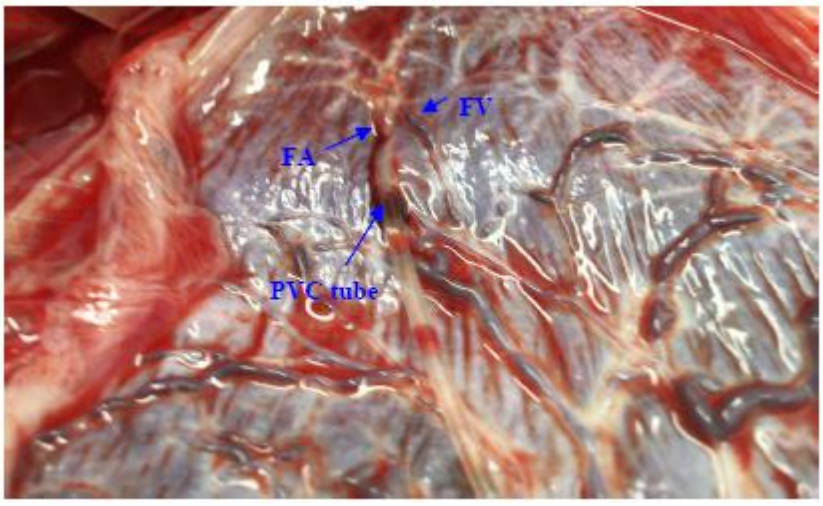


Figure 2-2 Insertion of a PVC / PE -tube with slow-flowing perfusate into the chorionic artery (FA)

The corresponding chorionic vein is identified here (FV).

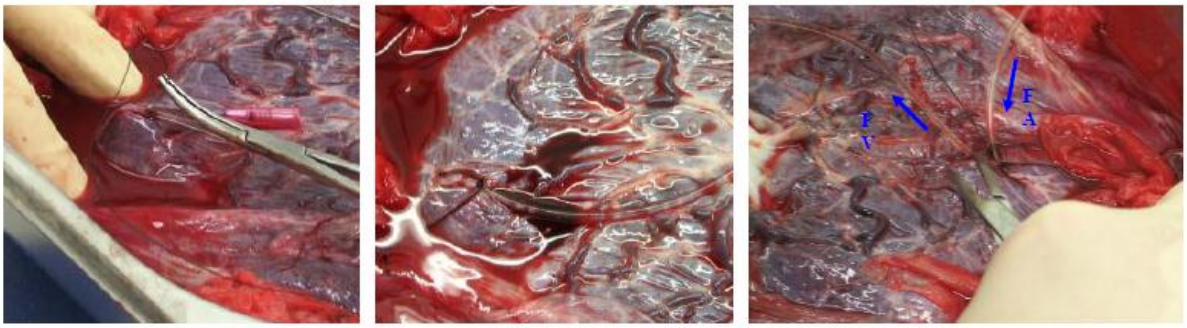


Figure 2-3 Suturing the tubes tightly but carefully into place

After inserting the tubes into the chorionic arteries they were fixed in place with the help of surgeon's knots. The procedure was repeated for the corresponding chorionic vein.

During this time, we prepared the reservoir for the maternal compartment (400 mL of perfusate in 500 mL glass bottle) by connecting either ends of tubes to the maternal reservoir and gassing with carbogen gas. The perfusion chamber was disassembled into its 3 components as shown in the figure 2-4. (It has a funnel-like base; spiked middle portion; and top portion).

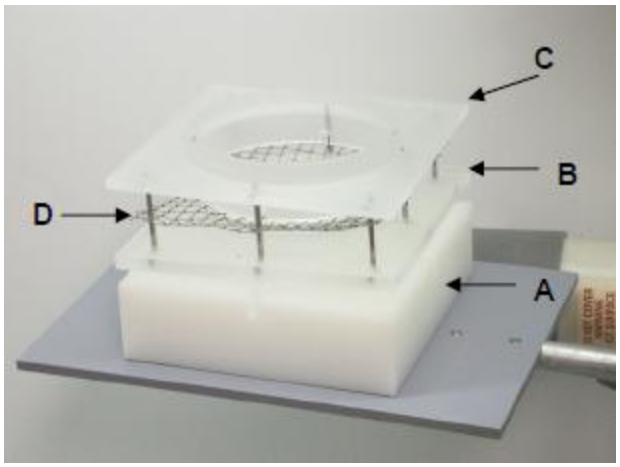


Figure 2-4 Perfusion block

(A) Funnel-like base; (B) spiked middle portion; (C) top portion; (D) mesh grid.

We carefully examined the maternal side and identified the cotyledon perfused by the chorionic vessels by observing the blanching pattern. We then cut around the cotyledon carefully, leaving sufficient surrounding tissue to avoid damage to the blanched cotyledon (Figure 2-5A). The cotyledon was carefully transferred onto a mesh grid in the middle portion of perfusion chamber with maternal side down resting in the grid (Figure 2-5B).

The excess tissue present in the cut end of placenta could be held in the spikes to secure the tissue in position. Initial standardizations were made by covering the fetal side with parafilm (Figure 2-5C) which helped to keep the tissue warm, and held the tissue in place when inverted it for catheterizing the maternal side. We did not cover the fetal side with parafilm when perfusion experiments were conducted with chemicals (BPA, 4-NP and genistein) under test conditions. A glass petridish was used to cover the fetal side in all our experiments. The top portion of the perfusion block was placed into its position as shown in Figure 2-5D.

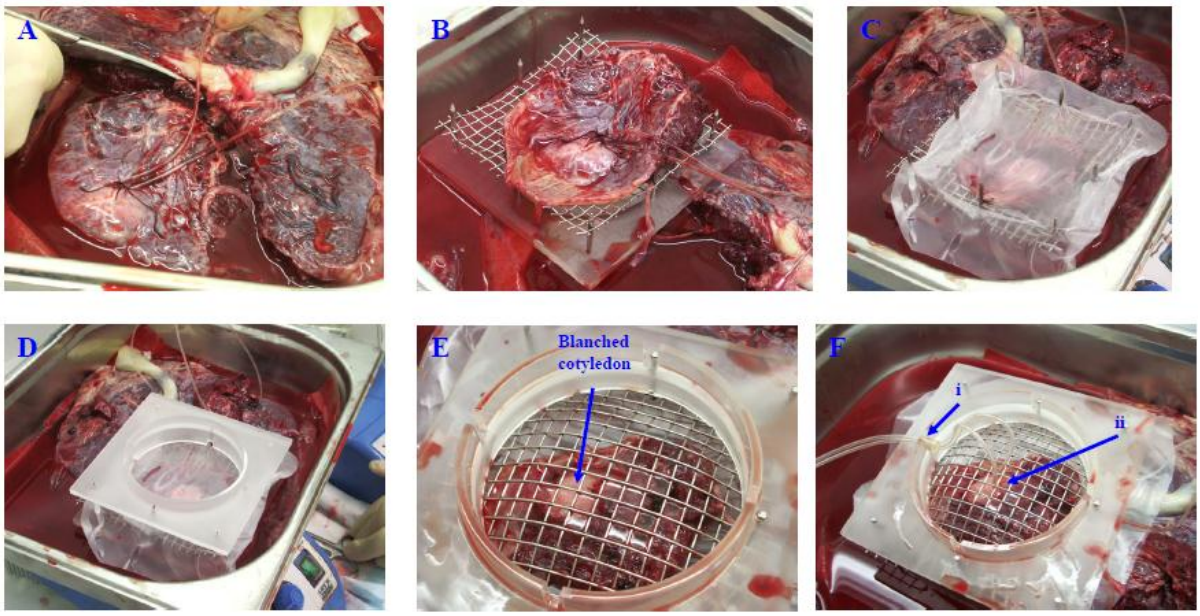


Figure 2-5 Trimming and mounting the cotyledon in perfusion block

The isolated cotyledon was carefully trimmed away (A) from the placenta and placed the maternal cotyledon side down into the centre of the mesh grid (B). The fetal surface of cotyledon was covered with parafilm using the spikes to secure it (replaced with glass petridish in experiments using chemicals of interest) (C). The top portion was secured into place (D) and the apparatus was gently inverted to reveal the maternal surface through the mesh (E). The maternal catheter was locked into place (Fi), then the blunt ended tubes were fed into 2 separate locations in the same blanched cotyledon (remnants of the spiral arteries) (Fii).

The top portion of the perfusion block with tissue in place was then inverted gently as shown in Figure 2-5E. The perfused cotyledons were then located with the help of blanching observed in the perfused areas. The remnants of spiral arteries (small openings)

in the decidual plates were cannulated with two Polyethylene (PE, ID 1.0 mm, OD 2.0 mm) cannulation tubes (Figure 2-6) that fit tightly into the block side-slits (Figure 2-5.Fi).

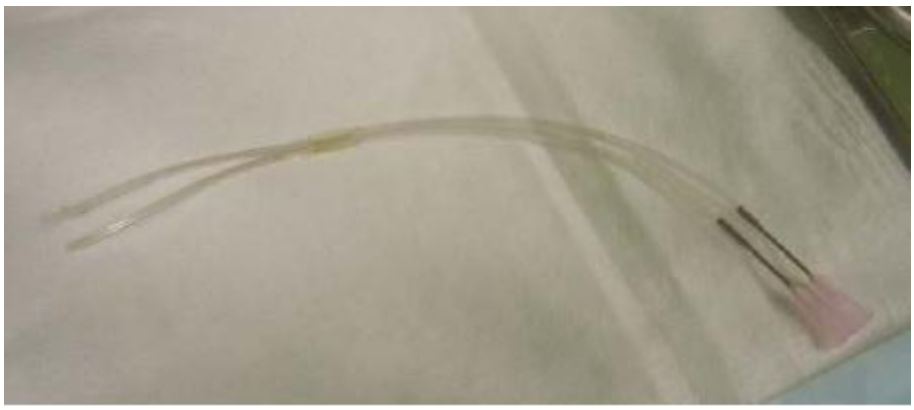


Figure 2-6(a)

Figure 2-6(b)

Figure 2-6 Maternal Catheters

(a) The Maternal Catheter, two PVC tubes (1.0 mm ID; 2.0 mm OD) approximately 15 cm in length, was fed through a small piece of thicker tubing (or held together with masking tape). (b) The blunt ends were inserted into the remnants of the maternal spiral arteries. Small cuffs near the end of the tube helped to hold the tubes in position.

This is designed to prevent the tubes from being pulled out of the tissue when manipulating the luer-lock end and to connect it to the maternal inflow line. The blunt ended tubes were inserted into each spiral artery (Figure 2-5.Fii), which allowed the support cuffs to grip the wall of the "pseudoartery". The tubing was slotted in the slits in the underside of the

middle portion of the chamber, and the funnel-base was carefully locked into place (Figure 2-7A). Although there should be no resistance for about 2-3 mm below the decidual plate, caution was taken when inserting the tubes into the spiral "pseudoartery" to avoid piercing and damaging the tissue. The fetal pressure fluctuations were carefully monitored while manipulating the tissue because major perfusion failures may occur on faulty tissue manipulations.

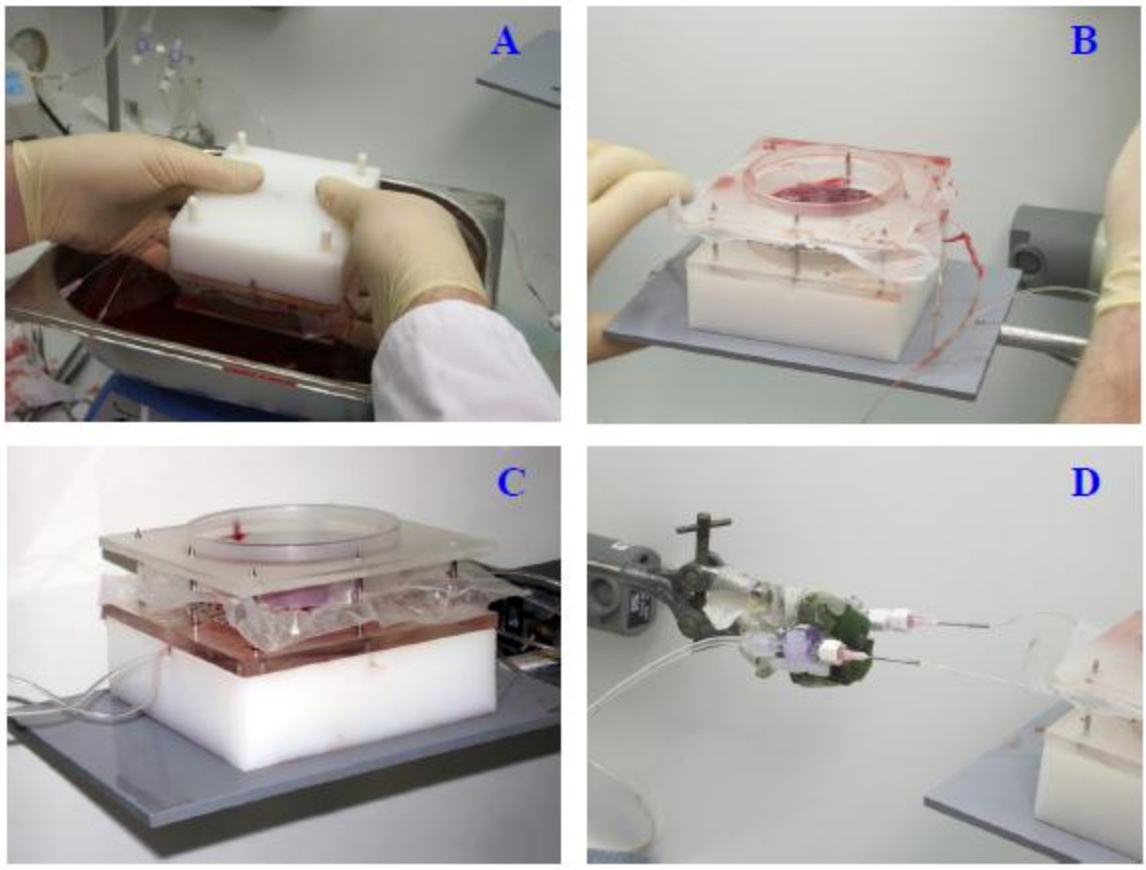


Figure 2-7 Placing the perfusion block in the perfusion chamber

(Above) – The funnel-base was clamped onto the bottom (A), the block was carefully inverted so that the fetal side was on top, and placed on the support arm (B). We made sure that the catheters are orientated appropriately (in this case, fetal on the right and maternal on the left) (C). After the maternal arterial line was flushed with oxygenated perfusate, the two maternal catheters were connected to the double-3-way taps

Figure 2-8 The overall perfusion set up

We carefully inverted the apparatus so that the fetal circuit was on top, and placed it on the support arm (Figure 2-7B). The arm was level with the fetal sphygmomanometer and the catheters protruding from the apparatus were correctly oriented (Figure 2-7C - fetal-right; maternal-left).

The maternal pumps were then operated with motor head speed set to about 32 rpm (10 mL/min – 2.0 mm ID pump tubing). The perfusates in the maternal compartment were allowed to circulate in an open-configuration for approximately 10 minutes to clean out the red blood cells. Once the circulation was established, the circuit was closed by attaching the maternal-effluent tube to the hole under the base (blue asterix in Figure 5E) of the funnel, with the pump output tube feeding back into the reservoir. PE tubes with large internal diameter (3.0 – 4.0 mm ID) were used to clear the maternal effluent out of the funnel , thus preventing a build-up of fluid.

2.3.1.6. Equilibration period (30 minutes to 1 hour)

Once maternal and fetal effluents were clear of RBCs, the maternal and fetal compartments were closed by allowing the perfusates to re-circulate through them. The pressure and flow rates were observed for 10 to 15 minutes and once stable values were reached we kept the re-circulating mode of perfusion running for a total of 30 to 60 minutes. Only pH was adjusted during this period with 1M NaOH or 1M HCl, if pH fluctuations were observed; they were measured at 10 minutes intervals.

2.3.1.7. Perfusion experiment

Initially, standardization was performed to establish a re-circulating mode of perfusion (closed – closed type) (Figure 2-9). Once the pressure and flow rates were established in equilibration period, the perfusates were changed with fresh bottles of perfusates containing the compounds of interest in the maternal compartment [400 mL of perfusate with added positive marker (antipyrine), negative marker (FITC-dextran – 4 KDa), compounds dissolved in methanol in 500 mL of autoclaved glass bottles]. Carbogen gas was introduced into the maternal compartment to oxygenate the perfusates. Control experiments without compounds were performed to check the background contamination of compounds of interest present in the placenta. The fetal compartment solution was replaced with 200 mL of perfusate in fresh 250 mL bottles. The fetal tubes were inserted in the fresh perfusates while the pumps were in operating mode to avoid any drastic changes in pressure. One of the problems to avoid at this point was the inadvertent release of air bubbles, and we needed to monitor if any had been released by opening the stop-cock region of the tubes. The perfusates were continuously mixed with magnetic stirrers. The perfusate in both compartments were monitored at regular intervals with pH meters (10-15 min) to detect any pH fluctuations and they were adjusted with 1M NaOH or 1M HCl and

the fetal compartment was monitored continuously for pressure fluctuations. Samples (7.5 mL perfusates from both maternal and fetal reservoirs) were collected at 0 and 10 minutes and at an interval of 30 minutes for 3 hours. The samples were split into 3 vials (glass vials for chemicals / conjugate analysis and amber / clear eppendorffs for FITC-DX, β-hCG, glucose and lactate estimation. 7.5 mL of blank perfusate was added to each compartment to make up the volume. The samples were immediately stored in -20°C (for chemicals) and -80°C (for the metabolites).

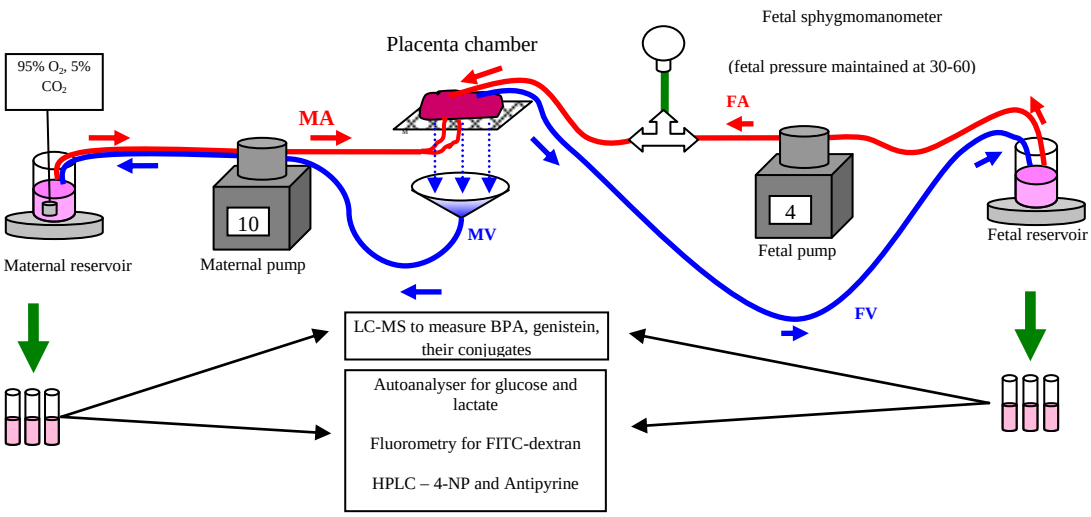


Figure 2-9 Re-circulating single cotyledon dual Placental Perfusion Model

After suitable lobules were selected, an appropriate arterial (FA) and venous (FV) pair of vessels on the fetal side (chorionic plate) were catheterized and slowly perfused with blank perfusate. Two blunt-ended cannulas were inserted into the intervillous space of the corresponding lobule on the maternal side to serve as maternal arteries (MA), through which oxygenated (Carbogen gas; 95% O2 and 5% CO2) perfusate was introduced. The venous effluent (dotted arrows; MV) was collected by gravity in a funnel and pumped back into the maternal reservoir for mixing and recirculation. Compounds of interest were added to the maternal reservoir, and samples taken from both reservoirs at t=0 min 10 min and at 30 min intervals thereafter. Samples were assayed for chemicals of interest, and for β-hCG, glucose and lactate levels. Red and blue arrows indicate the direction of flow.

2.3.1.8. Problems solved during method development

There were many challenges surpassed to standardize the perfusion method in our laboratory.

1. Time to transport placenta from hospital to the laboratory:

One of the major reasons for the failed perfusion was the latency observed in the collection of placenta from hospital. The normal procedure involved the despatch of autoclaved trays from the hospital reception approximately an hour before the actual time for caesarean. Immediately after delivery the receptionist made contact through mobile phone for picking up the placentae. Many times it was observed that the turgor of the fetal vessels was lost due to the time spent in the tray. The collection time was reduced by reaching the hospital 30 minutes prior to the time for caesarean section and immediately transporting the placenta to laboratory. It took only 10 minutes to transport the placentae from hospital to the laboratory. Once reached, the laboratory the tray containing placentae was immersed in separate tray containing distilled water maintained at 37°C. We immediately washed the placentae with 250 mL of sterile 1X PBS (maintained at 37°C) and again poured another 250 mL of sterile PBS over the placentae to reduce the tissue trauma. Examination time was reduced by keeping the placentae in the biohazard cabinet while examining the gross morphological and physiological integrity of the membranes and immediately catheterized via the chorionic vessels with perfusates flowing through the tubes. The earlier method [276] incorporated the removal of whole amnion from the fetal compartment before catheterizing the chorionic vessels. We found this procedure caused delay and may have traumatized the chorionic plate. Our modification of removing only the membrane lining the chorionic vessels (where a nick should be placed to pass the catheter in) was found to drastically improve the perfusion process. The membrane may have provided additional covering to the vessels and chorionic plate, thus preventing fluid loss from evaporation and maintaining the physiology throughout the perfusion. Moreover, the hanging ends of the membrane could be used to secure the cotyledon in perfusion chamber by attaching them to the spikes.

2. Feto-maternal fluid shift:

Another major reason for terminating the perfusion experiments was the feto-maternal fluid loss evidenced by the loss of fluid from the fetal compartment. One explanation for the feto-maternal fluid shift was the inadvertent entry of air into the chorionic vessels when the flow rate was increased with the manual pumps. This problem was taken care of by the use of digitally controlled peristaltic pumps, which were efficient in smoothly increasing the flow rate. The use of digitally controlled pumps also facilitated a reduction of pressure fluctuations in the fetal compartment during perfusion experiments. The second important reason for feto-maternal fluid shift was the presence of multiple terminal branches of the chorionic vessels supplying the adjacent cotyledons. This issue was remedied to a certain extent by carefully suturing the visible branches as shown in Figure 2-10.

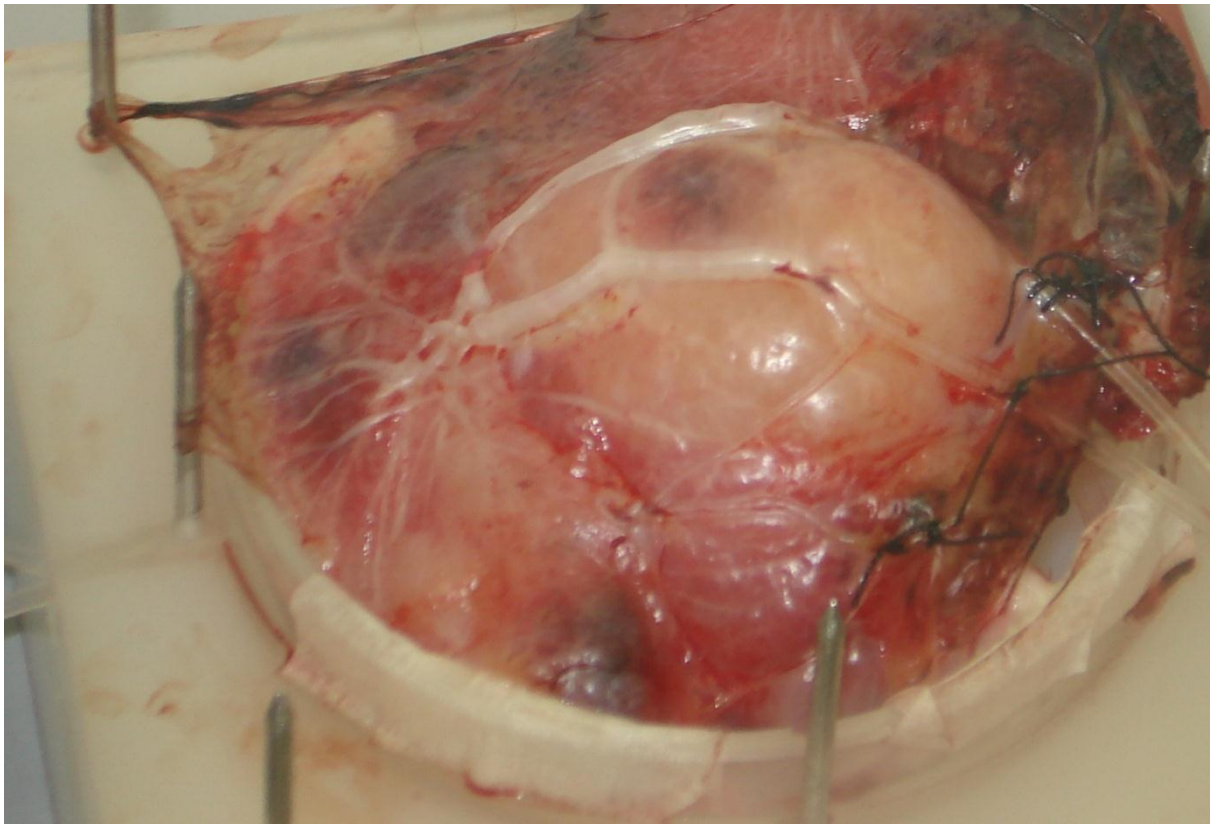


Figure 2-10 Suturing the multiple branches of chorionic vessels to prevent the feto-maternal fluid shift

3. Pressure and pH fluctuations:

Pressure fluctuations were evident during the initial periods of perfusion. As described earlier the use of digital pumps reduced the undue fluctuations in the pressure. The pressure immediately after passing the tubes in the chorionic arteries were in the range of 40 mm of Hg even with very low head speed of the pumps. This would fluctuate between 40 to 60 mm of Hg during the first 10 minutes of perfusion until the pump speed reached its maximum to deliver 4 mL/min. The tissue manipulation further deteriorated it (sometimes reaches above 80 mm of Hg and a total collapse was observed in such cases) and a few modifications drastically improved stabilizing pressure fluctuations during this period. Oxygenating fetal perfusates by very slow administration of carbogen gas could reduce the pressure in the fetal compartment. Another modification was to keep the excised tissue in the perfusion chamber and then carefully inserting the tubes through the remnants of spiral arteries with minimum digital pressure on the fetal side to fix it.

4. Changes in the equilibration time

Due to unacceptable levels of materno-fetal leakage after equilibration period, many perfusion experiments were forcefully terminated. On contact with experts in the field it was learned that increasing equilibration time to 2.0 hours might stabilize the leak (Prof. Richard K. Miller through personal communication). Introducing these protocols in our system has tremendously improved perfusion process. However further standardization could reduce the equilibration time to 30 to 60 minutes.

5. Changes in tubing

We tried using PVC and polyethylene tubes. To avoid any potential leach of BPA from PVC tubes we modify our perfusion experiments using Polyethylene tubes (PE tubes). PE tubes were rigid on comparison to PVC tubes and further care was taken while inserting it to the fetal vessels. Due to its rigid nature PE tubes were avoided in maternal

catheterization. We adopted Pharmmed tubes which are impermeable to gases and vapours.

6. Changes in perfusion block

New set of perfusion blocks were designed with Aluminium body for proper maintenance of temperature. Further we have provided the inlets for temperature and oxygen probes to connect and read the online measurements of oxygen consumption and temperature changes. This further modified our perfusion process.

2.3.2. High Performance Liquid Chromatography (HPLC) for detection of antipyrine

Two extraction methods were standardized in our laboratory to detect the antipyrine concentration in the maternal and fetal perfusates.

A) extraction with dichloromethane – n-pentane (50:50) [277]

B) extraction with ethyl acetate

2.3.2.1. Extraction with dichloromethane – n-pentane (50:50)

Antipyrine stock standards were prepared at 10 mg/mL in methanol. Phenacetin (internal standard) stock standards were prepared at 1 mg/mL in 50% ethanol (first to dissolve completely) and 50% methanol. Calibration standards (7 calibration standards were prepared by serial dilution) for antipyrine (1 µg/mL to 100 µg/mL) were then prepared in perfusate by diluting the methanolic standard 100 fold to get the final concentration (for example for preparing 100 µg/mL solution, 10 µL of 10 mg/mL stock standard was transferred to 990 µL of perfusate). Similarly Phenacetin stock standard was diluted 100 fold in perfusate to get the final working concentration.

250 µL of standards were added to 10 mL glass tubes. The samples in duplicate (perfusates from maternal and fetal compartments collected at pre-determined time points) were added

to the remaining glass bottles. Internal standard prepared in perfusate (100 µL of 1 µg/mL solution) was added to each tube and mixed thoroughly by vortexing. Dichloromethane – n-pentane (50:50) (4mL) was dispensed into each glass tube and screw capped, then mixed thoroughly for 15 minutes on a tube rotator. The tubes were centrifuged at 3000 g for 15 min (Beckman J6-MI) and stored at -80°C for 30 minutes. The supernatant (organic layer) was carefully poured into 5 mL glass test tubes and lyophilised. The dried extracts were re-dissolved in 200µL of mobile phase, mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thin septa and screw caps.

Chromatographic separations were performed on an HPLC system (Waters Alliance 2690 Waters Corporation, Milford, MA, USA). The column used to separate the compounds was Luna 3µ C18 (2) 100A, 250 X 4.6 mm (Phenomenex). The mobile phase was 6.7 mM phosphate buffer pH 7.2: acetonitrile (65:35). The separation was carried out in an isocratic mode at a flow rate of 0.7mL/min. 10µL of sample was injected and detection was carried out using a UV detector (Waters 996 photodiode array detector) at 254 nm. The data were analyzed using Millennium Chromatography Manager V4.

2.3.2.2. Extraction with ethyl acetate

This method was initially standardized with an objective of simultaneous detection and estimation of bisphenol A, 4-nonylphenol and antipyrine in the perfusates with the help of serially connected Fluorescence / Ultraviolet (UV) photodiode detector. The method optimization for BPA is described in detail in section 2.1.3.

This HPLC method was a modification of the method standardized in our laboratory [277]. Briefly 100µL of Phenacetin (internal standard, Serva, Feinbiochemica, USA), 500µL of 0.025N HCl and 500µL of acetonitrile were added to 500µL of perfusate and

mixed well by vortexing. Then 4 mL of ethyl acetate was added and the solution was mixed for 15 minutes in a tube rotator. The tubes were then centrifuged at 3000g for 10 minutes (Beckman J6-MI) and stored at -80°C for 30 minutes. The supernatant (organic phase) was transferred to a glass tube and lyophilized. The dried extracts were re-dissolved in 200µL of mobile phase, mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thin septa and screw caps.

Chromatographic separations were performed on an HPLC system (Waters Alliance 2690 Waters Corporation, Milford, MA, USA) as given above.

Both methods were comparable in sensitivity and robustness; hence the first method is used for majority of antipyrine detection in our studies. The methods have a limit of detection of 250 ng/mL with a signal to noise ratio of >10. The chromatogram for antipyrine and phenacetin were as given in the Figure 2-11. The standard curve was linear from 250 ng/mL to 100 µg/mL (Figure 2-12). The recovery percentage was more than 96% (Figure 2-13).

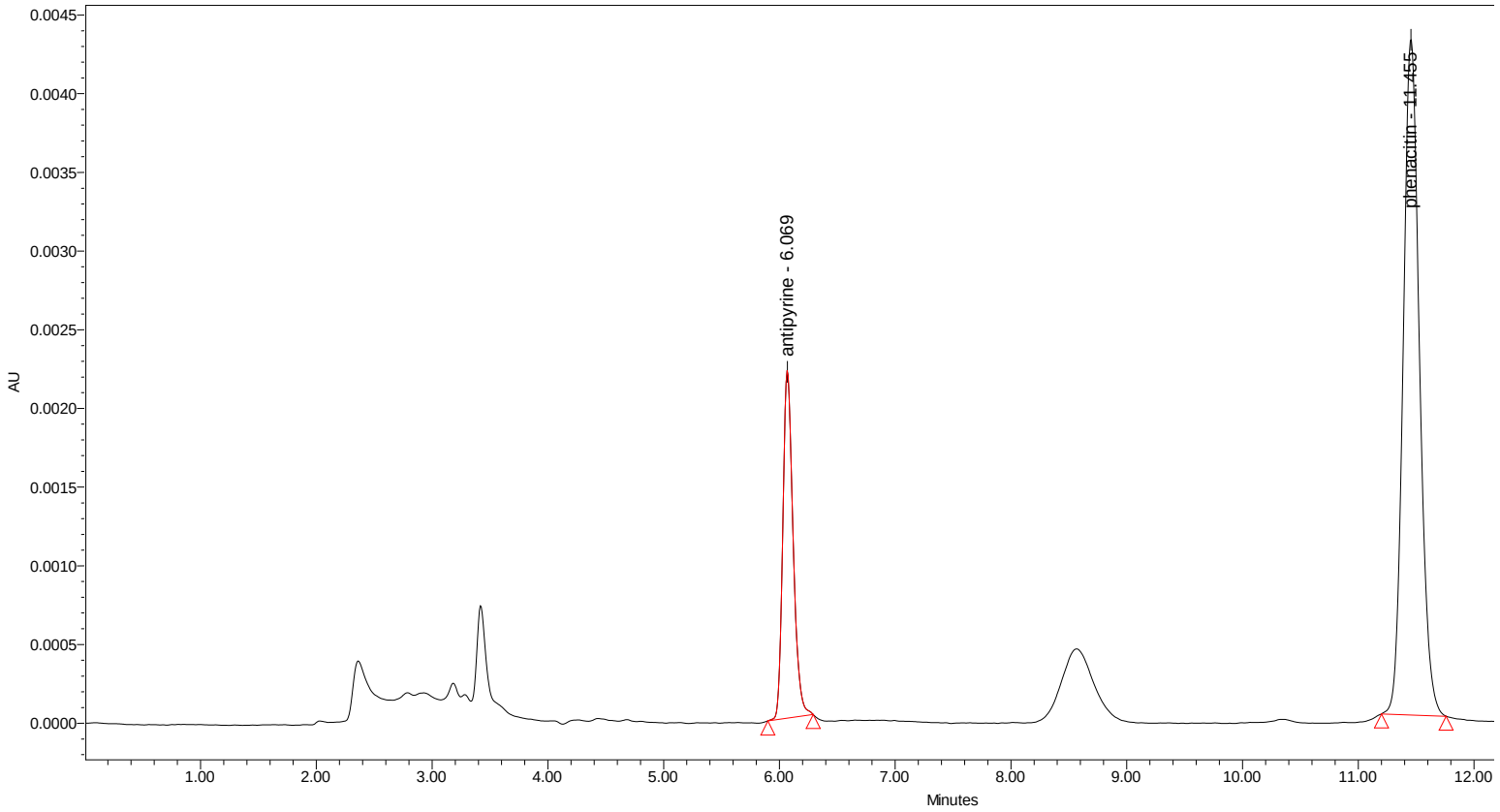


Figure 2-11 HPLC traces for phenacetin and antipyrine

Antipyrine eluted at 6.25 minutes and phenacetin eluted at 11.5 minutes

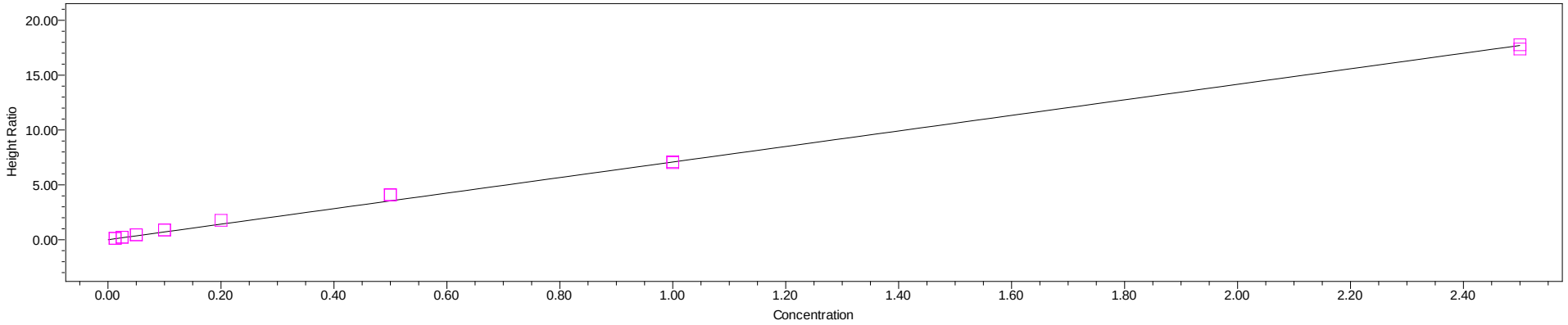


Figure 2-12 Standard curve for antipyrine

Serial dilutions were prepared from 250 ng/mL to 50 µg/mL in perfusate and extracted with dichloromethane – n-pentane (50:50) as mentioned in the materials and methods. The standard curve was linear from 250 ng/mL to 50 µg/mL with $r^2 = 0.999$.

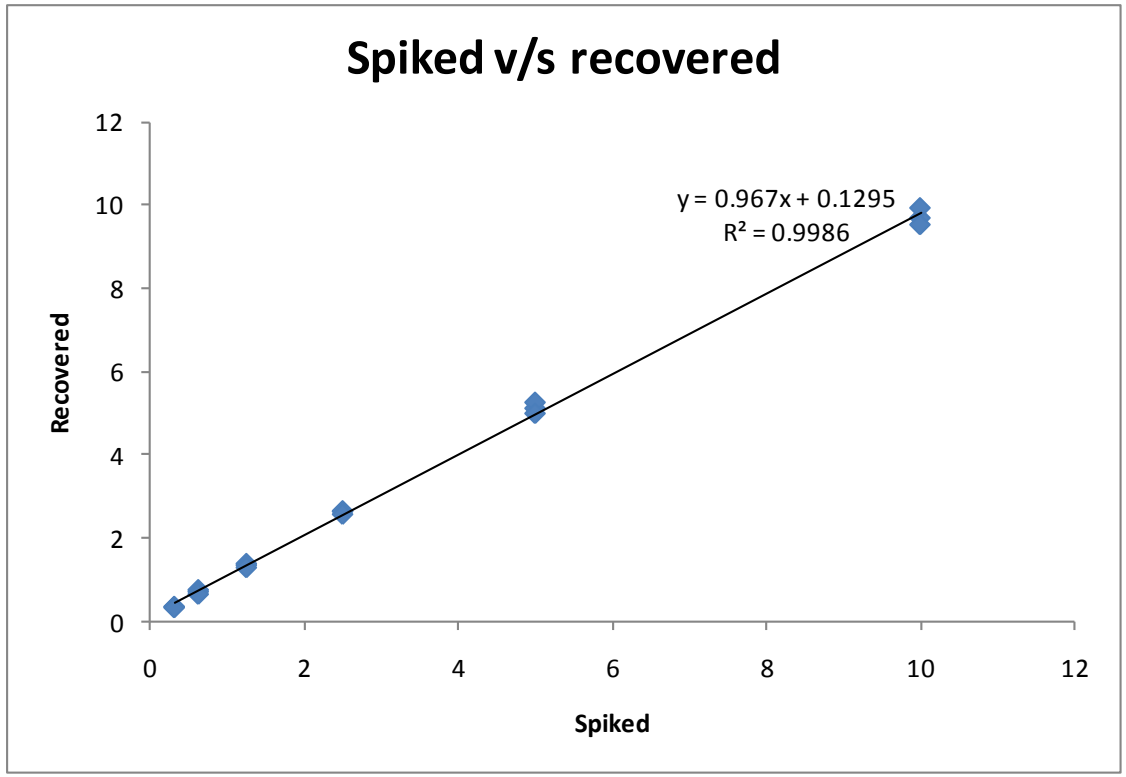


Figure 2-13 Efficiency of recovery

Perfusates were spiked with antipyrine at 0.3125, 0.625, 1.25, 2.5, 5 and 10 µg/mL. When a graph was plotted with the actual spiked concentration of antipyrine on X-axis with the recovered concentrations on Y-axis the trend line was linear with $r^2 = 0.9986$.

2.3.3. High Performance Liquid Chromatography (HPLC) for the detection of BPA in the perfusates

The HPLC method was a modification of the method developed by Shin *et al* (2004) [278]. This method was standardized to extract 4-nonylphenol, BPA and antipyrine simultaneously. BPA and 4-nonylphenol stock solutions were prepared at 10 µg/mL each in methanol. Butylparaben (internal standard) stock standards were prepared at 1 mg/mL in methanol. Nine calibration standards for BPA (100 pg/mL to 100 ng/mL) were then prepared in perfusates by diluting the methanolic standard 100 fold to get the final concentration. Similarly butylparaben stock standard was diluted to 100 fold in perfusate to get the final working concentration. In brief, 100µL of butylparaben, 500µL of 0.025N HCl and 500µL of acetonitrile were added to 500µL of perfusate and mixed by vortexing. Then 4 mL of ethyl acetate was added and the solution was mixed for 15 minutes in a tube rotator. The tubes were then centrifuged at 3000g for 10 minutes (Beckman J6-MI) and stored at -80°C for 30 minutes. The supernatant (organic phase) was carefully transferred to a glass tube and lyophilized. The dried extracts were re-dissolved in 100µL of mobile phase (1% acetic acid: acetonitrile at 70:30), mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thin septa and screw caps.

Chromatographic separations were performed on an HPLC system (Waters Alliance 2690 Waters Corporation, Milford, MA, USA). The column used to separate the compounds was Luna 3µ C18 (2) 100A, 250 X 4.6 mm (Phenomenex). Chromatographic conditions were studied using different mobile phases, gradient system, flow rates and excitation and emission fluorescence. The best mobile phase was a gradient mixture of acetonitrile and

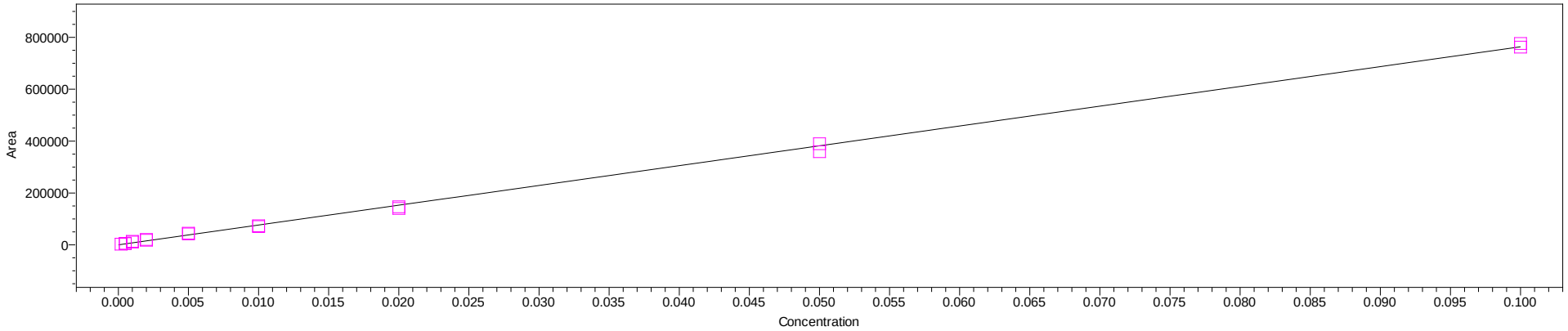
acetic acid (0.1%). The gradient conditions were standardized as in the table below (Table 2-2).

Table 2-2 Gradient parameters for detecting BPA in perfusates

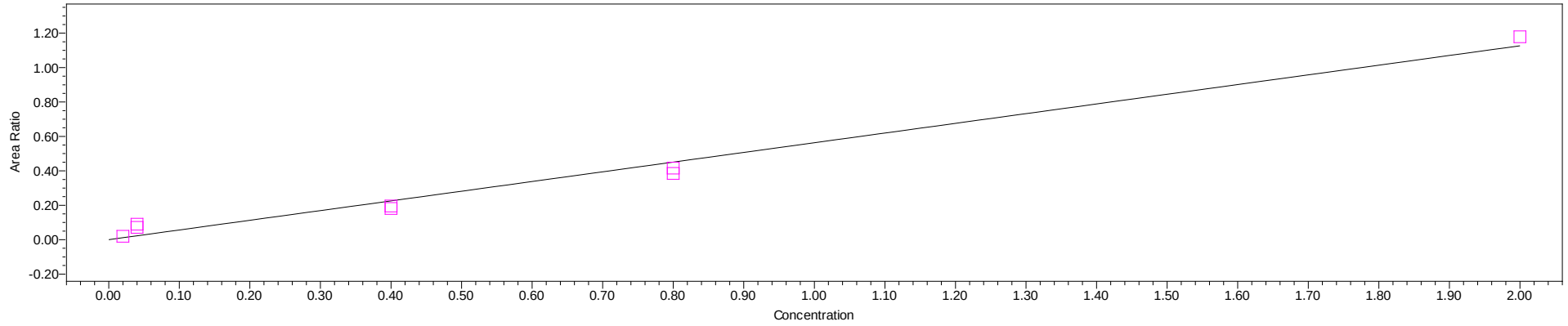
	Time	Flow	%A	%B	%C	%D	Curve
1		1.00	0.0	35.0	0.0	65.0	
2	1.00	1.00	0.0	35.0	0.0	65.0	6
3	9.00	1.00	0.0	10.0	0.0	90.0	6
4	10.00	1.00	0.0	10.0	0.0	90.0	6
5	15.00	1.00	0.0	35.0	0.0	65.0	6

A = Water, B = 0.1% acetic acid, C = methanol, and D = acetonitrile

40 µL of the extract was injected and the detection was carried out with a fluorescence detector at excitation wavelength of 315 and an emission wavelength of 278. The method had good sensitivity with a limit of detection of 1 ng/mL (Figure 2-14 a & b and Table 2-3). BPA eluted at 8.5 minutes and butylparaben eluted at 12.6 minutes (Figure 2-14). No peaks for 4-nonylphenol could be detected.



(a) The standard curve for un-extracted 8 standards in methanol. The curve was linear with $r^2 = 0.99$



(b) The standard curve after recovery from perfusates. The curve was linear with $r^2 = 0.97$

Figure 2-14 Standard curve for BPA (a) in methanol and (b) after extraction

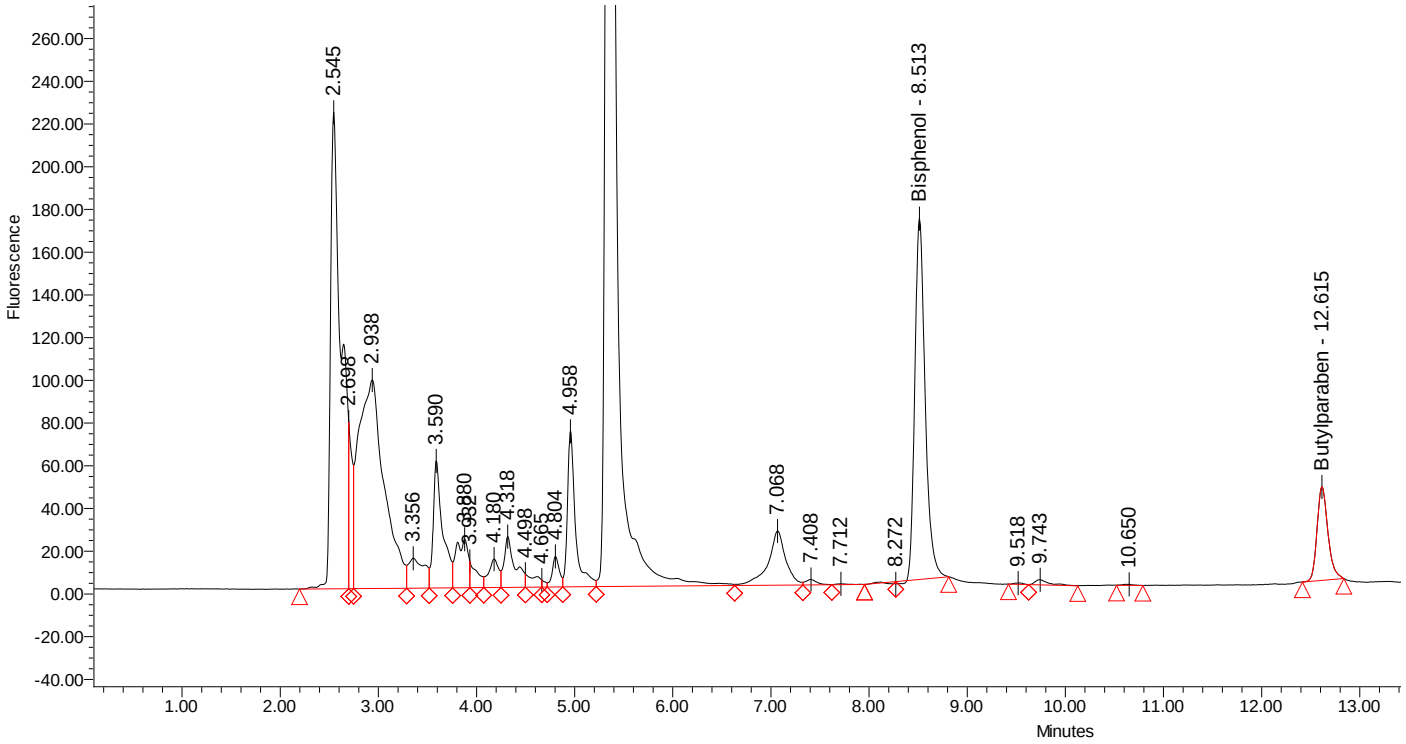


Figure 2-15 HPLC trace for BPA and butylparaben at 100 ng/mL.

The elution times for BPA was 8.5 min and for butylparaben was 12.6 min

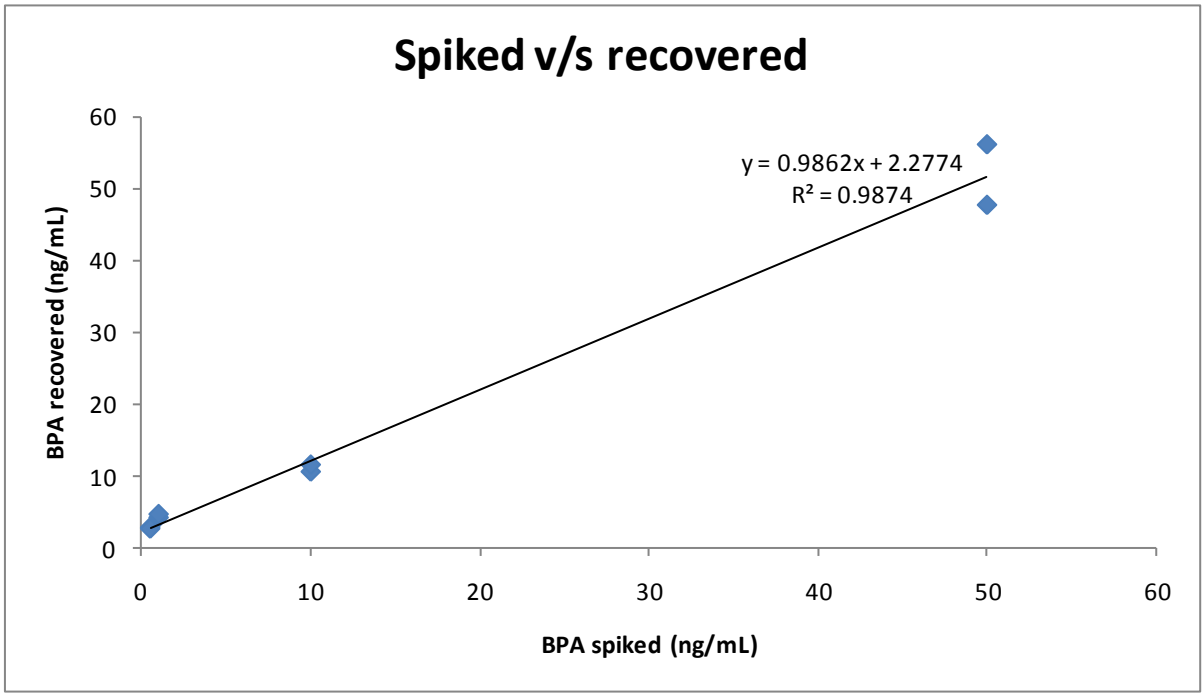


Figure 2-16 Recovery experiment BPA

Three QC samples were prepared in the perfusates (obtained after 3 hours perfusion with blank perfusates) and extracted with the method described in the text. The recovery was compared with spiked values and the recovery percentage was approximately 98%

Method	Limit of detection (ng/mL)	Accuracy	Interassay reproducibility (%CV)	Mean retention time	Linearity
HPLC-FD	1.0	Y = 0.79 +1.73	16.6 (n=8)	8.54 ± 0.1 (n=133)	0.989 ± 0.01 (n=6)

Table 2-3 Standardization parameters of BPA detection by HPLC – FD method

When this method was used for routine analysis of BPA from the perfusates, an interfering peak co-eluted with bisphenol peak interfered with its accurate estimation. When the reagents we used in chromatography were tested, most of them seemed to contain background levels of BPA interfering with its detection (Figure 2-17). Further HPLC was not sensitive enough to detect the conjugated form of these compounds as the enzyme itself showed many interfering peaks as shown in the Figure 2-18. Hence it was decided to

standardize Liquid Chromatography – tandem Mass Spectrometry to measure the concentrations of BPA and other xeno-estrogens in the perfusates.

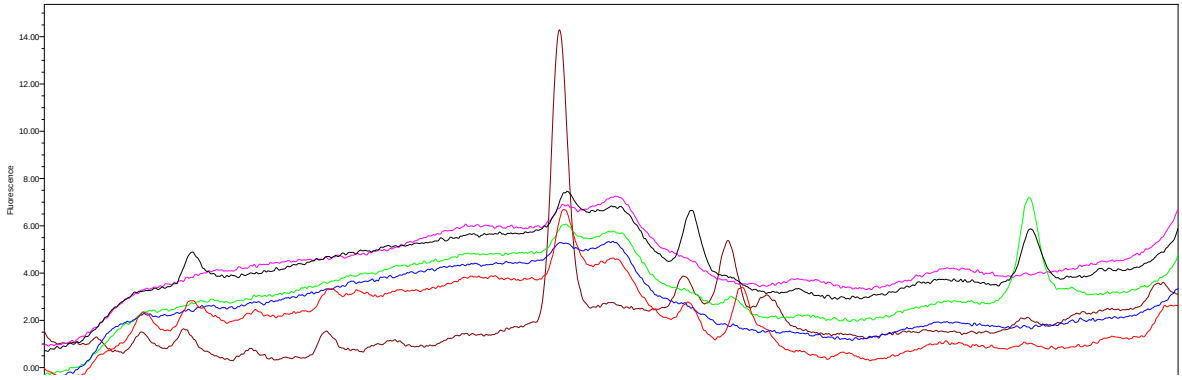


Figure 2-17 Blank BPA in the analytical reagents

The analytical reagents had background levels of BPA contamination, the colours in the chromatogram rpresents various analytical reagents. (Green = acetonitrile, blue and pink = ethyl acetate, black = perfusate, red = BPA at 2.5 ng/mL and Brown = BPA at 10 ng/mL)

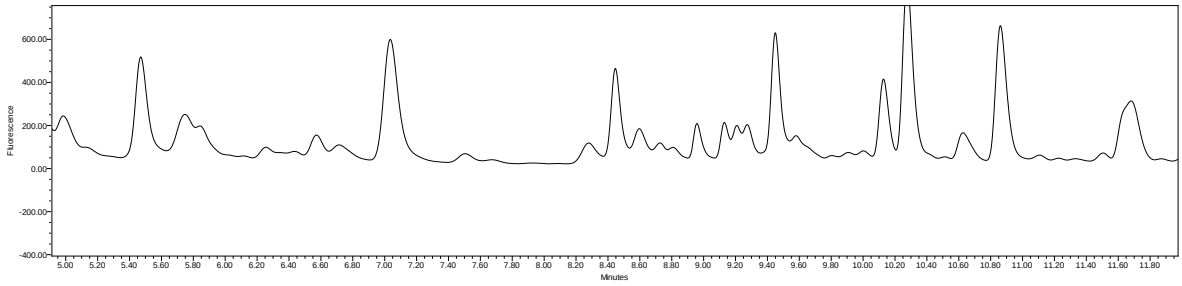


Figure 2-18 HPLC trace of perfusates after de-conjugation assay

The blank perfusates were treated with glucuronidase / sulphatase enzyme and extracted as described in the text. Chromatogram was noisy with many interfering peaks interfered with BPA detection.

On a literature survey and personal communication from overseas experts, it was inferred that the background contamination is a major problem and one of the causes include the reagents used for HPLC analysis. Based on the technical advice from Phenomenex Strata-X SPE cartridges (a patent pending solid phase extraction cartridge developed to remove phenolic compounds from water, and a gift from Phenomenex) were used to detect the concentration of BPA in HPLC reagents and milli Q water. Detectable quantities of BPA were present in all reagents tested. Strata –X SPE cartridges were effective in removing

BPA from all reagents. Their efficiency was further tested by spiking distilled water with different concentrations of BPA followed by screening the filtrates for any detectable peaks in chromatogram. All the filtrates were free from BPA and hence it was advised to use these or SDB-RPS (polystyrenedivinylbenzene) filters to filter all reagents before carrying out HPLC or LC-MS.

2.3.4. Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS) to detect BPA and estradiol in the perfusates

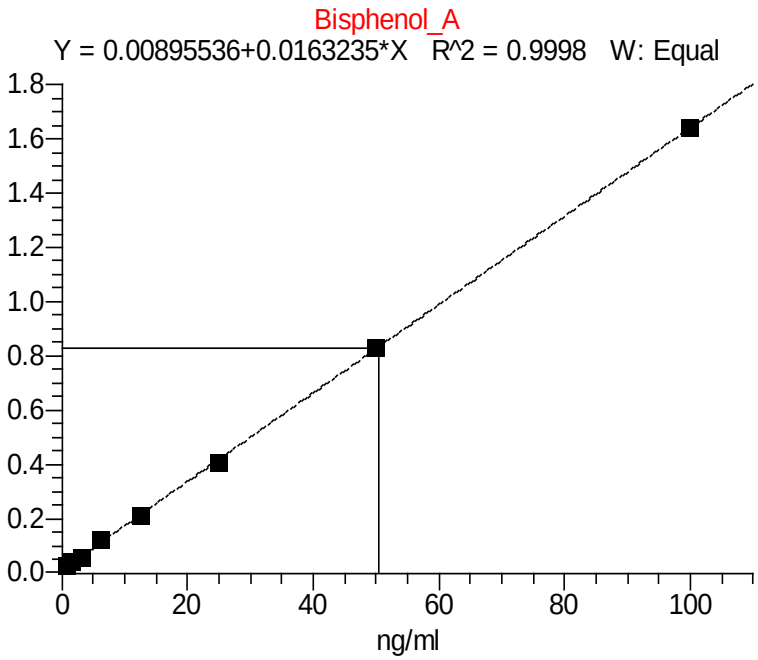
An LC-MS/MS method was optimized in our laboratory to measure the BPA content in the perfusates. Initial standardization was carried out using deuteriated estradiol (Estradiol d3) as an internal standard. Later BPA d16 (BPA d16) was procured to increase the sensitivity of BPA detection. The stock / working standard solutions of BPA and estradiol were prepared as described earlier. Estradiol d3 internal standard was prepared in 1 mg/mL in ethanol. This solution was added to the perfusate to get a final concentration of 10 ng/mL.

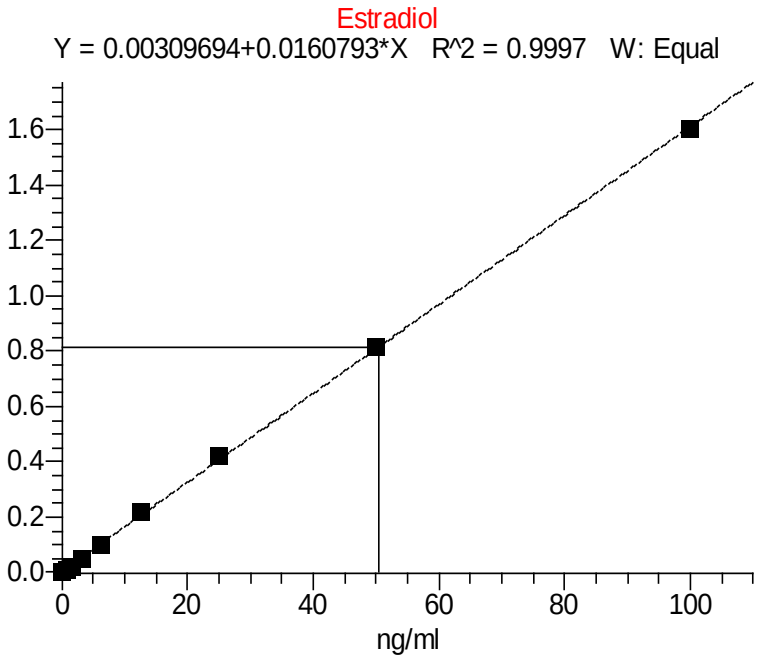
Initially an attempt was made to develop LC-MS detection with Electrospray Ionization (ESI) in negative ion mode. Mass spectrometric parameters were optimized for 17-β-estradiol and BPA to simultaneously detect both from the same samples. When the methanolic solution of 100 ng/mL of BPA was infused directly into the mass spectrometer, the sensitivity was poor with a signal of only $1e^2$. Various tune files were tried to optimize the detection however all these methods failed to increase the sensitivity further. Hence a detection based on Atmospheric Pressure Chemical Ionization (APCI) in negative ion mode was optimized in our laboratory. The ionization of BPA was poor and yielded low signal strength when 50:50 acetonitrile and 0.1% acetic acid were used as mobile phase. Replacing the acetonitrile considerably improved the ionization. The parent to daughter ion transition was 227.04 to 133.06m/z.

All the reagents used for analysis were filtered through Empore extraction discs (RP-SDB discs; Phenomenex, Torrance, CA) to prevent any potential BPA contamination.

Ethyl acetate was used for the extraction of BPA and 17-β-estradiol from the perfusates. In brief, 100 µL of a mixture of BPA d16 (30 ng/mL), estradiol d3 (10 ng/mL) was mixed in a tube with 200 µL of perfusate, and 1 mL of ethyl acetate was then added and the solution mixed by vortexing for 30 seconds. The tubes were then kept in a tube rack for 10 minutes. Each assay contained 8-10 serially diluted standards and 4 quality control samples.

The organic phase was collected in a glass tube and lyophilized. The dried extract was redissolved in 70 µL of mobile phase (methanol-water, 80:20), vortexed, and transferred to 200 µL PVC inserts in HPLC vials. Ten microliters of the samples were injected with an autoinjector. The column used for separating the compounds was a Phenomenex 100 × 3.0 mm 2.5µ C18 (2) HST. The HPLC system (Alliance 2690; Waters) was interfaced to a triple quadruple mass spectrometer (Finnigan TSQ Quantum Ultra AM; Thermo Electron Corp, San Jose, CA) and the detection was carried out with APCI ionisation source in negative ionization mode. The parent-to-daughter ion transition for BPA was 227.03-133.03 and for BPA d16 was 241.17-142.16. The retention time was 6.47 minutes for BPA and 6.39 minutes for BPA d16. The data were analyzed using Finnigan Excalibur software (Thermo Electron Corp) and the standard curves were extracted from the generated results (Figure 2.21). Co-extraction of genistein was also tried with this method. But the ionization was poor as observed from the poor standard curve (Figure 2.19). The method validation parameters are given in the Table 2.4.


Bisphenol_A
Y = 0.00895536+0.0163235*X R^2 = 0.9998 W: Equal
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
ng/ml


Estradiol
Y = 0.00309694+0.0160793*X R^2 = 0.9997 W: Equal
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
20
40
60
80
100
ng/ml

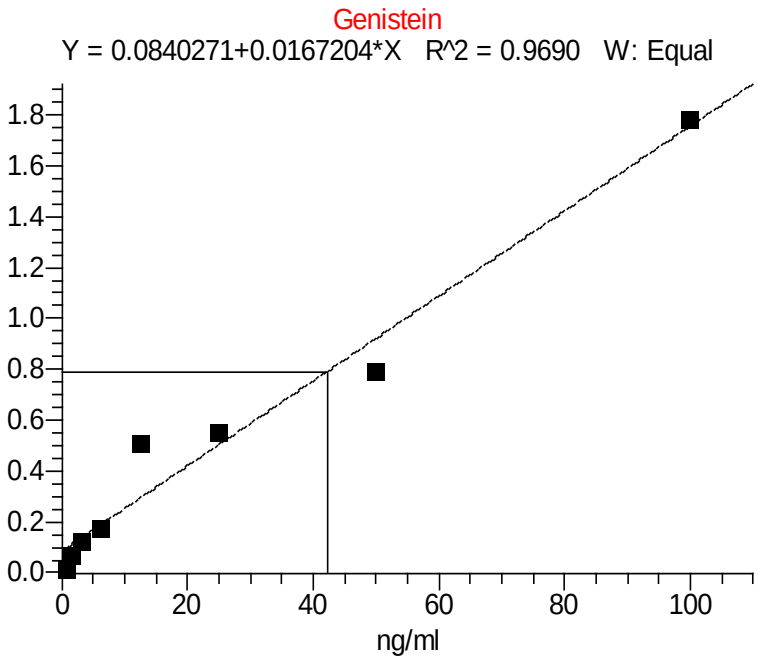


Figure 2-19 APCI – LC-MS/MS for bisphenol, estradiol and genistein

The standard curves were plotted using X-calibur software attached to the mass spectrometer. Both estradiol and BPA had good linearity from the concentration ranges. Linearity was not good for genistein due to the problems of poor ionization.

Compound	Limit of detection (pg/mL)	Accuracy	Intra-assay reproducibility (% CV)	Inter-assay reproducibility (% CV)	Mean retention time (min) [±SD]	Linearity [±SD]
BPA	62.5	Y = 1.05X−0.11	3.86 (n = 6)	18.5 (n = 5)	6.43 ± 0.02	0.989 ± 0.01

Table 2-4 Standardization parameters of LC-MS/MS for BPA and estradiol

2.3.4.1. De-conjugation enzyme assay to detect the glucuronide and sulphate conjugates of xeno-estrogens in the perfusates

To detect the conjugated levels of BPA, a deconjugation enzyme assay [279] was used with modification. Two hundred microliters of perfusate was buffered with 50 µL of 2 N

sodium acetate (pH 5.0). Glucuronidase/sulfatase (Crude extract from Helix pomatia; Sigma Aldrich) concentrations were optimized by using sodium salts of β-estradiol-17β-glucuronide and β-estradiol-3-sulfate and varying the concentration of enzyme and substrate as shown in Table 1.5. The enzyme at various concentrations were added to the tubes and incubated at 37°C in a water bath overnight (14 hours). The reaction was stopped by heating the tubes to 70°C for 10 minutes. The total estradiol was then extracted as per the protocol cited in section 1.1.4, and detection was carried out using LC-MS/MS as described in section 1.1.4. The estradiol detected before deconjugation (free estradiol) was deducted from estradiol detected after deconjugation (total estradiol), and the difference (conjugated estradiol) is expressed as a percent of total estradiol. 0.2 µL of enzyme (500 U of glucuronidase and 3.5 U of sulfatase) was effective in de-conjugating > 99% of conjugated glucuronide / sulphates (Table 2.5). This concentration was used to de-conjugate BPA conjugates from the perfusates. To detect whether enzyme assay buffer had any role in interfering with the mass spectroscopic detection of estradiol and BPA, a standard curve of estradiol and BPA was generated each time by spiking 8-10 standards in perfusate containing enzyme assay buffer. The linearity of the curve shows that the enzyme assay buffer had no role in interfering with the mass spectrometric detection of estradiol and BPA (Figure 2.20 &2.21).

Table 2-5 Enzyme de-conjugation assay standardization

Validation	Enzyme (µL)	Substrate (ng/mL)	% conjugation
Sulphatase	0.2	2.5	100.0
	0.2	10	99.5
	5	2.5	100.0
	5	10	99.2
	10	2.5	99.1
	10	10	99.6
Glucuronidase	0.2	2.5	99.0
	0.2	10	99.6
	5	2.5	100
	5	10	99.5
	10	2.5	100
	10	10	99.4

To estimate the optimum enzyeme substrate concentration, a deconjugation assay was optimized by incubating various concentrations of sodium salts of estradiol glucuronide and sulphate as shown in the table. The recovery percentage was >99% for all the concentrations of the enzyme used.

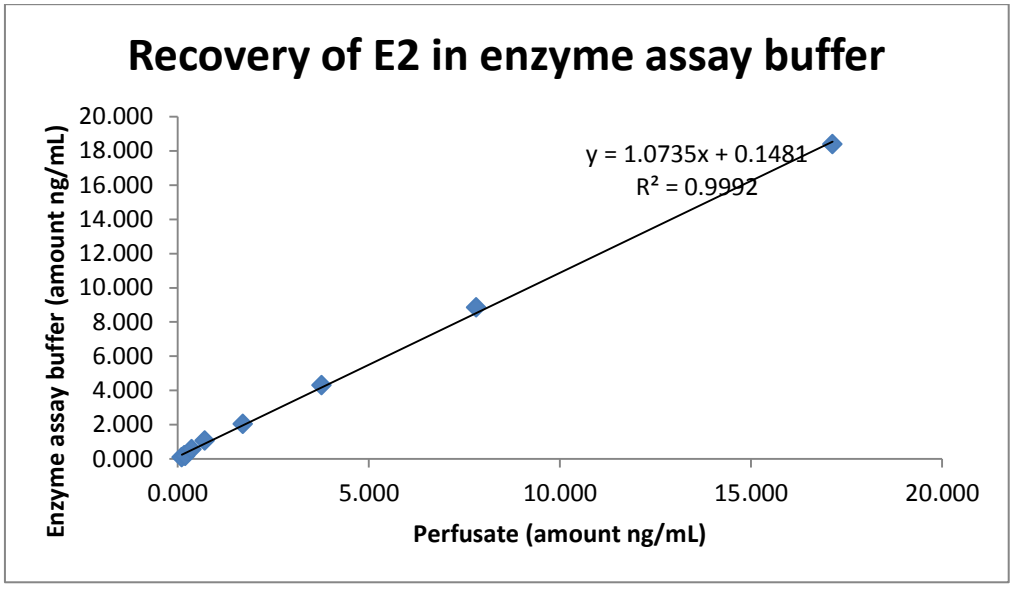


Figure 2-20 Recovery of estradiol standards in enzyme assay buffer

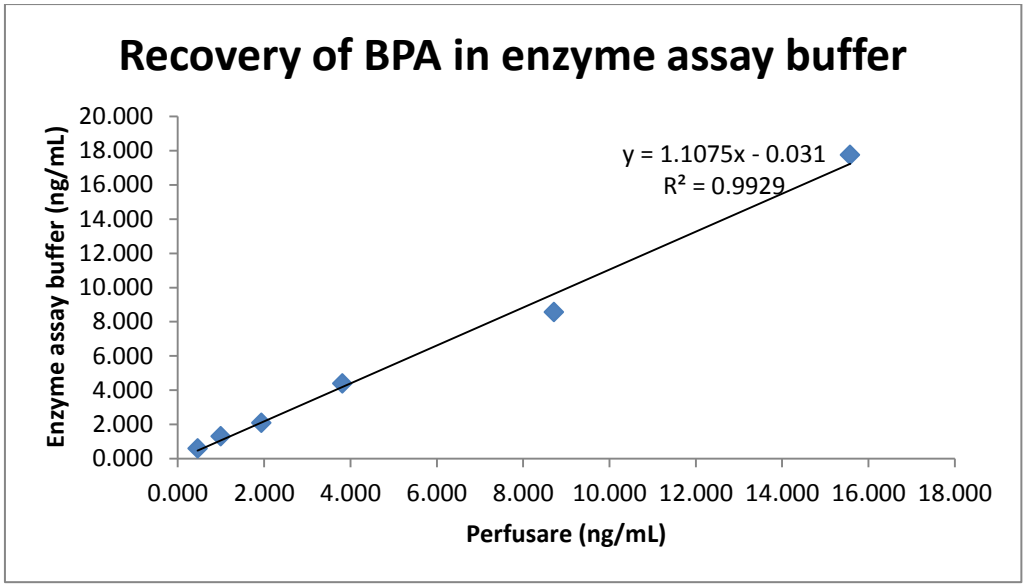


Figure 2-21 Recovery of BPA standards in enzyme assay buffer

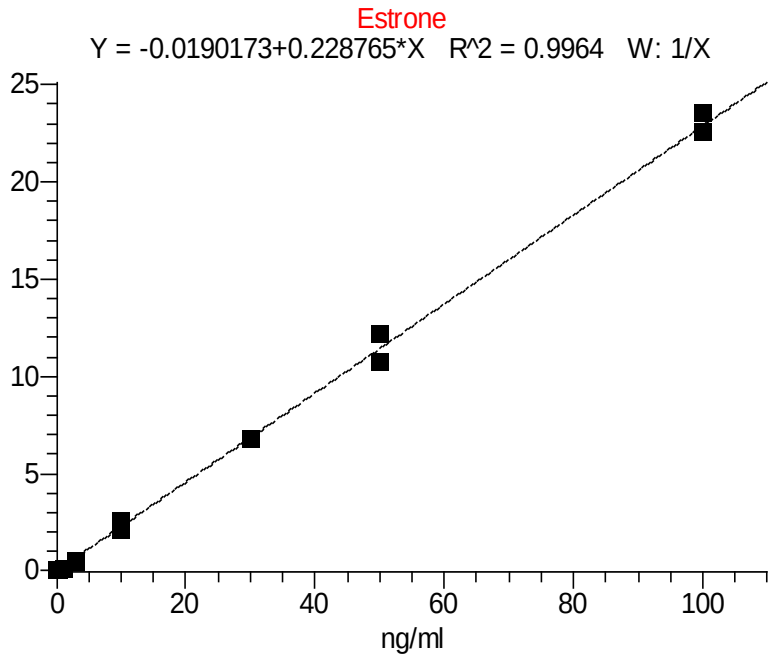
To test whether the components of enzyme assay buffer affects the ionization of BPA / estradiol (E2) a standards were spiked in enzyme buffers and the curve when plotted against perfusate spiked standards the slope had a linearity of >0.99.

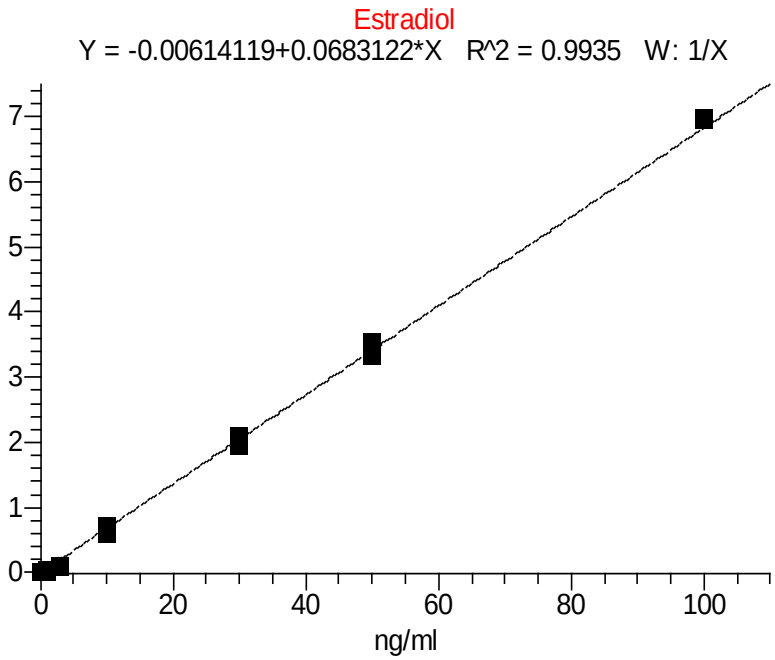
2.3.5. Development of Liquid Chromatography – tandem Mass Spectrometry (LC-MS/MS) to detect BPA, genistein, estrone, estradiol and estriol simultaneously in the perfusates

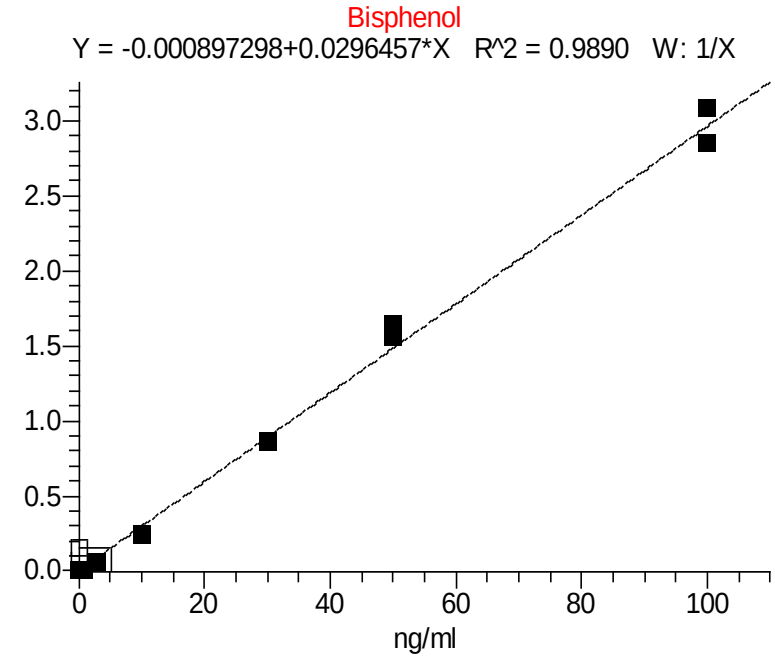
An LC–MS/MS method was optimized in our laboratory to measure the genistein, BPA, estrone, estradiol and estriol and their conjugates in perfusates.

Deuterium labelled standards for BPA (BPA d16), estradiol (E2d3) and genistein (genistein d4) were used as internal standards for BPA, estradiol, and genistein respectively. The stock and standard solutions were prepared according to the protocol described earlier in this section. In brief, 100 µL of internal standards were mixed in a tube with 200 µL of perfusates and / or standards (Each assay contained 8-10 serially diluted standards and 4 quality control samples.), 1 mL of ethyl acetate was then added and the solution mixed by vortexing for 30 s. The tubes were then kept in a tube rack for 10 min. The organic phase was collected in collection glass tube and lyophilized. The extract was re-dissolved in 100 µL of mobile phase (methanol:acetonitrile:water – 25:25:50), vortexed

and transferred to 200 µL PVC inserts in HPLC vials. 10 µL of the samples were injected with an auto-injector. The compounds were separated using a Phenomenex 100 × 3.0 mm 2.5µ C18 (2) HST column at 25 °C. The separation was carried out with a gradient system for 10 min. The HPLC system (Waters Alliance 2690) was interfaced to a triple quadruple mass spectrometer (Finnigan TSQ Quantum Ultra AM, Thermo Electron Corporation, CA, USA). For mass spectrometry Electrospray ionization (ESI) was used in negative ion mode. A standard curve was prepared by dissolving 8-10 different concentrations of standard in perfusate (Figure 2-22). Fragmentation plots and validation parameters are presented in the following figures (Figure 2-23 thorough 2-26 and tables 1-6 & 1-7).




Estradiol
Y = -0.00614119+0.0683122*X R^2 = 0.9935 W: 1/X
0
1
2
3
4
5
6
7
0
20
40
60
80
100
ng/ml


Bisphenol
Y = -0.000897298+0.0296457*X R^2 = 0.9890 W: 1/X
0.0
0.5
1.0
1.5
2.0
2.5
3.0
0
20
40
60
80
100
ng/ml

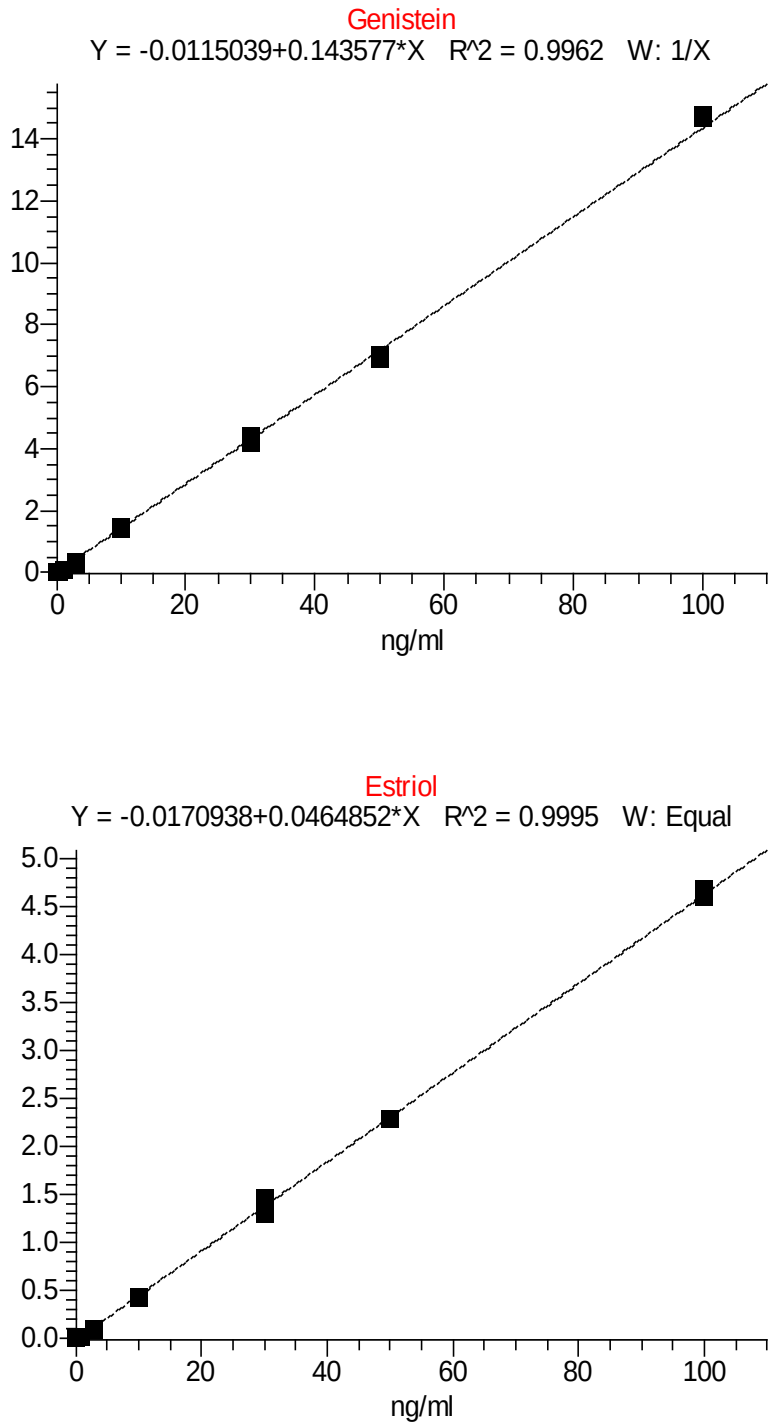


Figure 2-22 Standard curve for the analytes of importance (Estrone, Estradiol, genistein, BPAnd estriol)

LC-MS was re-standardized by combining many tune files to detect endogenous estrogens, genistein and BPA. The method was robustic and the standard curves for all the analytes were linear with $r^2 > 0.99$.

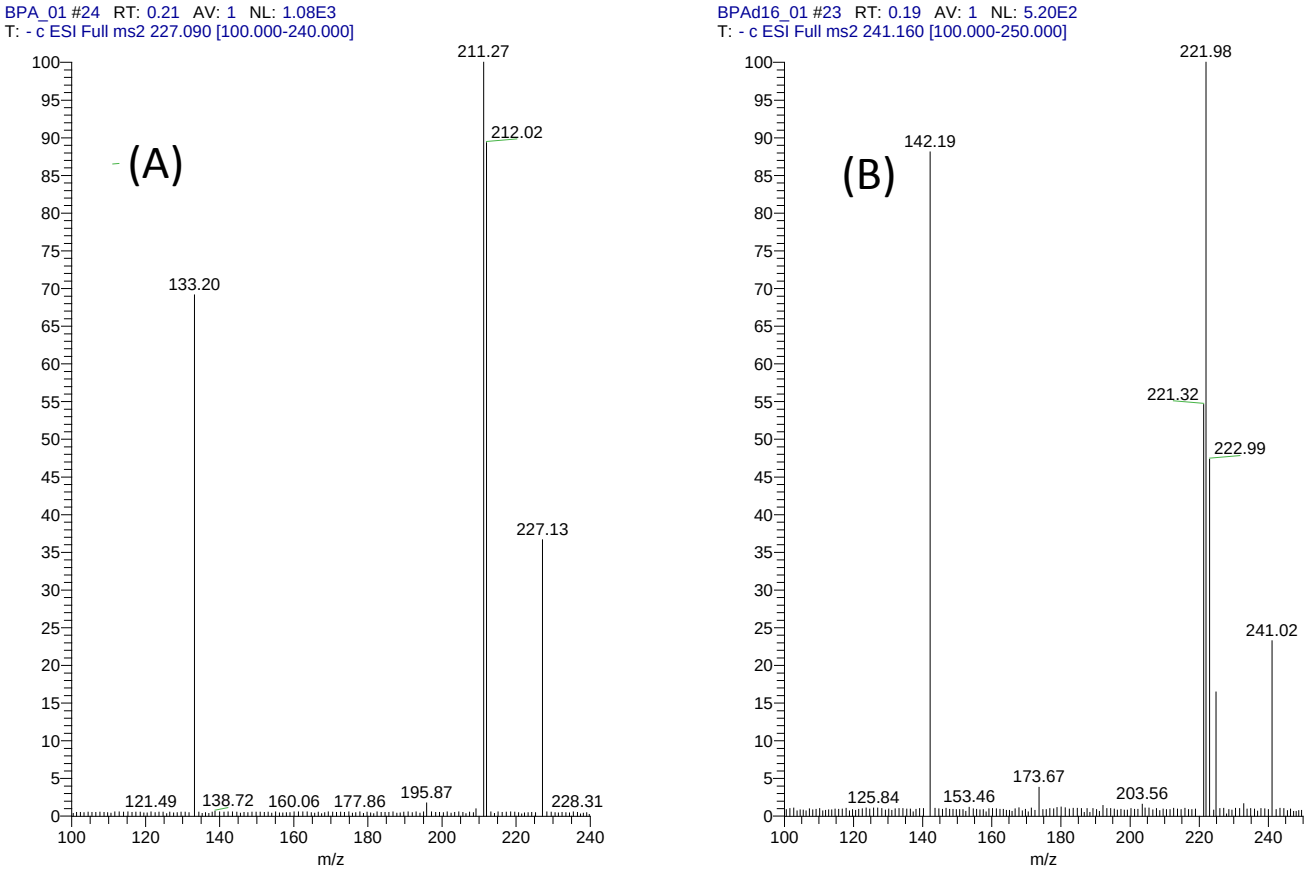


Figure 2-23 Fragmentation plots for BPA and BPA d_{16} of importance (LC-MS/MS-ESI)

The parent mass was 227.13 and the abundant daughter ion produced was 133.2 for BPA (A) and the same was 241.5 and 142.0 for BPA d_{16} (B).

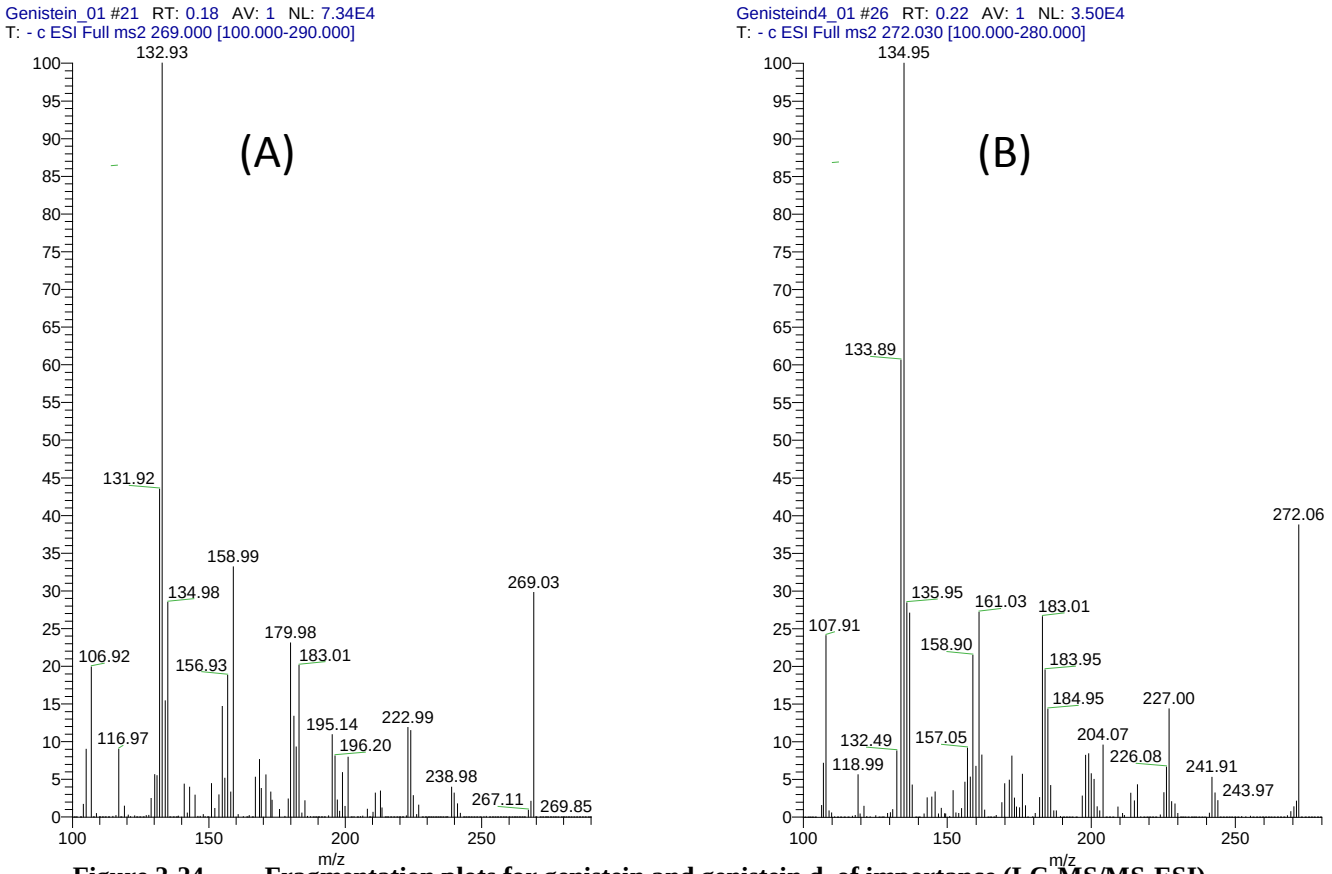


Figure 2-24 Fragmentation plots for genistein and genistein d_4 of importance (LC-MS/MS-ESI)

The parent mass was 269.03 and the abundant daughter ion produced was 132.93 for genistein (A) and the same was 272.06 and 134.95 for genistein d_4 (B).

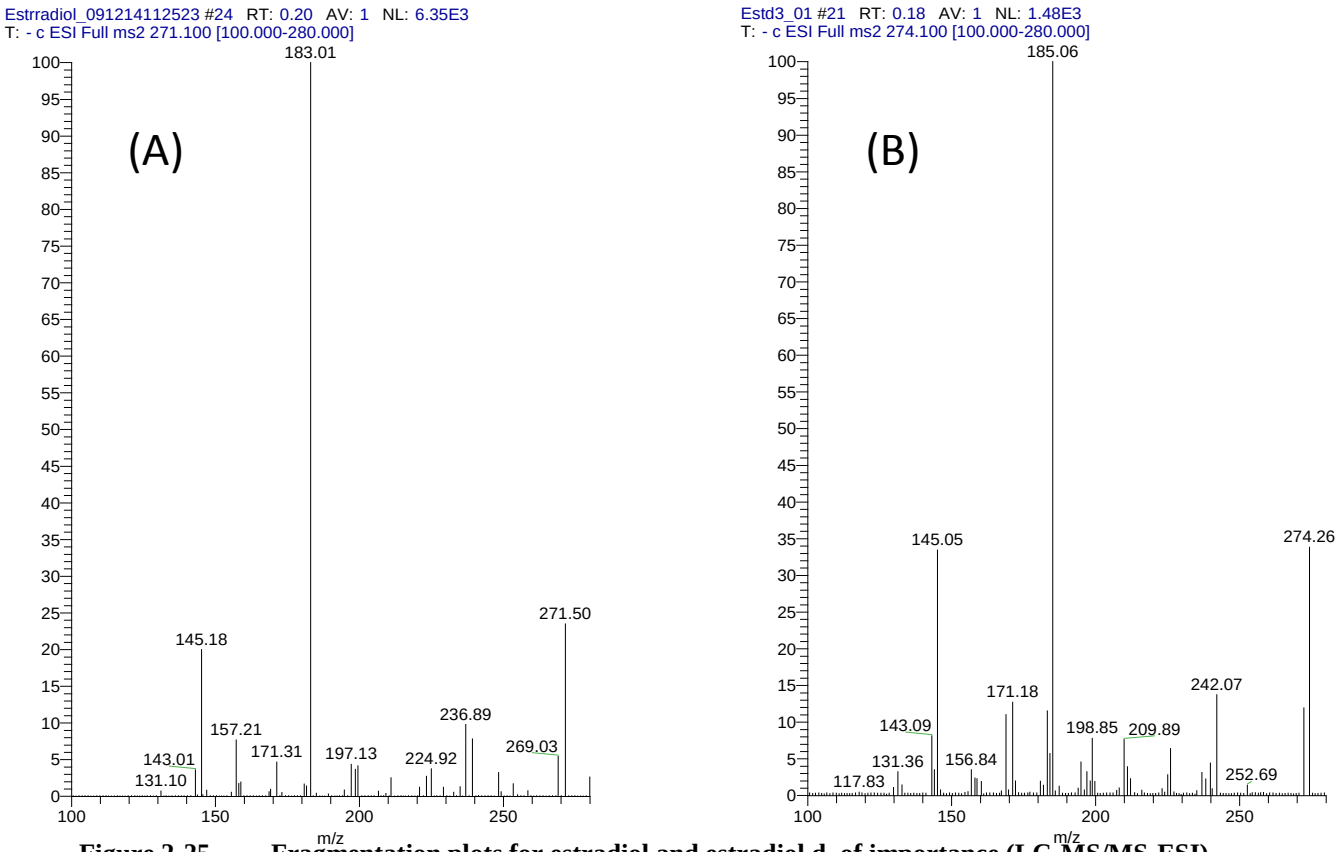


Figure 2-25 Fragmentation plots for estradiol and estradiol d_3 of importance (LC-MS/MS-ESI)

The parent mass was 271.5 and the abundant daughter ion produced was 183.01 for estradiol (A) and the same was 274.26 and 185.06 for estradiol d_3 (B).

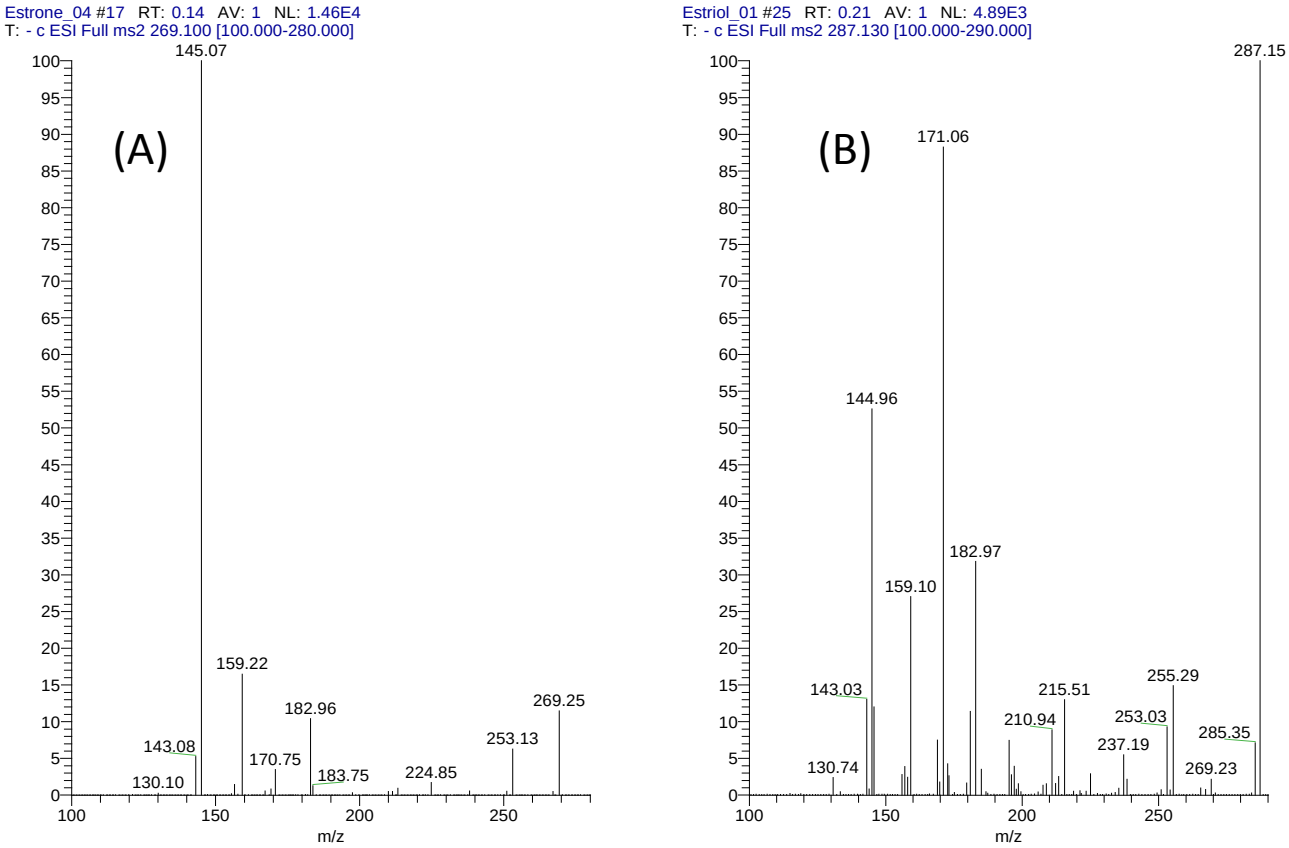


Figure 2-26 Fragmentation plots for estrone and estriol of importance (LC-MS/MS-ESI)

The parent mass was 269.25 and the abundant daughter ion produced was 145.07 for estrone (A) and the same was 287.15 and 171.06 for estriol (B).

Table 2-6 Parent to daughter ion transition under Selected Reaction Monitoring (SRM) for the selected compounds in LC-MS/MS (Electrospray Ionization-negative ion mode)

Compound	Parent mass	Daughter ion
Estradiol	271.0	183.0
Estradiol D3	274.0	185.0
Estriol	287.1	171
Estrone	269.1	145.0
Bisphenol A	227.08	133.1
Bisphenol A D16	241.17	142.16
Genistein	268.93	133.0
Genistein D4	272.01	135.0
4-nonylphenol*	219.13	106.2

Table 2-7 LC-MS/MS validation parameters for the selected compounds in ESI method

Compound	Limit of detection (pg/mL)	Accuracy	Inter-assay reproducibility (10 ng/mL) (%CV)	Retention time (min)	Linearity (r^2)
Genistein	100	y = 0.9851x + 0.0009	3.44 (n=5)	5.2	0.9942
Bisphenol A	100	y = 1.0911x - 0.0296	5.35 (n=5)	6.6	0.9921
Estradiol	100	y = 1.0382x - 0.0178	8.51 (n=5)	7.32	0.9927
Estriol	250	y = 0.9425x + 0.0023	3.57 (n=5)	3.4	0.9944
Estrone	100	y = 1.0211x + 0.0398	3.67 (n=5)	7.8	0.9947

2.3.6. Development of High Performance Liquid Chromatography to detect 4-nonylphenol concentrations from the perfusates

All the reagents used for HPLC were of HPLC grade (Merck, KGaA, Germany). The HPLC method was a modification of the method described recently [280]. Butyl Paraben was used as an internal standard. Briefly 100µL of 100ng/mL Butyl Paraben (Sigma) was added to 250µL of perfusate and mixed well by vortexing. Then 4 mL of Dichloromethane – n-Pentane (50:50) was added and the tube was mixed for 15 minutes on a tube rotator. The tubes were then centrifuged (Beckman J6-MI, Beckman Coulter, Inc, CA, USA) at 3500g for 30 minutes and stored at -80°C for 30 minutes. The supernatant (organic phase) was carefully transferred to a glass tube and lyophilized. The dried extracts were re-dissolved in 70µL of mobile phase, mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thick septa and screw caps.

Chromatographic separations were performed on an HPLC system (Waters Alliance 2690, Waters Corporation, Milford, MA, USA). The column used to separate the compounds was a Synergi 4µm POLAR-RP 80A 250x4.6 mm (Phenomenex, USA). The mobile phase was 0.1% acetic acid:acetonitrile. The column temperature was 40°C and the sample temperature was 10°C. The separation was carried out with a linear gradient at a flow rate of 1mL/min. The gradient started with 65% water (0.1% acetic acid) and 35% acetonitrile, proceeding to 65% acetonitrile over 4 minutes, held for a minute and then proceeding to 90% acetonitrile over the next 8 minutes, held for a minute, and then proceeding back to the initial conditions (65:35, water:acetonitrile) over the next 3 minutes. 35µL of sample was injected and detection was carried out using a Fluorescence Detector (Waters 2475) Ex λ 278 nm, Em λ 315nm.

Various extraction buffers were tested for optimum recovery of 4-NP from perfusates and Dichloromethane – n-pentane (50:50) was found to be the best (Table 2-8). The validation parameters were presented in Table 2-9 and Figures 2-27 & 2-28.

Table 2-8 Optimization of extraction solvents for 4-NP extraction

Solvents		CV (%) (n=6)
DCM-n-pentane (50-50)	Int Std (area)	2.8
	4-NP (area)	3.9
	Area ratio	1.3
Ethyl Acetate	Int Std (area)	24.0
	4-NP (area)	27.6
	Area ratio	13.2
DEE- n-hexane (30-70)	Int Std (area)	54.9
	4-NP (area)	68.5
	Area ratio	23.3
DEE- n-hexane (30-70) + Amm. Acetate	Int Std (area)	74.7
	4-NP (area)	83.5
	Area ratio	28.0

Many solvents were studied for extraction of 4-NP from perfusates. Recovery was consistent only with dichloromethane – n-pentane (50:50)

Table 2-9 Validation parameters of HPLC for 4-NP detection

Detection limit	Accuracy	Reproducibility (Intra assay CV%)	linearity
1.6 ng/mL	y=0.2723x-0.2293	1.80%	0.9948

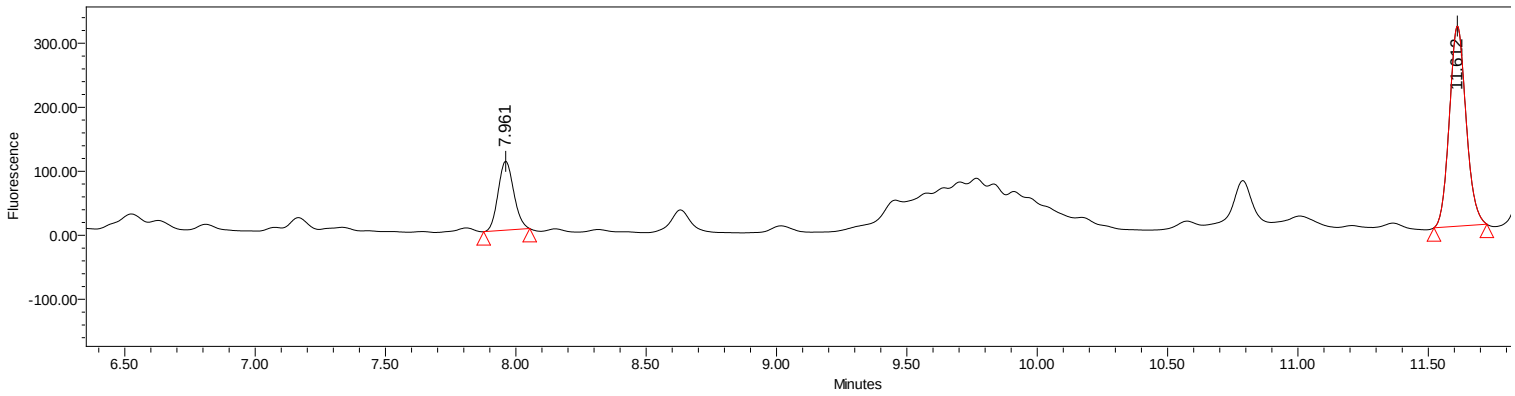


Figure 2-27 Chromatographic traces for 4-nonylphenol

The internal standard butylparaben was eluted at 7.9 minutes and the 4-NP was eluted at 11.6 min

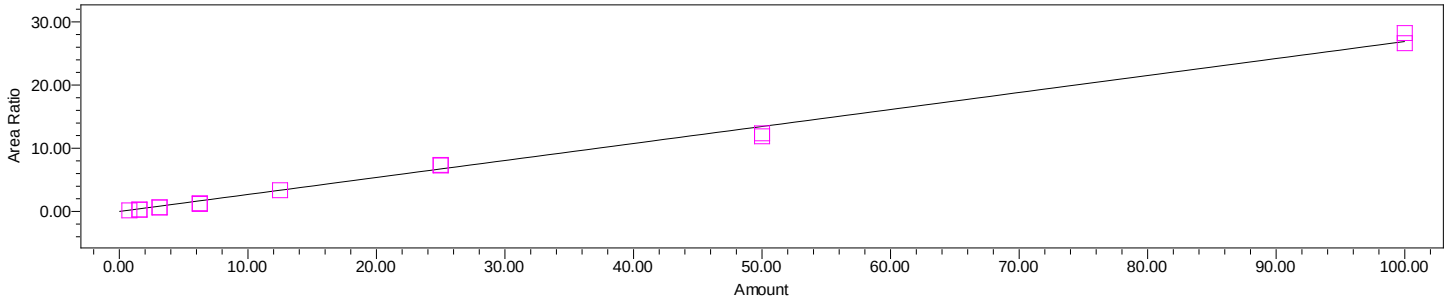


Figure 2-28 Standard curve of 4-NP

8 serially diluted standards were prepared in perfusates and the extraction was carried out as described in the text. the standard curve was linear with $r^2 = 0.99$

2.3.7. FITC –Dextran Assay

The working standard solution was prepared by dissolving 500 µg FITC-Dextran in 1mL perfusate and this was further serially diluted in perfusate to make 8 standards. 100 µL of each standard and sample were transferred to 96 well black FIA plates (Greiner Bio-one, Orlando, USA). The plates were read spectrofluorometrically with the help of Synergy 2 Multi-mode microplate reader (Bio Tek, VT, USA) (Ex λ 494nm, Em λ 521nm). A standard curve was generated with 5-6 concentrations and was found to be linear (Figure 2-29)

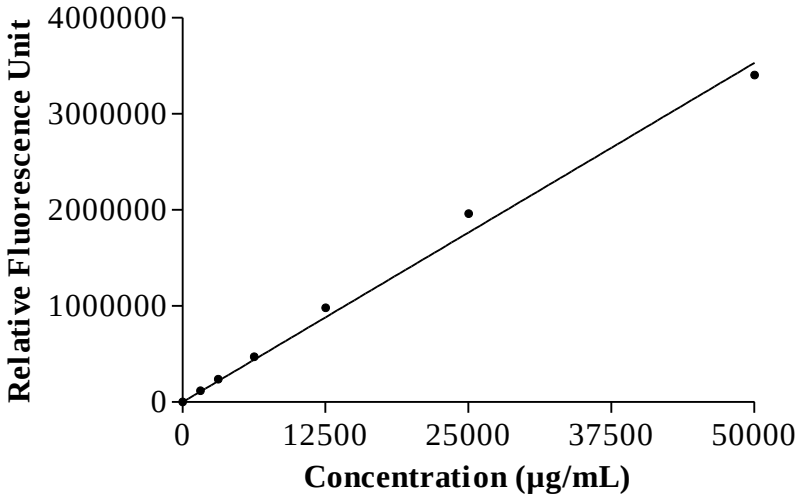


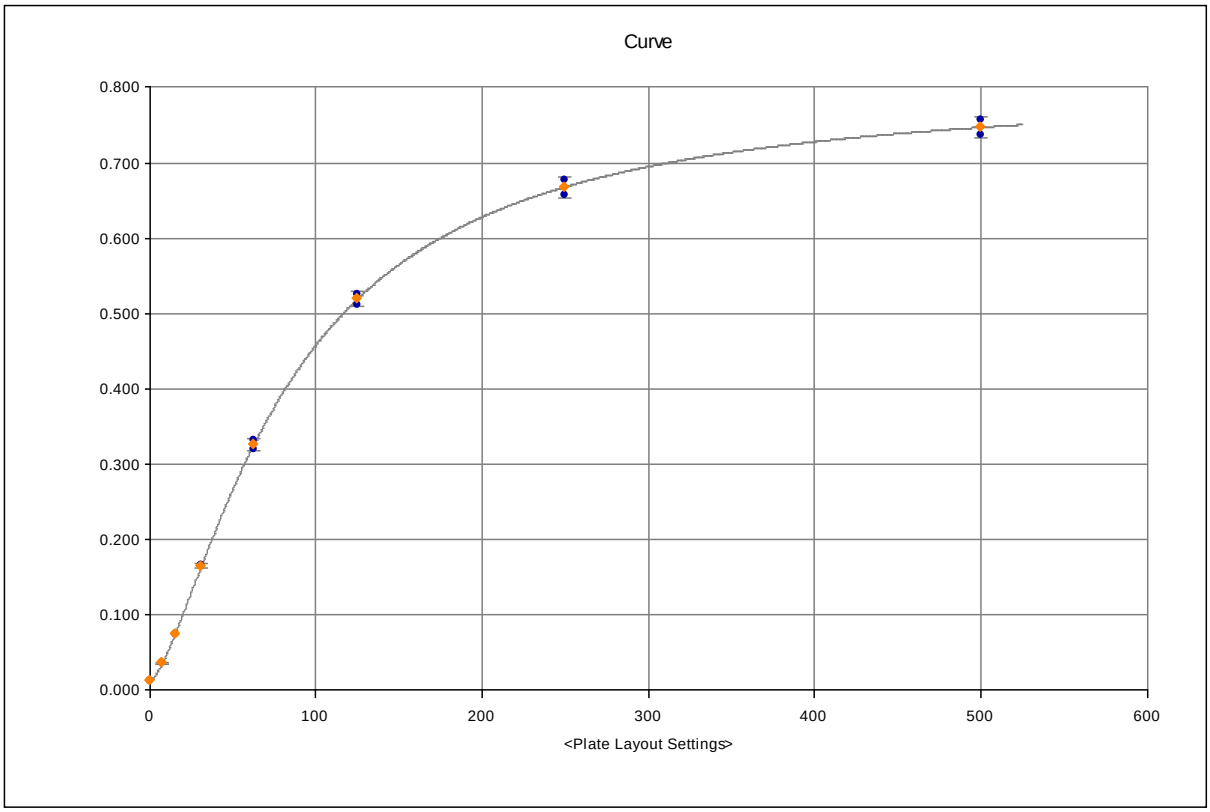
Figure 2-29 Standard curve for FITC-dextran assay

7 standards were prepared in perfusates and the fluorescence was measured with a spectrofluorometer. The standard curve was linear with $r^2 = 0.98$

2.3.8. β-hCG (human Chorionic Gonadotropin) Enzyme Linked Immunosorbent Assay (ELISA)

A sandwich ELISA standardized in our laboratory was used to measure the β-hCG concentration of the perfusates. In brief, 96 well Nunc Immuno plates (Maxisorp, Nunc, Thermo Fisher Scientific, NY, USA) were coated with 100 µL of primary antibody (2

µg/mL mouse anti hCG) and incubated overnight at 4°C. The plates were washed with washing buffer and 100 µL of standard (one in three fold serial dilutions were prepared from the stock solution (210,000 mIU/mL) or sample in duplicate and 10µL of Immunocoat Buffer (Sigma) was transferred to the plate and further incubated for two hours at room temperature. After washing with wash buffer, 100 µL of secondary antibody (rabbit anti hCG) in Immunocoat Buffer was added and incubated for one hour. After further washing, 100 µL of anti-rabbit HRPO conjugate diluted in Immunocoat Buffer was added and incubated for 30 minutes. Following another wash, 100 µL of OPD (o-Phenylene Diamine) was added and incubated for 15 minutes. The plates were read in a spectrophotometer plate reader at 480 nm with blank reduction at 520 nm. A standard curve was generated with 7-8 serially diluted hCG standards and the curve generated was as shown in the Figure 2.30.



Curve Name	Curve Formula	A	B	C	D	R^2
Curve	Y = (A-D)/(1+(X/C)^B) + D	0.0122	1.45	83.5	0.802	1

Figure 2-30 β-hCG ELISA standard curve

Standards were prepared in perfusates and the absorbance was read with the help of a spectrophotometer. A standard curve was generated with the help of inbuilt software. The standard curve has the properties given in the table above.

Declaration:-

I, Biju Balakrishnan, do hereby declare that the following chapter is an updated version of a published article, the details of which are as given below.

“Balakrishnan B, Henare K, Thorstensen EB, Ponnampalam AP and Mitchell MD. Transfer of bisphenol A across the human placenta. Am J Obstet Gynecol. 2010; 202(4):393 e1-7”.

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Biju Balakrishnan

Chapter 3. TRANSFER OF BISPHENOL A ACROSS THE HUMAN PLACENTA

3.1. INTRODUCTION

Bisphenol A (BPA) is a well-studied xenoestrogen with weak affinity for the estrogen receptor [7] that is used in the preparation of polycarbonate plastics, dental sealants and resins for can linings. Residual traces remain after manufacture and are slowly released, such that BPA in foodstuffs and drinking water is now the most significant source of exposure to synthetic xenoestrogen in New Zealand [41].

Recent influential publications associating BPA exposure with several diseases have led to the compound being banned in baby bottles in Canada, and to some manufacturers switching to BPA-free plastics [36, 90, 200, 215, 281, 282].

The estrogenic action of BPA has been demonstrated in both *in vivo* and *in vitro* studies [7, 36, 283]. BPA binds to both nuclear and membrane estrogen receptors and imparts both genomic and non-genomic steroid actions. Experiments in rodents have demonstrated that BPA can readily cross the placenta [90] and can cause developmental abnormalities in the offspring [200, 215]. The trans-generational effects of BPA on the expression patterns of some testicular steroidogenic co-regulators have been reported in the rat [281]. These and several other studies have shown the endocrine disrupting activity of BPA especially if there is fetal and / or neonatal exposure at vulnerable periods of development [284].

BPA has been detected in maternal and fetal plasma, placenta, amniotic fluid and follicular fluid [23, 25]. The Concentration of BPA is very variable and range from 0.3 to 18.9 ng/mL in maternal plasma, from 0.2 to 9.2 ng/mL in fetal plasma, and from 1.0 to 104.9

ng/g in term human placenta [23]. Studies have shown that hepatic UDP-glucuronosyl transferase (UGT) can conjugate BPA into its glucuronide form, which is devoid of estrogenic action [285, 286]. Recently detectable quantities of BPA were demonstrated in human fetal liver as early as 3 months of age. The study also reported the presence of free BPA and BPA glucuronide in both first trimester placenta and in fetal liver. This is the first report on the exposure of human fetus to BPA during early gestation [287]. If placental UGT could also conjugate BPA, then the developmental abnormalities in the fetus due to exposure to the potent form of BPA would be minimised. To the best of our knowledge none of the studies directly assessed the ability of the human placenta to conjugate BPA. We hypothesise that BPA at environmentally relevant concentration can transfer across the human placenta in an active un-conjugated form. Controlled experiments in pregnant mothers are lacking, since the consequences on the developing fetus of exposure to these compounds make such a study impossible in women. Placental perfusion of human placentae is the only viable alternative. We have previously validated a placental perfusion model to study the trans-placental transfer of AZT, an anti-HIV drug across the human placenta [276].

The aim of this study was to determine placental transfer and conjugation of BPA at environmentally relevant concentrations.

3.2. MATERIALS AND METHODS

3.2.1. Placental perfusion

Placentae were obtained from women undergoing elective caesarean section at term from Auckland City Hospital. Written consents were obtained from each pregnant woman for the use of placentae for research and the use was approved by the Regional Ethics

Committee. It was earlier reported from our laboratory that smoking could affect the expression levels of drug metabolizing enzymes in the placenta. Hence only placentae from healthy non-smoking mothers were used in the study.

A modified and fully validated dually perfused ex-vivo placental perfusion system was used to study the transfer of BPA across the human placenta [273, 275, 276, 288] . The placentae were transported to the laboratory within 30 minutes of delivery. Every placenta collected for perfusion was thoroughly examined visually for gross breakages of the villous structures to try to find suitable lobules for perfusion. Placentae with visible gross breakages were excluded. Perfusion was established in the placentae where a suitable lobule could be found. The chorionic vessels were catheterized with polyethylene tubes and the perfusate ([Phenol red free Medium-199 (M-3769, Sigma Aldrich, St. Luise, MO, USA) cell culture media supplemented with 25g/L Polyvinylpyrrolidone PVP-40 (Sigma Aldrich), 1g/L Bovine Serum Albumin – Fraction V (Sigma Aldrich), 2g/L Glucose (Sigma Aldrich), 20,000 IU/L Heparin (Multiparin, CP Pharmaceuticals Ltd, Wexham, UK) and 48 mg/L Gentamicin Reagent Solution (GIBCO, Invitrogen, NY, USA) was allowed to circulate through the fetal side by means of a digitally controlled pump. While perfusate circulated in the fetal side, the cotyledon was severed from the remaining placenta and mounted in the perfusion chamber, with the fetal side facing up. The maternal side was catheterized and the perfusate was allowed to circulate through it with the help of a digitally controlled pump. After 15 minutes both compartments were perfused in a re-circulating mode and the perfusion was started with maternal flow rate of 10mL/min and fetal flow rate of 4mL/min for 1 hr to equilibrate the *ex vivo* system. The maternal circuit was gassed with 95% O_2 and 5% CO_2 (Carbogen gas). After an hour of pre-perfusion (equilibration) the perfusate in the maternal compartment was replaced with

fresh perfusate containing BPA (Sigma Aldrich) at 10ng/mL, antipyrine (Sigma Aldrich) at 40µg/mL, and FITC-Dextran [FITC-DX, FD4 (Sigma Aldrich)] at 12.5µg/mL. The BPA concentration selected is within the range from the serum levels reported in pregnant women [23]. The perfusion was then continued for 3 hours and the samples from maternal and fetal reservoirs were collected at 30 min intervals and stored at -80°C. Perfusions were stopped in any placentae with visible or measured leakages that were ≥ 3ml/hr and were rejected from the study. Hence, every single placenta that was used to produce the data in this study has met all of our following quality control measures: 1) Fetal Flow Rate of 4 mL/min; 2) Maternal Flow Rate of 10 mL/min; 3) Fetal pressure 30 to 60 mm of Hg; 4) pH 7.2-7.4; 5) Feto-maternal fluid shift less than 3 mL/hr. The viability and metabolic activity were assessed by measuring glucose utilization, lactate production (Hitachi 902 auto-analyser, Hitachi High Technologies Corporation, Tokyo, Japan) and human Chorionic Gonadotropin (hCG) secretion (ELISA). The FITC –DX transfer was measured with a spectroflurometer and antipyrine transfer was measured by HPLC (UV detection). A LC-MS/MS method was optimized to measure the BPA levels.

3.2.2. Antipyrine detection with HPLC (UV)

All the reagents used for HPLC and LC-MS/MS were of HPLC grade (Merck, KGaA, Germany). The HPLC method was a modification of the method standardized in our laboratory [277]. Briefly 100µL of Phenacetin (100µg/mL,internal standard, Serva, Feinbiochemica, USA), 500µL of 0.025N HCl and 500µL of acetonitrile were added to 500µL of perfusate and mixed well by vortexing. Then 4 mL of ethyl acetate was added and the solution was mixed for 15 minutes in a tube rotator. The tubes were then centrifuged at 3000g for 10 minutes (Beckman J6-MI) and stored at -80°C for 30 minutes. The supernatant (organic phase) was carefully transferred to a glass tube and lyophilized.

The dried extracts were re-dissolved in 200µL of mobile phase, mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thin septa and screw caps.

Chromatographic separations were performed on an HPLC system (Waters Alliance 2690 Waters Corporation ,Milford, MA, USA). The column used to separate the compounds was Luna 3µ C18 (2) 100A, 250 X 4.6 mm (Phenomenex). The mobile phase was 6.7 mM phosphate buffer pH 7.2: acetonitrile (65:35). The separation was carried out isocratically at a flow rate of 0.7mL/min. 10µL of sample was injected and detection was carried out using a UV detector (Waters 996 photodiode array detector) at 254 nm. The data were analyzed using Millennium Chromatography Manager V4.

3.2.3. BPA detection with LC-MS/MS

An LC-MS/MS method was optimized in our laboratory to measure the BPA content in the perfusates. All the reagents used for analysis were filtered through Empore extraction disk (RP-SDB discs from Phenomenex, USA) to prevent any potential BPA contamination. Briefly 100µL of a mixture of BPA d_{16} (30ng/mL) (deuterium-labelled internal standard for BPA - Cambridge Isotope Laboratory, MA, USA) was mixed in a tube with 200µL of perfusate, 1mL of ethyl acetate was then added and the solution mixed by vortexing for 30sec. The tubes were then kept in a tube rack for 10 minutes. The organic phase was collected in collection glass tube and lyophilized. The extract was re-dissolved in 100µL of mobile phase (Methanol: Water – 80:20). Vortexed and transferred to 200µL PVC inserts in HPLC vials. 30µL of the samples were injected with an auto-injector. The column used for separating the compounds was Phenomenex 100 X 3.0 mm 2.5µ C18 (2) HST. The HPLC system (Waters Alliance 2690) was interfaced to a triple quadruple mass spectrometer. (Finnigan TSQ Quantum Ultra AM Thermo Electron

Corporation, CA, USA) The parent to daughter ion transition for BPA was 227.03 to 133.03 and for BPA d_{16} was 241.17 to 142.16. The retention time was 6.47min. for BPA and 6.39 min for BPA d_{16}. The data were analyzed using Finnigan Excalibur Software (Thermo Electron Corporation).

To detect the conjugated levels of BPA a deconjugation enzyme assay [279] was used with modification. 200µL of perfusate was buffered with 50µL of 2N Sodium acetate (pH 5.0). Glucuronidase / sulphatase (Crude extract from *Helix pomatia*, Sigma Aldrich) at pre-optimized concentration (500 units of glucuronidase and 3.5 units of sulphatase) was added to tubes and incubated at 37°C water bath overnight (14 hours). The reaction was stopped by heating the tubes at 70°C for 10 minutes. The total BPA was then extracted as per the above protocol and detection was carried out using LC-MS/MS as described above. The BPA detected before deconjugation (free BPA) was deducted from BPA detected after deconjugation (total BPA) and the difference (conjugated BPA) is expressed as a percent of total BPA.

3.2.4. Calculations

The formula used to calculate transfer percentage from maternal to fetal circulation was as follows: [67]

Transfer percentage = 100 X Fc X Fv/ [(Fc X Fv) + (Mc X Mv)]

Fc = Fetal concentration

Fv = Fetal volume

Mc = Maternal concentration

Mv = Maternal volume

Transfer index = Transfer percentage of BPA / Transfer percentage of antipyrine.

Values are expressed as mean ± standard error of mean

3.3. RESULTS

3.3.1. Placental perfusion

A reduction in fetal volume and a consequent surplus in maternal volume were observed whenever there was a leak in the system. All placentae selected for the study showed a feto-maternal fluid loss of less than 3 mL/hr for a total of 180 min perfusion. The recorded fetal pressure for all successful placentae was in the range of 25-40 mm Hg. None of the placentae showed fluctuations in fetal pressures once they had passed the equilibration period. The wet weight of the perfused cotyledon was 29.3 ± 2.95.

The placentae studied maintained viability as well as metabolic activity throughout the perfusion period as evident from constant glucose consumption, lactate production and β-hCG secretion . The values for glucose consumption and lactate production were 0.32 ± 0.06 µM/g/min and 0.52 ± 0.1 µM/g/min in the maternal compartment and 0.32 ± 0.06 µM/g/min and 0.35 ± 0.06 µM/g/min in the fetal compartment. β-hCG secretion was only observed in the maternal compartment (3.0 ± 0.9 mIU/g/min) and the values in the fetal compartment were below the detection level.

3.3.2. Antipyrine and FITC-DX Detection

HPLC-UV detection was used to measure antipyrine concentrations in maternal and fetal compartments. Antipyrine transfer from maternal to fetal compartment was evident from the gradual disappearance of antipyrine in maternal compartment and subsequent gradual appearance of antipyrine in the fetal compartment. Approximately 25% of initial maternal

concentration of antipyrine was found in the fetal compartment after 180 minutes of perfusion (Figure 3-1A). FITC Dextran transfer was negligible after 180 minutes of perfusion (<1% per hour) (Figure 3-1B).

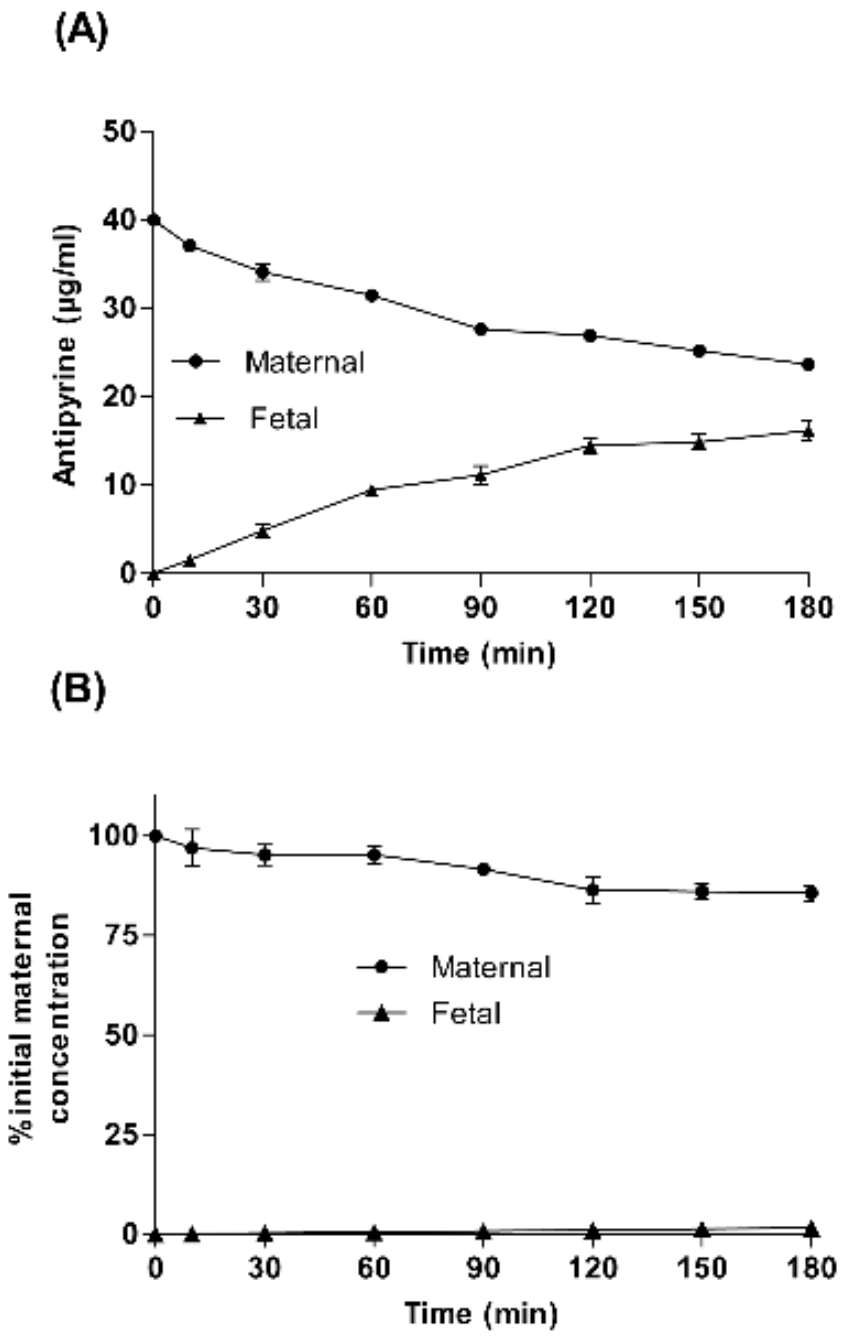

Figure 3-1 Antipyrine and FITC dextran transfer in perfused placenta.

(A) Antipyrine concentration after 3 hours of perfusion in maternal and fetal compartment. (B) FITC-DX concentration after 3 hours of perfusion in maternal and fetal compartment. (Data are presented as mean ± SEM; n=7)

3.3.3. Transplacental transfer of BPA

To measure BPA concentration accurately in maternal and fetal compartments a highly sensitive LC-MS/MS using Atmospheric Pressure Chemical Ionization (APCI) was validated and optimized in our laboratory. The validation parameters of the method are tabulated in Table 3-1. The standard curve was linear from 1 ng/mL to 100 ng/mL. The method is robust and sensitive with the Limit of Detection (LOD) = 62.5 pg/mL at signal to noise ratio of >3.

LC-MS/MS method was optimized to detect the BPA in maternal and fetal perfusates. The method has a high sensitivity to measure BPA in biological fluids.

Table 3-1 LC-MS/MS validation parameters for BPA

Compound	Limit of detection (pg/mL)	Accuracy	Intra-assay reproducibility (%CV)	Inter-assay reproducibility (%CV)	Mean retention time (min) [±SD]	Linearity [±SD]
Bisphenol A	62.5	Y = 1.05X-0.11	3.86 (n=6)	18.5 (n=5)	6.43 ± 0.02	0.989 ± 0.01

It was observed that there was a gradual decline in the concentration of BPA in the maternal compartment and a concomitant increase in the concentration of BPA in the fetal compartment. About 27% of BPA was detected in the fetal compartment within 180 minutes of perfusion. This clearly shows that BPA at low concentrations can cross the human placenta (Figure 3-2A). No evidence of BPA release from the placental tissues was observed when the perfusates from control placenta were analysed (Figure 3-2B). The transfer percentage of BPA was comparable to that of antipyrine for all the time intervals studied with a correlation coefficient of 0.995 ($p = 0.000003$) and transfer index was 1.1 ± 0.09 after 3 hours of perfusion (Table 3-2).

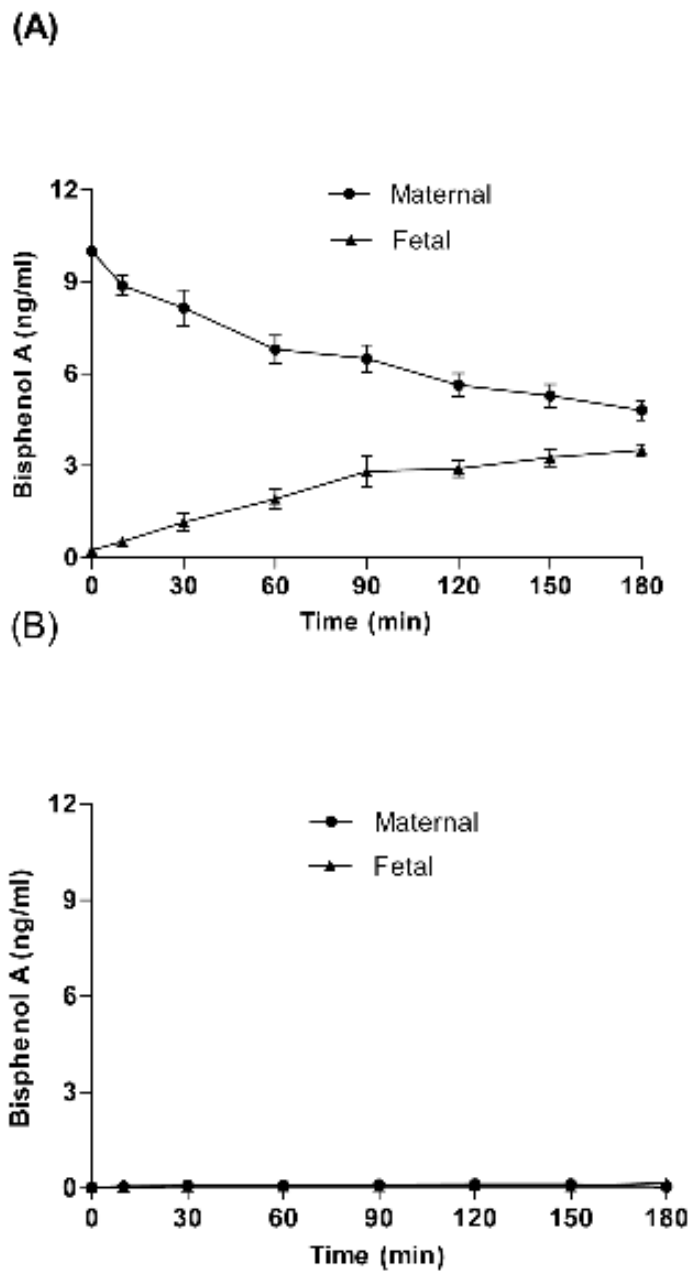

Figure 3-2 Transplacental transfer of BPA

BPA concentration in maternal and fetal compartment during 3 hours of perfusion as detected with LC-MS/MS (A) BPA transfer (n=7); (B) Control placenta (n=2). Data are presented as mean ± SEM.

Table 3-2 Transfer percentage and transfer index of BPA

Time (min)	Transfer Percentage		Transfer Index
	Antipyrine	Bisphenol A	Bisphenol A
10	2.0 ± 0.14	2.7 ± 0.92	1.3 ± 0.39
30	6.5 ± 0.80	6.4 ± 1.56	1.1 ± 0.29
60	13.0 ± 0.68	12.2 ± 1.72	0.95 ± 0.20
90	16.7 ± 1.14	17.9 ± 3.69	0.93 ± 0.15
120	21.1 ± 1.18	20.4 ± 1.34	0.96 ± 0.11
150	22.7 ± 1.00	23.6 ± 1.54	1.1 ± 0.11
180	25.5 ± 1.13	27.0 ± 1.88	1.1 ± 0.09

Transfer percentage and transfer index were calculated after 3 hours of perfusion, as described in materials section and tabulated as under (data are presented as mean ± SEM; n=7)

3.3.4. Placental biotransformation of BPA

It has been previously reported that the extraction reagents and / or incubation in assay buffer could spontaneously reverse the conjugated form back into non-conjugated form.[279]. Estradiol standards [sodium salts of β-estradiol-17-β-glucuronide and β-estradiol-3-sulphate)] were used in this study to evaluate this spontaneous hydrolysis and no detectable amounts of free estradiol were measured under the incubation and/or hydrolysis conditions. This further strengthens the quality of the deconjugation enzyme assay used in this study.

Three out of seven placentae showed less than 10 % conjugation in the fetal compartment and two out of seven perfused placentae showed the less than 2% conjugation in the maternal compartment (Figure 3). The levels of conjugation in the remaining placentae were lower than that of the limit of detection.

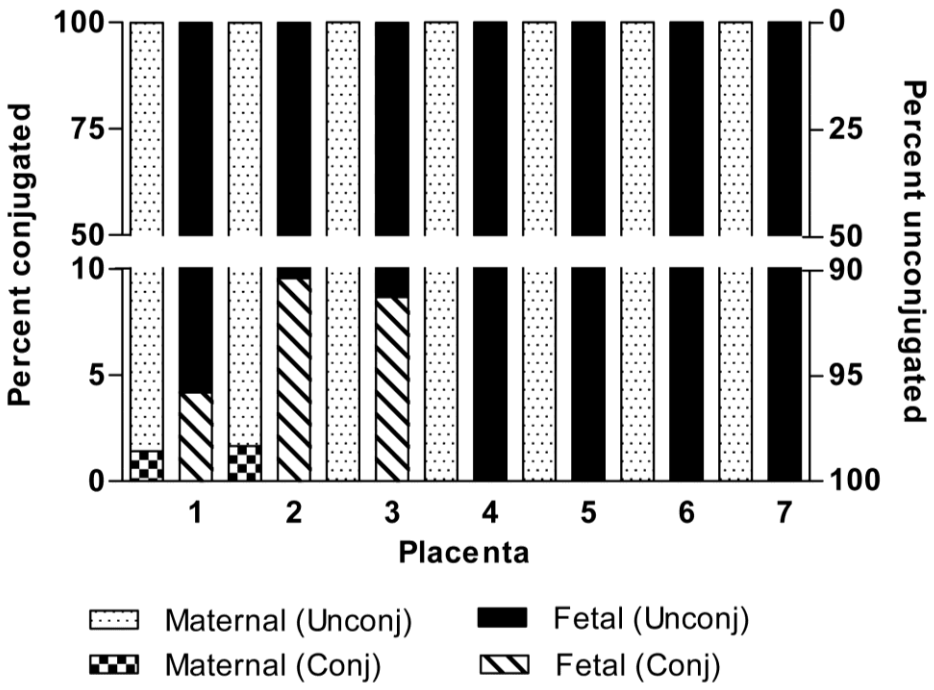

Figure 3-3 Percent conjugation of BPA

The samples were deconjugated with glucuronidase / sulphatase after 3 hours of perfusion and the free BPA was detected by LC-MS/MS.

3.4. COMMENT

The present study has demonstrated for the first time that BPA at environmentally relevant concentrations can transfer across the human placenta in an *ex-vivo* human placental perfusion model. The major fraction of the compound transferred was in un-conjugated form.

Substances transfer across the placenta either by simple diffusion or through active transporters. Transport of materials across the placenta is also dependant on the molecular weight of the compound, protein binding and lipophilicity. Our results indicate a rapid materno-fetal transfer of BPA across the human placenta. The transfer percentage and the feto-maternal ratio of both BPA and antipyrine (Table 3-2 and Figure 3-4) were

comparable for the entire time interval studied. Our results thus imply that BPA freely diffuses across the human placenta at a rate similar to that of antipyrine. BPA has been shown to be a substrate for the ABC-transporter, P-glycoprotein (P-gp) in the intestine *in vitro*[110]. BPA was also found to stimulate the drug efflux mechanism in a human placental cell line (BeWo a choriocarcinoma cell line) indicating the possibility of regulation through P-gp [111]. Specific P-gp inhibition was found to reduce the apparent diffusion from fetal model compartment to maternal model compartment in a BeWo choriocarcinoma cell line, which shows a possible protective role of P-gp during various stages of gestation [289]. But the role of P-gp in the efflux of BPA has not yet been studied in the placenta. However, it has also been shown that placental expression of P-gp gradually decreases with gestational age with lowest expression at term [290]. Hence, the apparent passive diffusion of BPA from maternal to fetal compartment in out term placentae may be due to the fact that the expression of any drug efflux pumps are lowest at this time. Studies in pregnant rodents have also shown that BPA reached the fetal compartment within an hour of oral or intravenous administration, mostly in un-conjugated form [90, 104, 291]. Recently a study compared the feto-maternal (F/M) ratio of hydroxylated polybrominated diphenyl ethers (OH-PCBs) and BPA by measuring the concentrations of both compounds in the maternal and cord serum and reported a higher ratio for OH-PCBs than BPA. This finding is important as the octanol water partition coefficient (log K_{OW}) of OH-PCBs (6.62) is higher than BPA (3.2) and hence a slow transfer rate was expected when compared to BPA. The authors further speculated that other mechanisms, in addition to lipophilicity could also be involved in the diffusional transfer of EDs across the human placenta (possibility for transthyretin as a career for OH-PCBs in this case) [292].

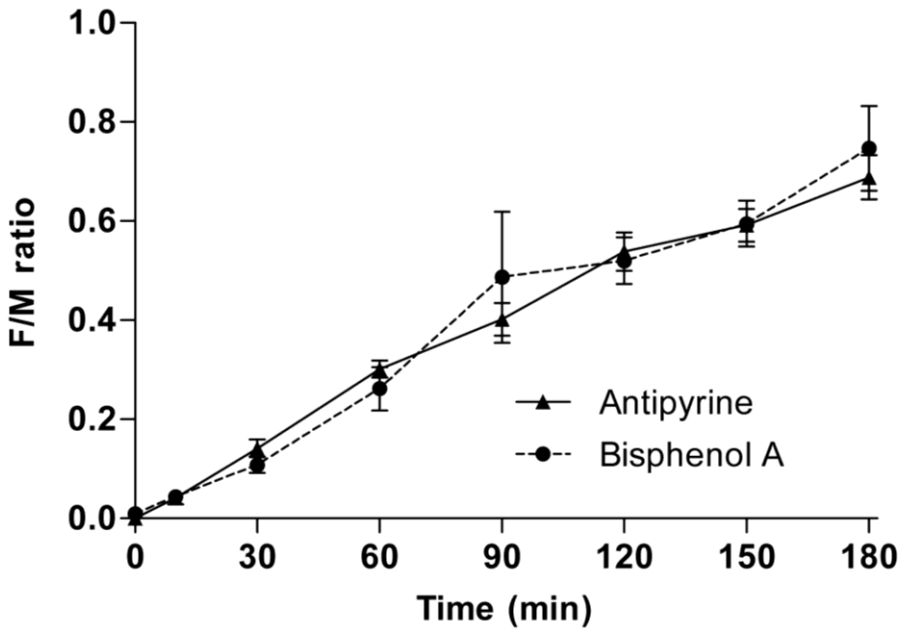

Figure 3-4 Feto-maternal ratio of antipyrine and BPA

The ratio becomes one when the concentrations at maternal and fetal compartments are equal. The figure shows the F/M ratio of antipyrine and BPA during 3 hours of perfusion. Data are presented as mean ± SEM (n=7)

BPA after oral absorption has been shown to be is conjugated either to glucuronide or sulphate form by hepatic enzymes[100, 285, 286]. Conjugation of BPA has been shown to ablate the estrogenicity of BPA *in-vitro* [293, 294]. Human term placenta expresses both Uridine 5' diphosphate glucuronyltransferases (UGT) and sulfotransferases required for the glucuronidation and sulphation of BPA[276, 295, 296]. Hence we explored the possibility of human placenta to convert BPA into its conjugates, thus protecting the fetus from the adverse affects of the xenoestrogen. However, our data conclusively imply that only negligible amounts of BPA are being conjugated by the placenta. The results further suggest that fetus is exposed to free BPA.

Placental perfusion studies have the limitation in that they do not take fetal metabolism into account. It is reported that fetuses and neonates have more glucuronidase and

sulphatase activity than gluronidation enzyme activity. A sluggish activity of glucuronyltransferase was reported in rodent / human fetuses [105, 297]. None of the rodent studies have addressed how BPA is glucuronidated in the fetus [104, 291]. Our data show more glucuronidated BPA in the fetal compartment than in the maternal compartment. This could be due to either the transfer of glucuronidated BPA from maternal to fetal compartment or glucuronidase activity in the fetal compartment. However, further studies are needed to elucidate the exact mechanism. To detect the transfer and metabolic fate of BPA glucuronide (BPA GA) a uterine perfusion study was conducted in pregnant rats and the transfer and a subsequent reactivation of BPA GA to BPA was demonstrated in the amniotic fluid and fetus. The transfer was mediated through influx and efflux transporters present in the placenta. On further evaluation the authors have observed higher levels of expression of glucuronidase in fetal liver and cardiac cells [298].

There was another study published recently on placental BPA transfer thorugh human placenta [289], which used 10 times higher dose of BPA compared to our experiments. However the results were comparable and they also observed a rapid transfer of BPA across the human placenta.

Our results show that 21.5 ± 1.5 % of total BPA was not accounted for when combining the BPA amount in the maternal and fetal compartments. This difference may accounted for BPA accumulation in the placenta or indicate that some of it is metabolized to unidentified products.

Our data clearly show that BPA at maternal serum concentrations can transfer across the human placenta and imply that placental glucuronyltransferase or sulfotransferase enzymes do not convert BPA into its conjugated form. The underdeveloped fetal liver [297] cannot

protect the fetus against this constant exposure. Hence the fetus is potentially very vulnerable to the adverse effects of BPA and it is critical to evaluate the harmful effects on health and well being of the fetus from such an exposure.

Declaration:-

I, Biju Balakrishnan, do hereby declare that the following chapter is an updated version of a published article, the details of which are as given below.

Balakrishnan B., Thorstensen EB., Ponnampalam AP and Mitchell MD (2010), 'Transplacental transfer and biotransformation of genistein in human placenta', Placenta, 31 (6), 506-511.

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Biju Balakrishnan

Chapter 4. TRANSPLACENTAL TRANSFER AND BIOTRANSFORMATION OF GENISTEIN IN HUMAN PLACENTA

4.1. INTRODUCTION

Estrogenic endocrine disruptors are abundant in the environment. Naturally occurring chemicals in plants that have estrogenic action are classified as phytoestrogens. Various degradation processes release synthetic estrogenic chemicals (xenoestrogens) into the environment eg. bisphenol A, alkylphenols and DDT. A significant correlation between developmental abnormalities, including carcinomas, on developmental exposure to diethylstylbesterol (a synthetic estrogen used previously as a treatment for threatening miscarriage) was observed in both humans and animals [299]. Researchers hypothesize that the endocrine disrupting action of these chemicals may be responsible for reproductive and / or developmental abnormalities observed in experimental and wild animals [36, 300].

Genistein is one of the phytoestrogens abundant in soy based foods and infant formulas. It has been proposed that isoflavones in soy based diets are beneficial to counteract certain cancers in humans [301], are osteoprotective [302] and can act as an antiobesity agent [303]. These observations led researchers to explore the use of genistein as a chemopreventive agent in many diseases, either alone or in combination with other chemotherapeutics. Genistein has been shown to possess several actions including inhibition of 'tyrosine phosphorylation, DNA topoisomerase activity, angiogenesis and cell growth'[304]. Genistein shares structure activity relationship with 17-β-estradiol (Figure 4-1). It can bind to both estrogen receptors, ER-α and ER-β with potencies 10^{-1} to

10^{-4} of 17-β-estradiol and can cause an effect (deleterious or favourable) based on the dose / tissue / exposure window [305, 306] . The deleterious effect of genistein such as tumorigenesis was more pronounced if the fetus is exposed *in-utero* while the favourable actions such as tumour protection were observed if exposed post-natally before puberty [307].

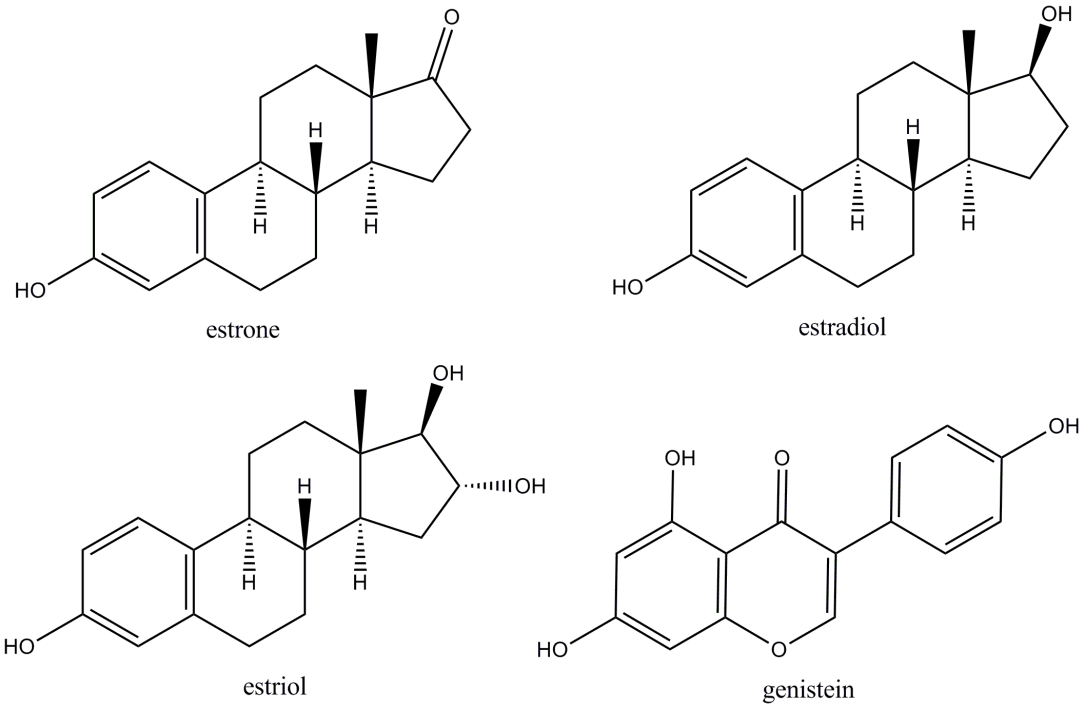

Figure 4-1 Structure activity relationship (SAR) of genistein and endogenous estrogens

Genistein shares structural similarity with 17-β estradiol and other endogenous estrogens, having a potency of 10^{-1} to 10^{-4} of 17-β-estradiol and can bind to both ER-α and ER-β. Due to SAR with 17-β-estradiol it may be recognized by steroid conjugating enzymes in the placenta.

Experiments in rodents have demonstrated that genistein can cross the placenta [99, 308] and cause developmental abnormalities at a dose relevant to human exposure [309]. Genistein has been shown to stimulate the growth of mammary tumour cells and uterine adenocarcinomas and stimulate uterine growth in immature or ovariectomized rodents [310, 311]. Prenatal exposure to genistein can also induce proliferative lesions in male mammary glands similar to the one induced by ethenyl estradiol [312]. Genistein was

found to inhibit progesterone synthesis and stimulate estrogen synthesis in human choriocarcinoma cell lines [313]. This finding suggests that there is a possibility of early fetal loss or abortion in pregnant mothers if exposed to high concentrations of genistein during gestation [313]. It also inhibits hCG production in these cell lines suggesting endocrine disrupting actions in placenta and thus enhancing the threat to the fetus [115]. A large longitudinal cohort study reported that the incidence of hypospadias in male children born to pregnant mothers on vegetarian food was significantly greater than mothers with omnivore diet (Incidence was 2.2 % vs. 0.6%). There was also an increase in the proportion of boys born with hypospadias when pregnant mothers had soy milk / soy food during pregnancy [314]. Due to the proposed health benefits of genistein in osteoprotection and/or cancer prevention dietary flavanoids have been in greater use [315].

The concentrations of genistein found in human is very variable and range from 0 to 59.2 ng/mL in maternal serum, from 0.6 to 87.9 ng/mL in umbilical cord, and from 0.22 to 7.88 µg/L in amniotic fluid of pregnant women [51-53, 263, 316]. It is also proposed that genistein tends to accumulate in the fetus [52, 53]. Studies have shown that both intestinal and hepatic Uridine diphosphate (UDP) glucuronyltransferase (UGTs) and sulfotransferases (SLUTs) can recognize and convert genistein to its glucuronide or sulphate forms [317]. Both glucuronide and sulphate forms are devoid of estrogenic activity [308]. It has also been suggested that the glucuronide form is unable to transfer across the placenta. Soucy *et al*. also demonstrated that the conjugated forms of genistein accumulated in fetal plasma and amniotic fluid indicating that genistein was conjugated after placental transfer [308]. Majority of the genistein administered was metabolized in liver and excreted through urine and feces. However pregnant rats when administered a dose mimicking human exposure levels rapidly transferred across the placenta and the fetal

concentration was sufficient to compete with estradiol for estrogen receptor – β (ER-β) activation [308]. Hence, if placental metabolizing enzymes could recognize and convert genistein to its glucuronide / sulphate forms, the exposure to the human fetus may be minimal. To the best of our knowledge none of the studies have directly assessed the ability of human placentae to conjugate genistein. In addition, controlled experiments in pregnant mothers are lacking, since exposure to these compounds may have adverse consequences with regard to the development of the fetus, making such a study impossible in women. As there is wide variation in placental histological structure across species, data from animals are difficult to extrapolate to humans. One of the best viable alternatives is performing placental perfusion studies. *Ex vivo* perfusion studies have been performed to study various drugs and toxicant transfer in human placenta under controlled conditions [318]. We have previously validated a placental perfusion model to study the trans-placental transfer of Azidothymidine (AZT), an anti-retroviral drug across the human placenta [276]. We further modified our perfusion model and reported the transfer of bisphenol A across the human placenta [319].

The present study hypothesises that genistein at environmentally relevant concentration can transfer across the human placenta in an active un-conjugated form. The aim of this study was to determine placental transfer and conjugation of genistein at environmentally relevant concentrations.

4.2. MATERIALS AND METHODS:-

4.2.1. Placental perfusion

Placentae were obtained from women undergoing elective caesarean section at term from Auckland City Hospital. Written consents were obtained from each pregnant woman for

the use of placentae for research and the use was approved by the Regional Ethics Committee. Placentae from healthy non-smoking mothers were used in the study.

A modified and fully validated dually perfused *ex-vivo* placental perfusion system was used to study the transfer of genistein across the human placenta [273, 275, 276, 288] . The placentae were transported to the laboratory within 30 minutes of delivery and after thorough physical examination, a suitable cotyledon was selected which was free from tears or pathological lesions. The chorionic vessels were catheterized with polyethylene tubes and the perfusate (Phenol red free Medium-199 (M-3769, Sigma Aldrich, St. Luise, MO, USA) cell culture media supplemented with 25g/L Polyvinylpyrrolidone PVP-40 (Sigma Aldrich), 1g/L Bovine Serum Albumin – Fraction V (Sigma Aldrich), 2g/L Glucose (Sigma Aldrich), 20,000 IU/L Heparin (Multiparin, CP Pharmaceuticals Ltd, Wexham, UK) and 48 mg/L Gentamicin Reagent Solution (GIBCO, Invitrogen, Auckland, New Zealand) was allowed to circulate through the fetal side by means of a digitally controlled pump. While perfusate circulated in the fetal side, the cotyledon was separated from the remaining placenta and mounted in the perfusion chamber, with the fetal side facing up. The maternal side was catheterized and the perfusate was allowed to circulate through it with the help of a digitally controlled pump. After 15 minutes both compartments were perfused in a re-circulating mode and the perfusion was started with maternal flow rate of 10mL/min and fetal flow rate of 4mL/min for 1 hr to equilibrate the *ex vivo* system. The maternal circuit was gassed with 95% O_2 and 5% CO_2 (Carbogen gas). Physical parameters used for selection criteria for a successful perfusion were as in Table 1 [273, 320]. After an hour of pre-perfusion (equilibration) the perfusate in the maternal compartment was replaced with fresh perfusate containing genistein (Sigma Aldrich) at 10ng/mL, antipyrine (Sigma Aldrich) at 40μg/mL, and FITC-Dextran [FITC-DX, FD4

(Sigma Aldrich)] at 12.5µg/mL. The genistein concentration selected is within the range reported in the serum of pregnant women [52, 53]. The perfusion was then continued for 3 hours and the samples from maternal and fetal reservoirs were collected at 30 min intervals and stored at -80°C. The viability and metabolic activity were assessed by measuring glucose utilization, lactate production (Hitachi 902 auto-analyser, Hitachi High Technologies Corporation, Tokyo, Japan) and human Chorionic Gonadotropin (hCG) secretion (ELISA). The FITC–DX transfer was measured with a spectroflurometer and antipyrine transfer was measured by HPLC (UV detection). A LC-MS/MS method was optimized to measure the genistein levels.

4.2.2. Antipyrine detection with HPLC (UV)

All the reagents used for HPLC and LC-MS/MS were of HPLC grade (Merck, KGaA, Germany). The HPLC method was a modification of the method standardized in our laboratory [277, 319]. Briefly 100µL of Phenacetin (internal standard, Serva, Feinbiochemica, USA), 500µL of 0.025N HCl and 500µL of acetonitrile were added to 500µL of perfusate and mixed well by vortexing. Then 4 mL of ethyl acetate was added and the solution was mixed for 15 minutes in a tube rotator. The tubes were then centrifuged at 3000g for 10 minutes (Beckman J6-MI) and stored at -80°C for 30 minutes. The supernatant (organic phase) was carefully transferred to a glass tube and lyophilized. The dried extracts were re-dissolved in 200µL of mobile phase, mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thin septa and screw caps.

Chromatographic separations were performed on an HPLC system (Waters Alliance 2690 Waters Corporation, Milford, MA, USA). The column used to separate the compounds was Luna 3µ C18 (2) 100A, 250 X 4.6 mm (Phenomenex, USA). The mobile phase was 6.7

mM phosphate buffer pH 7.2: acetonitrile (65:35). The separation was carried out isocratically at a flow rate of 0.7mL/min. 10µL of sample was injected and detection was carried out using a UV detector (Waters 996 photodiode array detector) at 254 nm. The data were analyzed using Waters Millennium Chromatography Manager V4.

4.2.3. Genistein detection with LC-MS/MS

An LC-MS/MS method was optimized in our laboratory to measure the genistein content in perfusates. Briefly 100µL of a mixture of genistein A d_4 (100ng/mL) (deuterium-labelled internal standard for genistein - Cambridge Isotope Laboratory, MA, USA) was mixed in a tube with 200µL of perfusate, 1mL of ethyl acetate was then added and the solution mixed by vortexing for 30sec. The tubes were then kept in a tube rack for 10 minutes. The organic phase was collected in aglass tube and lyophilized. The extract was re-dissolved in 100µL of mobile phase (Methanol: Acetonitrile: Water – 25:25:50), vortexed and transferred to 200µL PVC inserts in HPLC vials. 10µL of the samples was injected with an auto-injector. The compounds were separated using a Phenomenex 100 X 3.0 mm 2.5µ C18 (2) HST coloumn at 25°C. The separation was carried out with a gradient system for 10 minutes. The HPLC system (Waters Alliance 2690) was interfaced to a triple quadruple mass spectrometer. (Finnigan TSQ Quantum Ultra AM Thermo Electron Corporation, CA, USA) For mass spectrometry Electrospray ionization (ESI) was used in negative ion mode. A standard curve was prepared by dissolving 5-6 different concentrations of standard in perfusate.

To detect the conjugated levels of genistein, a deconjugation enzyme assay [279] was used with modification. 200µL of perfusate was buffered with 50µL of 2N Sodium acetate (pH 5.0). Glucuronidase / sulphatase (Crude extract from *Helix pomatia,* Sigma Aldrich) at pre-optimized concentration (500 units of glucuronidase and 3.5 units of sulphatase) was added

to tubes and incubated at 37°C in a shaking water bath for 2 hours. The reaction was stopped by heating the tubes at 70°C for 10 minutes. The total genistein was then extracted as per the above protocol and detection was carried out using LC-MS/MS as described above. The genistein detected before deconjugation (free genistein) was deducted from genistein detected after deconjugation (total genistein) and the difference (conjugated genistein) is expressed as a percent of total genistein.

4.2.4. Calculations

The formula used to calculate transfer percentage from maternal to fetal circulation was as follows: [67]

Transfer percentage = 100 X Fc X Fv/ [(Fc X Fv) + (Mc X Mv)]

Fc = Fetal concentration

Fv = Fetal volume

Mc = Maternal concentration

Mv = Maternal volume

Transfer index = Transfer percentage of genistein / Transfer percentage of antipyrine.

Values are expressed as mean ± standard error of mean

Glucose utilization = $(C_0 – C_{180}) / (W X t)$

Lactate production = $(C_{180} – C_0) / (W X t)$

B-hCG secretion = $(C_{180} – C_0) / (W X t)$

C_{180} = concentration of metabolites after 180 minutes of perfusion

C_0 = concentration of metabolites at time 0 minutes of perfusion

W = Wet weight of the perfused cotyledon

t = time in minutes

Data were analyzed and graphs were plotted using GraphPad prism 5 software (GraphPad Software Inc., CA, USA). The transfer rates of genistein and antipyrine for each time point were compared by two-way ANOVA with Bonferroni correction p values <0.05 were considered significant. Pearson's rank correlation was carried out to compare the FM ratio of genistein and antipyrine.

4.3. RESULTS

4.3.1. Placental perfusion

All the perfused placentae met the specifications underlined in the physical parameters mentioned in Table 4-1. A reduction in fetal volume and a consequent surplus in maternal volume were observed whenever there was a leak in the system. All placentae selected for the study showed a feto-maternal fluid loss of less than 3 mL/hr for a total of 180 min perfusion. The recorded fetal pressure for all successful placentae was in the range of 25-40 mm Hg. None of the placentae showed fluctuations in fetal pressures once they had passed the equilibration period. The placentae studied maintained viability as well as metabolic activity throughout the perfusion period as evident from constant glucose consumption, lactate production and β-hCG secretion (Table 4-2). β-hCG secretion was only observed in the maternal compartment and the values in the fetal compartment were below the detection level. FITC dextran transfer rate was less than 1% per hour (Table 4-2). The transfer percentage for antipyrine was 25.6 ± 1.40 after 3 hours of perfusion (Table 4-3).

Table 4-1 Physical parameters

Parameter	Values
Fetal Flow Rate	4.0 mL/min
Mternal Flow Rate	10.0 mL/min
Fetal Pressure	30-60 mm Hg
pH	7.2-7.6
Fluid Shift	< 3 mL/hr

The table shows the acceptable values for each parameter selected for a successful perfusion

Table 4-2 Biochemical parameters

Perfusion Parameters		
Weight of placenta (g)	572.1 ± 39.90 (n=6)	
Weight of perfused cotyledon (g)	28.1 ± 2.94 (n=6)	
Biochemical Parameters	**Maternal**	**Fetal**
Glucose consumption (μM/g/min)	0.34 ± 0.10	0.3 ± 0.08
Lactate production (μM/g/min)	0.49 ± 0.14	0.31 ± 0.06
h-CG (mIU/g/min)	2.5 ± 0.20	ND
FITC – DX transfer (% per hour)		<1

The table summarises the observed biochemical parameters for successful perfusion. Constant consumption of glucose, production of lactate and production of β-hCG demonstrates that the perfused lobule is metabolically active.

Table 4-3 **Transfer percentage and transfer index of genistein**

Time (min)	Transfer Percentage		Transfer Index
	Antipyrine	Genistein	Genistein
10	2.2 ± 0.14	1.1 ± 0.13	0.5 ± 0.06
30	5.5 ± 0.22	3.8± 0.26	0.7 ± 0.06
60	12.7 ± 0.74*	8.1 ± 0.72*	0.6 ± 0.07
90	15.4 ± 1.00	12.7± 1.34	0.8 ± 0.13
120	21.0 ± 1.36	17.0 ± 1.59	0.8 ± 0.09
150	21.9 ± 1.06	18.7 ± 1.35	0.9 ± 0.06
180	25.6 ± 1.4	22.1 ± 1.61	0.9 ± 0.04

*2-way anova with Bonferroni post test p value < 0.05

Transfer percentage and transfer index were calculated after 3 hours of perfusion, as described in materials section and tabulated as under (data are presented as mean ± SEM; n=4)

4.3.2. Transplacental transfer of genistein in human placenta

A highly sensitive LC-MS/MS method using Electrospray ionization (ESI) was validated and optimized in our laboratory to measure genistein concentration accurately in maternal and fetal compartments. The method is robust and sensitive with a limit of detection (LOD) of 100 pg/mL at signal to noise ratio >3 and the standard curve was linear from 100 pg/mL to 30 ng/mL (Figure 4-2). This protocol also has the advantage of detecting genistein and all three endogenous estrogens (estradiol, estrone and estriol) in the perfusate simultaneously.

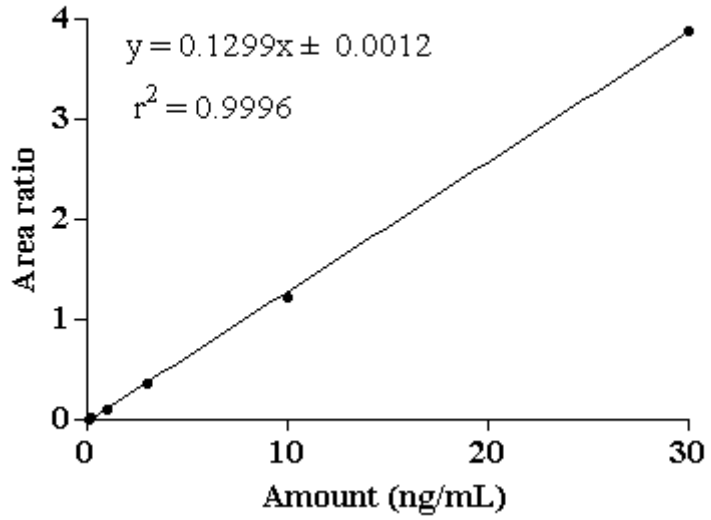

Figure 4-2 Standard curve for genistein

The perfusates were spiked with different concentrations (100 pg/mL to 30 ng/mL) of genistein and the calibration curves were constructed from the area ratio against the spiked concentrations. The relationship is linear between 100 pg/mL to 30 ng/mL.

The analysis shows a gradual decline in the concentration of genistein in the maternal compartment and a concomitant increase in the concentration of genistein in the fetal compartment. 1.1±0.13 % of genistein in unconjugated form was transferred to the fetal compartment within 10 minutes of perfusion. The transfer rate gradually increased and approximately 22.1±1.61% of genistein in unconjugated form could be recovered from the fetal compartment after 3 hours of perfusion. This clearly shows that genistein at low concentrations can cross the human placenta (Figure 4-3). The transfer index for genistein was 0.90 ± 0.04 after 3 hours of perfusion in human placenta. The transfer rates of genistein was significantly lower than that of antipyrine after one hour of perfusion, was gradually increased till 90 minutes and then remained stable up to 3 hours of perfusion (Table 4-3). There was a significant correlation between the fetomaternal ratios (Figure 4-4) of antipyrine and genistein during the 3 hours of perfusion (r^2 = 0.99 with p value < 0.0001).

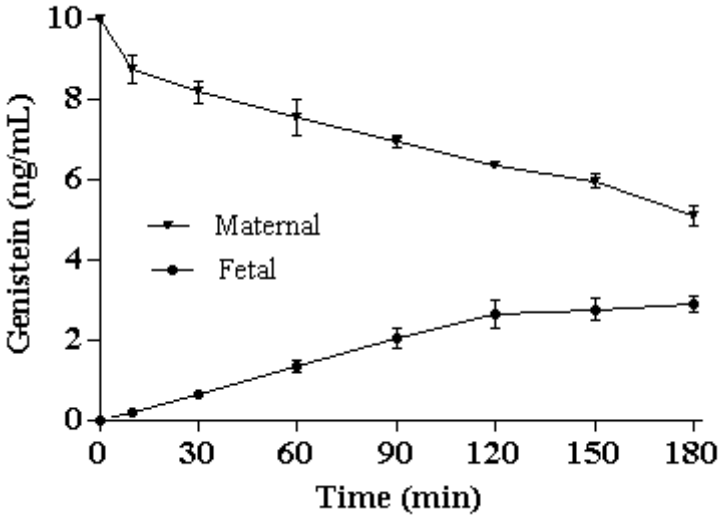

Figure 4-3 Transplacental transfer of genistein in human placenta

The gradual decline of genistein from the maternal compartment and subsequent gradual increase of genistein in the fetal compartment shows that genistein rapidly crosses the human placenta as detected with LC-MS/MS (data is presented as mean ± SEM; n=4)

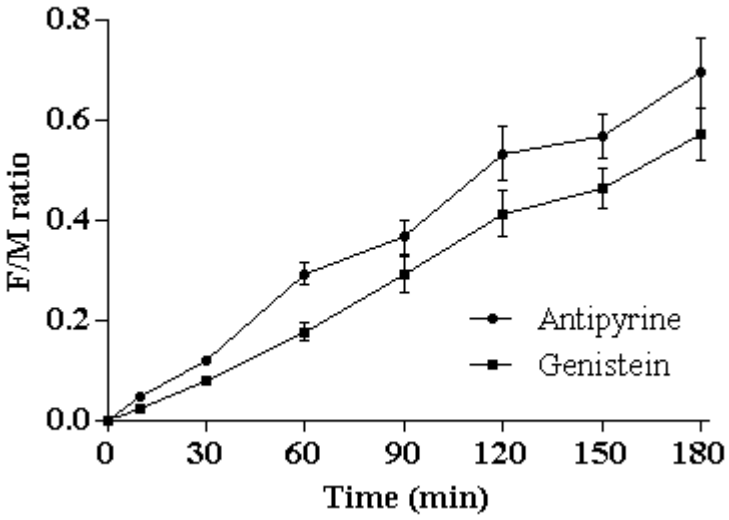

Figure 4-4 Feto-maternal ratio of antipyrine and genistein

The ratio becomes one when the concentrations at maternal and fetal compartments are equal. From the figure it is evident that both antipyrine and genistein have comparable F/M ratio and both are approaching equilibrium after 3 hours of perfusion. ($r^2 = 0.99$, $p < 0.0001$).

4.3.3. Placental biotransformation of genistein

It has been previously reported that the extraction reagents and / or incubation in assay buffer could spontaneously reverse the conjugated form back into non-conjugated form [279]. Estradiol standards (sodium salts of β-estradiol-17-β-glucuronide and β-estradiol-3-sulphate) were used in this study to evaluate this spontaneous hydrolysis and no detectable amount of free estradiol was measured under the incubation and/or hydrolysis conditions. This further strengthens the quality of the deconjugation enzyme assay used in this study.

All the four placentas showed evidence of conjugation in the fetal compartment and three out of four placentas showed evidence of conjugation in the maternal compartment after 3 hours of perfusion. Approximately 12.0 ± 2.4% of genistein in the fetal compartment and 7.4 ± 4.7% of genistein in the maternal compartment were in conjugated form (Figure 4-5).

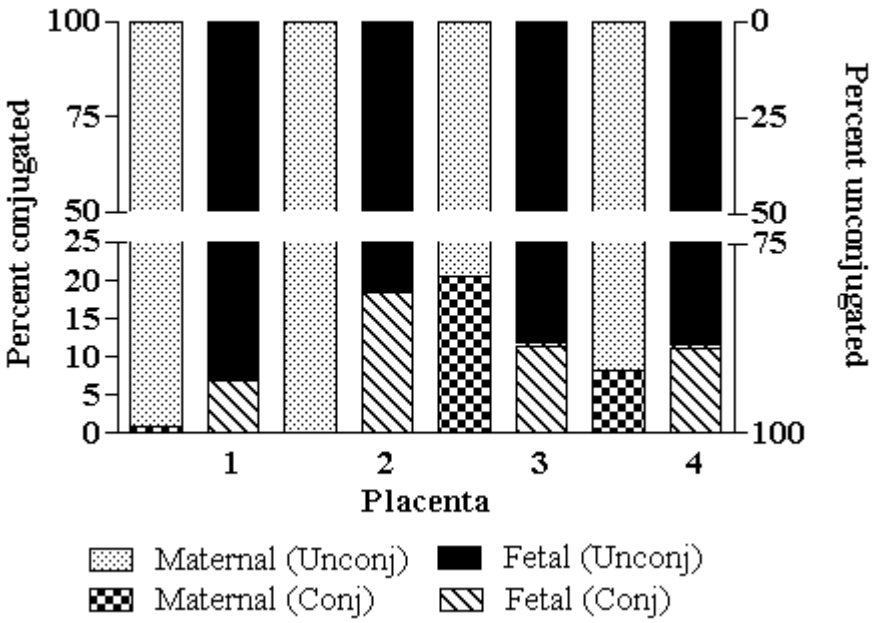

Figure 4-5 Percent conjugation of genistein

The samples were deconjugated with glucuronidase / sulphatase after 3 hours of perfusion and the free genistein was detected by LC-MS/MS. The graph is split into two halves to clearly show the percent conjugation levels. Each segment in the lower half of the graph represents 5% and each segment in the upper half of the graph represents 25% conjugated / unconjugated levels as the case may be.

4.4. DISCUSSION

This study has demonstrated for the first time that genistein, can transfer across the human placenta at environmentally relevant concentrations in an *ex-vivo* human placental perfusion model. Our data also show that approximately 12% of genistein transferred to the fetal compartment was in conjugated form.

Substances transfer across the placenta either by simple diffusion or through active transport. Transport of materials across the placenta is also dependant on the molecular weight of the compound, protein binding and lipophilicity. Our results indicate a rapid materno-fetal transfer of un-conjugated free genistein across the human placenta and the rate is significantly lower to that of antipyrine during the initial period of perfusion and then reaches a steady state, which follows the transfer rate as that of antipyrine. This shows that the method of transfer of genistein may be different from that of antipyrine, which is transferred across the placenta by simple diffusion. Genistein has been shown to be a BCRP (Breast Cancer Resistant Protein) substrate in a cancer cell line and was found to inhibit BCRP mediated drug efflux [321]. Since the placenta expresses high levels of BCRP [322, 323], it may regulate the transfer of genistein to the fetus. It has also been shown that genistein accumulates in BCRP knockout mice fetus when genistein was included in the diet of pregnant mice during gestation [324], which supports the involvement of BCRP in genistein efflux in mice. The detrimental effects of phyto- and xenoestrogens predominantly relate to fetal development in the first trimester. Expression of ATP- and related transporters is subject to maturational changes of the trophoblast [323,

325]. Hence the regulatory role of BCRP in genistein transfer in term placenta mandates further studies.

It is reported that the hepatic and intestinal Uridine 5' diphosphate glucuronyltransferases (UGT) isoforms, UGT1A1, UGT1A8, UGT1A9 and UGT1A10 could convert genistein into its glucuronide form [317, 326]. Genistein has been shown to be conjugated either to glucuronide or sulphate form by hepatic enzymes after oral absorption in humans [53]. Conjugated form of genistein has been shown to be devoid of any estrogenic action [53]. It is also suggested that genistein glucuronidates does not cross the placenta [308], while there is some evidence that the sulphated conjugates does cross the placenta [53]. Human term placenta expresses both the Uridine 5' diphosphate glucuronyltransferases (UGT) and sulfotransferases required for the glucuronidation and sulphation of genistein [276, 295, 296, 327]. Hence we explored the ability of human placenta to convert genistein into its conjugates, thus protecting the fetus from the adverse affects of the xenoestrogen. Our data imply that after three hours perfusion a small fraction of genistein in the fetal compartment is in the form of genistein conjugates. The sample volume was a limitation to detect the conjugated levels of genistein during various time intervals. The variations observed in the conjugation pattern between the placentae may be due to the inter-placental variation. It is also reported that the environmental exposure to certain chemicals, gestational maturity and alcohol consumption can affect the expression of phase 2 metabolizing enzymes including UGT [295]. Hence in future comparison on the expression levels of UGT isoforms in various compartments of perfused placentae would be required. Previous studies in rodent model have suggested that genistein glucuronide does not cross the placenta [308]. This suggestion is partly based upon the available literature and also upon the linear course of appearance of genistein conjugates in fetal

plasma while scanty appearance of the same in the placenta [308]. Our data imply that the placenta is capable of conjugating a fraction of genistein and that the conjugated genistein can transfer across the human placenta. However further perfusion studies using conjugated genistein in maternal perfusates would be required to confirm this. Placental perfusion studies have the limitation in that they do not take fetal metabolism into account. It is reported that fetuses and neonates have more glucuronidase and sulphatase activity than glucuronidation enzyme activity. A sluggish activity of glucuronyltransferase was reported in rodent / human fetuses [105, 297]. Hence the actual fraction of active unconjugated genistein reaching in various fetal tissues may vary.

About 30% of genistein is unaccounted for in our studies. This may be attributed to tissue binding in the placenta. It is shown that genistein tends to accumulate in the placenta within 4-6 hours of administration in rodents [308]. The potential endocrine disrupting actions of genistein in the placenta needs to be explored further [115, 313].

Our data clearly shows that genistein at low, environmentally relevant concentration can transfer across the human placenta at term. Due to the structure activity relationship with 17-β-estradiol a minor fraction of genistein is recognized by the placental conjugating enzymes and is converted to its glucuronide and or sulphate forms. The effective genistein concentration reaching on the fetus (~15 nanomols / mL) at this maternal exposure levels (~40 nanomols / mL) could be sufficient to elicit a nongenomic action mediated through estrogen receptor α (ER-α) [328]. Hence it may be possible that the fetus of a pregnant mother on high soy diet is constantly exposed to phytoestrogens at the vulnerable window of development and the effects on health and well being of the fetus from such an exposure has yet to be elucidated.

Declaration:-

I, Biju Balakrishnan, do hereby declare that the following chapter is the final version of a manuscript accepted for publication in Placenta, the details of which are as given below.

Balakrishnan B., Thorstensen EB., Ponnampalam AP., Mitchell MD, 'Passage of 4-nonylphenol across the human placenta' In Press, Placenta.

Biju Balakrishnan

Chapter 5. PASSAGE OF 4-NONYLPHENOL ACROSS THE HUMAN PLACENTA

5.1. INTRODUCTION

4-nonylphenol (4-NP) is one of the major microbial degradation products of alkylphenol ethoxylates used heavily in the production of industrial and household detergents. Nonylphenol derivatives are also used in certain cosmetics and as a spermicide in contraceptives [68]. It is estimated that the annual production of alkylphenols exceeds 650,000 tons globally [329]. 4-NP is more hydrophobic and less biodegradable than the parent alkylphenols [68]. Hence, it can accumulate and contaminate aquatic environments (marine, as well as fresh water fauna and flora) and can enter the food chain and drinking water; therefore making human exposure unavoidable.

In-vitro and *in-vivo* studies have shown that most of the alkylphenols and their metabolites including 4-NP possess weak estrogenic action[7]. 4-NP is a non-steroidal phenolic compound which shares structural similarity with 17-β-estradiol and diethylstilbestrol (the latter is an estrogen agonist and a proven teratogen and a carcinogen in humans [330]), due to the presence of an alkyl group in the para position (Figure 5-1) [331, 332].

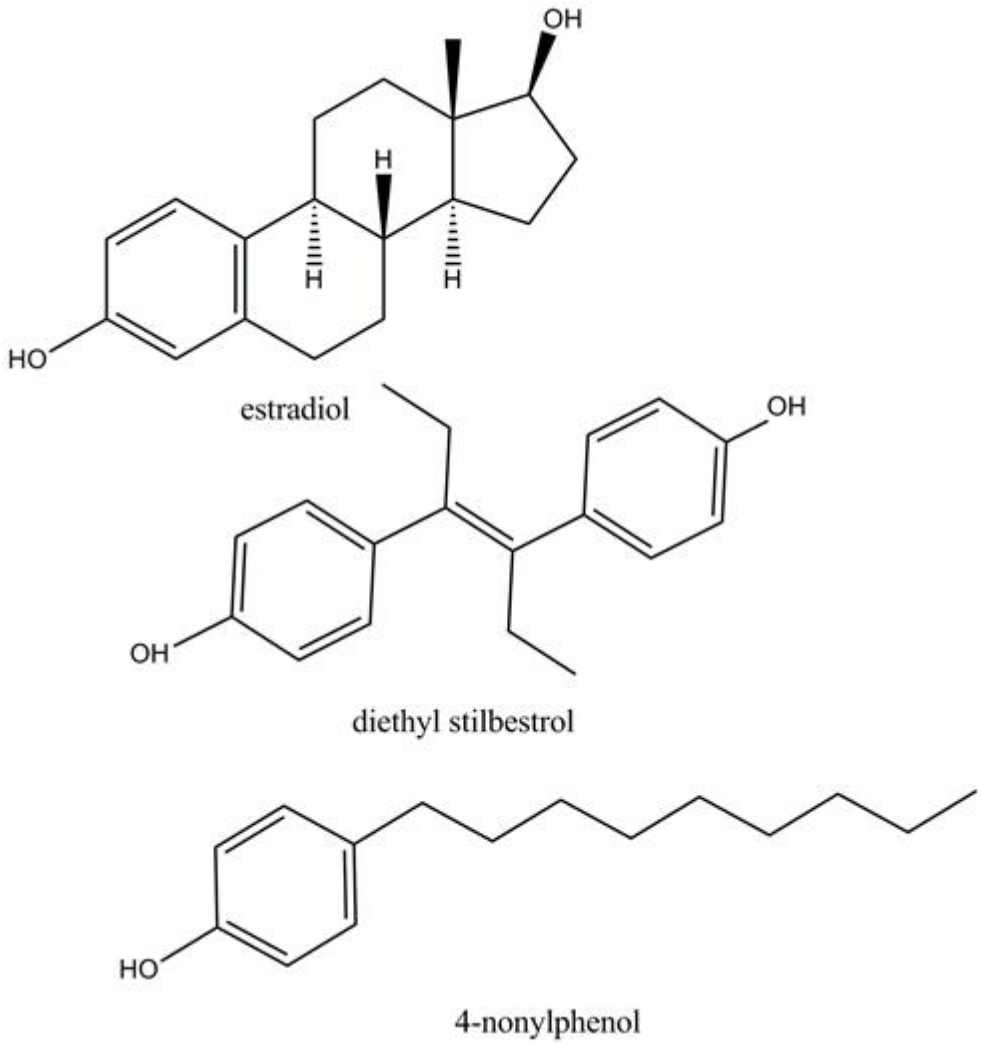

Figure 5-1 Structural similarities of 4-NP to 17-β-estradiol and diethylstilbestrol

Due to the presence of alkyl group in the para-position these compounds can interact with estrogen receptors to mediate an estrogen receptor mediated action.

It has been shown that 4-NP impairs reproductive functions through direct interaction with estrogen receptors [333], however, it could also act through membrane-bound estrogen receptors (non-genomic action) and some of its activities have been found to be equipotent to that of 17-β-estradiol [334]. It can bind to other steroid receptors like progesterone [262] and androgen receptors [335] with a lower affinity than their natural ligands. 4-NP has been shown to regulate the expressions of ER-α receptors, ER-β receptors, ERR (estrogen related receptors) –α, -β and γ [336, 337], growth hormone [338], p53 [339] and 11-β-Hydroxysteroid dehydrogenase [340]. Taken together these data suggest that 4-NP is

a potential endocrine disruptor and may cause developmental abnormalities if fetal exposure occurs at vulnerable windows of human development.

Studies have shown that humans are constantly exposed to 4-NP [341], with dermal absorption, ingestion or inhalation [329] being the main routes. It is estimated that an adult human's exposure to 4-NP through drinking water and food may be 0.7µg/L and 7.5µg/day, respectively. 4-NP has been shown to accumulate in human adipose tissues, possibly due to its lipophilic nature [342]. A high degree of correlation has been reported between the circulating levels of 4-NP in maternal and umbilical cord blood indicating the possibility of placental transfer and early fetal exposure [343].

Exposure to 4-NP during pregnancy can cause transgenerational adverse effects on reproductive function and organ failure in rats [209]. 4-NP has been shown to transfer across the placenta rapidly and accumulate in fetal brain in rodents [344], and has been shown to induce the expression of calbindin-D 9k (CaBP9K) in the maternal and fetal rat uterus [270]. Prenatal exposure to 4-NP has been reported to cause precocious puberty and sperm defects in rodents [345]. Gestational exposure to 4-NP has profound effects on reproductive and neurobehavioural development in male rat offspring and fetal growth and development [346].

To the best of our knowledge there is no direct characterization of human placental transfer of 4-NP. The aim of this study was to evaluate placental transfer of 4-NP at environmentally relevant concentrations using a well validated dually perfused human single cotyledon placental perfusion model.

5.2. MATERIALS AND METHODS

5.2.1. Placental perfusion

Placentae were obtained from women undergoing elective caesarean section at term from Auckland City Hospital, after informed consent. The research was approved by the Northern X Regional Ethics Committee. Placentae from singleton pregnancies of healthy non-smoking mothers were used in the study.

A modified and fully validated dually perfused *ex-vivo* placental perfusion system was used to study the transfer of 4-NP across the human placenta as described earlier [273, 275, 276, 288, 319, 347]. In brief, placentae after physical examination, were catheterized at chorionic vessels (fetal side) and remnants of spiral arteries (maternal side), while perfusates were flowing with the help of digitally controlled pumps [Phenol red free Medium-199 (M-3769, Sigma Aldrich, St. Luise, MO, USA) cell culture media supplemented with 25g/L Polyvinylpyrrolidone PVP-40 (Sigma Aldrich), 1g/L Bovine Serum Albumin – Fraction V (Sigma Aldrich), 2g/L Glucose (Sigma Aldrich), 20,000 IU/L Heparin (Multiparin, CP Pharmaceuticals Ltd, Wexham, UK) and 48 mg/L Gentamicin Reagent Solution (GIBCO, Invitrogen, NY, USA)]. Selection criteria for a successful perfusion were as described previously [273, 320]. After equilibration the perfusate in the maternal reservoir was replaced with fresh perfusate containing 4-NP (44-2873, Supelco, Sigma Aldrich) at 30ng/mL, antipyrine (Sigma Aldrich) at 40μg/mL, and FITC-Dextran [FITC-DX, FD4, MW 4 KDa, (Sigma Aldrich)] at 12.5μg/mL. The 4-NP concentration selected is within the range of observed human exposure [343]. The perfusion was continued in a re-circulating mode (both maternal and fetal reservoirs) for 3 hours and samples from maternal and fetal reservoirs were collected at 10 minutes and then at 30 min intervals and stored at -80°C. The viability and metabolic activity were assessed

by measuring glucose utilization, lactate production (Hitachi 902 auto-analyser, Hitachi High Technologies Corporation, Tokyo, Japan) and human Chorionic Gonadotropin (hCG) secretion (ELISA). Due to the limited transfer rate (<1% per hour), FITC – Dextran was used as a negative marker in perfusion experiments. Antipyrine can transfer freely across the placenta and is used extensively in perfusion experiments as a positive marker The FITC–DX transfer was measured with a spectrofluorometer and antipyrine transfer was measured by HPLC (UV detection) as described earlier [319, 347]. An HPLC method coupled with Fluorescence Detector was optimized in our laboratory to measure the 4-NP levels.

5.2.2. 4-NP detection with HPLC-FD

All the reagents used for HPLC were of HPLC grade (Merck, KGaA, Germany). The HPLC method was a modification of the method described recently [280]. Butyl Paraben was used as an internal standard. Briefly 100µL of 1µg/mL Butyl Paraben (Internal standard, Sigma) was added to 250µL of perfusate and mixed well by vortexing. Then 4 mL of Dichloromethane – n-Pentane (50:50) was added and was mixed for 15 minutes in a tube rotator. The tubes were then centrifuged (Beckman J6-MI, Beckman Coulter, Inc, CA, USA) at 3500g for 30 minutes and stored at -80°C for 30 minutes. The supernatant (organic phase) was carefully transferred to a glass tube and lyophilized. The dried extracts were re-dissolved in 70µL of mobile phase, mixed thoroughly by vortexing and transferred to 200µL PVC inserts in HPLC vials and sealed with thick septa and screw caps. Chromatographic separations were performed on an HPLC system (Waters Alliance 2690, Waters Corporation, Milford, MA, USA). The column used to separate the compounds was a Synergi 4µm POLAR-RP 80A 250x4.6 mm (Phenomenex, USA). The mobile phase was 0.1% acetic acid:acetonitrile. The column temperature was 40°C and the sample

temperature was 10°C. The separation was carried out with a linear gradient at a flow rate of 1mL/min. The gradient started with 65% water (0.1% acetic acid) and 35% acetonitrile, proceeding to 65% acetonitrile over 4 minutes, held for a minute and then proceeding to 90% acetonitrile over the next 8 minutes, held for a minute, and then proceeding back to the initial conditions (65:35, water:acetonitrile) over the next 3 minutes. 35µL of sample was injected and detection was carried out using a Fluorescence Detector (Waters 2475) Ex λ 278 nm, Em λ 315nm.

A deconjugation assay was performed in the maternal and fetal perfusates as described earlier [319, 347], the free 4-NP released into the perfusates was extracted and detected with HPLC as described above.

5.2.3. FITC Dextran Assay

The working standard solution was prepared by dissolving 500 µg FITC-Dextran in 1mL perfusate and this was further serially diluted in perfusate to make 8 standards. 100 µL of each standard and sample were transferred to 96 well black FIA plates (Greiner Bio-one, Orlando, USA). The plates were read spectrofluorometrically with the help of Synergy 2 Multi-mode microplate reader (Bio Tek, VT, USA) (Ex λ 494nm, Em λ 521nm).

5.2.4. β-hCG Assay

A sandwich ELISA standardized in our laboratory was used to measure the β-hCG concentration of the perfusates. Briefly 96 well Nunc Immuno plates (Maxisorp, Nunc, Thermo Fisher Scientific, NY, USA) were coated with 100 µL of primary antibody (2 µg/mL mouse anti hCG) and incubated overnight at 4°C. The plates were washed with washing buffer and 100 µL of standard [one in three fold serial dilutions were prepared from the stock solution (210,000 mIU/mL)] or sample in duplicate and 10µL of

Immunocoat Buffer (Sigma) were transferred to the plate and further incubated for two hours at room temperature. After washing with wash buffer, 100 µL of secondary antibody (rabbit anti hCG) in Immunocoat Buffer was added and incubated for one hour. After further washing, 100 µL of anti-rabbit HRPO conjugate diluted in Immunocoat Buffer (1 in 250) was added and incubated for 30 minutes. Following another wash, 100 µL of O-Phenylene Diamine (0.5 mg/mL OPD) (o-Phenylene Diamine) was added and incubated for 15 minutes. The plates were read in a spectrophotometer plate reader at 480 nm with blank reduction at 520 nm.

Calculations

The following formula was used to calculate transfer percentage from maternal to fetal circulation: [67]

Transfer percentage = 100 X Fc X Fv/ [(Fc X Fv) + (Mc X Mv)]

Fc = Fetal concentration

Fv = Fetal volume

Mc = Maternal concentration

Mv = Maternal volume

Transfer index = Transfer percentage of 4-NP / Transfer percentage of antipyrine.

Values are expressed as mean ± standard error of mean

Glucose utilization = $(C_0 – C_{180}) / (W X t)$

Lactate production = $(C_{180} – C_0) / (W X t)$

B-hCG secretion = $(C_{180} – C_0) / (W X t)$

C_{180} = concentration of metabolites after 180 minutes of perfusion

C_0 = concentration of metabolites at time 0 minutes of perfusion

W = Wet weight of the perfused cotyledon

t = time in minutes

Data Analysis

Statistical analysis was performed with Sigmaplot V10. The significance between the groups was evaluated with paired student's t test. The p-values of <0.05 were considered as statistically significant. Graphs were plotted using GraphPad prism 5 software (GraphPad Software Inc., CA, USA).

5.3. RESULTS

5.3.1. Placental perfusion

Six perfused placentae met the specifications outlined in the physical parameters mentioned previously [319, 347]. Fluid shifts from fetal to maternal reservoirs were observed when there was a leak in the system. All placentae selected for the study showed a feto-maternal fluid loss of less than 3 mL/hr for a total of 180 min perfusion. The recorded fetal-side inflow hydrostatic pressure (FIHP) for all successful placentae was in the range of 25-40 mm Hg. FIHP fluctuated (40-60 mm Hg) in the initial period of perfusion (equilibration period) and became steady for the rest of the perfusion. The biochemical parameters observed (glucose consumption, lactate production and β-hCG secretion) revealed that the placentae used in this study maintained viability as well as metabolic activity throughout the perfusion period. β-hCG secretion was only observed in the maternal reservoir. 43.7 % of initial maternal perfusate reservoir concentration of

antipyrine could be detected in fetal reservoir after 3 hours of perfusion. FITC dextran transfer rate was less than 1 percent per hour. This transfer rate was insignificant for a molecule of this size [288]. The physical and biochemical parameters observed were as shown as in Table 2 (a) and (b).

Table 5-1 Physical and Biochemical parameters observed for placentae perfused with 4-NP

Parameter	Values*
Fetal Flow Rate	3.96 ± 0.1 mL/min
Maternal Flow Rate	10.1 ± 0.05 mL/min
FIHP	30.2 ± 2.5 mm Hg
pH	7.48 ± 0.23
Fluid Shift	< 3 mL/hr

- The values are mean ± sem of 6 placentae perfused with 4-NP during post-equilibration period.

Perfusion Parameters		
Weight of the placenta (g)	565.9 ± 37.8 (n=6)	
Weight of the perfused cotyledon (g)	25.5 ± 1.9 (n=6)	
Biochemical Parameters	**Maternal**	**Fetal**
Glucose consumption (μM/g/min)	0.42 ± 0.08	0.31 ± 0.19
Lactate production (μM/g/min)	0.64 ± 0.35	0.32 ± 0.14
β-hCG (mIU/g/min)	2.63 ± 0.21	ND*
Antipyrine concentration after 3 hours of perfusion (% initial maternal concentration)	57.3 ± 1.42	43.7 ± 2.7
FITC – dextran transfer rate (% per hour)		<1

*ND = below detection limit

5.3.2. Transplacental transfer of 4-NP in human placenta

To measure 4-NP concentration accurately in maternal and fetal perfusates, a sensitive HPLC using Fluorescence Detector was validated and optimized in our laboratory. The standard curve was linear from 0.8 ng/mL to 100 ng/mL. The method is robust and sensitive with the Limit of Detection (LOD) of 1.56 ng/mL at signal to noise ratio >3 (Table 5-2).

Table 5-2 HPLC-FD validation parameters for 4-NP.

Detection limit	Accuracy	Reproducibility (Intra assay CV %)	Linearity
1.6 ng/mL	Y=0.2723x-0.2293	1.80%	0.9948

HPLC method was optimized to detect the 4-NP in maternal and fetal perfusates. The method has a detection limit of 1.6 ng/mL with a signal to noise ratio of >3.

A rapid decline in the concentration of 4-NP in the maternal reservoir was observed until 90 minutes of perfusion and then it declined at a constant rate until the end of perfusion (180 minutes). Detectable quantities of 4-NP appeared in the fetal reservoir after 30 minutes of perfusion. After 3 hours of perfusion, 2.0 ± 0.35 ng/mL (mean ± s.e.m, n=6) 4-NP could be recovered from the fetal reservoir and 3.9 ± 0.96 ng/mL 4-NP from the maternal reservoir (Figure 5-2). Antipyrine transfer was rapid and was detectable in the fetal reservoir within 10 minutes of perfusion (Table 5-3). The transfer percentage and feto-maternal ratio of 4-NP significantly differed from that of antipyrine till 60 minutes of perfusion (Table 5-3 & Figure 5-3). The transfer index of 4-NP reached approximately one at 90 minutes of perfusion. However the 4-NP passage after 90 minutes of perfusion is much more variable than that of antipyrine. About 84% of 4-NP could not be recovered

from the perfusates. A deconjugation assay was performed as described previously [347] but little variation in the 4-NP peak was found indicating conjugation of 4-NP was unlikely to explain the disappearance of 4-NP from the maternal reservoir or lack of its appearance in the fetal reservoir.

Table 5-3 Transfer percentage and transfer index of 4-NP

(data are presented as mean ± SEM; n=6)

Time (min)	Transfer Percentage		Transfer Index
	Antipyrine	4-NP	4-NP
10	3.05 ± 0.36	0.82 ± 0.34*	0.31 ± 0.14
30	7.25 ± 0.75	1.39 ± 0.62*	0.2 ± 0.10
60	14.22 ± 1.28	4.9 ± 1.5*	0.4 ± 0.16
90	16.24 ± 2.02	13.3 ± 3.2	1 ± 0.32
120	22.28 ± 1.89	17.6 ± 3.0	0.85 ± 0.21
150	24.08 ± 1.3	20.8 ± 3.8	0.85 ± 0.17
180	27.6 ± 1.20	22.75 ± 3.76	0.8 ± 0.12

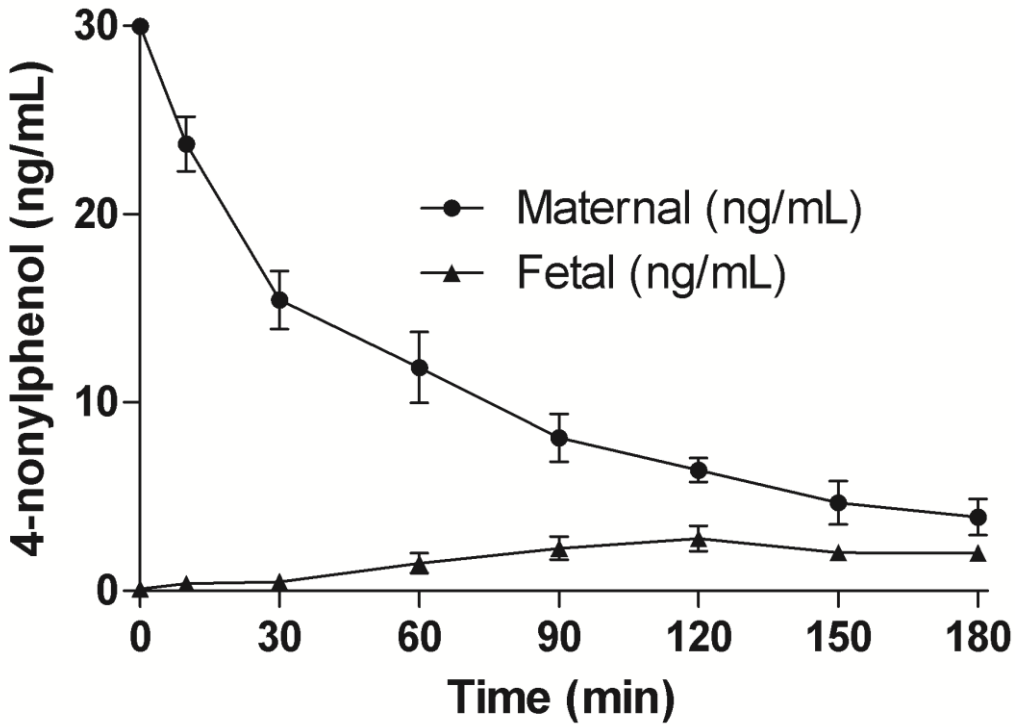

Figure 5-2 4-NP transfer in human placenta

The rapid decline of 4-NP from the maternal perfusate reservoir concentration and a later appearance of 4-NP in the fetal perfusate reservoir concentration shows that 4-NP transfers slowly across the human placenta (data is presented as mean ± SEM; n=6).

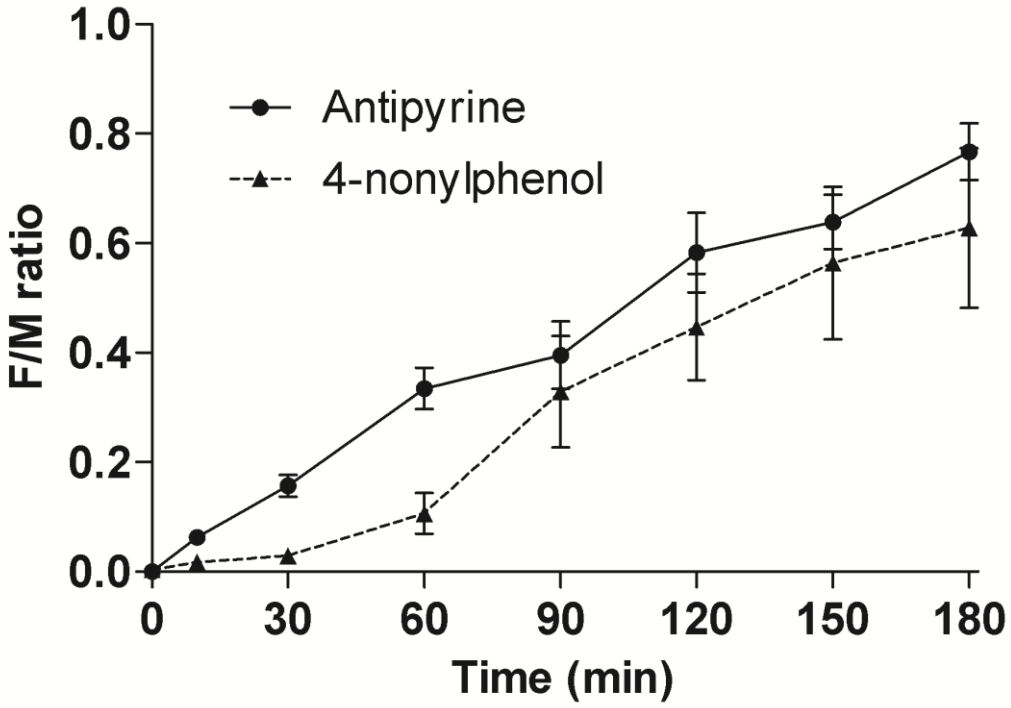

Figure 5-3 Feto-maternal ratio of antipyrine and 4-NP.

The ratio becomes one when the concentrations at maternal and fetal perfusate reservoir concentrations are equal. From the figure it is evident that both antipyrine and 4-NP have variable F/M ratio throughout 180 minutes of perfusion.

5.4. DISCUSSION

This study has demonstrated that 4-NP at environmentally relevant concentrations can transfer in the parent form across the human placenta in an *ex-vivo* human placental perfusion model. Our data also show that there was an initial rapid disappearance of 4-NP from the maternal reservoir without an equivalent rise in the fetal perfusate reservoir concentration. This might be due to tissue binding or the formation of an intermediary metabolite.

Our results indicate 4-NP can transfer across the human placenta. Antipyrine was used as a marker to compare and normalize the transfer parameters of 4-NP in these experiments, as it is an inert compound which diffuses freely across the human placentae [348]. FITC dextran (4 KDa) penetrates poorly through the placenta. If there is a compromise in the integrity of materno-fetal membrane FITC dextran transfers rapidly to the fetal reservoir. Our finding of a FITC – DX transfer rate <1% per hour are a good indication that the materno-fetal membrane retained its integrity throughout the experiment. Previously other researchers have used radio-labelled inulin of similar molecular weight to demonstrate this effect [288]. The transfer rate and transfer index of 4-NP during the first 60 minutes of perfusion was significantly lower than that of antipyrine (Table 5-3). This indicates that either the mechanism of 4-NP transfer is not similar to that of antipyrine or the changes in concentration of 4-NP occur mainly by tissue binding. In rats hepatic and enteric excretion of nonylphenol was delayed as it contained long alkyl chains [349, 350]. The possibility that ATP-dependent pumps (Multidrug resistance Protein 2, MRP2) are involved in the excretion of nonylphenol glucuronides was postulated as a possible mechanism for this delay [349, 350]. The same mechanism could be responsible for the slow transfer of 4-NP across the human placenta. However, the role if any, of ATP-dependent pumps in transport of xenoestrogens and their conjugates in human placentae is not well studied. A steep decline of 4-NP levels in the maternal perfusate reservoir, especially during initial period of perfusion (first 90 minutes) was observed (Figure 5-2). However, detectable levels of 4-NP in the fetal reservoir were only apparent after 30 minutes (Figure 5-2). We couldn't recover about 83.6 ± 3.6 % of 4-NP from the perfusates. The rapid decline in the maternal levels of 4-NP brought the feto-maternal ratio of 4-NP closer to one during the later stages of perfusion (after 90 minutes of perfusion) indicating the possibility of tissue binding or formation of an intermediary metabolite. It has been shown previously that the placental

transfer of compounds with a high octanol water partition coefficient (log pOW = 5.0 to 6.0, moderate lipophilicity) may be limited or very slow [270]. 4-NP mixtures have log pOW in the range of 4-6 [351], hence it is possible that a major portion of unrecovered 4-NP might have bound to the placental tissue.

Various *in-vitro* studies have shown the potential endocrine disruptive functions of 4-NP in human placentae [116, 352, 353]. Treatment of pre-term placental explants with environmentally relevant levels of 4-NP resulted in greater secretion of hCG than 17-β-estradiol [116]. In addition, the results suggested that 4-NP could activate similar apoptotic pathways in trophoblasts, to that of 17-β-estradiol [116]. Recently it has been reported that at environmentally relevant levels, 4-NP can cause a Th1/ Th2 cytokine imbalance in pre-term placental explants which also lead to increased production of hCG [353]. Hence if the rapid disappearance of 4-NP from the maternal reservoir observed in our studies were the result of placental binding, it may provide sufficient time for the chemical to interact with estrogen receptors in the placental cells to cause an effect.

It has been reported that the hepatic and intestinal Uridine Glucuronisyltransferase (UGT) isoforms can convert 4-NP to its glucuronide form [350], which is the predominant metabolite. Glucuronidation of 4-NP could lead to reduced toxicity as it is thought to abrogate the estrogenic actions of the compound [354]. Since placentae contain the UGT isoforms, it is possible that it could convert a portion of 4-NP to its glucuronide form. None of the studies have directly assessed the ability of placentae to conjugate 4-NP. We conducted a de-conjugation enzyme assay to evaluate the ability of human placentae to conjugate 4-NP in our perfusion model. We were unable to detect a change in the size of the peak corresponding to the 4-NP peak in the chromatogram following this assay, indicating that little or no glucuronidation of 4-NP was occurring in our model. Thus

indicating that the 84% unrecovered 4-NP in this study could not be explained by conjugation. The concentration of 4-NP we incorporated in our perfusates was very low, so as to mimic environmentally relevant levels. This low concentration combined with the large mass of placental tissue (~30g) meant it was impractical to attempt extracting the tissue bound fraction of 4-NP from perfused cotyledons. To detect the specific tissue binding a modified protocol using a higher concentration of 4-NP or using radiolabelled 4-NP would be required.

In a controlled study using human volunteers it was observed that 4-NP distributes to the lipid phase of the body within 2 hours of administration and the formation of conjugates / metabolites depends upon the route of administration [355]. Formation of unknown intermediates is possible in human placenta as it has the Phase I and II metabolizing enzymes, which could explain the initial rapid decline of 4-NP in the maternal reservoir. It has been recently shown that human Cytochrome P 450 (CYP) isozymes could bioactivate 4-NP to quinone metabolites [356]. As quinone metabolites could form DNA adducts leading to carcinogenesis, the potential for placental CYPs in bio-transforming 4-NP is a matter of concern. The concentration of 4-NP used in the present study is within the range observed in pregnant mothers [343] and it has recently been shown that this concentration can cause a derangement in the placental cytokine production in human pre-term placenta [116, 353].

Our data show that 4-NP at environmentally relevant levels could be transferred across the human placenta. In addition, our results suggest a rapid disappearance of 4-NP administered in the maternal reservoir possibly through the formation of an intermediary metabolite or high tissue binding. Hence human placenta may not protect the fetus against exposure to 4-nonylphenol. In addition, the possibility of formation of intermediary

metabolites are of concern as hepatic transformation could produce carcinogenic quinone metabolites. Further studies are needed to identify these issues.

Chapter 6. IN-VITRO ACTIONS OF ENDOCRINE DISRUPTORS

6.1. INTRODUCTION

The widespread observation of developmental anomalies in the wild animals led scientists at the Wingspread conference held in Racine, WI in 1991 to coin the term 'endocrine disruptors' to describe the chemicals responsible for these anomalies [2]. An Endocrine disruptor is defined as "an exogenous agent that interferes with the production, release, transport, metabolism, binding action or elimination of natural hormones responsible for the maintenance of homeostasis and the regulation of developmental process" [40]. There are many *in vitro* assays developed to measure the estrogenic activity of environmental endocrine disruptors. E-screen assay (an *in vitro* assay for screening compounds with estrogen like activity) utilizing the proliferative response of estrogen receptor (ER) positive MCF7 cells (breast cancer cell line) to estrogens at different concentrations is one of the most sensitive methods to determine the estrogenic actions of various environmental chemicals [357]. Most of the chemicals tested by these and rodent uterotrophic assays (*in vivo* method to measure the estrogenicity) showed much lower potency as compared to the natural hormone 17-β-estradiol.

Estrogens and estrogenic chemicals mediate many of their effects through the classical nuclear receptor pathway by binding to estrogen receptors -α and –β [358]. Microarray experiments with ER-α knockout / ovariectomised mice necessitate the requirement of nuclear ER-α for both early and late gene responses [359]. It is also reported that the estrogen mediated effects differ between tissues and even cells within tissues depending on the developmental stages [360].

It is well established that estrogen can mediate its effect either through nuclear receptor pathway (classical genomic pathway) or membrane mediated receptor pathway (rapid nongenomic pathway) [361, 362]. Lately xeno-estrogens has also been shown to mediate its effect through non-genomic pathway [328]. Xeno-estrogens has been shown to be equipotent with 17-β-estradiol in Ca / prolactin release in neuronal cells through a membrane bound non-genomic pathway [328, 363].

BPA is one of the well studied xenoestrogen. Estrogen related receptor – γ (ERR-γ) is considered to be a putative receptor for BPA and it is thought to be involved in many actions mediated on exposure to BPA [364]. X-ray crystallographic studies have shown that binding affinities of compounds with a phenol group are equipotent to that of BPA and hence ERR- γ may be a putative endogenous receptor for phenolic compounds [364]. The fact that these receptors are highly expressed in human placenta [365] and fetal brain [366] mandates further inquiry into the physiological function of these receptors to see whether the low dose effects of BPA (and possibly other phenolic estrogenic compounds) might be mediated through these receptors.

We hypothesize that the estrogen responsive genes in the placenta could be regulated by the estrogenic endocrine disruptors when exposed to environmentally relevant concentrations. Our collaborators have identified many oncogenes in estrogen receptor-α (ER-α) positive breast cancer cell lines, which are involved in the initiation and propagation of mammary carcinogenesis [367-372]. As there are some common pathways shared by carcinogenesis and placental development [373], we have selected some of these genes as a starting point to evaluate the expression pattern in a breast carcinoma cell line and human choriocarcinoma on exposure to environmentally relevant concentrations of selected endocrine disruptors. We observed only marginal variation in the expression

levels of genes studied in the choriocarcinoma (BeWo cells) cell line. The expression levels of ER-α were very low in BeWo cell lines. This coupled with our findings on the transplacental transfer of BPA has furthered our hypothesis to explore the *in vitro* actions of BPA on the placenta and fetal membrane (choriodecidua). Hence we furthered our study to evaluate *in vitro* actions of BPA on placental villous explants and choriodecidual explants, the end points being changes in gene expressions of key genes involved in the placental development. Since a balanced cytokine environment is a pre-requisite for normal placental and fetal development and recent studies with xeno-estrogens have shown an imbalance in cytokine secretion by placental villous explants [353], as an additional start-up analysis we have explored the secretory patterns of a pro- and anti- inflammatory cytokine secretion on exposure to the environmentally relevant concentrations of BPA. The concentration range of BPA used was from a range detected previously in the human placentae (1 pM, 100 pM, 10 nM and 1 μM) [23]. Similar concentration ranges of 17-β-estradiol were used to find any regulation observed with the natural ligand for the time period studied. To find whether the regulation was mediated through estrogen receptor signalling pathway we used a 'pure' antiestrogen Faslodex at a concentration of 100 nM. Faslodex is an estrogen receptor downregulator, which is considered as 'pure' antiestrogen without any agonistic activity and functions by binding to estrogen receptors, blocking ER dimerization and thus preventing the binding of receptor complex to estrogen receptor elements in the nucleus and degrading the bound receptors through proteosomal pathway [374, 375]. It can antagonize ER-α and ER-β [374]. We selected this concentration (100 nM Faslodex) based upon a previous standardization in our laboratory [369]. It was also reported that no toxicity was observed when using Faslodex at this concentration in specifically inhibiting cytotrophoblasts from human placenta [376].

6.2. MATERIALS AND METHODS

6.2.1. Sample Collection

Term placentae from healthy patients undergoing caesarean sections at the Auckland City Hospital were used in this study. The ethical approval to conduct such a study was obtained from Northern X Regional Ethics committee. Written consents were obtained from the patients taken part in the study.

The explants were prepared as per the protocol developed in our laboratory [377, 378] with modifications.

6.2.1.1. Villous Explants

After the removal of the basal plate from placental lobules, tissue cubes of ~ 2 cm^3 were dissected from at least several randomly selected sites free of calcification across the placenta. The tissue was transferred to a sterile container in phosphate-buffered saline (PBS) and washed thoroughly to remove the blood. Chorionic villi (10 to 20 mg) from the collected tissue were dissected, rinsed once with PBS, and twice with Hams F12-DMEM culture medium (Invitrogen). Three explants were cultured in 6 well clear individually packed sterile tissue culture plates (BD Falcon, BD New Zealand, Auckland, New Zealand) in 2.0 mL culture medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Each of the three disks in a single well was cut from random areas of the membranes to minimize the effects of any regional differences in gene expression / cytokine production present in these tissues. The following day, media were replaced with serum-free media containing antibiotics. Explants were then treated with the various test substances or the appropriate vehicle control. After an additional 24 hours, the tissue was lysed for RNA extraction; cDNA synthesis and the mRNA level expression of selected

genes were quantified using Q-RT-PCR. The media were collected and cytokine production rates were derived and normalized to the wet weight of the explants in the individual wells.

6.2.1.2. Choriodecidual Explants

After the amnion was manually removed, choriodecidual membranes were washed carefully in media to remove residual red blood cells without causing damage to the integrity of the membrane. Disks of tissue (6 mm) were excised using a sterile cork borer, transferred to twelve-well culture plates (three disks per well, three wells per treatment) and equilibrated for 24 h in Hams F12-DMEM supplemented with 10% FCS and antibiotics at 37°C in a humidified atmosphere of 5% $C0_2$-95% air. Each of the three disks in a single well was cut from random areas of the membranes to minimize the effects of any regional differences in gene expression patterns in these tissues. The following day, media were replaced with serum-free media containing antibiotics. Explants were then treated with the various test substances or the appropriate vehicle control. After an additional 24 hours, the tissue was lysed for RNA extraction; cDNA synthesis and the mRNA level expression of selected genes were quantified using Q-RT-PCR.

6.2.2. RNA extraction

TissueLyser II (QIAGEN, Auckland, New Zealand) was used to lyse the tissue for RNA extraction according to the manufacturer's protocol. The tissues were placed in a 2 mL eppendorff tube containing 1 stainless steel bead (7 mm mean diameter). Placed the tubes at room temperature and immediately added 1 mL of Trizol reagent. Placed the tubes in tissue lyzer adapter 2X24 and operated the tissue lyzer for 2 minutes at 30 Hz. Disassembled the adapter set, rotated the rack of tubes so that the tubes nearest to the

TissueLyser became the outermost, and reassembled the adapter set. Operated the TissueLyser for another 2 min at 30 Hz. RNA was extracted with Trizol® reagent according to the manufacturer's recommendation. The homogenized sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. Then 0.2 mL of chloroform was added to the tubes and mixed vigorously. The tubes were incubated for 3 minutes at room temperature and then centrifuged at 12,000 x g for 15 minutes at 4°C. Carefully aspirated the aqueous phase of the sample was placed it into a new tube. 0.5 mL of 100% isopropanol was added to the aqueous phase and incubated at room temperature for 10 minutes. The samples were then centrifuged at 12,000 × g for 10 minutes at 4°C. The supernatant was carefully removed from the tube without disturbing RNA pellet. The pellet was washed with 1 mL of 75% ethanol. Thoroughly mixed the samples by vortexing and then centrifuged the tubes at 7500 x g for 5 minutes at 4°C.

After carefully removing the supernatant without disturbing the pellet and air dried the RNA pellet for 10 minutes. The pellet was resuspended in 15µL of RNase-Free water and stored at -80°C until use. The purity of RNA was determined by checking the 260/280 ratios in a nanodrop.

6.2.3. cDNA synthesis

To remove any genomic DNA contamination we treated 1 µg of RNA with 1 µL of DNase I enzyme in a PCR tube as stated below:-

1 µg RNA sample

1 µl 10X DNase I Reaction Buffer

1 µl DNase I, Amp Grade, 1 U/µl

DEPC-treated water to make the final volume to 10 µl.

The above reaction was incubated at room temperature for 15 minutes. Later the DNase I was inactivated by incubating it with 1 µL of 25 mM EDTA solution and heated at 65°C for 10 minutes. The cDNA was synthesized by using SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen) following the manufacturer's recommendation. 1 µL of random hexamers and 1 µL of 10 mM dNTP mixture was added to the 10 µL of DNase I treated RNA and incubated at 65°C for 5 minutes. During this time a master mix was prepared by adding the kit components (for single reaction) in a 0.5 mL tube.

Reverse Transcriptase Buffer	4 µL
0.1 M DTT	1 µL
RNase Out	1 µL
Superscript Reverse Transcriptase II	1 µL

7 µL of master mix was then added to the tubes and incubated in a thermocycler using the program given below:-

25°C for 5 minutes

50°C for 60 minutes

70°C for 15 minutes

4°C hold.

6.2.4. Real time PCR

The ABI 7700 real-time PCR system ((Applied Biosystems, Foster City, CA) was used for the real-time PCR and analysis. Selected estrogen responsive genes and a housekeeping gene (TATA Box Binding Protein, TBP) were used for real-time PCR analysis using the EXPRESS SYBR® GreenER™ qPCR SuperMix Universal (Invitrogen). 1 μL of cDNA (1 in 10 diluted) synthesized from each 1 μg of RNA was added to a 10 μL reaction mix containing EXPRESS SYBR® GreenER™ qPCR SuperMix Universal and 200 nM of each primer. Triplicate reactions were performed for each marker in a 384-well plate using a two-step amplification program of initial denaturation at 95° C for 20 seconds, followed by 40 cycles of 95° C for 1 sec and 60° C for 20 sec. A melting curve analysis step was carried out at the end of the amplification. Standard curves were generated from each experimental plate using serial 10-fold dilutions of untreated cDNA. The mean of the cycle threshold (Ct) value for each reaction was calculated. Amplification efficiencies were calculated according to the equation $E = 10^{(-1/\text{slope})}$ and ranged from 90–104% for some of the selected genes; no nonspecific amplification or primer dimer was observed in any of the reactions as confirmed by the melting curve analysis.

Table 6-1 Details of the primers used for real time PCR

Gene name	Primer sequence (5' to 3')	Amplicon size
TBP	Forward: GGGCACCACTCCACTGTATC	289
	Reverse: CTCATGATTACCGCAGCAAA	
ER-α	Forward: CCACCAACCAGTGCACCATT	108
	Reverse: GGTCTTTTCGTATCCCACCTTTC	
ER-β	Forward: AGAGTCCCTGGTGTGAAGCAAG	143
	Reverse: GACAGCGCAGAAGTGAGCATC	
ERR-γ	Forward: GCACATGGATTCGGTAGAACTTTG	215
	Reverse: GGTTGAACTGTAGCTCCCACTG	
IGF-1	Forward: TGGTGGATGCTCTTCAGTTC	191
	Reverse: GACAGAGCGAGCTGACTTG	
ARTN	Forward: ATGAACACTACAGTGGTGAGG	132
	Reverse: AGCTCCCATGAGTGAGTACAGG	
CYP 450 (aromatase)	Forward: TGGACGTGGTGACCCTCATG	224
	Reverse: CCACCAGAATTTCGATCTCGT	

6.2.5. Cytokine ELISA

Human Interleukin-1β (IL-1β) ELISA kit and Interleukin-10 (IL-10) ELISA kit from BD Biosciences (BD OptEIA) were used for estimating the concentrations of IL-1β and IL-10in the supernatants of villous explants using the protocols recommended by the manufacturer. The sensitivity of the assay for IL-1β is 0.8 pg/mL and for IL-10 is 2 pg/mL

6.2.6. Statistical analysis

For explants, the data are mean ± s.e.m for three independent placental explants in triplicate treated with the four concentrations of BPA. The data were normalized to the control and the fold change was plotted against the concentration with Graphpad prism V-5 software. The statistical comparisons were carried out using student's t test using

sigmastat. p–value of <0.05 were shown with an asterisk and were considered as statistically significant.

6.3. RESULTS

6.3.1. Gene expression changes in Villous explants culture

6.3.1.1. Estrogen Receptor – α (ER-α)

A significant downregulation of ER-α gene expression was observed in response to 10 nM concentration BPA (Figure 6-1A). There was a trend towards down regulation of ER-α gene expression in response to 100 pM BPA treatment, with results approaching significance at p = 0.07. ER-α gene expression was significantly downregulated in response to 100 pM BPA in combination with 100 nM Faslodex (Figure 6-1B). However, No significant difference was found on ER-α gene expression in response to BPA at 1 pM, 10 nM and 1 µM concentrations in combination with 100 nM Faslodex (Figure 6-1B). When the data were plotted a non-monotonic pattern was observed in the ER-α gene expression in response to 17-β-estradiol for the concentration ranges studied. However due to interplacental variability none of the results were statistically significant (Figure 6-1C).

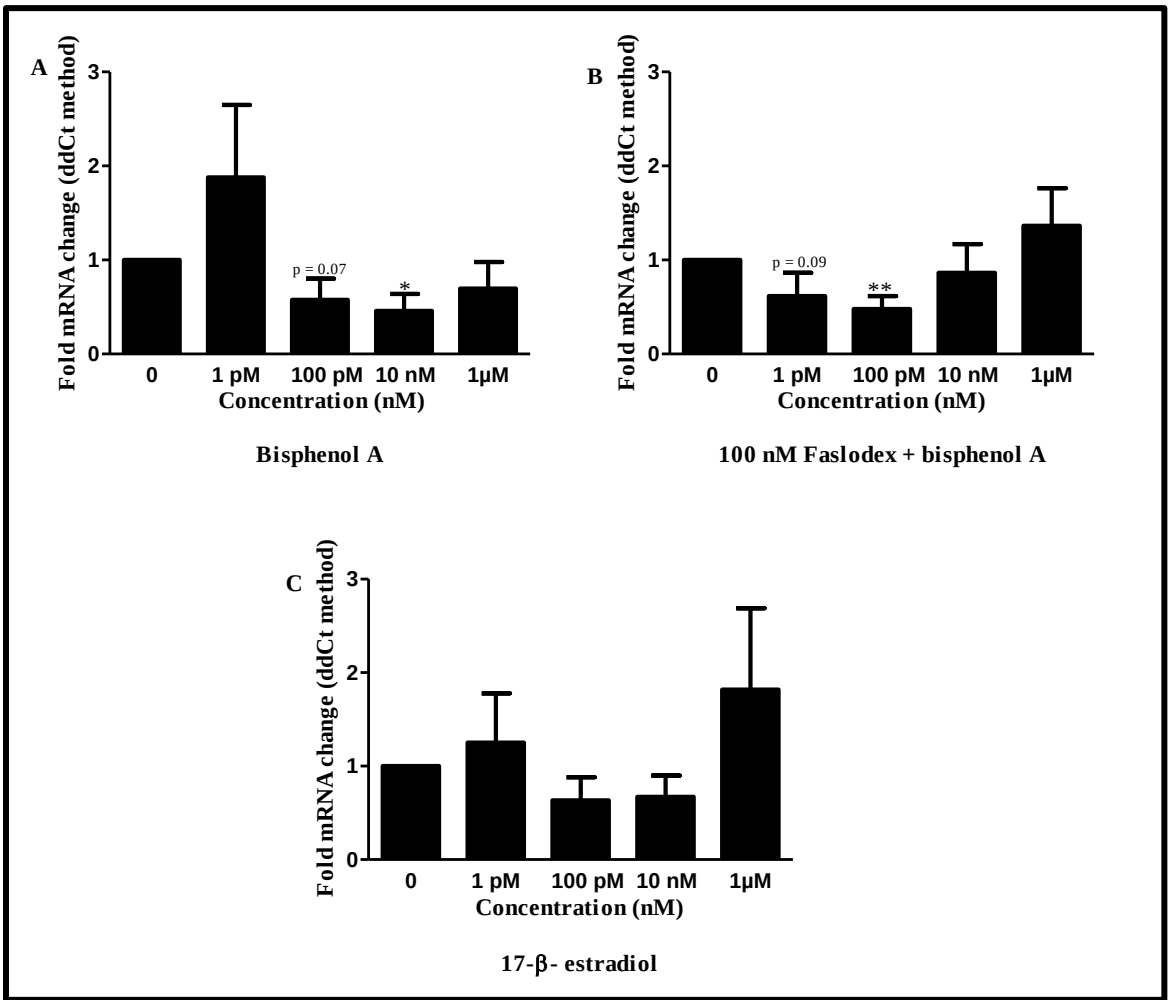

Figure 6-1 mRNA level regulation of Estrogen Receptor - α (ER-α) at various concentrations of BPA and 17-β-estradiol in term placental villous explants.

Term placental villous explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ER-**α** gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$, **$p<0.005$).

6.3.1.2. Estrogen Receptor – β (ER-β)

When the data were plotted a non-monotonic pattern was observed in the ER-β gene expression in response to BPA for the concentration ranges studied. However a significant change was observed only in response to BPA at 10 nM. At this concentration BPA significantly downregulated the expression of ER-β gene in villous explants (Figure 6-2A). ER-β gene expression was significantly downregulated in response to only 1 µM BPA in combination with 100 nM Faslodex (Figure 6-2B). No significant difference was observed

on ER-β gene expression in response to 17-β-estrdiol at any of the concentrations studied (Figure 6-2C).

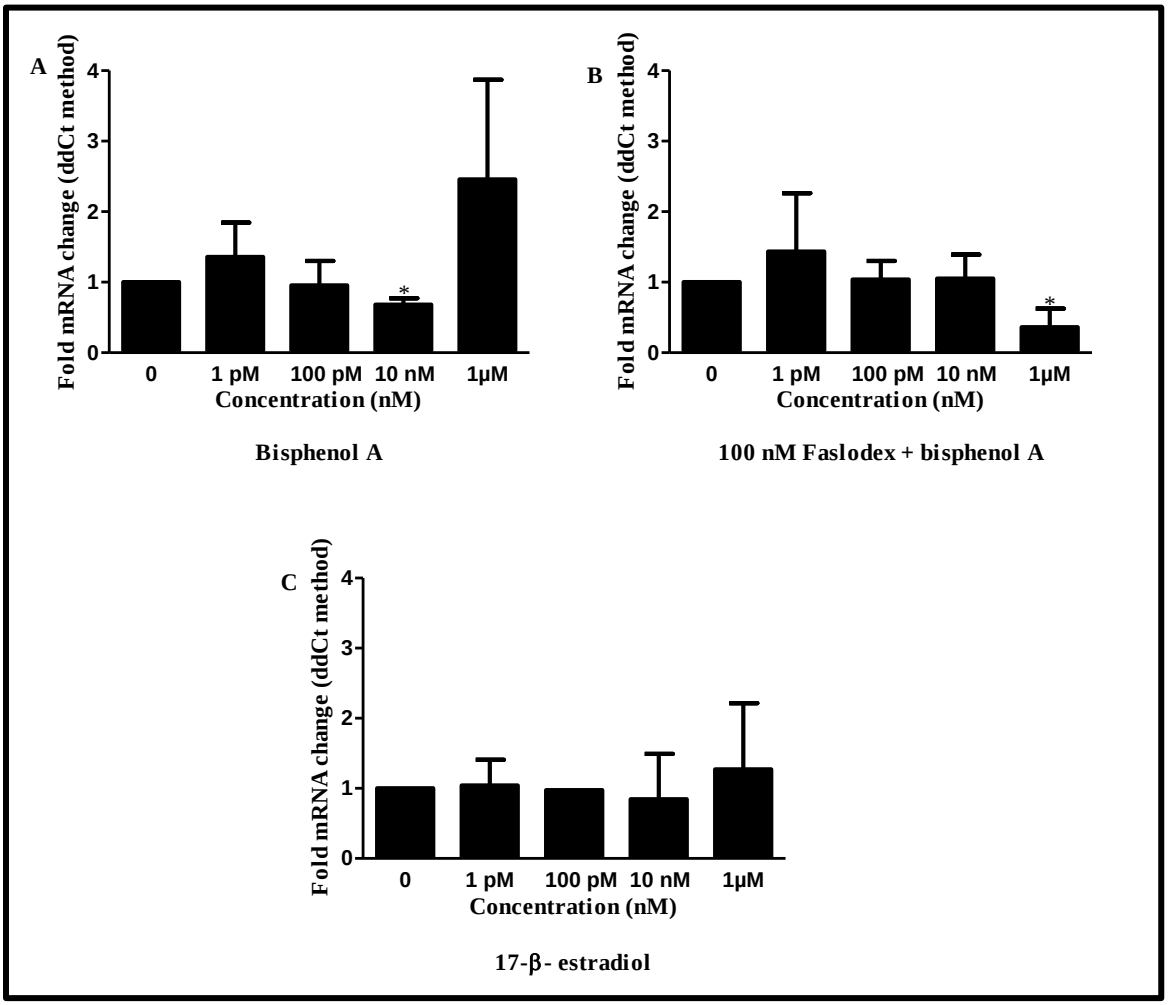

Figure 6-2 mRNA level regulation of Estrogen Receptor - β (ER-β) at various concentrations of BPA and 17-β- estradiol in term placental villous explants

Term placental villous explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ER- β gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$).

6.3.1.3. Cytochrome P450 aromatase (aromatase)

BPA at 1 pM significantly upregulated the expression levels of aromatase gene in villous explants (Figure 6-3A). However BPA at 100 pM, 10 nM and 1 µM had no effect on the expression levels of aromatase transcription. 100 pM BPA in combination with 100 nM

Faslodex significantly downregulated the expression levels of aromatase gene in villous explants (Figure 6-3B). 17-β-estradiol at 100 pM and 10 nM concentrations significantly downregulated the expression of aromatase gene (Figure 6-3C).

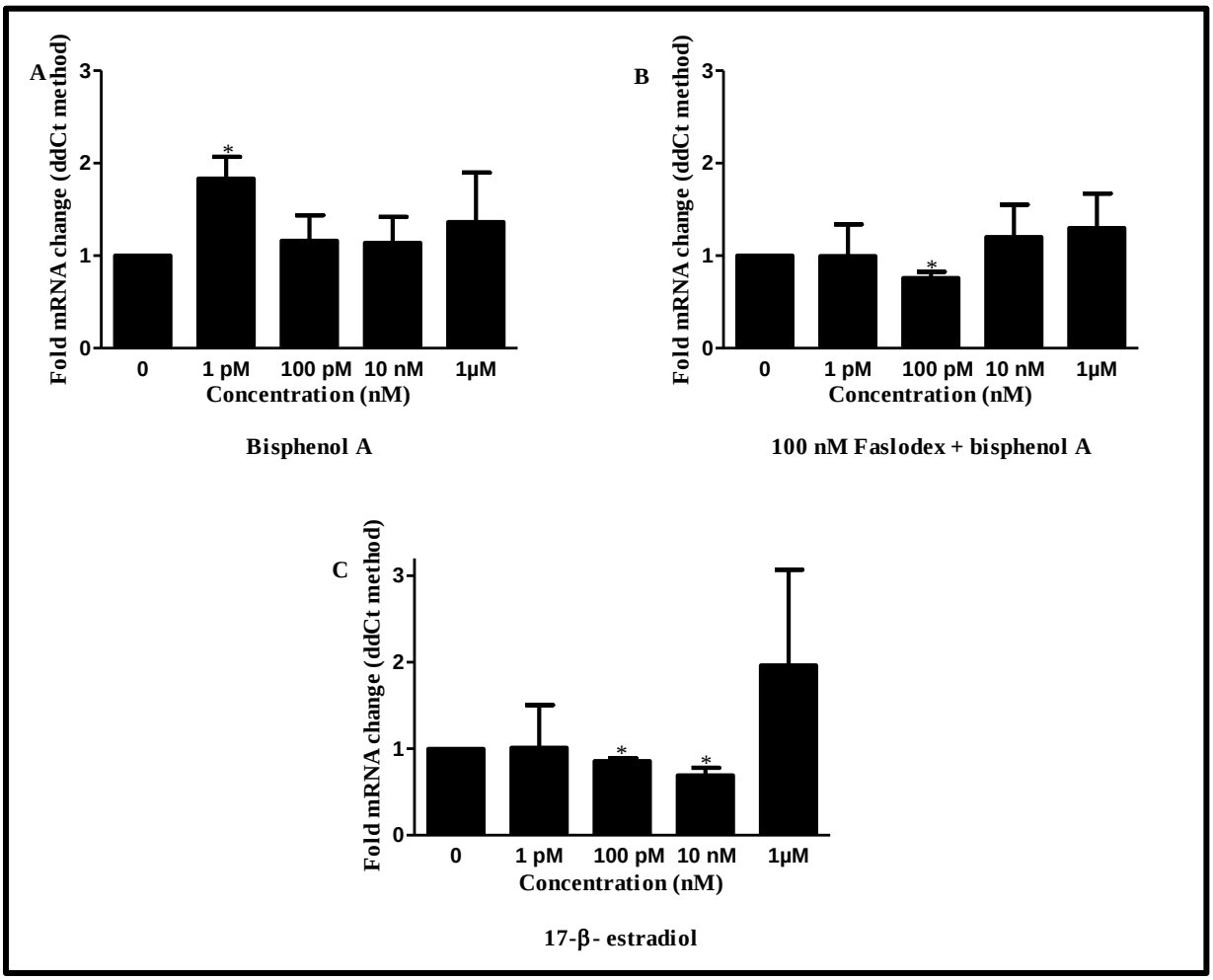

Figure 6-3 mRNA level regulation of CYP-450 at various concentrations of BPA and 17-β- estradiol in term placental villous explants

Term placental villous explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of CYP-450 gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$)

6.3.1.4. Artemin (ARTN)

BPA significantly downregulated the expression levels of ARTN gene at a concentration of 10 nM in villous explants (Figure 6-4A). No significant difference was found on ARTN gene expression in response to BPA in combination with 100 nM Faslodex at any of the

concentrations tested (Figure 6-4B). No significant difference was observed on ARTN gene expression in response to 17-β-estrdiol at any of the concentrations studied (Figure 6-4C).

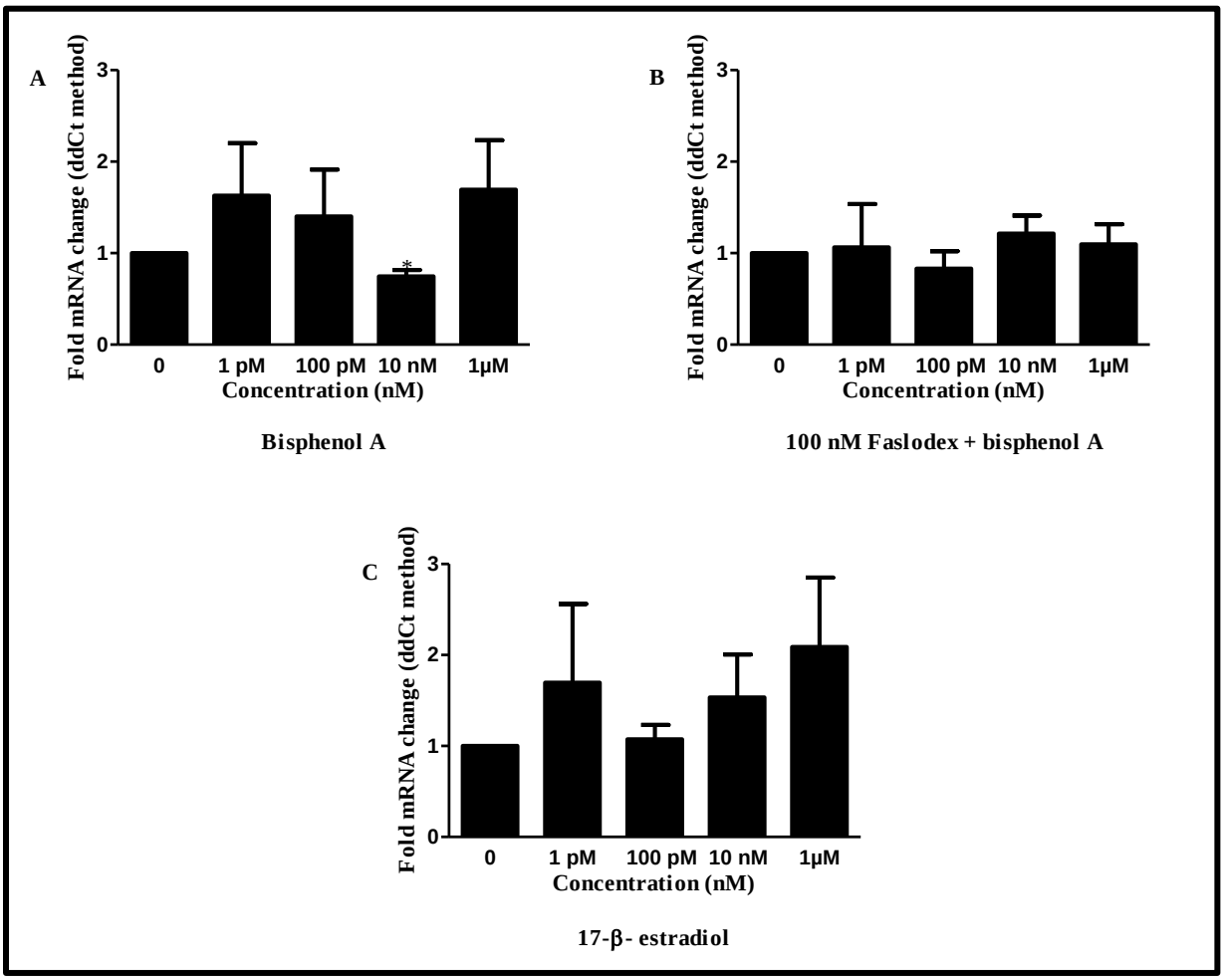

Figure 6-4 mRNA level regulation of ARTN at various concentrations of BPA and 17-β- estradiol in term placental villous explants

Term placental villous explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ARTN gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$).

6.3.1.5. Insulin like Growth Factor – I (IGF-I)

BPA at 10 nM and 1 µM significantly downregulated the expression levels of IGF-1 gene in villous explants (Figure 6-5A). The combination of 100 nM Faslodex with BPA at 100 pM and 10 nM significantly reversed this effect (Figure 6-5B). No significant changes on

the expression patterns of IGF-1 gene were observed with any concentrations of 17-β-estradiol in villous explants (Figure 6-5C).

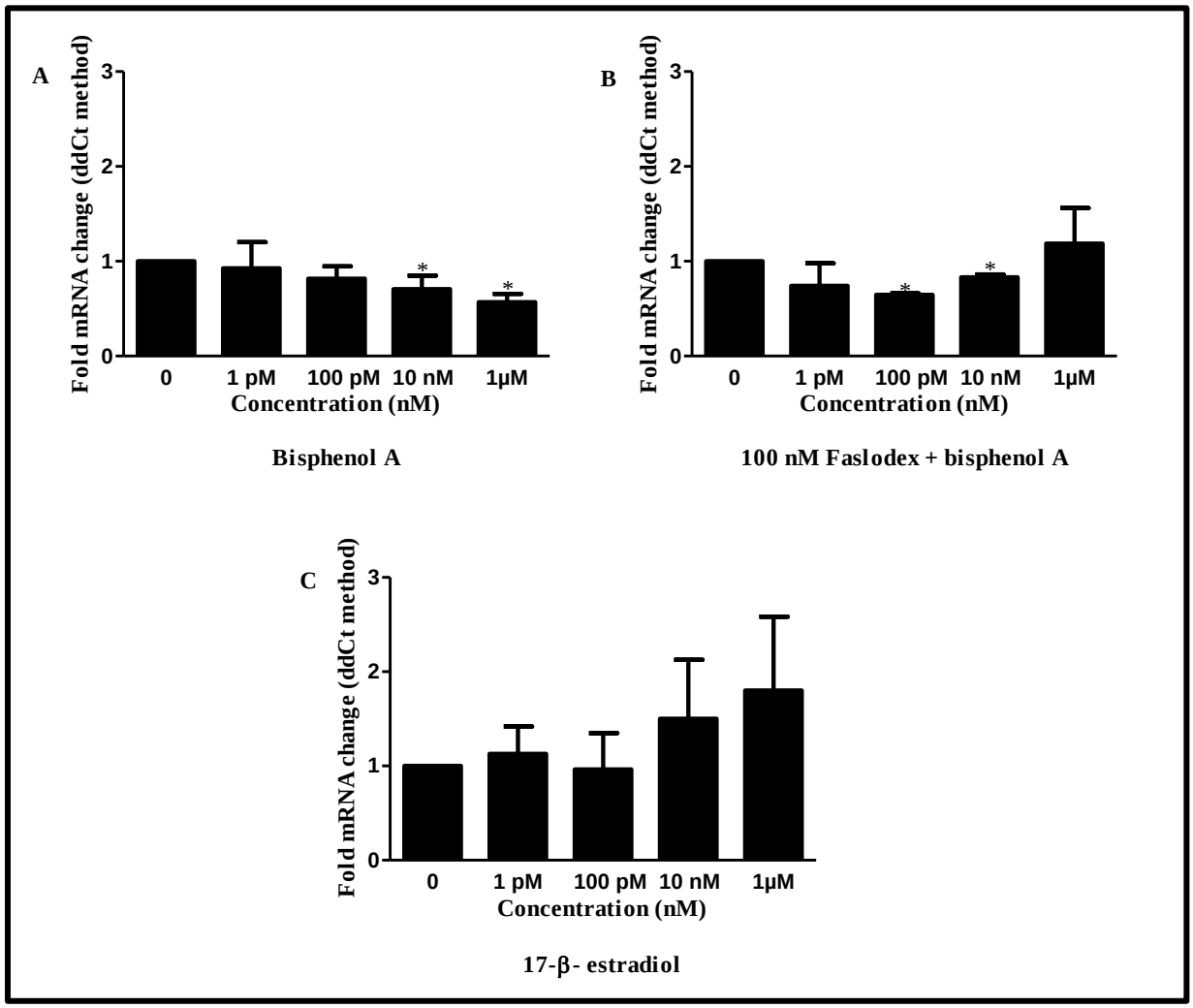

Figure 6-5 mRNA level regulation of Insulin like Growth Factor -1 (IGF-1) at various concentrations of BPA and 17-β- estradiol in term placental villous explants

Term placental villous explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of IGF-1 gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$).

6.3.2. Gene expression changes in Choriodecidual explants

6.3.2.1. Estrogen receptor – α (ER-α)

BPA at 10 nM and 1 µM concentration significantly downregulated the expression levels of ER-α in choriodecidual explants (Figure 6-6A). No significant changes on the expression levels of ER- α gene were observed when a combination of BPA and Faslodex was incubated in choriodecidual explants (Figure 6-6B). 17-β-estradiol at 1 pM, 100 pM and 1 µM concentrations significantly downregulated the expression levels of ER-α gene (Figure 6-6C).

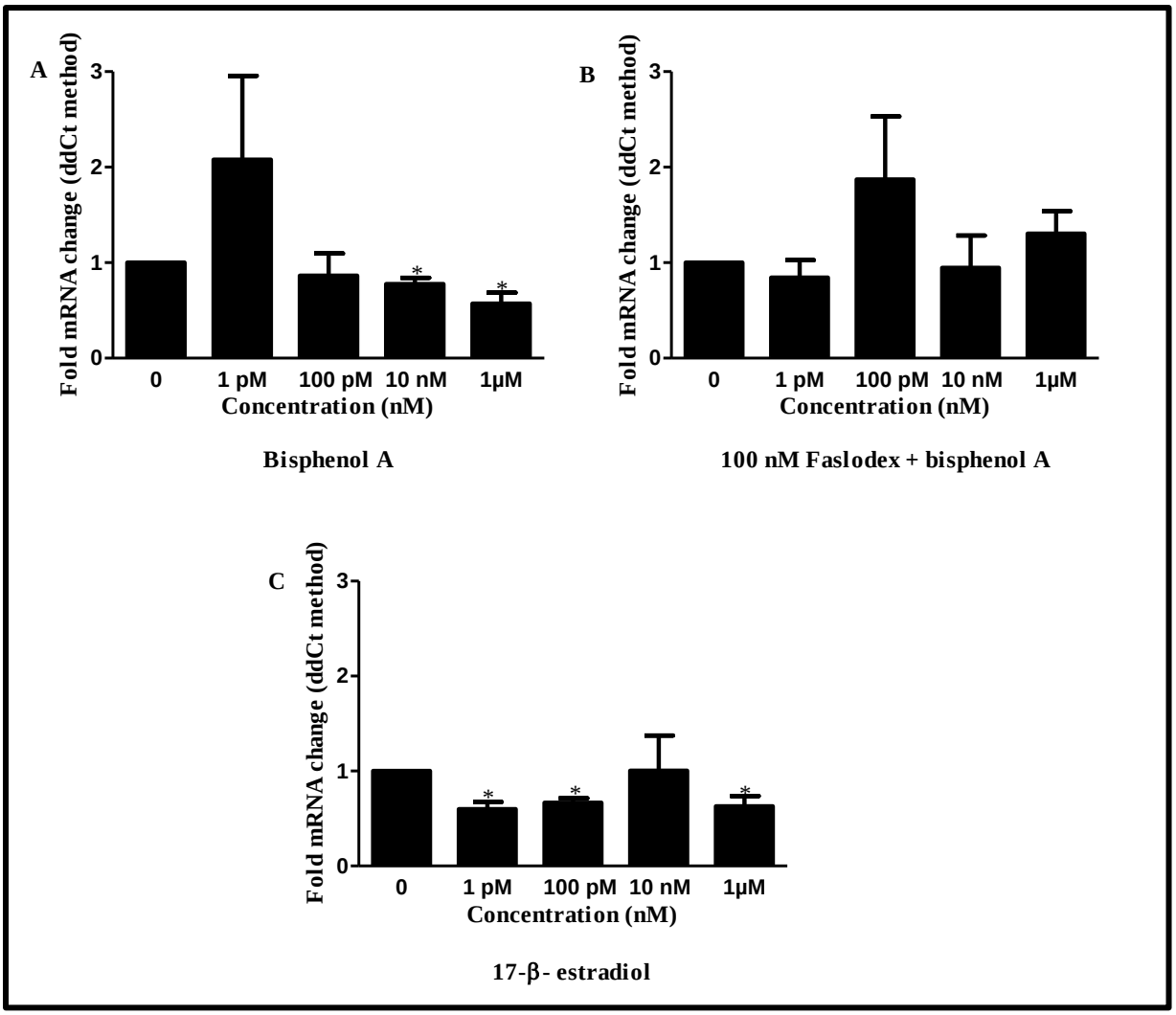

Figure 6-6 mRNA level regulation of Estrogen Receptor – α (ER – α) at various concentrations of BPA and 17-β- estradiol in term placental choriodecidual explants

Term placental choriodecidual explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ER-α gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$)

6.3.2.2. Estrogen receptor – β (ER-β)

None of the treatments had any significant changes in ER-β gene expression in choriodecidual explants (Figure 6-7A, B & C).

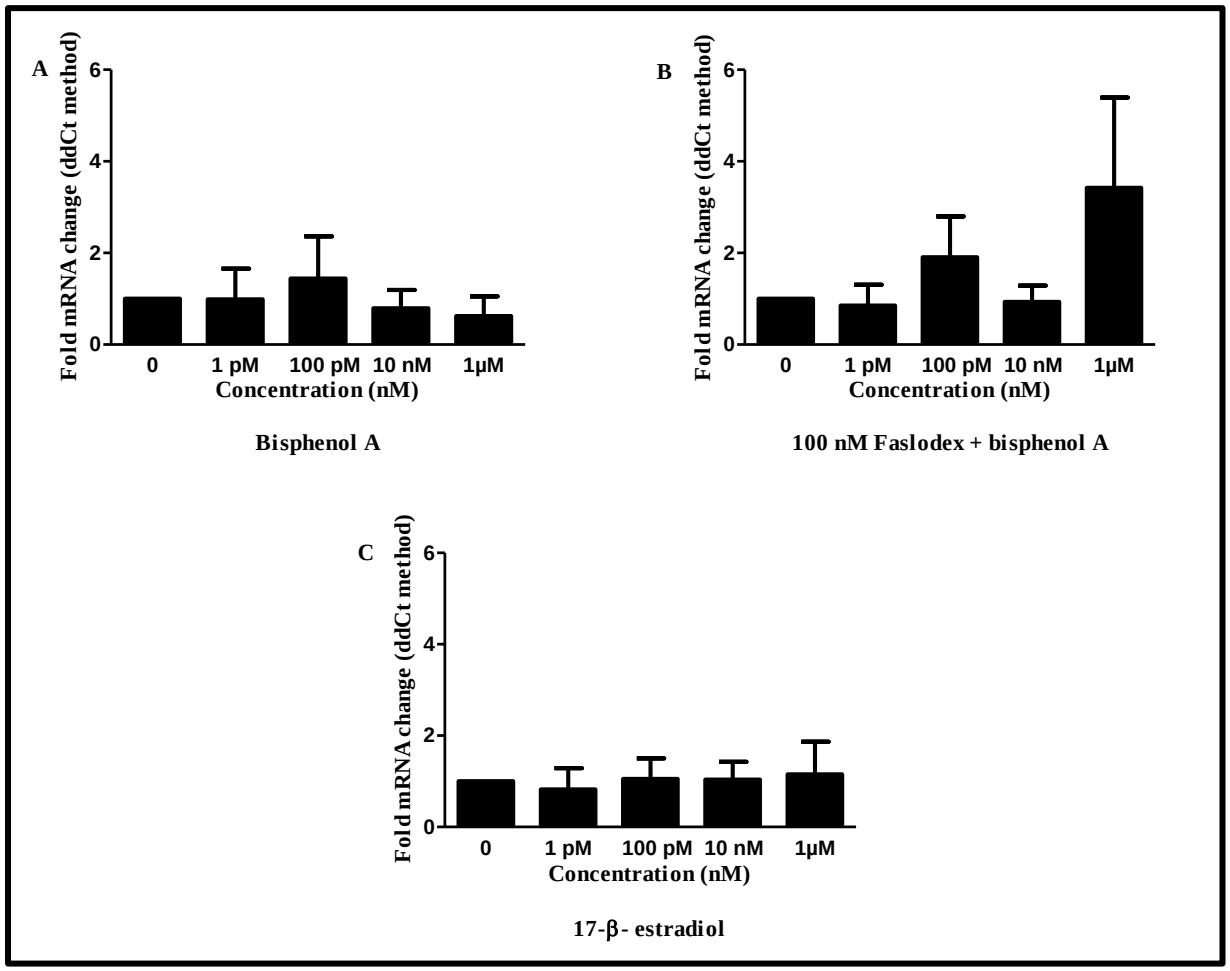

Figure 6-7 mRNA level regulation of Estrogen Receptor – β (ER – β) at various concentrations of BPA and 17-β- estradiol in term placental Choriodecidual explants

Term placental choriodecidual explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ER-β gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$)

6.3.2.3. Estrogen related receptor – γ (ERR-γ)

BPA significantly downregulated the expression levels of ERR-γ gene at 1 pM and 10 nM concentrations (Figure 6-8A). A trend towards down regulation was also observed in response to 100 pM BPA (Figure 6-8A). 100 nM Faslodex in combination with 100 pM BPA was found to significantly downregulate ERR-γ gene expression (Figure 6-8B). 17-β-estradiol significantly downregulated the expression levels of ERR-γ gene at all the four concentrations studied in choriodecidual explants (Figure 6-8C).

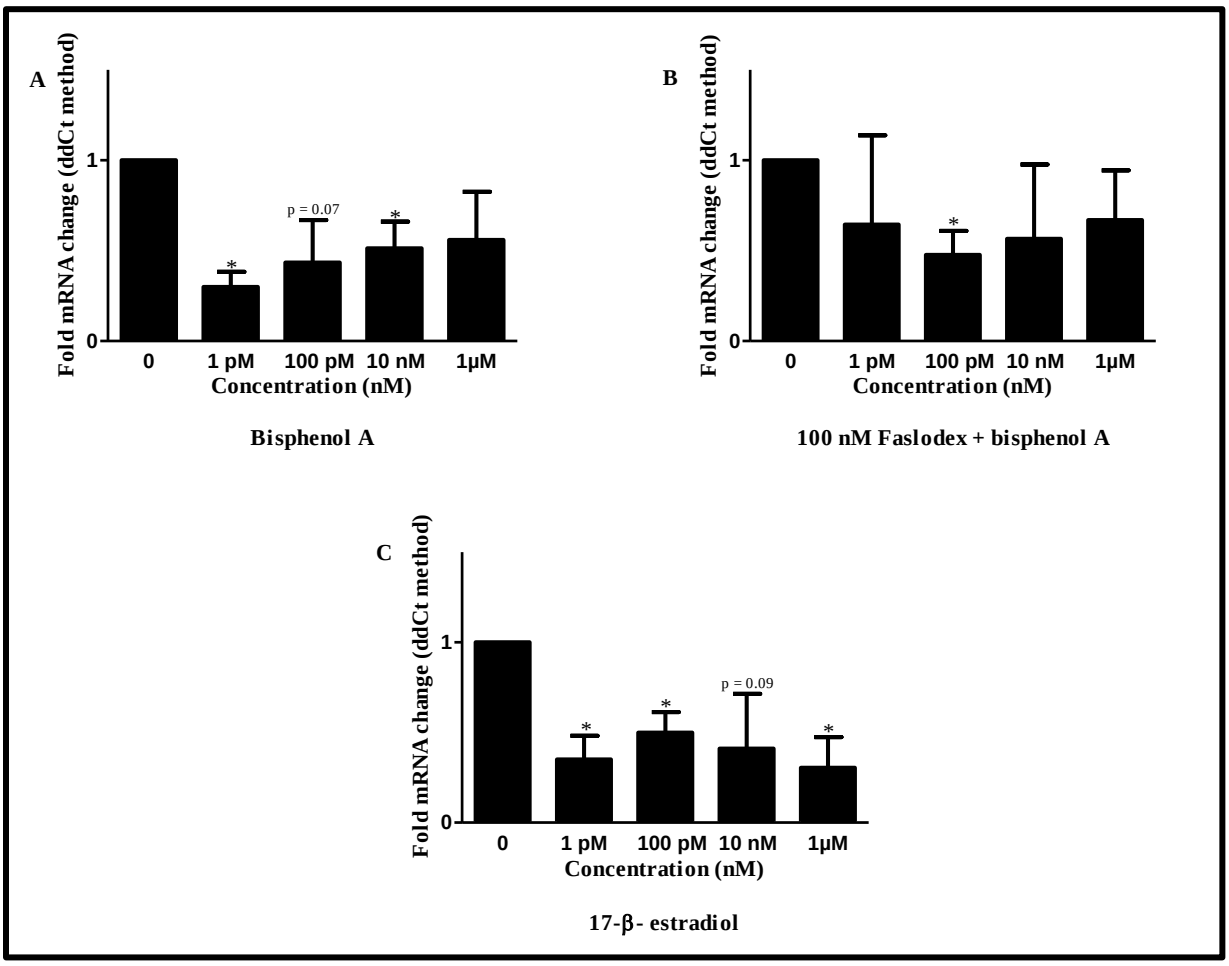

Figure 6-8 mRNA level regulation of Estrogen Related Receptor – γ (ERR-γ) at various concentrations of BPA and 17-β- estradiol in term placental Choriodecidual explants

Term placental choriodecidual explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ERR-γ gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).(* $p<0.05$)

6.3.2.4. Artemin (ARTN)

BPA upregulated the expression levels of ARTN gene at concentrations 100 pM and 1 µM (Figure 6-9A). The combination of Faslodex and 1 µM BPA significantly upregulated the expression of ARTN gene (Figure 6-9B). 17-β-estradiol significantly upregulated the expression of ARTN gene at 10 nM concentrations (Figure 6-9C). A trend towards up regulation was also observed 17-β-estradiol at 1 pM concentrations (Figure 6-9C).

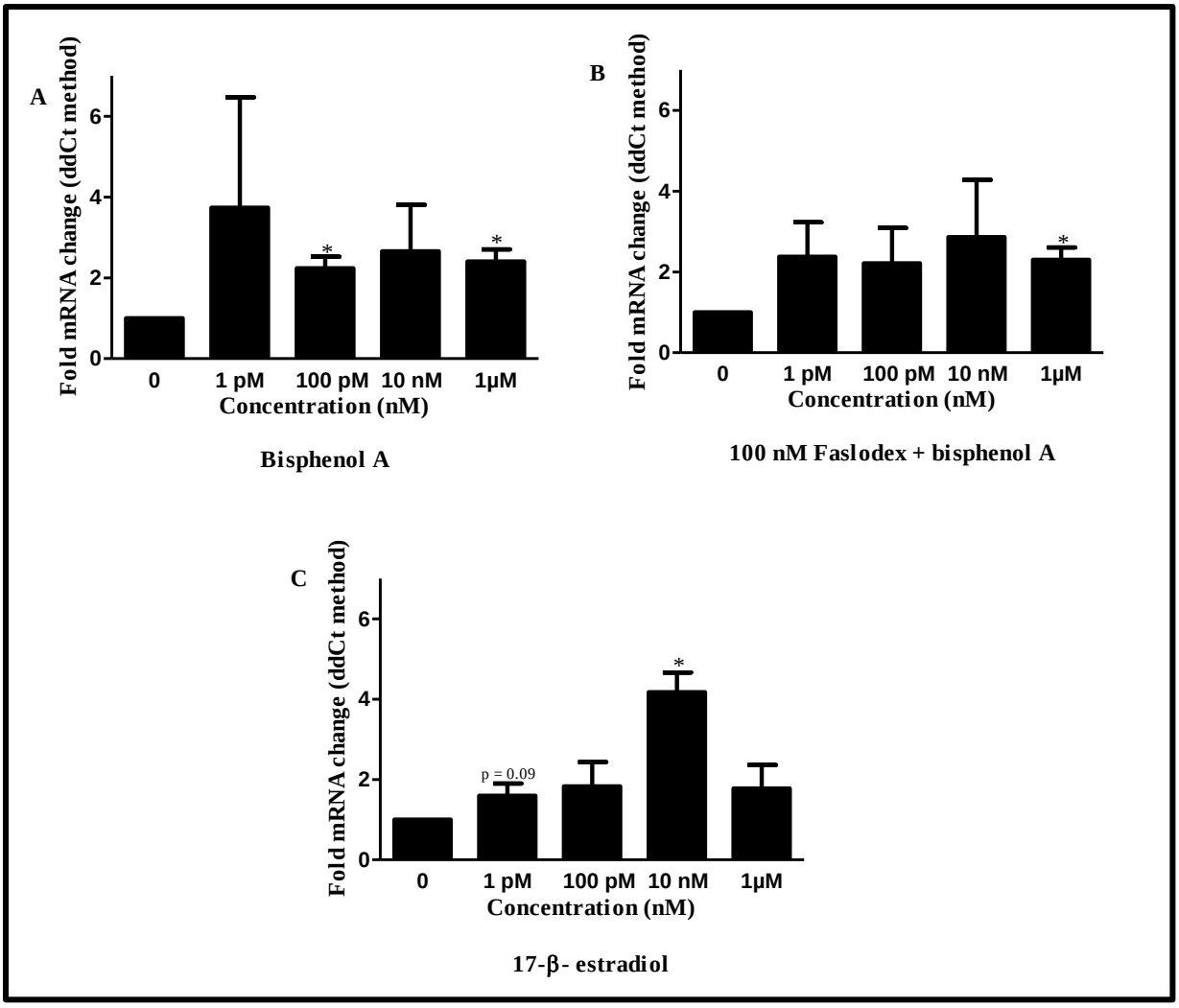

Figure 6-9 mRNA level regulation of Insulin ARTN at various concentrations of BPA and 17-β-estradiol in term placental Choriodecidual explants

Term placental choriodecidual explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of ARTN gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).

6.3.2.5. Insulin like Growth Factor – I (IGF-I)

BPA significantly upregulated the expression levels of IGF-1 gene at 1 pM concentrations in choriodecidual explants (Figure 6-11A). Faslodex in combination with 100 pM and 10 nM BPA significantly upregulated the expression of IGF-I gene (Figure 6-11B). 17-β-estradiol at 100 pM significantly upregulated the expression levels of IGF-1 gene in choriodecidual explants (Figure 6-11 C).

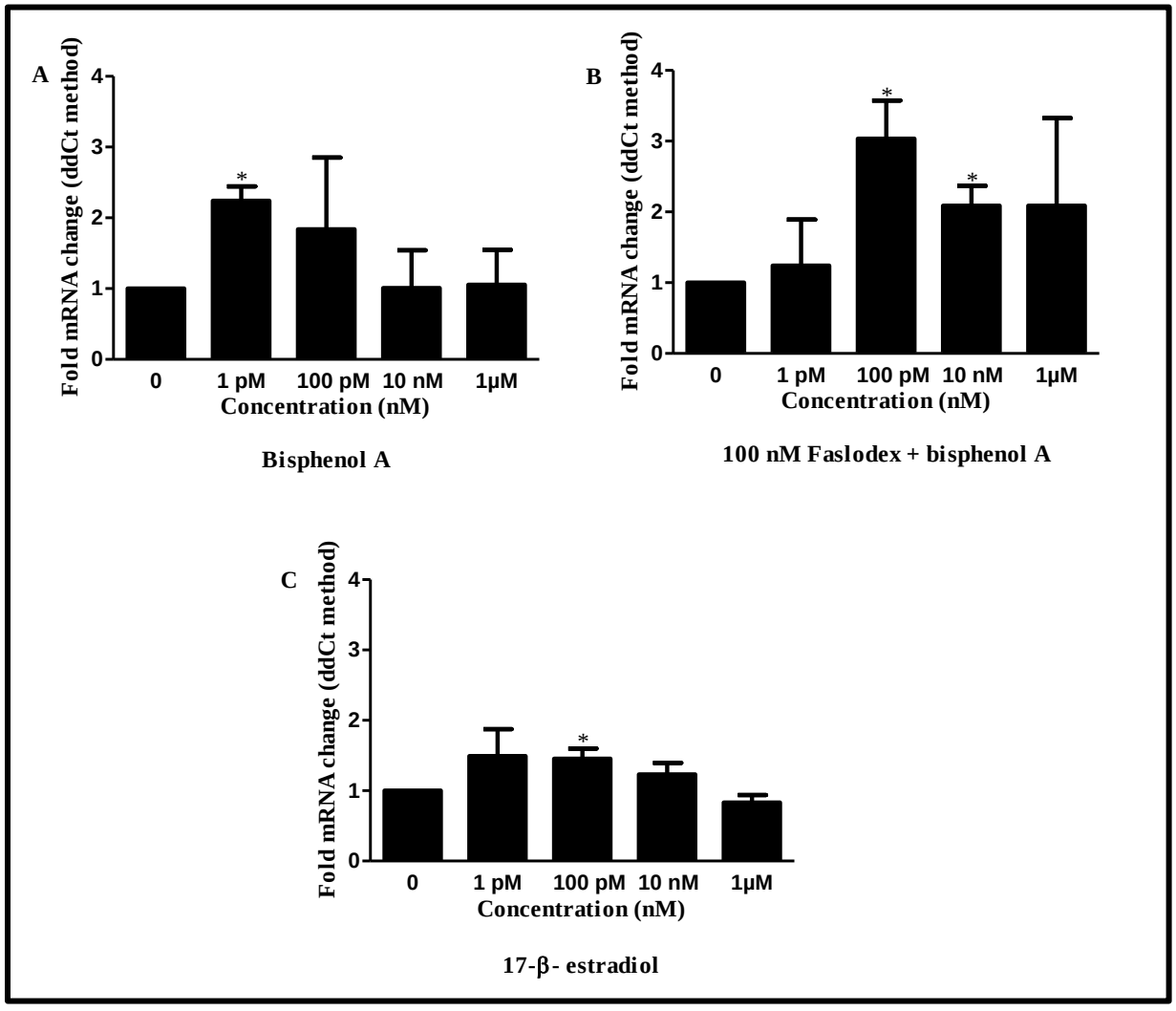

Figure 6-10 mRNA level regulation of Insulin Insulin like Growth Factor (IGF-1) at various concentrations of BPA and 17-β- estradiol in term placental choriodecidual explants

Term placental choriodecidual explants were treated with BPA or BPA and Faslodex or 17-β-estradiol at four concentrations overnight. The m-RNA level expression of IGF-1 gene were normalized with TBP (housekeeping gene) and vehicle control and the fold mRNA change was calculated (ddCt method). Data are mean ± s.e.m (n=3).

6.3.3. Cytokine ELISA (term villous explants)

6.3.3.1. Interleukin – 10 (IL-10) secretion by villous explants

BPA at concentrations of 1 pM and 100 pM significantly inhibited the production of IL-10 in villous explants and at concentrations of 10 nM and 1 µM significantly stimulated the production of IL-10. 17-β-estradiol at 1 pM, 100 pM and 10 nM significantly inhibited the production of IL-10 in term placental villous explants (Figure 6-12).

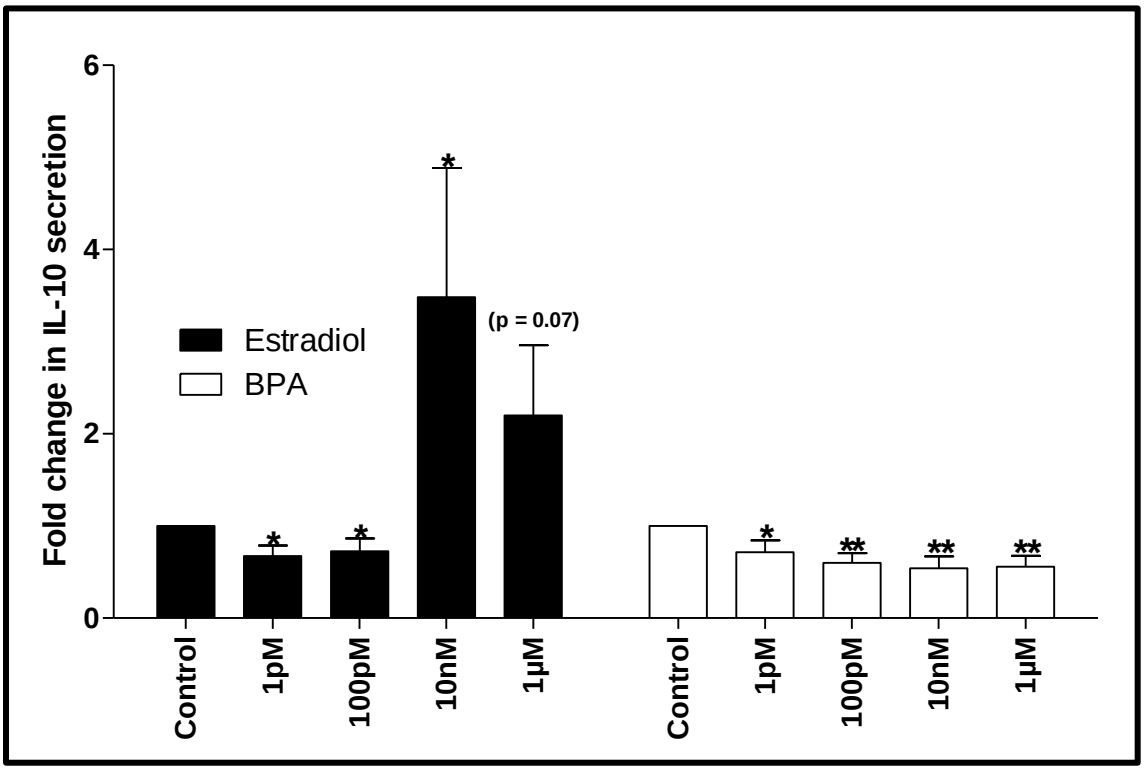

Figure 6-11 Effect on Interleukin – 10 (IL-10) secretions at various concentrations of BPA and 17-β-estradiol in term placental villous explants

Term placental villous explants were treated with BPA or 17-β-estradiol at four concentrations overnight. IL-10 secreted to the media were analysed by ELISA. The data was normalized to vehicle control and the fold change was calculated. Data are mean ± s.e.m (n=5).

6.3.3.2. Interleukin - 1β (IL-1β) secretion

There was a trend towards a stimulation of IL-1β production in villous explants with 10 nM and 1 µM BPA and with 17-β-estradiol at 1 pM and 1 µM concentrations. The effects were not significant for other concentrations studied (Figure 6-12).

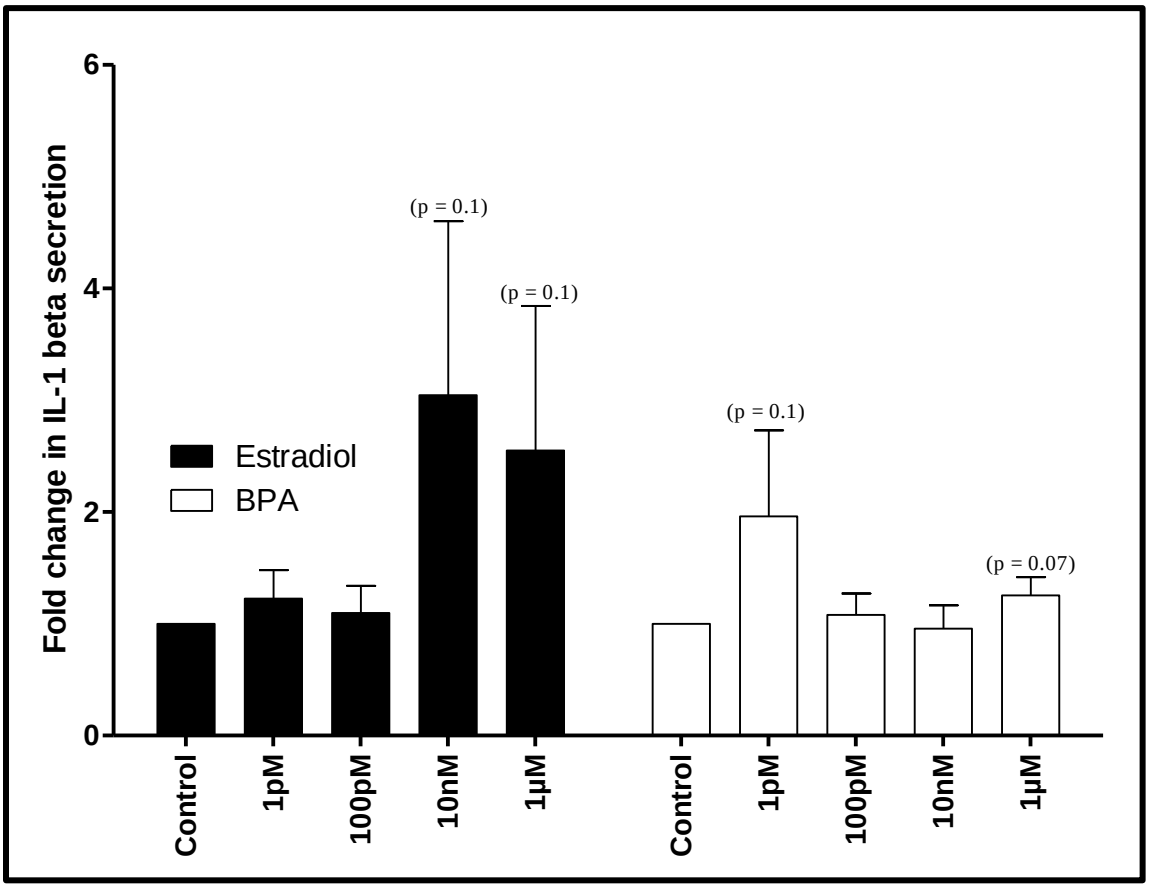

Figure 6-12 Effect on Interleukin – 1β (IL – 1β) secretion at various concentrations of BPA and 17-β-estradiol in term placental villous explants

Term placental villous explants were treated with BPA or 17-β-estradiol at four concentrations overnight. IL-1β secreted to the media were analysed by ELISA. The data was normalized to vehicle control and the fold change was calculated. Data are mean ± s.e.m (n=5).

6.4. DISCUSSIONS

We used environmentally relevant concentration ranges of BPA to explore the changes in estrogen responsive gene expression patterns in the human placenta *in vitro*. We also used Faslodex in combination with BPA at various concentrations to find whether the effects are mediated through estrogen receptors. We first examined the gene expression patterns of ER-α on exposure to four concentrations of BPA. BPA at 10 nM significantly downregulated the expression of ER-α gene in villous and choriodecidual explants. Co incubation of 10 nM BPA with Faslodex did not show any significant changes in the

expression patterns of ER-α in villous or choriodecidual explants. However a significant downregulation in the expression levels of ER-α gene was observed when choriodecidual explants were incubated with 1 pM, 100 pM and 1 µM concentrations of 17-β-estradiol. An interesting finding was a significant downregulation of ER-β expression when the choriodecidual explants were treated with a combination of 100 nM Faslodex and 100 pM BPA. This suggests the possibility of estrogen modulating activity of Faslodex in human placenta and this requires further studies. BPA at 10 nM concentration significantly downregulated the expression of ER-β gene in villous explants while none of the concentrations affected ER-β gene expression in choriodecidual explants. Various studies have also shown a regulatory role of BPA on the expression levels of ER-α and ER-β [379-381]. Yu *et al*., (2010), has also shown the tissue specificity for BPA in regulating ER-α expression pattern (either downregulation or upregulation depending on the tissue). It has been shown that in a prostatic cancer cell line BPA at 1 nM concentrations downregulated mRNA and protein levels of expression of ER-β gene [380]. Taken together our studies suggest a regulatory role of BPA on estrogen receptors α and β with major action towards ER-α in term placental villous explants. However the significance of this change is unclear at this stage.

Estrogen Related Receptor – γ is an orphan nuclear receptor highly expressed in placenta and this is suggested to be a putative receptor for BPA [364, 365]. We then sought to explore the regulatory role of BPA on the expression patterns of ERR-γ in the choriodecidual expalants. BPA at all concentrations showed a trend to downregulate ERR-γ gene expression in choriodecidual explants with a significant inhibition at 1 pM and 10 nM. Similarly 17-β-estradiol also had a trend to downregulate ERR-γ gene expression with a significant inhibition at 1 pM, 100 pM and 1 µM. Our results suggest a role in the

regulation of ERR-γ gene expression on exposure to environmentally relevant concentrations of BPA. However the combination of BPA and Faslodex did not reverse any of BPA mediated ERR-γ gene downregulation in choriodecidual explants. Recently the role of ERR-γ in the induction of aromatase in cytotrophoblastic differentiation was reported [382]. Hence if BPA could affect the transcription of ERR-γ, it could adversely affect the fetal development.

We next examined the expression patterns of aromatase on exposure to various concentrations of BPA. Placental aromatase converts the fetal adrenal androgens such as dihydroepiandrostenidione sulfate (DHEAS) and 16-hydroxy DHEAS to estrogens (estrone, estradiol, and estriol) at the same time reducing the production of testosterone [383] and hence the expression levels during pregnancy are very important for the proper development of fetus. BPA at 1 pM significantly upregulated the expression of aromatase gene, while 17-β-estradiol significantly downregulated its expression in villous explants. This shows a possible estrogen antagonistic effect of BPA in regulating the expression of aromatase gene in villous explants. It has previously been shown that BPA at higher concentration can downregulate aromatase in a choriocarcinoma cell line [384]. The combination of BPA and Faslodex was ineffective in reversing BPA mediated upregulation. On the other hand when the explants were incubated with 100 pM BPA and 100 nM Faslodex, the expression levels of aromatase gene was significantly downregulated. However our studies indicate a possibility for regulating the expression levels of aromatase in human placenta. This further demands the exploration of any estrogen receptor modulating activity of Faslodex at this concentration in villous explants.

We next explored whether artemin, an estrogen-inducible oncogene [368] is present in the placental explants and whether there is any regulation on its expression on exposure to

BPA. The expression of artemin was demonstrated in embryo and pregnant uterus and it is also reported to be involved in embryogenesis [385]. BPA at 10 nM was found to significantly downregulate the m-RNA level expression of artemin in villous explants. However 17-β-estradiol at any concentration did not elicit any regulation on expression of artemin in villous explants. On the other hand, BPA significantly upregulated the m-RNA level expression of artemin gene in choriodecidual explants. 17-β-estradiol at 10 nM concentrations upregulated artemin gene expression in choriodecidual explants. Faslodex was ineffective in reversing the effects of BPA, however in combination with Faslodex BPA significantly upregulated artemin expression in choriodecidual explants. The antagonistic effect of BPA on regulating the expression of artemin gene in villous and choriodecidual explants might be due to the variation in the tissue specificity. However this should be explored further.

Insuline like Growth Factor – 1 (IGF-1) is highly expressed in placentae and has a role in placental and fetal growth [386]. It was reported that IGF-1gene is expressed in both placental and fetal tissues and its deletion retards fetal growth [387]. We sought to explore whether BPA regulate the expression patterns of IGF-1 in term human placental explants. BPA at 10 nM and 1 μM significantly downregulated the expression of IGF-1 gene in villous explants. This effect was reversed by the addition of Faslodex, which is indicative of the signalling through estrogen receptor mediated pathway. BPA also upregulated the expression levels of IGF-1 gene in choriodecidual explants, but at a lower concentration than that required to regulate IGF-1 gene expression in villous explants. Faslodex in combination with BPA also found to upregulate the expression of IGF-1 gene in choriodecidual explants. This again shows the possibility of tissue variability in Faslodex action and this requires further investigation. 17-β-estradiol significantly upregulated the

expression of IGF-1 in choriodecidual explants. Recently a positive correlative trend was reported between IGF-1 and ER-α expression levels in placentae of normal control Swedish population [388]. Although the authors were unable to find any significant correlation of the two in placentae of growth restricted population, they suggested further studies to rule out the possibility. However the recent report on the positive correlation on intra uterine exposure to BPA and growth restricted fetal outcome further necessitates the exploration of possible regulatory role on placental and fetal IGF-1 expression [389].

The results of our preliminary studies are suggestive of BPA at environmentally relevant concentrations can cause subtle changes in the expression levels of key genes on involved in placental development. Further standardizations using various concentrations, incubation times etc are required to measure a sensitive end point specifically regulated by BPA on human placenta. Our studies with Faslodex indicate that the effective concentration to downregulate estrogen receptors were not achieved in the media and further standardizations are required. However its synergestic effect in combination with BPA further indicates a possibility for Faslodex to modulate estrogen receptors in placenta and this requires further exploration.

We also observed a change in the secretion patterns of two cytokines on exposure to environmentally relevant levels of BPA on villous explants. Due to the limitations of time we decided only to detect the secretion patterns of a pro-inflammatory (IL-1β) and an anti-inflammatory (IL-10) cytokines produced by villous explants on exposure to environmentally relevant concentration of BPA. BPA at 10 nM and 1 µM concentration tends to induce the secretion of IL-1β in villous explants ($p = 0.1$). 17-β-estradiol tends to induce the secretion of IL-1β at 1 pM and 1 µM concentrations ($p = 0.1$ and 0.07). BPA at very low concentrations significantly inhibited the secretion of IL-10 and at higher

concentrations had a trend to induce IL-10 production showing a non-monotonous dose response. 17-β-estradiol at all concentrations significantly inhibited the production of IL-10 secretion in villous explants. These findings suggest that BPA at environmentally relevant concentrations could affect the secretion patterns of TH1 / TH2 cytokines. Further studies are required to support these findings. Recently another xenoestrogen at very low concentration was reported to alter TH1 / TH2 cytokine secretion in pre-term placental explants [353]. This combined with our findings indicate a role for xenoextrogens in controlling the levels of cytokine secretions in human placenta.

6.5. SUMMARY

We sought to investigate the action of environmentally relevant concentrations of BPA on the expression patterns of estrogen responsive genes in the placenta. We observed BPA at 1 pM and 10 nM could significantly regulate the expression of some of the selected genes. The observed response for gene expression pattern on exposure to BPA was more evident in choriodecidual explants than villous explants. These genes are important in development perspective as some of them regulate estrogen receptor mediated signalling pathways and are very important as far as placental / fetal development are considered. Our studies also suggest the possibility for a shift in the TH1 / TH2 cytokine production by term placenta on exposure to environmentally relevant concentrations of BPA. Since the expression patterns of many genes including ER-α and –β vary based on the maturational process of placenta, the importance of our results in the time of gestation when the exposure occurs should be cautioned.

Chapter 7. GENERAL CONCLUSIONS AND SUMMARY

7.1. CONCLUSIONS AND SUGGESTIONS FOR FUTURE STUDY

7.1.1. Development of placental perfusion model

We developed a well validated ex-vivo perfused single cotyledon human placental perfusion model in our laboratory to study transfer and biotransformation of selected endocrine disruptors in human placenta. We received Prof. Leslie Myatt's expertise in standardizing the model in our laboratory. The present model was a modification of placental perfusion model developed previously in our laboratory [276]. One of the major drawbacks of perfusion experiments is that the techniques used by many laboratories vary and this makes difficulty in interlaboratory data comparison. Hence the requirements for proper validation and standardization within the laboratory are very much demanded. The major challenge of the system was to keep the tissue viable throughout the perfusion period. One of the pre-requisites for maintaining tissue viability was to reduce the time of transport of the organ from hospital to laboratory and early establishment of the perfusion circuit to provide adequate nutrients and oxygen to the tissue. Keeping the tissue warm throughout the period of gross examination and catheterization helped us to increase the number of successful perfusions. Feto-maternal leak was another reason for majority of the failures in the perfusion experiment. Using digital pumps and preventing air bubbles entering the fetal circuit reduced the incidence of leakage. Suturing the chorionic vessels supplying nearby cotyledons further helped in reducing the leakage. Using fluorescent tagged high molecular weight dextran (4 Kda) in the maternal compartment (FITC-dextran) was effective in identifying the materno-fetal fluid loss. The consistent utilization of glucose, production of lactate and secretion of β-hCG were suggestive of metabolic activity of placenta throughout the perfusion period. Our findings were comparable to

those reported by previous investigators [274-276]. The major discrepancies observed in glucose utilization and lactate production across the laboratories were a result of perfusion techniques used [390]. We modified the perfusion chamber with aluminium body to dissipate heat uniformly. We also modified it to incorporate the oxygen, pH and temperature probes when the parameters would be studied online by connecting to Mac-Lab system. The absence of online monitoring of pH and metabolites was one of the limitations in our system. We constantly monitored the pH and pressure fluctuations with the help of pH meter and sphygmomanometer and remedied any deviations immediately. However, in the future the use of an online monitoring system would be recommended.

The success rate of perfusions varies from laboratory to laboratory. After gross examination, only 50% of placentae received (approximately 180) for our studies were suitable for perfusion. Approximately 60% of placentae failed during equilibration due to a feto-maternal fluid loss of more than 3 mL/hour. Another 10% failed due to pressure fluctuations during equilibration period. The remaining 25-30 % of placentae was successfully perfused for at least 3 hours duration. We were successful in perfusing one placenta for 24 hours and another for 12 hours. The utility of peforming perfusion in a single placenta with various concentrations of the drugs were reported earlier [391]. We also advocate this methodology for studies examining different doses of endocrine disruptors in the future to explore various pharmacological / physiological functions mediated by such compounds on human placenta.

7.1.2. Development of High Performance Liquid Chromatography and Liquid Chromatography – tandem Mass Spectrometry to study low concentrations of selected endocrine disrupting chemicals

We optimized an HPLC method in our laboratory to measure the concentrations of BPA in perfusates. The method was modification of a previously published method and initially developed to extract BPA, 4-nonylphenol (4-NP) and antipyrine simultaneously from the perfusates. The method was sensitive in detecting low concentrations of BPA from the perfusates (Limit of detection 1 ng/mL at a signal to noise ratio of >3). But an interfering peak co-eluted with BPA and significantly interfered with BPA detection. Later it was inferred that the milli Q water and the reagents used for HPLC might contain an interfering phenolic compound which affected the sensitivity of the assay. We used empore filters to filter the reagents and water to remove the interfering peaks. For measuring the sulphate / glucuronide conjugated levels in perfusates we used crude glucuronidase / sulphatase enzyme from the snail, *Helix pomatia* to deconjugate BPA and the free BPA measured by HPLC [279]. When we injected the extracted deconjugated perfusates, many interfering peaks appeared in the chromatogram that may have been coming from the hemolymph of the snail. None of the methods tested could remove the interefering peaks and hence it was decided that more sensitive methods for the detection of BPA and other endocrine disruptors and their conjugated metabolites were needed. Moreover this method also failed to detect 4-NP from perfusates.

We standardized LC-tandem mass spectrometry method (Atmospheric Pressure Chemical Ionization, APCI, negative ionic mode) to measure the concentrations of BPA and estradiol in the perfusates. Deuteriated estradiol was used as an internal standard for initial experiments. The method had a sensitivity of 65 pg/mL and the sensitivity remained

unaltered when we used deuteriated BPA (BPA d16) as an internal standard. We tried to modify the method by incorporating other endocrine disruptors like genistein (GEN) and 4-NP in the same assay. But the ionization was poor for GEN and 4-NP. Hence LC-MS using Electrospin Ionization (ESI with negative ionic mode) was developed to detect the compounds in the perfusates. Using the ESI probe significantly increased the ionization of genistein and hence its signal strength. The advantage of this method was that it could detect BPA, genistein, 17-β-estradiol, estrone and estriol from the perfusates. It reduced the sensitivity of BPA by a magnitude of 2. 4-NP again failed to ionize properly and hence it was decided to develop HPLC to detect 4-NP and its conjugates.

Recently empore filters became available in 96-well format as solid phase extraction cartridge kit, which could be used in the future to increase the sensitivity of BPA.

7.1.3. Transfer of BPA across the human placenta

BPA is ubiquitous in nature due to its industrial use in the production of polycarbonated plastics and dental sealants, the decomposition of the latter can liberate BPA into the environment. BPA is a well-studied endocrine disruptive chemical which has shown to impart its action by binding to estrogen receptors [7]. Experiments in animal models have shown that embryo and fetal stages are the most vulnerable period for the actions of BPA [392]. The studies on human exposure assessment have demonstrated the compound in various body fluids and tissues at varying concentrations [23, 25] and possible fetal exposure. However none of the studies available during that period have directly assessed the transfer and biotransformation of BPA in human placenta. We conducted placental perfusion experiments by incorporating environmentally relevant levels of BPA (serum concentrations reported from pregnant mothers, 10 ng/mL) [23] to measure its transfer and conjugation patterns in human placenta. Our results have demonstrated that BPA can

transfer across the human placenta rapidly with a rate that is similar to that of antipyrine transfer. Our observations further suggest a simple diffusion as the mode of BPA transfer in term placenta. The transfer index was 1.1 after 3 hours of perfusion. The conjugated (glucuronide / sulphate) BPA is devoid of estrogenic action and hence we examined the ability of placentae to conjugate BPA in our placental perfusion model. The greater portion of the BPA extracted from fetal compartment was in unconjugated free active form of the compound. Based upon this finding we suggest a limited ability of human placentae to detoxify the parent BPA into its conjugated forms. We also found an inter-placental variation in conjugating BPA which further mandates the evaluation of the expression status of xenobiotic metabolizing enzymes in human placenta. This could cause a concern in that the developing fetus could be exposed to unconjugated free form of BPA at a concentration similar to that of maternal serum concentrations. Moreover recently it has been shown in a uterine perfusion rat model that the BPA glucuronide could be transferred across the placenta and could be reactivated to BPA by fetal glucuronidase enzymes [298]. Another finding in the same study was the presence of low levels of UGT2B (glucuronidyl transferase isoform in placenta required for BPA glucuronidation) in the fetal liver. These findings opened another controversy in considering BPA safe as the defence was based on the conversion of major fraction of BPA into its inactive conjugate form immediately after the exposure. The transfer was found to be rapid and without the acute accumulation either in the maternal or fetal compartments. This suggests that the ATP Binding Cassette transporters (ABC transporters, the xenobiotic efflux pumps) in the term placenta might not protect the fetus against the exposure to BPA. However the expression patterns of ABC transporters are maturation dependent and it has previously shown that the activity of such pumps were reduced when nearing term [393]. In the future it would be interesting to find role if any of specific ABC transporters in the efflux functions of BPA.

7.1.4. Transplacental transfer and biotransformation of genistein in human placenta

Genistein is a phytoestrogen with specific Tyrosine Kinase inhibitory activities [394]. Genistein is present in high concentration in soy based foods. Due to its proposed beneficial effects on postmenopausal osteoprotective [395] and cardiac protective [396] functions it is included in nutriceutical prescriptions. However the mounting evidence on endocrine disruptive potential of estrogenous compounds has given a warning on the use of genistein / soy based food during pregnancy. Genistein shares structural activity relationship with that of 17-β-estradiol. Studies on experimental animals have shown that genistein can transfer across the placenta and cause developmental abnormalities in the fetus [397, 398]. It has been shown by different research group that humans are constantly been exposed to genistein [51-53, 263, 316].There is a study which showed that the children born to mothers on high soy diet during pregnancy had reproductive abnormalities [314]. None of the studies have directly assessed the transfer and biotransformation of genistein in human placenta. We conducted placental perfusion studies by incorporating environmentally relevant levels of genistein (10 ng/mL; reported range from the serum of pregnant women) [52] to study transfer and biotransformation of genistein in human placenta. Our results have demonstrated for the first time in a placental perfusion model that genistein at environmentally relevant concentration can transfer rapidly across the human placenta. The transfer rate significantly altered from that of antipyrine during the first one hour of the transfer and then the rate of transfer is comparable to that of antipyrine for the rest of the perfusion period. This might be due to tissue binding of genistein during the initial period of perfusion or formation of an unidentified metabolite. The consumption of genistein during pregnancy was considered as safe based upon the fact that majority of absorbed genistein would convert to inactive glucuronide / sulphate metabolites, as the latter are devoid of estrogenic action. The finding in rodent models that genistein

glucuronides couldn't get transferred across the placenta further strengthens the safety if a major portion of genistein could be converted to its glucuronide form [308]. Our results have demonstrated that human placenta can conjugate a portion of genistein and was able to transfer a portion of conjugated genistein (about 12% of genistein in the fetal compartment was in the conjugated form). A greater variation on conjugating ability was observed between the placentae and this might be due to the different levels of expressions of UGT (glucuronidyl transferase) isoforms in the perfused placentae.

Genistein was shown to be a substrate for Breast Cancer Resistant Protein (BCRP, a member of ABC transporter) [321]. Placenta expresses high levels of BCRP and when pregnant gene knockout mice were fed with genistein, it tends to get accumulated in the fetus suggesting a protective role for BCRP in preventing the transfer of genistein across the placenta and / or accumulation of genistein in the fetus [322-324]. However in our studies we couldn't find accumulation of genistein either on maternal or on fetal compartment, which rules out the involvement of BCRP in the transfer of genistein in term human placenta. The expression of ABC transporters are subjected to maturational development of trophoblast and hence further studies are required to explore the role of BCRP in protecting the fetus against the exposure to genistein.

7.1.5. Passage of 4-nonylphenol across the human placenta

4-nonylphenol (4-NP) is one of the microbial degradation products of alkylphenols which are used in the industrial and household detergents. Its annual global production exceeds 650,000 tons and due to which they contaminate aquatic flora, fauna, surface water and enters in the food chain [329]. Its estrogenic and endocrine disrupting actions were demonstrated by both i*n vitro* and *in vivo* studies [7, 262, 333, 334]. Studies have shown that humans are constantly exposed to 4-NP [329, 341]. In experimental animals 4-NP can

transfer across the placenta rapidly and can cause developmental disorders in fetus [344, 346]. None of the studies have directly assessed the rate of transfer of 4-NP in human placenta. Hence we conducted placental perfusion study to assess the transfer and biotransformation of 4-NP at environmentally relevant levels [343] (30 ng/mL; reported range in the serum of pregnant mother) in human placenta. Our study has demonstrated for the first time in an *ex vivo* perfused placental perfusion model that 4-NP can transfer across the human placenta. We also observed a rapid disappearance of 4-NP from the maternal compartment without concomitant appearance in the fetal compartment. The transfer rate and transfer percentage were significantly lower than that of antipyrine. Based upon these findings we could infer that the mode of transfer of 4-NP is entirely different from that of antipyrine, the latter diffuses freely across the placenta. The rapid disappearance from the maternal compartment might be due to the binding and sequestration of 4-NP in the placental tissue. A delay in the excretion from liver and intestine was reported after perfusing a specific quantity of 4-NP in rodent liver [38]. The authors then compared their results with different compounds and the reason for the delay in excretion was partially attributed to the presence of long alkylchain in 4-NP. Another reason for the delay was the requirement of ABC transporter in excretion of conjugated metabolites of 4-NP [349, 350]. The octanol water partition coefficient of 4-NP suggests it to be moderately lipophilic. Taking all these factors and provided placenta expresses ABC transporters it could be inferred that tissue binding and subsequent requirement of ABC transporters might one of the reasons behind the rapid disappearance of 4-NP from the maternal compartment. Also we were unable detect any conjugated fraction in the perfusates, suggesting the inability of placentae to conjugate 4-NP or sequestration of conjugated fraction within the tissues. This opens another interesting area of research in the future. The rapid disappearance might also be due to the formation of an unidentified intermediate metabolite in the placenta as human

hepatic Cytochrome P 450 (CYP) isozymes could bioactivate 4-NP to quinone metabolites [356]. Further standardization of HPLC / LC-MS would be required to identify metabolites if any formed in the placenta. Our study clearly shows that 4-NP at environmentally relevant concentration can transfer across the human placenta at a slower rate.

7.1.6. *In-vitro* actions of endocrine disruptors in human placenta

We initially screened the regulation of estrogen responsive genes in human choriocarcinoma cell line (BeWo) on exposure to environmentally relevant concentrations of selected endocrine disruptors [viz. BPA (BPA), 4-nonylphenol (4-NP) and genistein (Gen)]. The expression levels of ER-α gene in BeWo choriocarcinoma cell lines were very low as the signal was obtained only after 36 cycles of PCR or more. We then extended our studies to identify the gene regulation pattern in villous and choriodecidual explant culture. We conducted our preliminary studies in explants using BPA as the model endocrine disruptor. We incubated the explant preparations with four different concentrations of BPA, four different concentrations of BPA with 100 nM Faslodex (anti-estrogen) and four concentrations of 17-β-estradiol. We selected Estrogen Receptor – α (ER-α), Estrogen Receptor – β (ER-β), Estrogen Related Receptor – γ (ERR-γ), Artemin (ARTN), Cytochrome p 450 (Aromatase) and Insulin Like Growth Factor – 1 (IGF-1). A wide variation due to heterogeneity of placenta could be observed. However our preliminary results indicate the BPA can cause subtle changes in the gene expression levels of key genes required for the placental development. Some of these effects could be reversed with the addition of an antiestrogen (Faslodex) which further indicates the possibility of signal transduction through Estrogen Receptors. However further elaborate standardizations are required to measure subtle changes caused by this very low concentrations of endocrine disruptors in human placenta.

7.2. SUMMARY

We have developed a fully validated *ex vivo* dually perfused single cotyledon human placental perfusion model in our laboratory. We also have found that this model could be used for conducting long hours of continuous perfusion (24 hours continuously). A highly sensitive Liquid Chromatography – Tandem Mass Spectrometry was standardized in our laboratory to detect low levels of BPA and genistein from perfusates. This has further extended to include endogenous estrogens and the technique can simultaneously detect BPA, genistein, estradiol, estrone and estriol from the perfusates. We conducted placental perfusion studies with three endocrine disruptive chemicals abundant in New Zealand diet. We used environmentally relevant concentrations of these compounds (selected from the range reported from pregnant mother serum). Our results were indicative of a transplacental transfer of all the three compounds studied. However the transfer rate and the ability of the placenta to metabolize the compounds vary tremendously between the compounds studied. Hence we conclude that placenta could not protect fetus against the exposure to BPA, 4-NP and genistein. We conducted preliminary studies by incubating different concentrations of BPA in choriocarcinoma cell lines, villous and choriodecidual explants. Our results were indicative of a non-monotonic pattern of regulation of key genes on exposure to environmentally relevant concentrations of these endocrine disruptive agents. Based upon our results we recommend the possible measures to be followed to avoid exposure to these agents during pregnancy.

APPENDIX – I (PUBLICATIONS)

1. Publications arisisng from this thesis

1. Shaw IC., **Balakrishnan B** and Mitchell MD (2009) “The effect of dietary endocrine disruptors on the developing fetus” In Endocrine-disrupting chemicals in food, Ed. Ian Shaw, Woodhead Publishing Limited, UK. pp 3-35.
2. **Balakrishnan B**., Henare K., Thorstensen EB., Ponnampalam AP and Mitchell MD (2010) “Transfer of bisphenol A across the human placenta” American Journal of Obstetetrics and Gynecology 202 (4), 393 e1-e7.
3. **Balakrishnan B**., Thorstensen EB., Ponnampalam AP and Mitchell MD (2010), 'Transplacental transfer and biotransformation of genistein in human placenta', Placenta, 31 (6), 506-511.
4. **Balakrishnan B**., Thorstensen EB., Ponnampalam AP., Mitchell MD, ‘Passage of 4-nonylphenol across the human placenta’ Placenta, 32 (10) 788-92.

2. Conference presentations arising from this thesis

1. **Balakrishnan B**., Henare K., Thorstensen E and Mitchell, MD (2008) "Transfer of bisphenol A across the human placenta" Reproductive Sciences, 15 (2), 141A-142A. (Poster presented in Society for Gynaecologic Investigation 55th Annual Scientific Meeting, San Diego, CA, USA, March 26-29, 2008)
2. **Balakrishnan B**., Thorstensen E and Mitchell, MD (2009) "Xenoestrogens cross the human placenta at varying rates and can be mainly in unconjugated form" Reproductive Sciences, 16 (3), 342A. (Poster presented in Society for Gynecologic Investigation 56th Annual Scientific Meeting, Glasgow, UK., March 18-21, 2009)
3. **Balakrishnan B**., Pandey V., Perry J., Lobie PE and Mitchell MD (2009) "Bisphenol A at low concentrations regulates the expression of growth hormone in a breast cancer and placental cell line", Reproductive Sciences, 16 (3), 149A-150A. (Poster presented in Society for Gynecologic Investigation 56th Annual Scientific Meeting, Glasgow, UK., March 18-21, 2009).
4. **Balakrishnan B**., Thorstensen E and Mitchell, MD (2009) "Xenoestrogens cross the human placenta at varying rates and can be mainly in unconjugated form" (Poster presented in Liggins Institute Research Day 2009).
5. **Balakrishnan B**., Thorstensen, E and Mitchell, MD (2009), "Transplacental transfer of xenoestrogens across the human placenta", National Research Centre for Research and Development (NRCGD) Science Symposium 2009 abstracts p 47. (Oral presentation in NRCGD science symposium McMeekan Centre, Ruakura, New Zealand., Sep 30-Oct 2, 2009)
6. **Balakrishnan B**., Shaw, IC., Thorstensen EB., Ponnampalam AP., Mitchell MD (2010) "Endocrine Disruptors: - Transfer and actions in Human Placenta", NRCGD Science Symposium 2010 (Oral presentation in NRCGD science symposium, Old Government House, University of Auckland, New Zealand., 4th & 5th August, 2010)

APPENDIX – II (REAGENTS)

A) Perfusate preparation

We used Phenol Red free M199 tissue culture media for preparing perfusates.

To Make 1 L perfusate from M199 powder the following constituents were added as given below:-

M199 powder without phenol red (9.8 g/L)

L-glutamine (100 mg/L)

NaHCO3 (2.2 g/L)

D-glucose (2 g/L)

BSA (1 g/L)

PVP-40 (25 g/L)

Added appropriate volume of autoclaved Milli Q water

Then added the following:

Heparin - anticoagulant (20,000 IU/L)

Gentamicin - antibiotic (48 mg/L)

Adjusted the pH to 7.4 with NaOH or HCl

Filter sterilized and stored at 4° C.

B) Reagents for hCG ELISA

1. Preparation of Coating Buffer

Dissolved the following in 1L MilliQ grade water:

2.0 g NaHCO3

4.2 g Na2HPO4 (anhydrous)

8.0 g NaCl

0.1 g Thiomerasol

Adjusted the pH to 9.0-9.5

2. Preparation of Wash Buffer (for use in ELISA plate washer)

Dissolved the following in IL MilliQ grade water:

80.4 g NaCl

60.0 g Tris

5.0 ml Tween-20

Adjusted pH to 7.2-7.5 using 10N HCl.

This is a 10x solution. Diluted in MilliQ prior to use.

3. Preparation of Citrate-Phosphate Buffer

0.51 g Citric Acid

0.72 g Na2HPO4

0.1 g Thiomerasol

Dissolved in 100 ml MilliQ water. The pH was adjusted to ~5.5

4. Preparation of OPD (colour) Reagent

For a total volume of 2 ml, add-

1-2 crystals O-phenylenediamine (OPD)

2ml citrate/phosphate buffer pH 5.5

2µl Hydrogen peroxide (30% v/v)

Adjust quantity of crystals and Hydrogen peroxide according to total volume.

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