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LIPIODOL FERTILITY ENHANCEMENT IN UNEXPLAINED AND ENDOMETRIOSIS-RELATED INFERTILITY

NEIL PHILIP JOHNSON

LIPIODOL FERTILITY ENHANCEMENT IN UNEXPLAINED AND ENDOMETRIOSIS-RELATED INFERTILITY

ASSOCIATE PROFESSOR NEIL PHILIP JOHNSON

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DEDICATION

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SUBMITTED PAPERS ARISING FROM THIS THESIS

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AWARDS ARISING FROM RESEARCH IN THIS THESIS

2005: The Shan S Ratnam 'Young Gynaecologist' Award for demonstrating leadership in research at the 19th Asia Oceania Congress of Obstetrics & Gynaecology (Seoul, Korea).

2005: Award for the Best Free Communication at the Australian Gynaecological Endoscopy Society 15th Annual Scientific Meeting (Perth, Australia) for 'Poppy seed oil or surgery: is lipiodol a more effective fertility treatment than laparoscopic endometriosis surgery?'

2004: New Zealand Committee RANZCOG Young Gynaecologist Award for outstanding contributions to gynaecology research in New Zealand.

2003: Prize for Best Clinical Paper by a Young Clinician at Fertility Society of Australia ASM (Perth, Australia) for 'The FLUSH Trial – RCT of lipiodol flushing'.

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ABBREVIATIONS USED IN THIS THESIS

AIH	Artificial insemination by husband
ART	Assisted reproductive technology
CC	Clomiphene citrate
CD	Cluster determinant
CI	Confidence interval
CSF-1	Colony stimulating factor-1
DC	Dendritic cell
df	Degrees of freedom
DNA	Deoxyribonucleic acid
FLUSH	Flushing with lipiodol for unexplained subfertility by hysterosalpingography
FSH	Follicle stimulating hormone
g	Grams
HSG	Hysterosalpingogram
ICSI	Intracytoplasmic sperm injection
Ig	Immunoglobulin
IGFBP-1	Insulin-like growth factor 1 binding protein
IL-6	Interleukin-6
IQR	Inter-quartile range
IUI	Intrauterine insemination
IVF	In vitro fertilisation
LH	Luteinising hormone
MHC	Major histocompatibility complex
ml	Millilitre
mmol	Millimoles
NK	Natural killer
NNT	Number needed to treat
NRR	National Research Register
OR	Odds ratio
OSCM	Oil soluble contrast medium
p	Probability value
PBS	Phosphate buffered saline
pmol	Picomoles
r-ASRM	Revised American Society of Reproductive Medicine
RP	Relative probability
RR	Relative risk
TNF-α	Tumour necrotising factor-alpha
RevMan	Review Manager computer software
RCT	Randomised controlled trial
sd	Standard deviation
VAS	Visual analogue scale
WHO	World Health Organisation
WMD	Weighted mean difference
WSCM	Water soluble contrast medium

SYNOPSIS

This thesis sought to investigate the 'old clinician's tale' that having the fallopian tubes flushed in a tubal patency test (with lipiodol, an oil soluble contrast medium) improves a woman's fertility. It focused upon (a) pre-existing evidence; (b) mechanisms of effect of lipiodol; (c) generating further evidence through a definitive randomised controlled trial; (d) adoption of lipiodol treatment into clinical practice.

EXECUTIVE ABSTRACT

Objectives

This thesis had the following objectives:

1) To assess the existing evidence base for the effectiveness of tubal flushing as a treatment for infertility (Section II, Chapters 4 and 5).
2) To assess current practice and prior beliefs amongst Australasian fertility specialists concerning the role of tubal flushing as a treatment for infertility (Section III, Chapter 6).
3) To investigate the possible mechanism of the fertility enhancing effect of the oil soluble contrast medium lipiodol, and specifically whether there is an effect on the endometrium (Section IV, Chapter 7).
4) To generate definitive evidence from a randomised controlled trial for the effectiveness of lipiodol flushing as a treatment for infertility (Section V, Chapters 8 and 9).
5) To evaluate the adoption of lipiodol flushing as an innovative treatment into clinical practice (Section VI, Chapter 10).

Methods

The work undertaken in this thesis was based on prospective study protocols using the following research methodologies:

- Systematic reviews and meta-analyses of treatment efficacy to meet objective 1.
- Two types of structured survey questionnaires with a Bayesian analysis to meet objective 2.
- A randomised animal study involving 60 Swiss white mice, combined with genital flushing procedures under anaesthesia, some of which involved

microsurgical techniques, followed by genital tissue harvesting, tissue preparation and immunohistochemistry studies to meet objective 3.

- An open, parallel group, single centre, randomised controlled trial involving 158 women with unexplained and endometriosis-related infertility to meet objective 4.
- A survival analysis of women in the lipiodol flushing randomised trial to meet objectives 4 and 5.
- A prospective observational study of the first 100 women to undergo lipiodol flushing in clinical practice to meet objective 5.
- A clinical hysterosalpingogram procedure to meet objective 4 and 5.

Results

1) Eight randomised controlled trials involving 1,971 women were identified and included in the systematic review. Tubal flushing with oil soluble contrast media versus no intervention was associated with a significant increase in the odds of pregnancy (Peto odds ratio [OR] 3.57, 95% confidence interval [CI] 1.76 to 7.23) but there were no data for live birth. There were no data from RCTs to assess tubal flushing with water-soluble media versus no intervention. Tubal flushing with oil soluble contrast media was associated with a significant increase in the odds of live birth versus tubal flushing with water soluble contrast media (OR 1.49, 95% CI 1.05 to 2.11) but the odds of pregnancy showed no significant difference (OR 1.24, 95% CI 0.97 to 1.57) and there was evidence of statistical heterogeneity for these two outcomes. The addition of oil soluble contrast media to flushing with water soluble contrast media (water plus oil soluble contrast media versus water soluble contrast media alone) showed no significant difference in the odds of live birth (OR 1.06, 95% CI 0.64 to 1.77) or pregnancy (OR 1.16, 95% CI 0.78 to 1.70).

2) Nineteen Australasian fertility specialists returned survey questionnaires. Eighteen of the 19 specialists believed that lipiodol flushing was more likely to be beneficial than harmful. The most widely held prior belief, reflected in both textual and numerical responses, was that lipiodol was likely to produce a small beneficial response. The credible limits of this belief were compatible with a reasonable fertility benefit, as more than 50% believed that a 1.5-fold increase in pregnancy rate was plausible. The two surveys found that a 1.2-fold or 1.4-fold increase in pregnancy rate was the median expected level of benefit at which clinicians would have been inclined to recommend lipiodol flushing to their patients (combined range 1.1 to 2.3-fold). Individual and collective equipoise was justification to proceed with a definitive randomised controlled trial.

3) The mean number of cluster determinant (CD) 205^{+} uterine dendritic cells decreased significantly in mice following lipiodol treatment compared to sham treated and saline treated mice, particularly in endometrial and sub-endometrial tissues. The mean number of $CD1^{+}$ uterine dendritic cells increased significantly following lipiodol treatment compared to sham-treatment. No significant differences were found in the mean nurnber of total leukocytes or macrophages in the murine uterus between the three treatment groups.

4) Six month follow up of the randomised trial of 158 women showed that lipiodol flushing resulted in a significant increase in pregnancy (48.0% versus 10.8%, RR 4.44, 95% CI 1.61-12.21) and live birth (40.0% versus 10.8%, RR 3.70, 95% CI 1.30-10.50) rates versus no intervention for women with endometriosis (n=62), although there was no significant difference in pregnancy (33.3% versus 20.8%, RR 1.60, 95% CI 0.81-3.16) or live birth (27.1% versus 14.6%, RR 1.86, 95% CI 0.81-4.25) rates for women with unexplained infertility without confirmed endometriosis (n=96). Survival analysis up to 24 months showed a significant benefit in

overall pregnancy rate following lipiodol treatment (hazard ratio 2.0, 95% confidence interval [CI] 1.3 to 3.2) for the combined endometriosis and unexplained infertility populations. Amongst women with endometriosis, the benefit in pregnancy rate seen in the first 6 months following lipiodol (hazard ratio 5.4, 95% CI 2.1 to 14.2) was not present at 6 to 24 months following lipiodol (hazard ratio 0.6, 95% CI 0.2 to 2.1). There was a more consistent effect of lipiodol on fertility throughout the 24 month follow up amongst women with unexplained infertility (hazard ratio 2.0, 95% CI 1.1 to 3.5).

5) Six month follow up in the observational study of 100 women undergoing lipiodol flushing as an innovative treatment in clinical practice showed an overall pregnancy rate 30% and live birth or ongoing pregnancy rate 27% six months after the procedure. For women under 40 years old, a 32% pregnancy rate and 25% live birth or ongoing pregnancy rate was seen in women with unexplained infertility; a 50% pregnancy rate and 47% live birth or ongoing pregnancy rate was seen in women with endometriosis. Of women aged 40 years and older, the pregnancy rate was 13% and the live birth or ongoing pregnancy rate was 13%. The pregnancy rates included those occurring after additional interventions, such as intrauterine insemination and in-vitro fertilisation, accounting for 12 of the 30 pregnancies. There were no treatment complications.

Conclusions

The conclusions of this thesis are as follows.

- Lipiodol flushing is a simple, inexpensive, effective fertility treatment, which carries a very low chance of complications and no increased chance of multiple pregnancy.

- Lipiodol treatment is particularly effective in the short term for women with endometriosis who have normal patent fallopian tubes.
- The fertility benefit from lipiodol treatment lasts longer for women with pure unexplained infertility than for women with endometriosis.
- The level of benefit from lipiodol treatment for women with unexplained and endometriosis-related infertility is of sufficient magnitude to convince most fertility specialists surveyed that it is a worthwhile treatment to offer routinely in clinical practice; however complex factors govern the implementation of an innovative fertility treatment.
- Observational study of the first 100 women to undergo lipiodol treatment in clinical practice has provided further evidence of the efficacy and safety of this approach.
- Uterine dendritic cell changes following lipiodol flushing in mice suggest that the mechanism of the fertility enhancing effect might be an immuno-biologic effect on the endometrium that could improve the receptivity of the endometrium (rather than a mechanical tubal flushing effect), although this hypothesis requires further exploration in women.

SECTION I: INTRODUCTION

CHAPTER 1: INFERTILITY AND TUBAL PATENCY TESTING

1.1 Definition of Infertility

Infertility is defined as the failure to achieve pregnancy after 12 months of unprotected sexual intercourse. In the context of at least one previous pregnancy, irrespective of the outcome of previous pregnancies, this is known as secondary infertility; where a woman has not previously been pregnant, this is known as primary infertility.

1.2 Epidemiology of Infertility

Infertility affects 1 in 6 of all couples at some point in their relationship. A 'male factor' contribution to infertility is present in up to half of all couples presenting with infertility.

The demographics of couples presenting with infertility has been influenced most in the last two decades by couples electing to postpone their childbearing until women are older, without sufficient awareness of the age-related decline in female fertility, leading to an increase in involuntary infertility consequent upon ovarian reserve problems.[1] In addition to the other causes of infertility, an impact of diminished ovarian reserve is common. As many as 10% of women will experience the reduced fertility potential that accompanies diminishing ovarian reserve by age 32 years.[2]

1.3 Causes of Infertility

Women may have the following factors, detectable by appropriate investigation:

- Ovulation factor – where an egg is either not released from the ovary (anovulation) or released only infrequently (oligo-ovulation).
- Diminished ovarian reserve – where the remaining egg numbers are low and the quality of those remaining is generally poor, often with a high proportion of aneuploid eggs (with abnormal numbers of chromosomes).
- Tubal factor – the fallopian tubes may be affected by adhesions that reduce their mobility, may have damage to the delicate micro-architecture of their internal lumen, or may be blocked, sometimes in association with a fluid-filled distension known as hydrosalpinx.
- Endometriosis (see chapter 3).

Men may be affected by:

- So-called 'male factor' infertility – this encompasses problems with sperm delivery or with the sperm itself, including a low sperm count (oligospermia), reduced sperm motility (asthenospermia), increased proportions of abnormal sperm morphology (teratospermia), or absence of sperm from the ejaculate (azoospermia).

It is not uncommon for mild female subfertility factors and mild male subfertility factors to potentiate the tendency to infertility when they occur in partners. For example, there is an over-representation of women with mild endometriosis presenting at fertility clinics whose partners have mild oligospermia.

1.4 Investigation of Infertility

In New Zealand, in common with most developed countries, couples usually present first to their general practitioner with the complaint of delay in conception. At this stage a thorough medical history should be taken from both partners, focussed to assessing risk factors for the various causes of infertility. In primary care, the women should be assessed for cervical smear history, have genital

tract swabs taken (as further fertility investigation and treatment often requires trans-cervical procedures, and exclusion of sexually transmissible infection carrier status ensures a low risk of provoking acute pelvic inflammatory disease through cervical cannulation). The GP should ensure that the couple have an understanding of the fertile phase of the cycle, have no problems with sexual intercourse and that sexual frequency is adequate – our current advice is that the most fertile frequency of sex is daily for the 5 days preceding and including the day of ovulation. The woman should also be made aware of the value of pre-conception and first trimester folate supplementation for fetal neural tube defect prevention.

Baseline investigations for the woman include the following: antenatal blood tests including rubella immunity status; follicle stimulating hormone (FSH) taken in the early follicular phase on day 2 or 3 of the menstrual cycle; progesterone level in the mid-luteal phase of the cycle. If a woman is anovulatory or oligo-ovulatory, further diagnostic testing should be undertaken, including thyroid stimulating hormone (TSH), prolactin, an androgen screen and transvaginal pelvic ultrasound scan (if available).

Baseline investigations for the man include a semen analysis.

Ideally 100% of couples should be investigated and managed as above, before first attending a fertility clinic, however our data suggest disappointingly low levels of appropriate action prior to fertility clinic attendance.[3]

If the above investigations fail to reveal the cause for infertility, or if the choice of fertility treatment depends on the integrity of the woman's fallopian tubes and there is reason to doubt this, a tubal patency test should be considered.

1.5 Tubal Patency Testing

The choice of tubal patency test depends on the choice of the woman herself in the context of the likelihood of pathology.

Chlamydia trachomatis antibody testing is insufficiently accurate as a diagnostic test for tubal pathology to be useful in clinical practice; nor is there clear evidence that detection of a positive result influences management of infertility.[4]

The options for tubal patency testing are:

- hysterosalpingogram (HSG);
- laparoscopy with tubal dye studies
- other imaging techniques, including hystero-contrast-salpingography (Hy-Co-Sy)

1.5.1 Hysterosalpingography

A hysterosalpingogram (HSG) is a radiologic tubal patency test. It is usually performed with the patient fully conscious and without local anaesthetic, but women may benefit from oral non-steroidal analgesic medication prior to the procedure to minimise crampy abdominal discomfort. In the supine or left lateral position, a speculum is placed in the vagina and instrumentation applied to the cervix to obtain a satisfactory cervical seal. Under intermittent fluoroscopy X-ray screening, contrast medium is then gently instilled into the uterus until bilateral spill of contrast is seen from the fallopian tubes. Traditionally HSGs were performed using oil soluble contrast media (OSCM), but two decades ago, a switch was made to the use of water soluble contrast media (WSCM) for diagnostic HSGs. The advantages of HSG are that it is inexpensive, minimally invasive compared to laparoscopy, and it gives information about the uterine cavity. Disadvantages include intravasation (contrast media entering the vascular system). It has been suggested that HSG is an appropriate first line screening test for tubal pathology.[5]

1.5.2 Laparoscopy

Laparoscopy with tubal dye studies is performed under general anaesthesia, usually as a day stay surgical procedure. It typically involves two skin incisions (umbilical and suprapubic) to allow full pelvic inspection. Camera laparoscopy allows detailed inspection not only of the pelvic organs, but also of the upper abdomen (including the liver, where there may be perihepatic adhesions reflecting Fitz-Hugh-Curtis Syndrome as a sequel of prior Chlamydia trachomatis pelvic inflammation with perihepatitis). Methylene blue dye, instilled transcervically, allows for tubal patency testing. This approach has the added advantage over HSG of identification of endometriosis and non-occlusive pelvic adhesions, which may be treated laparoscopically in the same procedure.[4]

1.5.3 Hysterosalpingo-Contrast-Salpingography

Hysterosalpingo-Contrast-Sonography (Hy-Co-Sy) involves the transcervical instillation of a galactose microbubble contrast agent that is visible on transvaginal ultrasound. The procedure is similar to HSG and was promoted as a less invasive alternative, although it is now little used, as the procedure is typically more painful than a standard HSG.

CHAPTER 2: UNEXPLAINED INFERTILITY

2.1 Epidemiology

Where no cause can be found for infertility, in spite of completion of the usual investigation for infertility (1.3 and 1.4), this is known as unexplained infertility. Unexplained infertility is present in 10-25% of all couples with infertility.

2.2 Diagnosis

Unexplained infertility is thus a diagnosis of exclusion. It does not mean that there is no cause, simply that no cause can be determined by the routine investigations.

2.3 Possible Reasons

There has been much speculation to explain the unexplained! The reality is that unexplained infertility is likely to be due to a multiplicity of subtle factors that reduce the likelihood of a successful pregnancy, sometimes multiple subtle subfertility factors existing within any particular couple, whereas with another 'more fertile' partner, subtle factors affecting only one partner might not lead to infertility. A Medline search linking causes with unexplained infertility over ten years from 1997 to 2007 has yielded the following 'explanations'.

General:

- Stress.[6]
- Other environmental factors, including smoking, body weight, environmental pollutants, alcohol and caffeine consumption.[7]

Ovum and ovulation factors:

- Borderline ovarian reserve.[8]
- Granulosa cell apoptosis.[9]
- Meiosis dysfunction.[10]
- Luteinising hormone (LH) hypersecretion.[11]

Tubal:

- Fimbrio-ovarian inaccessibility.[12]
- Abnormal tubal peristalsis or cilial activity.[13]

Sperm:

- Sperm deoxyribonucleic acid (DNA) fragmentation.[14]
- Antisperm antibodies.[15]
- Sperm motion, binding, acrosome reaction and penetration abnormalities.[16]
- Easily decapitated sperm defect.[17]

Immunological:

- Antiphospholipid syndrome.[18]
- Gonadotrophin and ovarian autoantibodies.[19]
- Endometrial autoantibodies.[20]

The intra-peritoneal environment:

- Microscopic endometriosis.[21]
- Sub-optimal peritoneal natural killer (NK) cell function.[22]

Fertilisation failure.[23]

Implantation:

- Reduced uterine perfusion.[24]

- Abnormal endometrial integrins.[25]
- Endometrial leukaemia inhibitory factor.[26]
- Abnormal endometrial apoptosis.[27]
- Intrinsic endometrial factor ovum captor defect.[28]
- 'Bacteria endometrialis'.[29]

Subtle luteal phase hormonal defects[30] possibly leading to failure of hormonal support to the implanting conceptus.

2.4 Current Management

The prognosis for couples with unexplained infertility is reasonable – after 2 years of unexplained infertility, approximately 50% of couples will achieve a pregnancy within the next 12 months.[31] For younger women, where the duration of infertility is short, the best advice is therefore sometimes for no specific treatment (expectant management). Longer durations of unexplained infertility are associated with a poorer prognosis for conceiving a pregnancy without treatment.

Other treatments in current use are the following:

- Empirical clomiphene citrate (CC) – CC tablets, taken from day 2-6 or day 5-9 of the cycle usually provide an 'ovulatory boost' and may correct subtle disorders of ovulation not detectable on routine tests. Empirical CC treatment was suggested by a systematic review to provide a small beneficial effect in unexplained infertility.[32] This is an inexpensive and low invasive treatment, but carries an increased chance of multiple pregnancy (6-7% compared to just over 1% with natural conceptions), estrogen deficiency side effects (usually mild and transient), and should not be continued for more than 10-12 cycles owing to a possible increased long term risk of ovarian cancer with prolonged use.[33] The value of empirical

CC treatment for unexplained infertility has recently been challenged by the completion of a large, well powered and designed, multicentre randomised controlled trial (RCT) that did not show significant benefit over expectant management.[34] It is therefore possible that anti-estrogenic effects of CC on the endometrium, uterine blood flow and cervical mucous might limit its ability to improve pregnancy rates.

- Intrauterine insemination (IUI) – also known as artificial insemination by husband (AIH), the principle is to obtain the highest quality sperm and inject them into a woman's uterus around the time of ovulation in a tracked cycle. Such IUI cycles may be either natural (unstimulated) or may utilise ovarian stimulation with either CC tablets or gonadotrophin injections. Whilst a systematic review has shown higher live birth rates with stimulated IUI,[35] a recent RCT has shown no significant benefit of unstimulated IUI over expectant management[34] and a further RCT found no advantage of gonadotrophin-stimulated IUI over expectant management in couples with unexplained infertility considered to have a good chance of spontaneous pregnancy.[36]
- In vitro fertilisation (IVF) – the most expensive and invasive treatment for unexplained infertility. Some authorities recommend intracytoplasmic sperm injection (ICSI) – where a single sperm is micro-injected into each egg for lengthy unexplained infertility to minimise the chance of complete failure of fertilisation as sometimes occurs with standard fertilisation techniques in vitro, although the benefit of ICSI over IVF remains unproven.[37] Again, clear RCT evidence of benefit of IVF over an expectant approach to unexplained infertility is lacking.[38]

CHAPTER 3: ENDOMETRIOSIS

3.1 Definition and Epidemiology

Endometriosis is a disease characterised by extra-uterine endometrium.

It occurs commonly in women of reproductive age in up to 1 in 6 of all women.[39] It is particularly common in women with chronic pelvic pain or infertility. The cause is unknown, but various pathogenetic mechanisms have been proposed, including:

(i) retrograde menstruation (implantation of endometrial cells after retrograde passage of endometrium through the fallopian tubes into the peritoneal cavity);

(ii) coelomic metaplasia (where extra-uterine cells change into endometrial type cells, perhaps as a result of genetic pre-programming);

(iii) embolic phenomena (where endometrial cells transfer to sites distant from the endometrium via the bloodstream or lymphatics);

(iv) immunologic factors.

3.2 Diagnosis

The diagnosis is suspected in women who experience dysmenorrhoea, non-menstrual pelvic pain (including mid-cycle pain related to ovulation and non-cyclical pain), dyspareunia (painful sexual intercourse) or dyschezia (painful defaecation), especially if physical examination reveals pelvic tenderness, a retroverted immobile uterus, or nodularity in the uterosacral ligaments, cul-de-sac or rectovaginal septum.[39] A bood test showing slightly elevated levels of the marker CA125 raises the suspicion of endometriosis; a pelvic ultrasound scan is seldom helpful, although it can show features of adenomyosis (ectopic endometrium within the uterine myometrial wall) or suggest the presence of an

endometrioma (an endometriotic cyst on the ovary). The diagnosis is confirmed only by the visual appearance at surgical laparoscopy, and is more secure if laparoscopic biopsies are taken and evaluated histologically. A further advantage of laparoscopic surgery is that endometriosis can also be surgically removed at the same procedure, by either excision or ablation of endometriotic lesions.[39]

The appearance of endometriosis at laparoscopy can be scored, based on the extent of endometriotic disease and the extent of associated adhesions, giving a revised American Society of Reproductive Medicine (r-ASRM) score:[40]
Stage 1 (minimal endometriosis) = r-ASRM score 1-5;
Stage 2 (mild endometriosis) = r-ASRM score 6-15;
Stage 3 (moderate endometriosis) = r-ASRM score 16-40;
Stage 4 (severe endometriosis) = r-ASRM score greater than 40.
There is only moderate correlation of the r-ASRM score or disease stage with severity of symptoms.

3.3 Pathophysiology of Infertility Related to Endometriosis

Infertility consequent upon the more severe forms of endometriosis (stage 3 and, particularly, stage 4 disease) is easy to understand. It results from occlusion (blockage) of the fallopian tubes, non-occlusive tubal damage, pelvic adhesions, ovarian endometrioma formation, anatomical distortion of pelvic anatomy (especially the relationship of the fallopian tube to the ovary), and in its most severe form is known as a 'frozen pelvis'.

Does mild or minimal endometriosis (stage 1 and 2) cause infertility? It has been hotly debated whether stage 1 and 2 endometriosis, with normal fallopian tubes and ovaries, in the context of otherwise unexplained infertility, should be simply regarded as unexplained infertility and managed as such. In mild or minimal endometriosis, particularly where the fallopian tubes and ovaries are completely

normal, mechanisms underlying reproductive failure are subtle and remain controversial. However, amongst such women undergoing donor insemination, there is increasing evidence that women with minimal and mild endometriosis have approximately half the chance of success of women without endometriosis.[41, 42]

There has been conflicting evidence whether results from assisted reproductive treatments (ART), such as in vitro fertilization (IVF) or intracystoplasmic sperm injection (ICSI), are compromised in women with endometriosis. Whilst some studies reported significantly diminished pregnancy and implantation rates in women with endometriosis-associated infertility treated with IVF compared to tubal factor and/or unexplained infertility,[43-46] other studies found no statistically significant effect on the pregnancy and implantation rates in those women.[47-58] A telling meta-analysis of 22 published studies found that the chance of achieving pregnancy was significantly lower for women with endometriosis (odds ratio, 0.56; 95% confidence interval, 0.44-0.70) when compared to women with tubal factor infertility, after controlling for other confounding factors.[59] Multivariate analysis also demonstrated a decrease in fertilisation and implantation rates, and a significant decrease in the number of oocytes retrieved amongst women with endometriosis. Pregnancy rates for women with severe endometriosis were also significantly lower than for women with mild endometriosis (odds ratio, 0.60; 95% confidence interval 0.42-0.87).[59] Furthermore, a recent systematic review of RCTs has suggested a significant improvement of IVF outcome in women with moderate and severe endometriosis undergoing prolonged hormonal suppression of endometriosis with gonadotrophin releasing hormone analogue (GnRHa) treatment for 3-6 months prior to IVF treatment.[60]

Endometriosis has a recognised association with autoimmune disease, including the antiphospholipid syndrome. The possible underlying factors related to infertility are those interfering with egg quality, fertilisation, embryo quality or embryo implantation. Such factors might have an influence on the egg's

developing environment in the ovarian follicle, the intraperitoneal environment, or the endometrial environment. Factors influencing gamete or zygote quality even early in this process may have a 'knock-on' effect downstream, for example a poor quality egg may lead to a poor quality embryo that has a reduced implantation potential. Egg quality might affect the ability of the egg to be fertilized, or once fertilized, to implant.

3.3.1 Egg Quality

Factors postulated as compromising egg quality in women with endometriosis:

(1) Women with endometriosis are often 'poor responders' even when other indicators of ovarian reserve (including FSH levels) are normal – indeed there are reduced numbers of primordial follicles when ovarian endometriosis is present.

(2) Follicular environment:[61] compared to controls, the follicular fluid of women with endometriosis has:
    (a) increased progesterone concentration;[62]
    (b) increased concentration of the cytokine interleukin-6 (IL-6);[63]
    (c) lower levels of cortisol;[64]
    (d) lower concentrations of insulin-like growth factor 1 binding protein (IGFBP-1).[65]

(3) Cultured granulosa cells from women with endometriosis show:
    (a) increased expression of the tumour necrotising factor-alpha (TNF-α);[66]
    (b) increased rate of apoptosis (cell death) in granulosa cells, possibly mediated by elevated concentrations of soluble Fas ligand in the serum and peritoneal fluid.[67]

3.3.2 Fertilisation

Intraperitoneal factors might affect sperm function or the ability of sperm to fertilise the egg. Studies have shown that peritoneal macrophages from women with endometriosis have enhanced ability to phagocytose sperm.[68]

3.3.3 Embryo Quality

Factors postulated as compromising embryo quality in women with endometriosis are:

(1) Poor egg quality (embryo quality is inherently dependent on the quality of the egg from which the embryo arises);

(2) 'Toxic' intraperitoneal, intratubal, or endometrial environment. The intraperitoneal environment shows the following characteristics:

   (a) increased inflammatory, proteolytic and angiogenic activity of the peritoneum and peritoneal fluid;[69]

   (b) elevated interleukin-6 (IL-6) levels;[70]

   (c) increase in peritoneal macrophage numbers;[71]

   (d) increased peritoneal expression of macrophage secreted products, such as growth factors and cytokines;[72]

   (e) increased peritoneal expression of haptoglobins;[73]

   (f) altered immune surveillance of the peritoneal cavity;[74]

   (g) sperm-egg interaction problems: fertilisation rates have been shown to be reduced in women with endometriosis undergoing assisted reproductive technology (ART) treatment.[46-48, 50, 75-78]

3.3.4 Implantation

Factors postulated as compromising embryo implantation in women with endometriosis:

(1) Poor embryo quality resulting from poor egg quality or genital tract toxicity[43-45, 79, 80] – resultant poor quality embryos may have a compromised viability potential with a lower chance of successful implantation;

(2) Compromised endometrial receptivity. Unique endometrial immuno-biologic factors occurring in women with endometriosis include:

(a) increased formation of antibodies to endometrial antigens;[81]

(b) aberrant cell adhesion molecules;

(c) microarray studies have shown differential gene expression in the endometrium from women with endometriosis compared to that from women without endometriosis.[82, 83]

It is recognised in IVF treatment that embryos from women with endometriosis have lower implantation potential. The standard method of ascertainment whether reduced implantation potential is due to embryo factors or emdometrial factors is to examine results from egg donation treatment – egg/embryo factors will be apparent if results are compromised where women with endometriosis act as egg donors; endometrial factors will be apparent if results are compromised where women with endometriosis are the recipients of embryos from egg donation treatment. There is less evidence to support a role of endometrial receptivity in decreased implantation rates in women with endometriosis treated with IVF.[58, 84] In a cross-over experiment, Pellicer et al compared fertility parameters in different groups of women receiving donor oocytes.[85] They noted that when donor oocytes came from women without known endometriosis, embryo development and implantation rates were similar for patients with and without endometriosis. However, when the results of oocyte donation were classified according to the nature of the oocytes donated, women who received embryos derived from oocytes from women with endometriotic ovaries showed a significantly reduced implantation rate compared to the controls.[85]

3.4 Current Management

Treatment of endometriosis depends on whether fertility optimisation or relief from pelvic pain and improved quality of life is the desired result. There is good

evidence that laparoscopic surgical removal of endometriosis, by either excision or ablation (or both) is effective for resolution of chronic pelvic pain.[39] There is also clear evidence of effectiveness of numerous hormonal drug treatments (the combined oral contraceptive pill, progestogens, gestrinone, danazol, gonadotrophin releasing hormone analogues for chronic pelvic pain, although insufficient evidence of relative benefits of any of these treatments over one another).[39]

Laparoscopic surgical removal of endometriosis has been demonstrated to be effective in improving fertility by a systematic review of two RCTs,[86] one demonstrating an effect in its own right,[87] the other being a smaller trial that did not show a significant benefit.[88] Stripping (excisional treatment) for endometriomas has been shown by a systematic review[89] of two RCTs[90, 91] to improve fertility outcomes compared to endometrioma drainage and coagulation (ablative treatment). There are few data to support the use of empirical clomiphene citrate treatment or intrauterine insemination (IUI). IVF is used as the 'default treatment', in the event of other unsuccessful treatments, or if tubal damage is present, but outcomes are poorer for more severe endometriosis.

There is debate of the value of aggressive surgical treatment of endometriosis and of medical treatment prior to IVF. There are no randomised trials assessing the surgical removal of endometriomas prior to IVF or ICSI. Other studies have produced conflicting results, some suggesting a decreased success rate of assisted reproduction following endometrioma removal.[92-94] Other studies have found that laparoscopic cystectomy for endometriomas had no significant effect on the ovarian response for IVF.[95, 96] Similarly, laser vaporization (cauterization) of the internal wall of endometriomas prior to IVF did not impair the outcome of IVF.[97] Finally, ultrasound-directed endometriotic cyst aspiration performed prior to IVF is associated with mixed results, with some groups reporting significantly higher clinical pregnancy rates per cycle,[98] and others reporting the opposite effect.[99] Furthermore, endometriotic cyst aspiration prior or during IVF seems to

be associated with an increased incidence of infection and tubo-ovarian abscess formation.[100-104]

There is evidence suggestive of improved IVF/ICSI outcome in women with endometriosis undergoing hormonal suppression of endometriosis GnRH analogues for 3-6 months prior to IVF/ICSI, from a systematic review of three small RCTs.[60]

SECTION II: EXISTING EVIDENCE SUPPORTING TUBAL FLUSHING FOR INFERTILITY

CHAPTER 4: NARRATIVE REVIEW OF NON-RANDOMISED STUDIES

4.1 Historical and Anecdotal Aspects

A possible therapeutic effect of diagnostic tubal patency testing has been debated in the literature for half a century, the first reports being a doubling of conception rate in the four months after HSG with oily media compared with the four months before the procedure.[105] Most gynaecologists are aware of patients who have conceived immediately after a diagnostic tubal patency test following lengthy infertility.

Historically a variety of agents have been used to 'flush' the fallopian tubes. Some of these agents have been used primarily for diagnostic purposes in assessing tubal patency, such as methylene blue water-soluble dye in conjunction with laparoscopy and the water-soluble contrast media (WSCM) and oil-soluble contrast media (OSCM) used for a hysterosalpingogram (HSG). Other agents have historically been used primarily for therapeutic purposes, such as carbon dioxide tubal insufflation and oil injection, although this does not form part of current routine practice in most centres.

Lipiodol, an OSCM, is an iodised poppy seed oil, obtained by substitution of ethyl esters for the glyceryl esters of lipiodol. One ml of Lipiodol Ultra Fluide (Guerbet, France) contains 0.48g iodine. Traditionally HSGs were performed with OSCM. Lipiodol was one of the commonest OSCM used for diagnostic HSGs with OSCM in previous decades. Their use was gradually replaced by WSCM for a number of reasons.

(i) WSCM permit better imaging of the tubal mucosal folds and ampullary rugae (internal architecture of the fallopian tubes) than OSCM.[106]

(ii) OSCM have a high viscosity, which results in slow filling of the fallopian tubes, often necessitating an inconvenient late film after 24 hours.

(iii) OSCM reabsorption is slow, leading to prolonged persistence of OSCM within

the pelvic cavity.
(iv) When there is accumulation of OSCM within a blocked fallopian tube, a chronic inflammatory reaction called a lipo-granuloma may occur, although this has not been reported in women with patent fallopian tubes and is not known to have long-term consequences.[107]
(v) The potential consequences of intravasation of OSCM into the pelvic blood vessels and lymphatics are allergic reactions or anaphylaxis. There were several reports of severe adverse reactions after the use of oily media in radiology (before the use of fluoroscopy screening) but none were reported after 1967.[108]
(vi) WSCM are generally cheaper than OSCM.
One of the most elegant descriptions of a possible beneficial therapeutic effect of OSCM came from a radiologist.[109] Gillespie (1965) implemented a practice change from OSCM to WSCM for safety reasons. A decreased pregnancy rate from 41% to 27% over the following 12 months prompted a change back to the use of oily media and the pregnancy rate rose again to 44%.[109] Much debate has surrounded whether oil-soluble or water-soluble contrast media might have the bigger fertility-enhancing effect.

It is reassuring that the advent of fluoroscopy screening appears to have abolished severe adverse reactions following the use of oily media in radiology[108] and the safety of HSGs with OSCM in this context has been confirmed.[110]

Non-randomised comparative studies (section 4.2) and randomised controlled trials (RCTs) (Chapter 5) have formed more recent evidence in the evolution of studies of the efficacy of tubal flushing in improving fertility.

4.2 Evidence from Non-Randomised Studies

Many uncontrolled reports on the therapeutic aspect of oil-soluble contrast media have been published since the 1960s. A gradual stream of non-randomised

controlled studies[107, 111-114] also fuelled the hypothesis of the fertility-enhancing effect of OSCM and its superiority compared to the use of WSCM. A further study was described as 'pseudo-randomised', employing alternate assignment of participants.[115] However, this is not an adequate method of sequence generation in a truly randomised trial, as patient allocation is never concealed, and such studies are prone to all the biases of non-randomised studies.

Typically non-randomised studies (and RCTs where allocation concealment is inadequate) tend to over-estimate the effect of treatments.[116] Any study employing a control group that has not been truly randomised, is prone to substantial selection bias. There is now consensus that the only acceptable test of the effectiveness of an intervention is that from RCT study design.

CHAPTER 5: SYSTEMATIC REVIEW OF RANDOMISED TRIALS

5.1 Introduction

The randomised trial is the gold standard assessment of the effectiveness of any intervention. Whilst non-randomised studies suggested possible fertility benefit from the use of OSCM for tubal flushing, a systematic review of randomised trials was necessary to assess the best available evidence for the effect of tubal flushing on fertility.

The first systematic review of non-randomised studies and RCTs in this field was published several years ago.[117] The original Cochrane Review,[118] first published in 1996, was an expansion and update of that overview. This systematic review, from data current to 2002, is an update of that review.

5.2 Objectives

To evaluate the effect of tubal flushing:

- OSCM versus no intervention;
- WSCM versus no intervention;
- OSCM versus WSCM.

5.3 Methodology

5.3.1 Criteria for studies

The criteria for considering studies for this review are as follows.

Types of studies

Randomised controlled trials (RCTs) where a potential therapeutic effect of tubal flushing on subsequent pregnancy outcomes could be studied.

Types of participants

Women in a relationship where infertility is present.

Sub-group analysis by cause of infertility was planned.

Types of interventions

Tubal flushing by means of hysterosalpingography (HSG).

Tubal flushing at the time of laparoscopy.

Any other tubal flushing procedure with visualisation of the fallopian tubes.

The following comparisons were considered:

- Tubal flushing with OSCM versus no treatment.
- Tubal flushing with WSCM versus no treatment.
- Tubal flushing with OSCM versus WSCM.
- Tubal flushing with OSCM and WSCM versus WSCM alone. This comparison was included as a separate comparison to allow access to all relevant data (and not included as a comparison of OSCM versus no intervention in the presence of concurrent WSCM, since it was unclear whether the presence of WSCM might alter the effect of OSCM).

Types of outcome measures

The following primary outcomes were considered:

- pregnancy per woman;
- live birth per woman.

In addition, the following secondary outcomes were considered:

- miscarriage per pregnancy;
- ectopic pregnancy per woman;

- procedural pain - immediate and delayed;
- intravasation;
- infection;
- haemorrhage;
- image quality - of the uterine cavity and of the tubal ampulla;
- long term complications.

5.3.2 Search strategy for identification of studies

Publications with the intervention of tubal flushing with visualisation in women with infertility were obtained using the following search strategy.

1) Regular searches of The Menstrual Disorders & Subfertility Group's Specialised Register of clinical trials were performed (using the search strategy developed by the Cochrane Menstrual Disorders & Subfertility Group).

2) The following electronic databases were searched using Ovid software:
Medline - 1966 to 2002;
Embase - 1980 to 2002;
Biological Abstracts - 1980 to 2002.

The following terms were used in this electronic search strategy:
1. HYSTEROSALPINGOGRAPHY/ or hysterosalpingography.mp. or hysterosalpingog$.tw.
2. salpingog$.tw.
3. HSG.tw.
4. laparoscopy adj3 dye).tw.
5. LAPAROSCOPY/
6. Fallopian Tube Patency Tests/
7. tubal adj flush$).mp. [mp=ti, kw, ab, bc, bt, bo, sh, hw, tn, ot, dm,mf, rw]
8. tub$ adj patency).tw.

9. or/1-8
10. OILS/
11. Ethiodized Oil/
12. Iodized Oil/
13. IODIPAMIDE/
14. WATER/
15. Contrast Media/
16. water adj soluble).tw.
17. oil adj soluble).tw.
18. lipiodol.tw.
19. OSCM.tw.
20. WSCM.tw.
21. Or/10-20
22. 9 and 21
23. exp clinical trials/
24. exp research design/
25. clinical trial.pt.
26. randomized controlled trial.pt.
27. (singl$ or doubl$ or trebl$ or tripl$).tw.
28. (mask$ or Blind$).tw.
29. 27 and 28
30. placebos/ or placebo.tw.
31. 23 or 24 or 25 or 26 or 29 or 30
32. 22 and 31

3) The National Research Register (NRR), a record of ongoing and recently completed research projects funded by, or of interest to, the United Kingdom's National Health Service, with entries from the Medical Research Council's Clinical Trials Register, and details on reviews in progress collected by the NHS Centre for Reviews and Dissemination, was also searched for any trials with the following key-words:

1. Hysterosalpingogram, HSG or salpingogram
2. Lipiodol or ethiodol
3. Water soluble contrast media, WSCM, oil soluble contrast media or OSCM
4. Tubal flushing.

The Clinical Trials Register, a registry of federally and privately funded clinical trials in the United States of America was also searched for the same key-words.

4) Citation lists of included trials, eligible studies and relevant review articles were also searched. The first or corresponding author of trials eligible for inclusion were contacted to ascertain if they were aware of any ongoing or unpublished trials.

Abstract booklets from scientific meetings, including the European Society of Human Reproduction and Embryology, the World Congress of IVF and Reproductive Genetics, the British Fertility Society, the Fertility Society of Australia and the British Congress of Obstetrics & Gynaecology, were also searched.

The search for this update of the review was carried out in December 2002.

5.3.3 Methods of the review

a) Selection of trials

The selection of trials for inclusion in the review was performed independently by two of three reviewers (NJ, PV and AW[119]) after employing the search strategy described previously. Differences of opinion were resolved by consensus after consultation with the other reviewers.

Trials were excluded from the review if they were not truly randomised, if they made comparisons other than those specified above or if the quality of the trial was inadequate. These were detailed in the table of excluded trials.

b) Quality assessment

Included studies were assessed independently by three reviewers (NJ, PV and AW[119]) for the following quality criteria and methodological details. This information is presented in a table describing the included studies and provided a context for assessing the reliability of results.

(A) Trial characteristics

a) Method of randomisation - from Lilford's classification, in order of preference as follows, only categories (i) and (ii) were considered suitable for inclusion in the meta-analysis, whilst category (iii) quasi-randomised studies, were excluded:

(i) third party randomisation, for example by pharmacy, computer or telephone

(ii) true randomisation by carer, for example by opaque numbered envelope or register

(iii) quasi (pseudo) randomisation

b) Study design:

(i) blinding

(ii) duration of follow-up

(iii) type of follow-up

c) Size of study:

(i) number of women recruited

(ii) number of women randomised

(iii) number of women excluded

(iv) number of women withdrawn and lost to follow-up

(v) number of women analysed

d) Study setting

(i) Single-centre or multicentre

(ii) Location

(iii) Timing and duration

(iv) Source of funding stated or not

e) Analyses

(i) Whether a power calculation was performed and adhered to

(ii) Whether 'intention to treat' analysis was performed by authors, possible from data but not performed by authors, not possible or uncertain

(B) Characteristics of the study participants

(i) Age

(ii) Primary or secondary infertility

(iii) Duration of infertility

(iv) Investigative work-up - baseline follicle-stimulating hormone (FSH), semen analysis, previous tubal patency test, confirmatory test of ovulation

(v) Breakdown by cause for infertility

(vi) Previous treatments

(vii) Exclusion criteria

(C) Interventions

(a) Timing of flushing

(b) Nature of flushing including single or multiple

(c) Absence of other interventions in treatment and control group

(d) Criteria for tubal flushing

(i) Primarily as a diagnostic procedure

(ii) Primarily as a therapeutic procedure

(D) Outcomes

(a) Primary

(i) Pregnancy (and diagnosis of pregnancy)

(ii) Live birth

(b) Secondary

(i) Miscarriage

(ii) Ectopic pregnancy
(iii) Procedural pain
(iv) Intravasation
(v) Infection
(vi) Haemorrhage
(vii) Image quality - uterine cavity, ampulla
(viii) Long term complications

c) Data Management

All data were extracted independently by at least two of three reviewers (NJ, PV and AW[119]) and differences of opinion were resolved by consensus. Additional information on trial methodology or actual original trial data was sought from the corresponding author of trials which appeared to meet the eligibility criteria, when aspects of methodology were unclear, or where data were in a form unsuitable for meta-analysis. Reminder correspondence was sent if a reply was not received within four weeks

d) Statistical Analysis

Statistical analysis was performed in accordance with the guidelines for statistical analysis developed by the Menstrual Disorders and Subfertility Group. Statistical heterogeneity between the results of different studies was examined by inspecting the scatter in the data points on the graphs and the overlap in their confidence intervals and, more formally, by checking the results of chi-squared tests. The outcomes were pooled statistically where clinical and statistical heterogeneity was absent.

Dichotomous data were expressed as an odds ratio with 95% confidence intervals and combined for meta-analysis with RevMan software using the Peto-modified Mantel-Haenszel method with a fixed effects model. An increase in the odds of a particular outcome (which may be beneficial, for example in the case of live birth, or detrimental, for example in the case of a complication) was displayed

graphically in the meta-analyses to the right of the centre-line and a decrease in the odds of an outcome was displayed graphically to the left of the centre-line.

It was planned to perform sensitivity analyses to examine the stability of the results in relation to a number of factors including study quality and the source of the data (published or unpublished) where more than 10 randomised trials were included in the meta-analysis; additional sensitivity analyses were planned to examine the stability of the pregnancy outcome results when trials in which the tubal flushing performed primarily as a diagnostic procedure and those in which it was performed primarily as a therapeutic procedure were considered separately.

Ongoing searches for new trials were planned to be undertaken every two years, with update of the review accordingly if new trials are found.

5.4 Description of studies

5.4.1 Included and excluded studies

Eight randomised trials with a total of 1,971 analysed participants were identified and included in this review. A further three trials[120-122] were added in this update[119] from the previous version of the review.[118] One previously included trial[115] has now been excluded as it was not truly randomised (see below). Two 'trials' included in a previous version of the review[118] have now been categorised as separate reports[108, 123] of a single trial.[123] The characteristics of the included trials are summarised in Table 5.1.

Eight studies were excluded from the review, one of which was not truly randomised with the use of alternate assignment,[115] five were non-randomised comparative studies of HSG with OSCM versus WSCM,[107, 109, 112-114] one was a

three-way non-randomised comparative study of HSG with OSCM versus WSCM versus no treatment[111] and one did not report pregnancy outcomes.[124]

Of the included trials, two trials including 223 analysed participants assessed tubal flushing with OSCM versus no treatment;[121, 125] five trials with 1,241 participants assessed flushing with contrast which included OSCM versus flushing with WSCM alone (OSCM versus WSCM).[120, 123, 126-128] The latter of these trials[120] included a group receiving tubal flushing with both WSCM and OSCM - this group was considered in a separate comparison versus the WSCM group and this data pooled for meta-analysis with data from the final trial[122] which made a similar comparison of OSCM+WSCM versus WSCM tubal flushing: 502 participants were involved in this comparison.

The included studies and their methodological details are summarised comprehensively in the table of included studies.

5.4.2 Quality assessment of included studies

The overall quality of the included trials was variable. The method of randomisation (an important potential source of bias) was not stated in four trials[122, 123, 125, 127] and not fully described in two trials,[126, 128] leading in each case to an allocation score B (where adequate concealment of assigned treatment prior to allocation was unclear). Adequate concealment of assigned treatment prior to allocation was unequivocal in two trials,[120, 121] which received an allocation score A. Randomisation was undertaken some time in advance of the tubal flushing procedure itself (at referral and at scheduling) in two trials[123, 125] and subsequently a number of participants were withdrawn before they underwent the HSG because they had conceived, changed their mind about undergoing the procedure or participating in the trial, or were subsequently found not to fulfil the criteria for the trial. In both trials where patients were withdrawn before the intervention, 15% of the participants were affected in this way. Randomisation immediately before the procedure is more appropriate.

Withdrawals and losses to follow-up after HSG varied from 0%,[121, 122] 1%,[120] 9%,[123] 19%,[126] 28%[128] and 37%[125] of participants who underwent the procedure; this was unclear for one trial.[127] The highest withdrawal rate of 37%[125] was due to the fact that patients underwent the HSG (or not) before any results of their other investigations was known, and only patients with proof of ovulation in all four cycles of follow-up were retained in the analysis. Incompleteness or loss to follow-up accounted for approximately one half of the withdrawals in the other trials. Only one trial[126] specified outcome details for patients withdrawn from each randomised group. Recalculation of the OR including these subjects has little effect on the conclusions of this trial (OR 1.31, 95%CI 0.51-3.04 for all subjects versus OR 1.31, 95%CI 0.56-3.09 after exclusion). Other than in the trials where all randomised participants were analysed, it is impossible to recalculate the treatment effect based on the originally randomised groups (using the 'intention to treat' principle). In fact, it is not obvious that the 'intention to treat' principle is the best approach to analysis given the poor design (randomisation before eligibility established) of some of the trials. If an intention to treat analysis was to be performed, assumptions would have to be made, for example that equal numbers of patients were allocated to each arm for the study denominator; for the numerator, it would be reasonable to assume either that no additional pregnancies occurred in withdrawn participants or that the proportion of pregnancies in those withdrawn was equal to that of the included participants overall (not differentiating between treatment arms). Owing to the necessity for multiple assumptions and the uncertainty of the appropriateness of the approach in this case, an intention to treat analysis was not performed.

Power calculations were mentioned in two trials.[120, 121] The size of the study suggested by power calculations was not achieved in one trial[121] owing to a slower recruitment rate than was anticipated and the investigators running out of time for further recruitment. Where trials have been stopped early, particularly those with small numbers where the statistical significance could be dramatically

altered by, for example, one pregnancy in the control group, interpretation of the results must be cautious. The numbers suggested by the power calculation were attained in two of the three groups in the other trial, which had the highest number of participants of all the trials in this meta-analysis,[120] with the third group closed by the monitoring committee at the request of the investigators owing to the adverse effect of the third group on recruitment to the trial.

The source of funding was stated for one trial[123] who acknowledged a source of support by a company manufacturing (water soluble) contrast media. Although a potential conflict of interest exists, this particular contrast medium turned out to be inferior.

One trial was multi-centre;[120] the other trials were single-centre. The timing and duration of the trial was stated for all trials except one.[128]

None of the trials mentioned blinding, although participant blinding would have been possible in trials where different contrast media were compared; all trials could have been single-blind for the investigators assessing outcomes - scope for bias from not blinding includes the possibility of more thorough follow-up by investigators to find outcomes in couples not attending follow-up clinics.

The important prognostic factor of the women's ages was stated in all but two trials[123, 125] - for details, refer to Table 5.1. Infertility was defined as >12 months in 5 RCTs, >8 months in one RCT,[122] >6 months in one RCT[127] and unspecified in one RCT.[125] The mean duration of infertility was similar across randomised groups in all eight RCTs. Only one trial[120] specified the proportion of women with primary and secondary infertility. Detail on the investigative work-up was not well described in four trials.[120, 122, 123, 125]

The cause of infertility was defined as purely unexplained in three trials.[121, 127, 128] Two trials specified a breakdown of the causes of infertility.[122, 126] Three trials did not specify a breakdown of the causes of infertility.[120, 123, 125]

The intervention was intended primarily as a diagnostic procedure in six trials[120, 122, 123, 125-127] and a therapeutic procedure in two trials.[121, 128] The technique for the HSG was similar in all studies and was done under fluoroscopic control, usually in the first half of the cycle. No study mentioned the use of prophylactic antibiotics. One trial specified the use of ultrasound and a pregnancy test in the diagnosis of pregnancy[126] and several others specified ultrasound, although specific ultrasound criteria were not stated in any trial. Live birth is the best outcome measure in infertility trials, as this is what eventually matters for the patient. This outcome was specified by two trials.[120, 123]

5.5 Results

The results are shown graphically in meta-analysis plots in Figures 5.1 to 5.18.

1. Tubal flushing with OSCM versus no treatment

Tubal flushing with OSCM was associated with a significant increase in the odds of pregnancy (OR 3.57, 95%CI 1.76 to 7.23).

Sub-group analysis analysis by cause for infertility showed the odds of pregnancy for couples with unexplained infertility were significantly increased following OSCM tubal flushing versus no treatment, although numbers were small and the confidence interval therefore wide (OR 9.74, 95%CI 1.50 to 63.17).

The sensitivity analysis for whether the interventions in the trials were performed primarily for diagnostic or therapeutic purposes gave the following results. For trials where the intervention was intended primarily as a diagnostic test,[125] pregnancy OR 3.02 (95%CI 1.41 to 6.48); no data available for live birth; for trials

where the intervention was intended primarily as a therapy,[121] pregnancy OR 9.74 (95%CI 1.50 to 63.17); no data available for live birth.

2. Tubal flushing with WSCM versus no treatment

There were no data from RCTs to assess this intervention.

3. Tubal flushing with OSCM versus WSCM

Tubal flushing with OSCM was associated with a significant increase in the odds of live birth versus tubal flushing with WSCM (1.49, 95%CI 1.05 to 2.11) but the odds of pregnancy showed no statistically significant difference (OR 1.23, 95%CI 0.95 to 1.60). There was evidence of statistical heterogeneity in the meta-analyses of tubal flushing with OSCM versus WSCM for the outcome pregnancy (chi-square test p-value 0.01; I^2 test 65.3%) and live birth (chi-square test p-value <0.0001; I^2 test 93.6%). There were no significant differences in the odds of miscarriage per pregnancy (OR 0.82, 95%CI 0.41 to 1.64) or ectopic pregnancy (OR 0.49, 95%CI 0.10 to 2.42). The odds of obtaining a satisfactory image were significantly decreased for OSCM versus WSCM for both the uterine cavity (OR 0.18, 95%CI 0.12 to 0.26) and the tubal ampulla (OR 0.05, 95%CI 0.04 to 0.07). The odds of the complication intravasation were significantly higher with OSCM (OR 5.41, 95%CI 2.57 to 11.37), although there were no serious sequelae from this; the odds of post-procedure bleeding were significantly lower with OSCM (OR 0.22, 95%CI 0.15 to 0.31); there was no significant difference in the odds of infection (OR 0.34, 95%CI 0.11 to 1.05). No serious complications were seen in any of the studied participants. The odds of experiencing procedural pain were significantly less for OSCM versus WSCM procedures, whether immediate procedural pain within the first 24 hours (OR 0.53, 95%CI 0.34 to 0.84) or prolonged pain lasting more than 24 hours from the procedure (OR 0.26, 95%CI 0.15 to 0.45) was considered.

Sub-group analysis by cause for infertility showed no statistically significant differences in pregnancy outcomes within any of the diagnostic category sub-groups.

The sensitivity analysis for whether the interventions in the trials were performed primarily for diagnostic or therapeutic purposes gave the following results: for trials where the intervention was intended as a diagnostic procedure,[108, 120, 123, 126, 127] live birth OR 1.49 (95%CI 1.05 to 2.11); for trials where the intervention was intended primarily as a therapy,[128] pregnancy OR 3.47 (95%CI 0.70 to 17.19); no data available for live birth.

4. Tubal flushing with OSCM+WSCM versus WSCM

There were no significant differences for any of the outcomes for which data were available for the comparison of these interventions, including pregnancy (OR 1.16, 95%CI 0.78 to 1.70), live birth (OR 1.06, 95%CI 0.64 to 1.77), miscarriage (OR 1.14, 95%CI 0.53 to 2.48) and ectopic pregnancy (OR 0.54, 95%CI 0.08 to 3.45).

Sub-group analysis analysis by cause for infertility showed no statistically significant differences in pregnancy outcomes within any of the diagnostic category sub-groups.

In both trials in this comparison,[120, 122] the tubal flushing was performed primarily for diagnostic purposes, so no further sensitivity analysis was necessary.

5.5 Discussion

This systematic review of randomised trials has yielded some evidence that tubal flushing with oil-soluble contrast media might increase the odds of pregnancy versus no intervention. The limited evidence of an increase in the odds of live birth from tubal flushing with oil-soluble contrast media versus water-soluble contrast media must be interpreted cautiously. In 5 RCTs the analysis could be broken down by sub-groups according to the cause of infertility,[121, 122, 126-128] although different diagnostic criteria were used. The existence of data for specific sub-groups within trials might be an aspect of publication bias. Thus sub-group analysis must be interpreted with extreme caution in both primary

research and meta-analysis. In fact, although data from non-randomised studies and previous meta-analyses have suggested that the greatest effect of OSCM tubal flushing occurred in the sub-group with unexplained infertility,[117] none of the diagnostic category sub-groups demonstrated a statistically significant difference in this meta-analysis.

The strength of this analysis is that it is the totality of all randomised trial data in studies that have addressed the question of efficacy of tubal flushing in improving fertility. There are obvious weaknesses: many trials were underpowered to detect clinically meaningful differences; non-blinded trials are prone to bias through confounding variables such as sexual frequency, which could have been different in treatment and control groups; only a rninority of trials reported live birth, obviously the outcome of importance to couples with infertility.

Inclusion of the new data available since publication of the previous version of this review[118] has raised more questions than answers! The quality and reliability of the primary studies are important factors in weighing the conclusions of an overview. Despite some methodological concerns about the trials included in the meta-analysis, the studies comparing OSCM with WSCM showed a consistent and homogeneous therapeutic effect of oily media prior to this review update.[118] Results from non-randomised studies have also suggested that OSCM tubal flushing increases the pregnancy rate and that the pregnancy rate following OSCM tubal flushing exceeds that following WSCM tubal flushing.[117] However one of the largest and methodologically most robust trials in this update of the meta-analysis failed to show a benefit of OSCM over WSCM.[120] Non-randomised trials have now been excluded from the meta-analysis and thus the influence (or weight) of this trial on the pooled odds ratio is considerable, meaning that the effect of OSCM versus WSCM, based on the meta-analysis, is of borderline statistical significance. Inclusion of this trial has also introduced statistical heterogeneity, meaning that the conclusions drawn from the meta-

analysis, with regard to the comparison OSCM versus WSCM, must be guarded. Clearly there remains doubt over the relative efficacy of OSCM and WSCM.

Theories have been proposed to explain the possible fertility enhancing effect of OSCM. Less well supported theories include 'straightening' of fallopian tubes, disruption of peritubular adhesions, stimulation of tubal ciliary action, improving cervical mucus and an iodine-induced bacteriostatic action on mucous membranes.[126] It has even been suggested that the iodine component of OSCM such as lipiodol might improve fertility by correcting a subclinical iodine deficiency. The more plausible theories are:
1) OSCM may be more effective than WSCM in 'flushing out' debris from otherwise undamaged fallopian tubes and although this debris may not necessarily block the tubes, it may hinder conception or embryo transport;[121]
2) OSCM may affect peritoneal immuno-biology, possibly via an effect on peritoneal macrophages[129] through an alteration of interleukin and prostaglandin production by peritoneal macrophages[130] or a modulation of macrophage activity in phagocytosis of sperm.[131]
3) OSCM could affect endometrial immuno-biology, producing a direct endometrial effect that enhances the receptivity of the endometrium to an implanting embryo, although this has never previously been suggested.

There has been an international trend for centres to move towards the use of laparoscopy with tubal patency testing rather than HSG, owing to the increased information available concerning tubo-peritoneal abnormalities (including endometriosis) from laparoscopy and the possibility of treating endometriosis and adhesions surgically.[4] Currently those centres performing HSGs tend to use WSCM rather than OSCM for the reasons outlined in the background section. On the other hand, irrespective of subsequent pregnancy rates, OSCM offer some advantages over WSCM, as follows.
(i) The slow filling of the fallopian tubes owing to the higher viscosity of OSCM often necessitates a 'late' film, but some authorities regard the 24-hour film as an

advantage because of the additional information this gives, mainly in the evaluation of adhesions after slow peritoneal spillage.[132]
(ii) Some studies have demonstrated a sharper radiographic image of the uterus and easier detection of uterine anomalies,[127] although this review suggested that the odds of obtaining an acceptable uterine image were greater with WSCM.
(iii) Less pain occurs with OSCM than with WSCM, probably because of less chemical irritation of the peritoneum.[106]

Whilst there have been data to suggest a fertility-enhancing effect of tubal flushing, particularly with OSCM, this does not form part of routine current practice. There has been a reluctance to embrace this as a standard treatment, possibly relating to:
a) prior beliefs amongst clinicians which have not, to date, been sufficiently swayed by available data - criticisms have included that data on sexual frequency were not available for the 'flushing' versus 'no treatment' trials hence the notion that the increased pregnancy rate might be due simply to an increased sexual frequency in the group who received treatment, and that much of the data were from trials where the interventions were performed as diagnostic tests rather than as therapeutic interventions (prior beliefs of clinicians are the focus of Chapter 6);
b) a trend towards IVF as the panacea for all causes of infertility.

Conclusions
The evidence that tubal flushing with oil-soluble contrast media might increase the odds of pregnancy versus no intervention is worthy of further investigation. The limited evidence of an increase in the odds of live birth from tubal flushing with oil-soluble contrast media versus water-soluble contrast media must be interpreted cautiously.

Further robust randomised trials comparing oil-soluble versus water-soluble media and comparing each versus no intervention are required to provide

convincing evidence as to whether the technique should be accepted into widespread clinical practice as a therapy. Owing to methodological flaws in the trials to date, further research is required, where live birth is considered as the primary outcome and where confounding variables, such as sexual activity, must be accounted for. Indeed this systematic review has defined the research agenda for a definitive randomised trial of lipiodol flushing (described in Chapter 8). Further scientific research on the possible OSCM-related improvement in fecundity may clarify its working mechanism and explain some cases of hitherto 'unexplained' infertility (see Chapter 7).

Table 5.1 Characteristics of included studies

Study	Methods	Participants	Interventions	Outcomes	Notes	Allocation concealment
Alper 1986	Method of randomisation: random number table. Time of randomisation: at HSG itself. No mention of blinding. 131 women recruited and randomised. No exclusions before HSG. 13 (9.9%) withdrawn after HSG. 12 (9.2%) lost to follow-up. 106 women analysed. Duration of follow-up: 6 months Single-centre: Ottowa Civic Hospital, Ottowa, Canada. Duration of trial: recruitment 1 April to 31 December 1984. Source of funding not stated. No mention of power calculation.	Mean age 29.3 (SD 4.6) WSCM; 29.1 (SD 2.9) OSCM. Primary or secondary infertility for more than 12 months (mean or range of duration of pre-existing infertility not stated, but duration and proportion of primary/secondary similar in two groups). Investigative work-up: semen analysis, PCT, BBT and endometrial biopsy; diagnostic laparoscopy prior to HSG in most women. Breakdown specified by cause for infertility. Previous fertility treatments not specified. Women with bilateral tubal blockage withdrawn after HSG; no other exclusions specified.	HSG with OSCM Ethiodol (Savage Laboratories, Missouri City, USA) versus WSCM Renographin (ER Squibb & Sons, Princeton, USA). A volume of 10 to 20 ml of contrast medium was used. Timing: any day of menstrual cycle. No co-interventions. Primarily intended as diagnostic procedure.	Pregnancy (diagnosis based on urine hCG or serum beta-hCG plus ultrasound, although ultrasound criteria not specified). Volume of contrast medium required. Pain during HSG. Intravasation.	Unclear whether the assigned treatment was adequately concealed prior to allocation.	B

	Intention to treat analysis not performed but possible from the data.					
De Boer 1988	Method of randomisation not stated; time of randomisation not stated. No mention of blinding. Number of women randomised not stated. Number of women excluded not stated. Number of women withdrawn and lost to follow-up not stated. 175 women analysed. Duration of follow-up: 6 months. Single centre: St Radboud University Hospital, Nijmegen, Holland. Duration of trial: February 1985 to October 1986. Source of funding not stated. No mention of power calculation.	Mean age 29 (range 19-44) years. Primary or secondary infertility for more than six months; mean infertility duration 37 (SD 26.2) months. Investigative work-up: normal PCT and/or sperm penetration test and BBT. Breakdown by cause for infertility: unexplained only. Previous fertility treatments not specified other than exclusion for women with previous infertility surgery.	HSG with OSCM Ethiodol (Guerbet, France) versus WSCM Iopamidol (Bracco, Italy). A volume of 10 ml contrast medium was used. Timing: day 6-13 of menstrual cycle. No co-interventions. Primarily intended as diagnostic procedure.	Pregnancy rate (diagnosis based on ultrasound, although ultrasound criteria not specified). Quality of visualisation of uterine cavity. Quality of visualisation of ampullary tubal folds. Time for contrast medium to disperse from pelvis.	Unclear whether the assigned treatment was adequately concealed prior to allocation.	B

	Intention to treat analysis unclear.					
Letterie 1990	Method of randomisation: random number scheme. Time of randomisation: during laparoscopy. No mention of blinding. 40 women recruited and randomised (from a total investigated population of 225, 185 were found to have pelvic abnormatilies at diagnostic laparoscopy and excluded from trial entry). No exclusions before HSG; exclusion criterion iodine allergy. 11 withdrawn after randomisation (8 inadequate follow-up and 3 "inadequate coital exposure"). No other losses to follow-up specified. 29 women analysed. Duration of follow-up: 12 months. Single-centre: Tripler Army Medical Centre, Honolulu, Hawaii, USA.	Mean age 27 (SD 3.5) years OSCM; 25 (SD 4.1) years WSCM (not significant). Unexplained infertility of mean duration 24 (SD 14.5) months OSCM; 28 (SD 13.9) months WSCM; inclusion criterion > 12 months. Investigative work-up: normal semen analysis; ovulatory confirmation based on BBT and serum progesterone and/or secretory phase; normal prolactin, thyroxine and TSH; normal pelvis and bilateral tubal patency at laparoscopy. Breakdown by cause for infertility: unexplained only. Previous fertility treatments not specified. Exclusions specified: where cause for infertility diagnosed; iodine allergy.	Tubal flushing during laparoscopy, after standard dye studies, with OSCM Ethiodol (Savage Laboratrories) versus WSCM Conray-60 (Mallinckrodt Inc.). A volume of 20 ml of contrast medium was used. Timing not specified wrt menstrual cycle. No co-interventions. Primarily intended as therapeutic procedure.	Pregnancy (diagnostic criteria not specified). Ectopic pregnancy.	Unclear whether the assigned treatment was adequately concealed prior to allocation.	B

	Duration and timing of the trial not stated. Source of funding not stated. No mention of power calculation. Intention to treat analysis not performed nor possible.					
Nugent 2002	Method of randomisation: sequentially-numbered sealed opaque envelopes with randomised allocation on paper inside. Time of randomisation: usually several days before HSG. Not blinded. 34 women recruited and randomised. No exclusions before HSG. No women withdrawn or lost to follow-up. 34 women analysed. Duration of follow-up: 6 months. Dual-centre: Leeds General Infirmary and	Women mean age 30.6 years (eligibility criterion <36 years). Unexplained primary or secondary infertility (proportion of primary/secondary not stated) for more than 12 months (mean duration of pre-existing infertility 49 months). Investigative work-up: normal semen analysis by WHO criteria, ovulatory confirmation by serum progesterone or serial scanning, normal fallopian tubes at laparoscopy and dye insufflation or HSG. Breakdown by cause for infertility: unexplained only, all other causes for infertility excluded. Previous fertility treatments not specified.	HSG with OSCM Lipiodol versus no treatment. Timing wrt menstrual cycle not specified. Information sheet on fertile phase of the cycle given to both groups; no other co-interventions. Primarily intended as therapeutic procedure.	Pregnancy rate (diagnosis based on positive pregnancy test). Viable pregnancy (diagnosis based on fetal heart on ultrasound). Adverse events.	Assigned treatment was clearly adequately concealed prior to allocation.	A

	Princess Royal Hospital, Hull, UK. Duration of trial: 18 months Source of funding: none (a pragmatic trial conducted in a National Health Service outpatient setting). Power calculation specified a requirement for 180 recruits but trial terminated early owing to slow recruitment rate and running out of time. Intention to treat analysis performed.					
Ogata 1993	Method of randomisation: not stated. Time of randomisation: at initial visit to clinic. No mention of blinding. 302 women randomized, but only 190 fulfilled entry criteria and completed follow-up. Duration of follow-up: 4 months. Single-centre:	Mean age not specified; said to be similar between the 2 groups. Primary or secondary infertility (proportion not specified) having first visit to infertility clinic; duration of infertility not specified but said to be similar between the 2 groups. Investigative work-up: not specified, but rate of male infertility and PCT results said to be similar between the 2 groups. Breakdown by cause for infertility not specified.	HSG with oil-soluble contrast medium Lipiodol (Ultra-Fluid) versus no HSG (the HSG was delayed for 4 months until after the analysis). Volume of contrast medium not specified. Timing wrt menstrual cycle not specified. No co-interventions. Primarily intended as diagnostic procedure.	Pregnancy (method of diagnosis not specified).	Unclear whether the assigned treatment was adequately concealed prior to allocation.	B

	University of Kyusyu, Fukuoka, Japan. Duration of trial: between November 1989 and February 1991. Source of funding not stated. No mention of power calculation. Intention to treat analysis not performed nor possible.	Previous fertility treatments not specified. No exclusion criteria specified.				
Rasmussen 1991	Method of randomisation: not stated. Time of randomisation: at referral for HSG. No mention of blinding. 507 women recruited and randomised. 78 (15.4%) excluded before HSG. 31 (6.1%) withdrawn after HSG. 14 (2.8%) lost to follow-up. 398 patients analysed. Duration of follow-up: 9 months. Single-centre: Odense	Mean age not stated. Primary or secondary infertility for more than 12 months (mean or range of duration of pre-existing infertility not stated). Investigative work-up: not stated, although stated that the distribution of the diagnostic categories of infertility were similar between the groups. Breakdown by cause for infertility not specified. Previous fertility treatments not specified. Exclusion criteria: pregnant prior to HSG; HSG declined; technical difficulties leading to unsuccessful HSG; HSG not performed by authors.	HSG with OSCM Lipiodol (Laboratoire Guerbet, France) versus 3 types of WSCM: Iohexol (Omnipaque 350, Nycomed, Oslo), Ioxaglate (Hexabrix 320, Laboratoire Guerbet, France), Diatrizoate (Urografin, Schering, Berlin). As there were no outcome differences between the 3 groups using WSCM, they were combined in the analysis of results. A volume of 5-10 ml of contrast medium was used. Timing wrt menstrual cycle not specified. No co-interventions. Primarily intended as diagnostic procedure.	Pregnancy (method of diagnosis not specified). Other outcomes of this trial (reported image quality, pain, infection, haemorrhage and intravasation) are reported in a separate publication (Lindequist 1991).	Unclear whether the assigned treatment was adequately concealed prior to allocation.	B

	University Hospital, Odense, Denmark. Duration of trial: 4 years between 1985 and 1988. Source of funding not stated. No mention of power calculation. Intention to treat analysis not performed nor possible.		Outcomes Pregnancy (method of diagnosis not specified). Other outcomes of this trial (reported image quality, pain, infection, haemorrhage and intravasation) are reported in a separate publication (Lindequist 1991).			
Spring 2000	Method of randomisation: computer-generated random numbers in blocks of 9 at each site. Time of randomisation: at HSG itself. No mention of blinding. 673 women recruited and randomised. No exclusions before HSG. No withdrawals after HSG. 7 lost to follow-up. 666 women analysed. Duration of follow-up: 12 months. Multi-centre: 10 centres	Mean age 29.3 (SD 4.6) years WSCM; 29.1 (SD 2.9) years OSCM. Primary or secondary infertility (OSCM 35.0%, WSCM 37.1%, WSCM+OSCM 34.8% primary infertility). Mean duration of infertility: OSCM 3.13 (SD 3.03) years, WSCM 3.15 (SD 3.18) years, WSCM+OSCM 3.09 (SD 3.61); eligibility criterion > 12 months. Investigative work-up: not specified. Breakdown by cause for infertility not specified. Previous fertility treatments not specified. No exclusions specified.	HSG with OSCM Ethiodol (Savage Laboratories, Melville, USA) versus WSCM Diatrizoate and Iodipamide (Bracco Diagnostics, new Brunswick, USA) versus both WSCM and OSCM. Volume WSCM mean 9.4 (range 2-75) mls; OSCM mean 8.6 (range 1-55) mls; both - WSCM mean 8.2 (range 1-30) mls and OSCM mean 6.0 (range 1-20) mls. Timing wrt menstrual cycle not specified. Co-interventions: artificial insemination performed in 25.3% OSCM; 24.6% WSCM; 24.8% WSCM+OSCM. Primarily intended as diagnostic procedure.	Pregnancy (diagnostic criteria not specified). Live birth. Miscarriage. Ectopic pregnancy.	Assigned treatment was clearly adequately concealed prior to allocation.	A

	co-ordinated by the Kaiser Permanente Medical Care Program Infertility Work Group, California, USA. Duration of trial: December 1993 to July 1996. Source of funding not stated. Power calculation suggested a requirement for 257 women per contrast group (achieved for 2 groups and recruitment abandoned for third group owing to difficulty recruiting). Intention to treat analysis not performed.					
Yang 1989	Method of randomisation: not stated. Time of randomisation: at time of HSG. Double blind. 109 women recruited and randomised. No exclusions, withdrawals or losses to follow-up stated.	Age range 22-44 years; mean age WSCM 30.1 years, WSCM+OSCM 30.0 years.. Primary or secondary infertility for more than 12 months (mean or range of duration of pre-existing infertility not stated). Investigative work-up: not stated. Breakdown specified by cause for infertility.	HSG with WSCM Telebrix Hystero (Laboratoire Guerbet) versus WSCM Telebrix Htstero followed by OSCM Lipiodol Ultrafluide (Laboratoire Guerbet). A volume of 10 mls WSCM and 5 mls OSCM were used. Timing wrt menstrual cycle not specified. No co-interventions. Primarily intended as diagnostic procedure.	Pregnancy (method of diagnosis not specified).	Unclear whether the assigned treatment was adequately concealed prior to allocation.	B

	109 women analysed. Duration of follow-up: 8 months. Single-centre: Mackay Memorial Hospital, Taipei, China. Duration of trial: recruitment October 1986 to March 1987. Source of funding not stated. No mention of power calculation. Intention to treat analysis not mentioned (all randomised women were analysed).	Previous fertility treatments not specified.				

Key to Table 5.1

Allocation concealment category:
A = Adequate; B = Unclear; C = Inadequate; D = Not used.

Table 5.2: Characteristics of excluded studies

Study	Reason for exclusion
Acton 1988	Non-randomised study comparing HSG with OSCM versus WSCM in 420 women.
Barwin 1971	Non-randomised study comparing HSG with OSCM versus WSCM in 248 women.
DeCherney 1980	Non-randomised study comparing HSG with OSCM versus WSCM in 339 women.
Gillespie 1965	Non-randomised study comparing HSG with OSCM versus WSCM in 271 women.
Mackey 1971	Non-randomised study of HSG with OSCM versus WSCM versus no treatment in 523 women. [Showed no therapeutic effect of HSG with WSCM (OR 0.87, 95%CI 0.47 to 1.59), but a significantly higher pregnancy rate after HSG with OSCM (OR 1.60, 95%CI 1.09 to 2.35).]
Schwabe 1983	Described as 'pseudo-randomised' with alternate assignment (thus not a truly randomised trial and therefore excluded), studied HSG with OSCM versus WSCM in 198 women (121 analysed). [Showed no significant difference in the odds of pregnancy for OSCM versus WSCM (OR 2.00, 95%CI 0.74 to 5.45).]
Wolf 1989	Double-blind RCT of HSG with Iotrolan (WSCM) versus Iohexol versus Diatrizoate assessing image quality and pain, but not pregnancy outcomes, in 60 women. A potential therapeutic effect on subsequent pregnancy outcomes could not therefore be studied.
Yaegashi 1987	Non-randomised study of HSG with OSCM versus WSCM in 224 women. The details of this study were confirmed after commissioning a translation from the original Japanese publication.

Figures 5.1 to 5.18: Meta-analysis Graphs of the Effect of Tubal Flushing with Oil or Water Soluble Contrast Media

(Adapted from meta-analysis graphs, undertaken using RevMan software, courtesy of the Cochrane Collaboration, adapted from meta-analysis graphs from Johnson et al [2005].[119] Copyright Cochrane Library, reproduced with permission.)

Figure 5.1: Meta-analysis Graph for Effect of OSCM versus No Treatment on Pregnancy

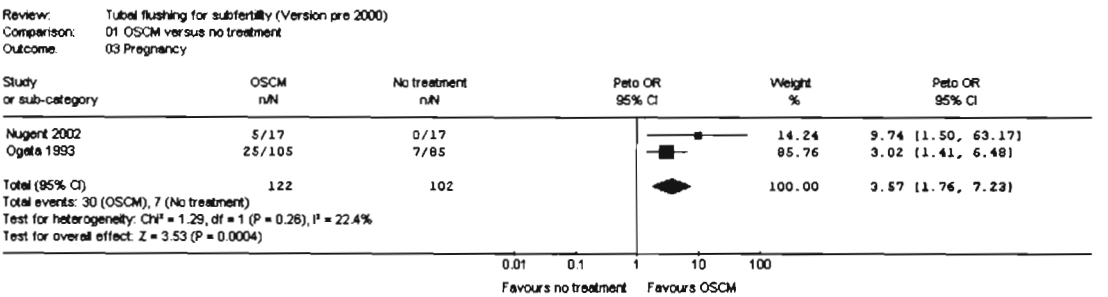


Figure 5.2: Meta-analysis Graph for Effect of OSCM versus No Treatment on Pregnancy Amongst Subgroups

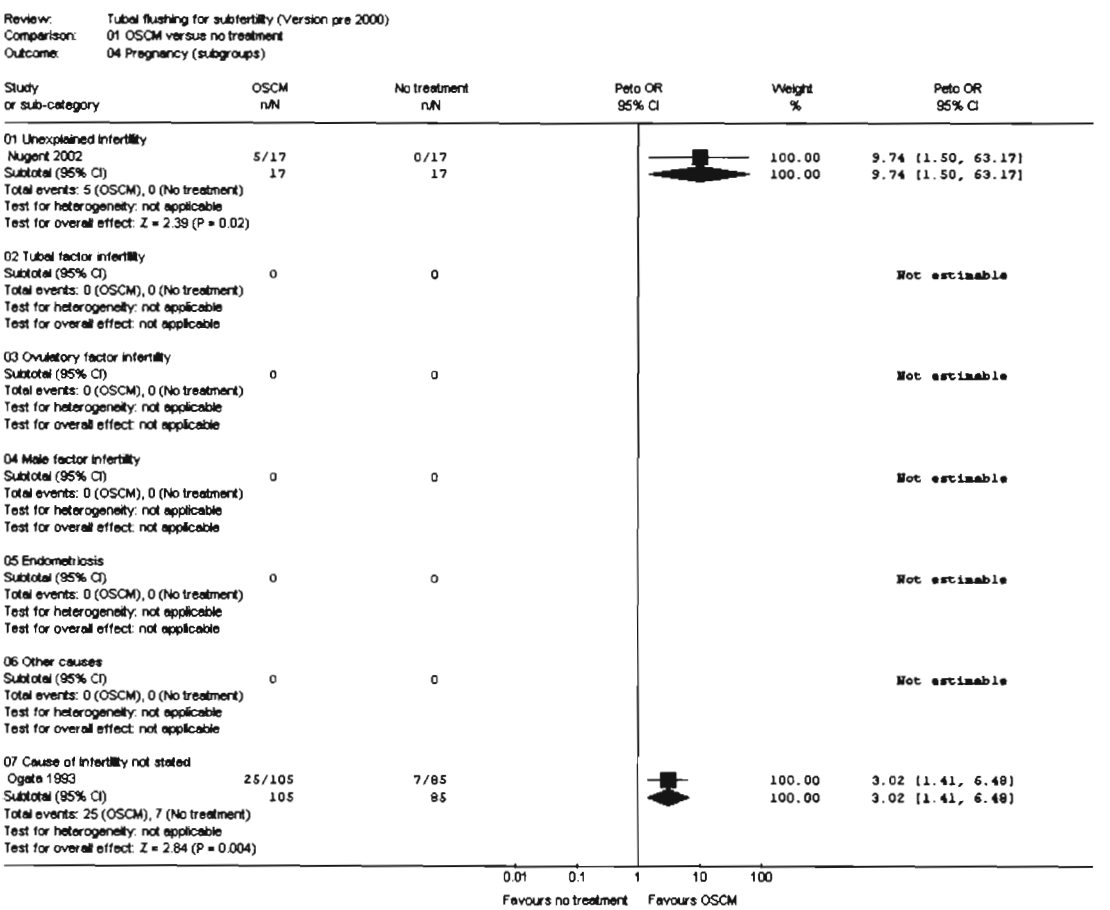


Figure 5.3: Meta-analysis Graph for Effect of OSCM versus WSCM on Live Birth

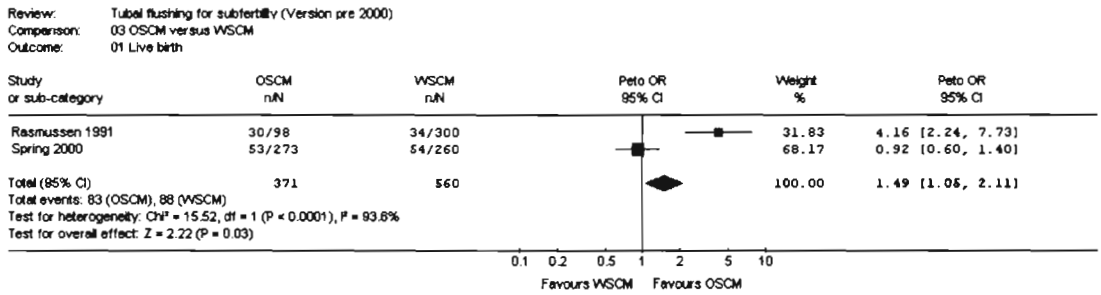


Figure 5.4: Meta-analysis Graph for Effect of OSCM versus WSCM on Live Birth Amongst Subgroups

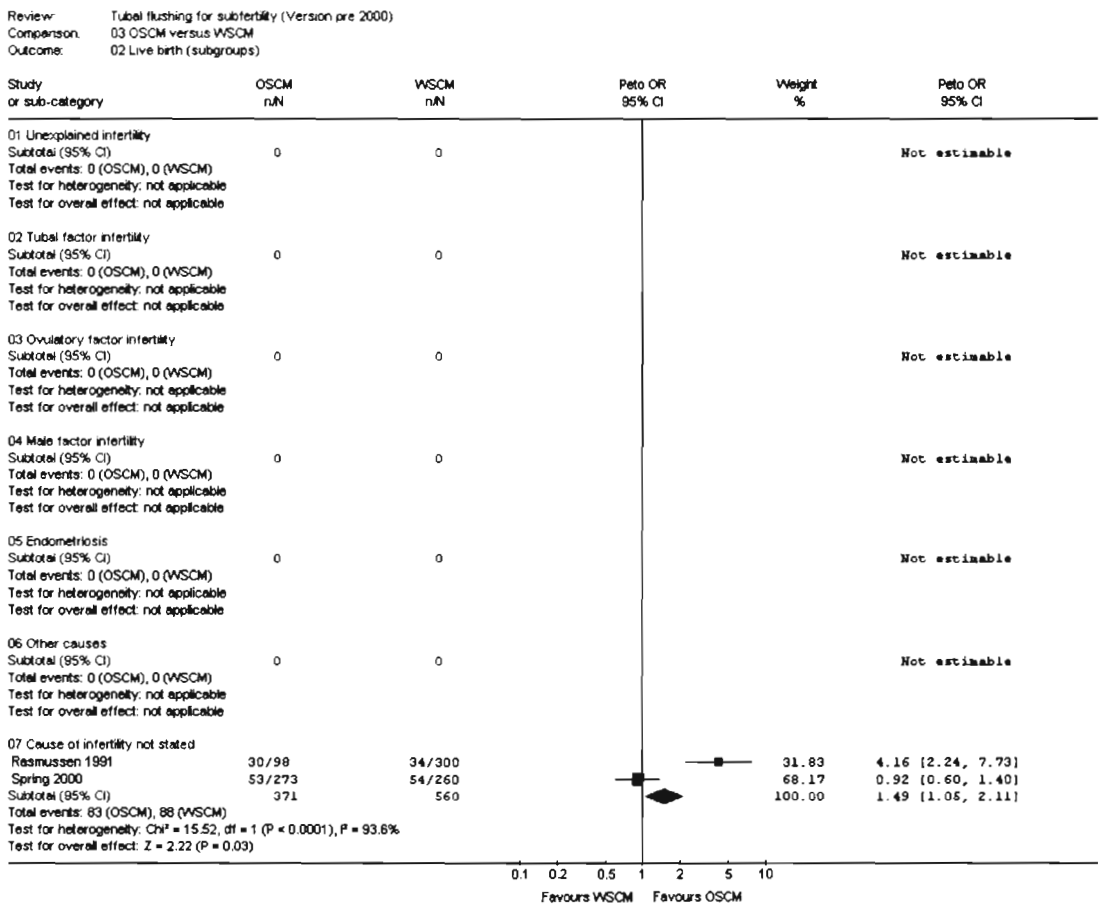


Figure 5.5: Meta-analysis Graph for Effect of OSCM versus WSCM on Pregnancy

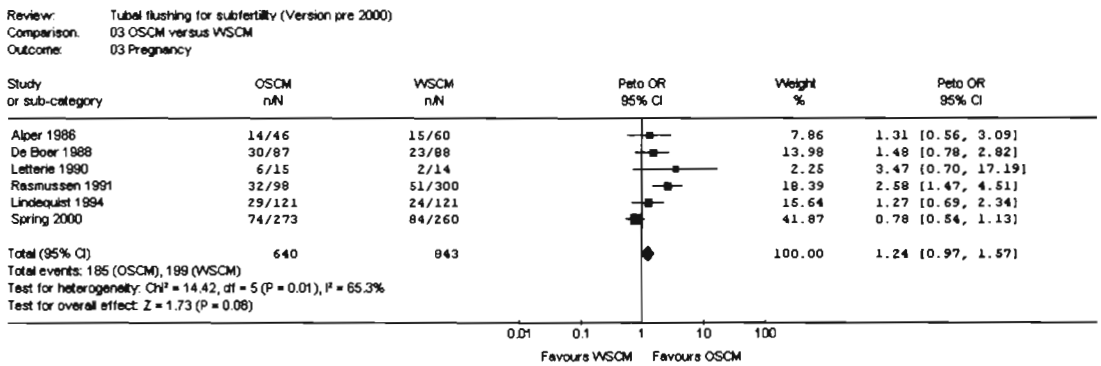


Figure 5.6: Meta-analysis Graph for Effect of OSCM versus WSCM on Pregnancy Amongst Subgroups

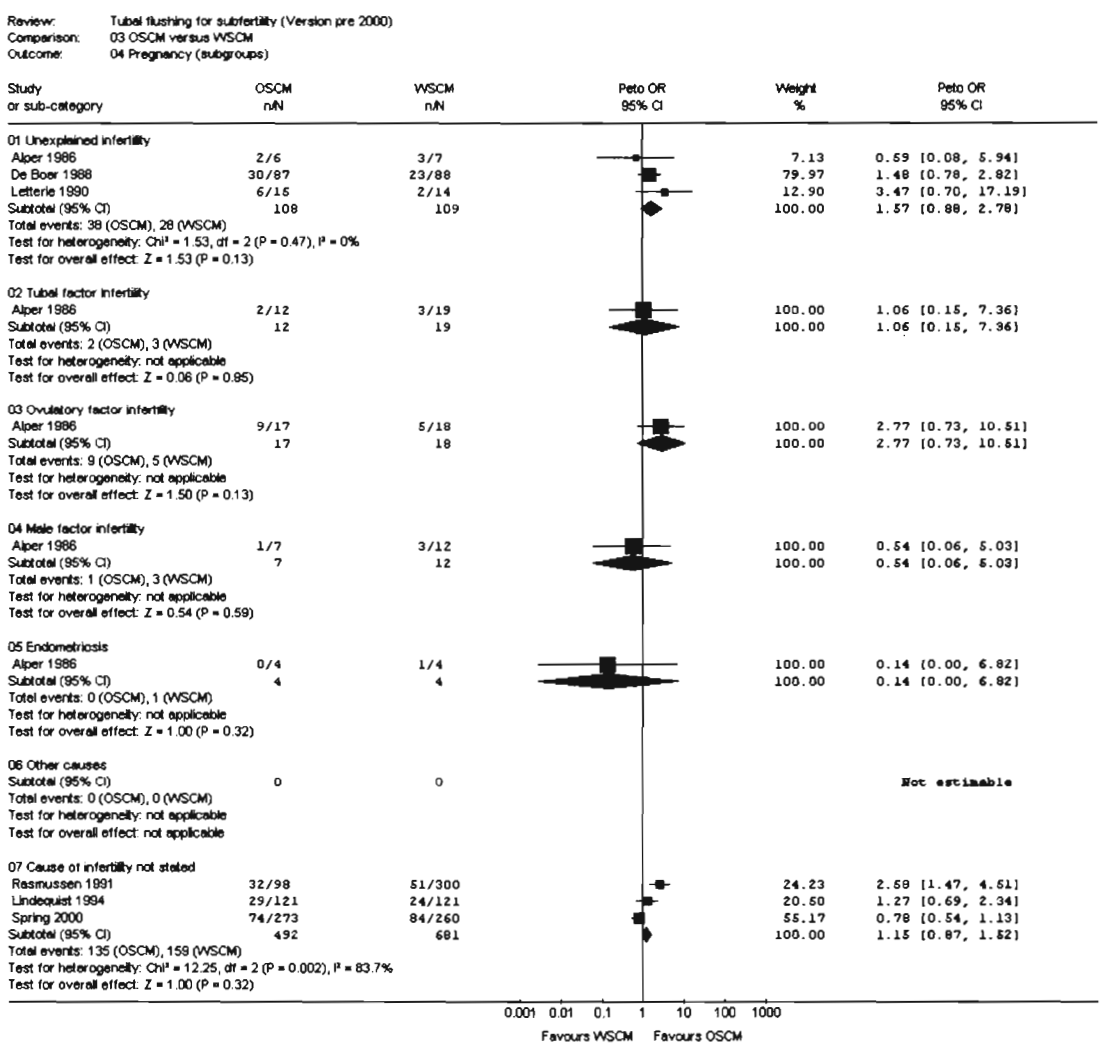


Figure 5.7: Meta-analysis Graph for Effect of OSCM versus WSCM on Miscarriage

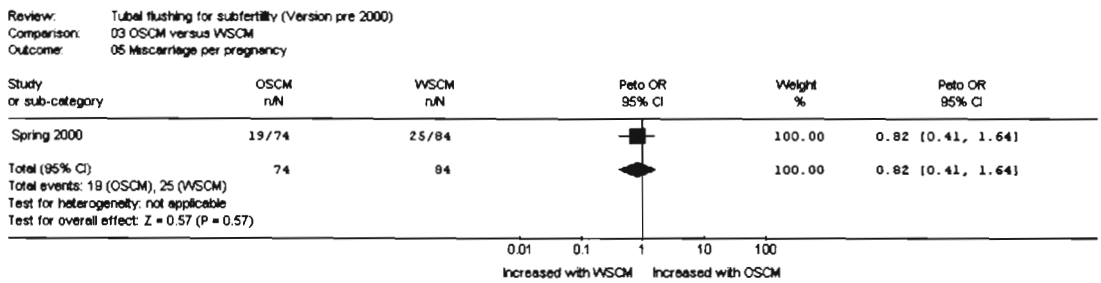


Figure 5.8: Meta-analysis Graph for Effect of OSCM versus WSCM on Ectopic Pregnancy

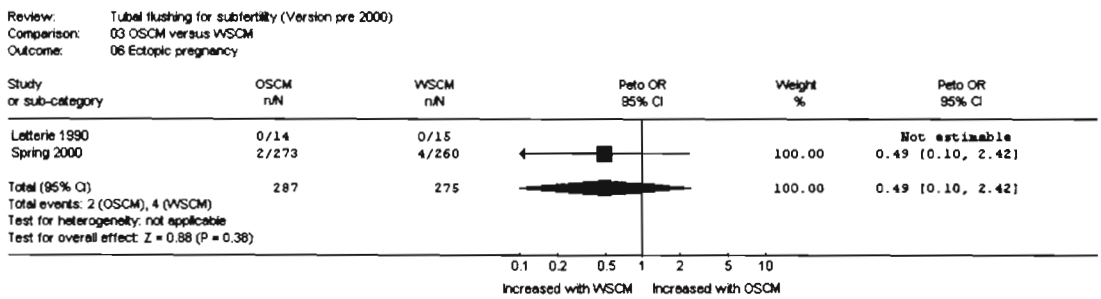


Figure 5.9: Meta-analysis Graph for Effect of OSCM versus WSCM on Pain (continuous variable)

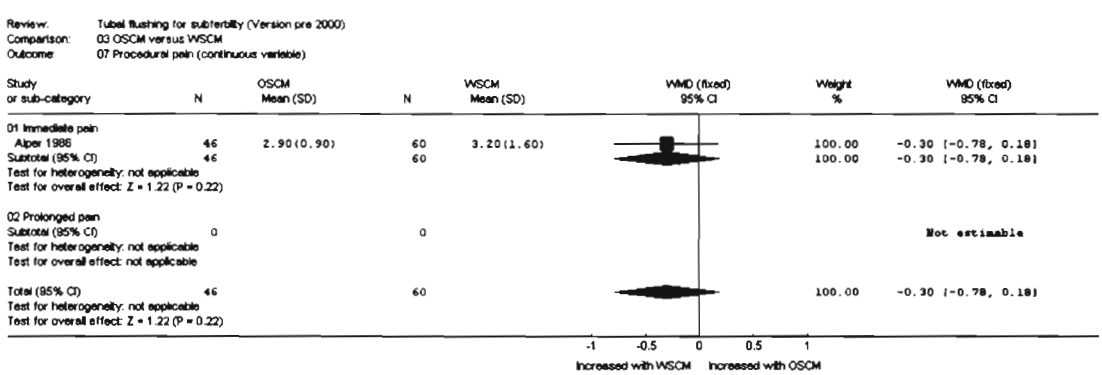


Figure 5.10: Meta-analysis Graph for Effect of OSCM versus WSCM on Pain (dichotomous variable)

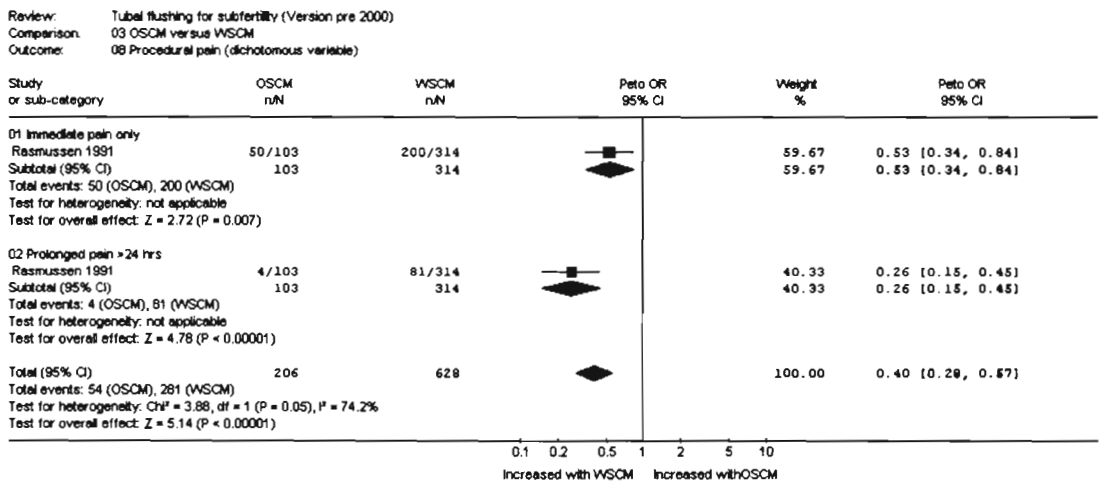


Figure 5.11: Meta-analysis Graph for Effect of OSCM versus WSCM on Complications

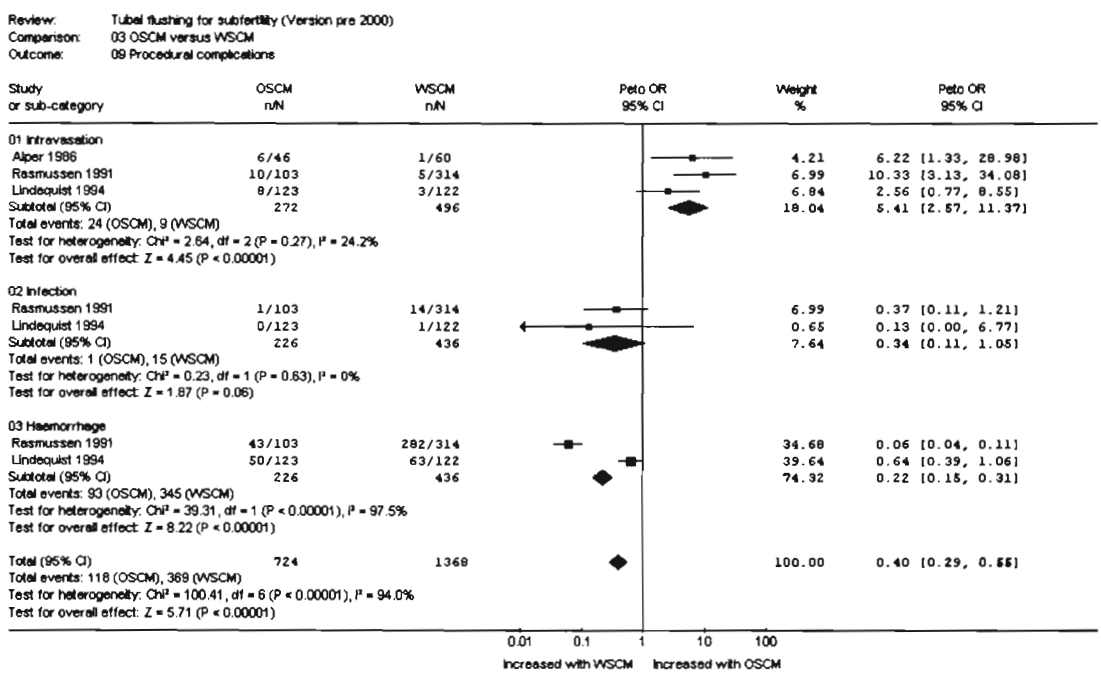


Figure 5.12: Meta-analysis Graph for Effect of OSCM versus WSCM on Image Quality

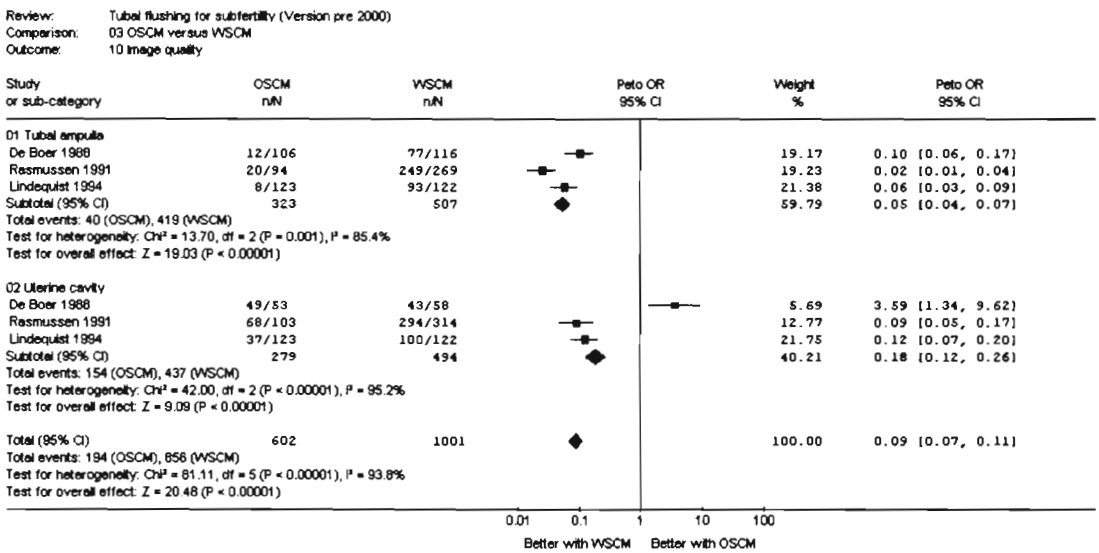


Figure 5.13: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Live Birth

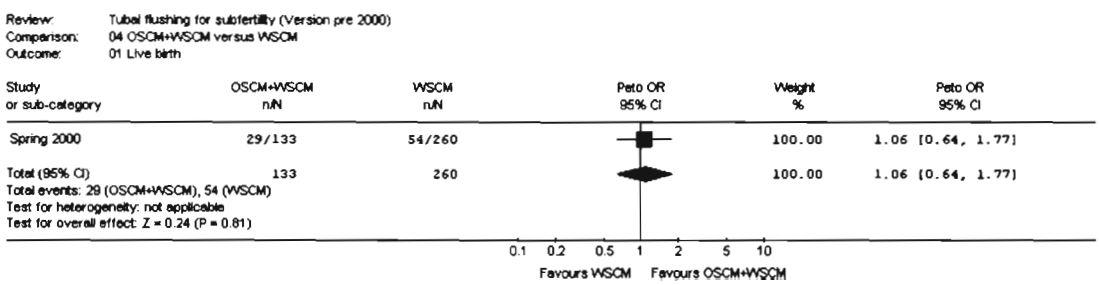


Figure 5.14: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Live Birth Amongst Subgroups

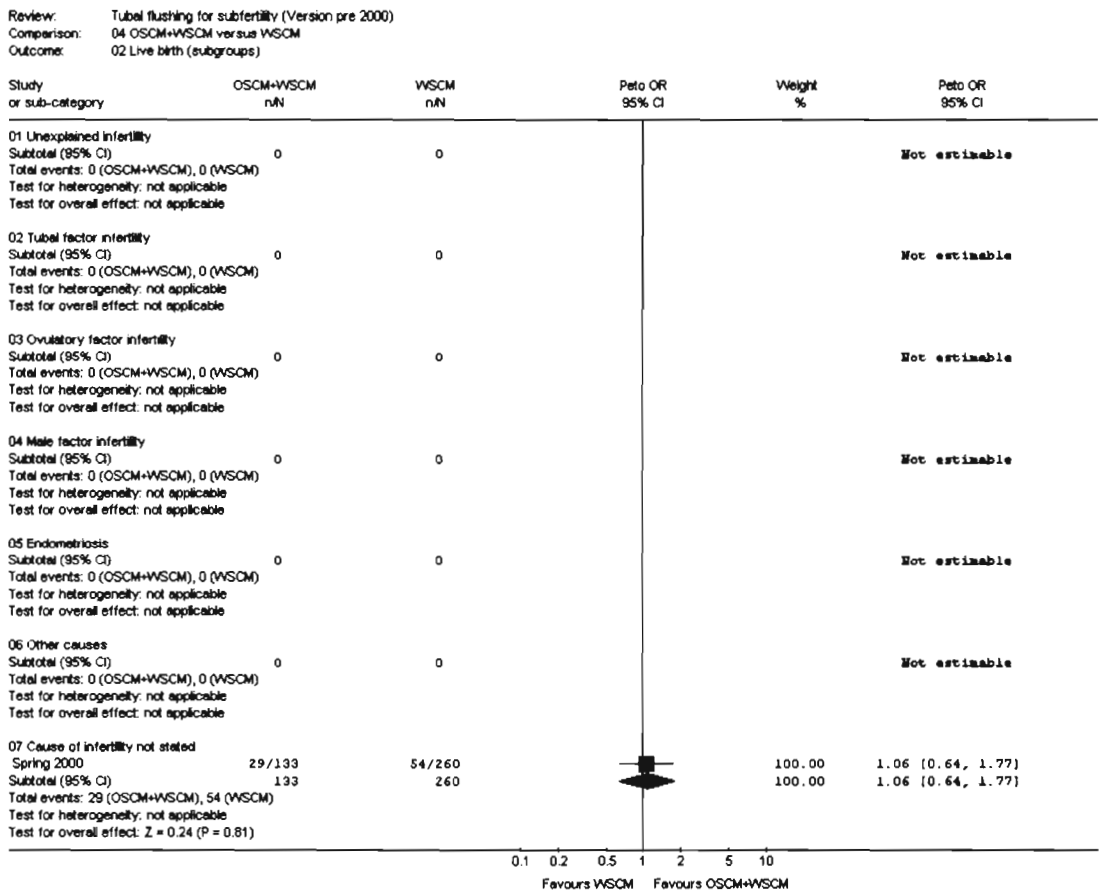


Figure 5.15: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Pregnancy

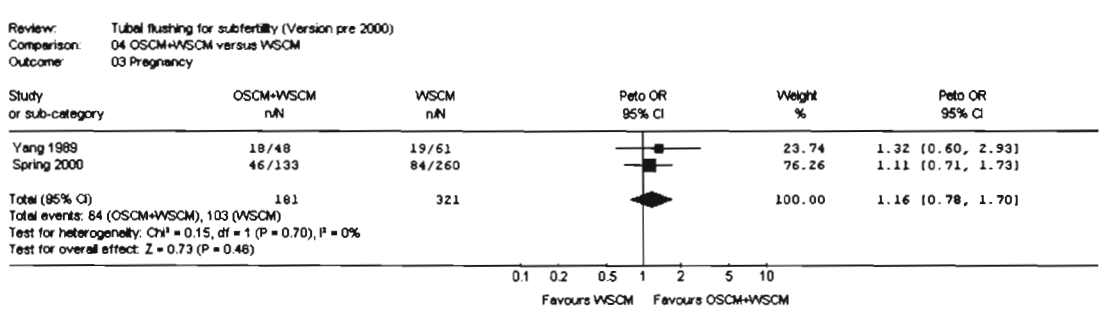


Figure 5.16: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Pregnancy Amongst Subgroups

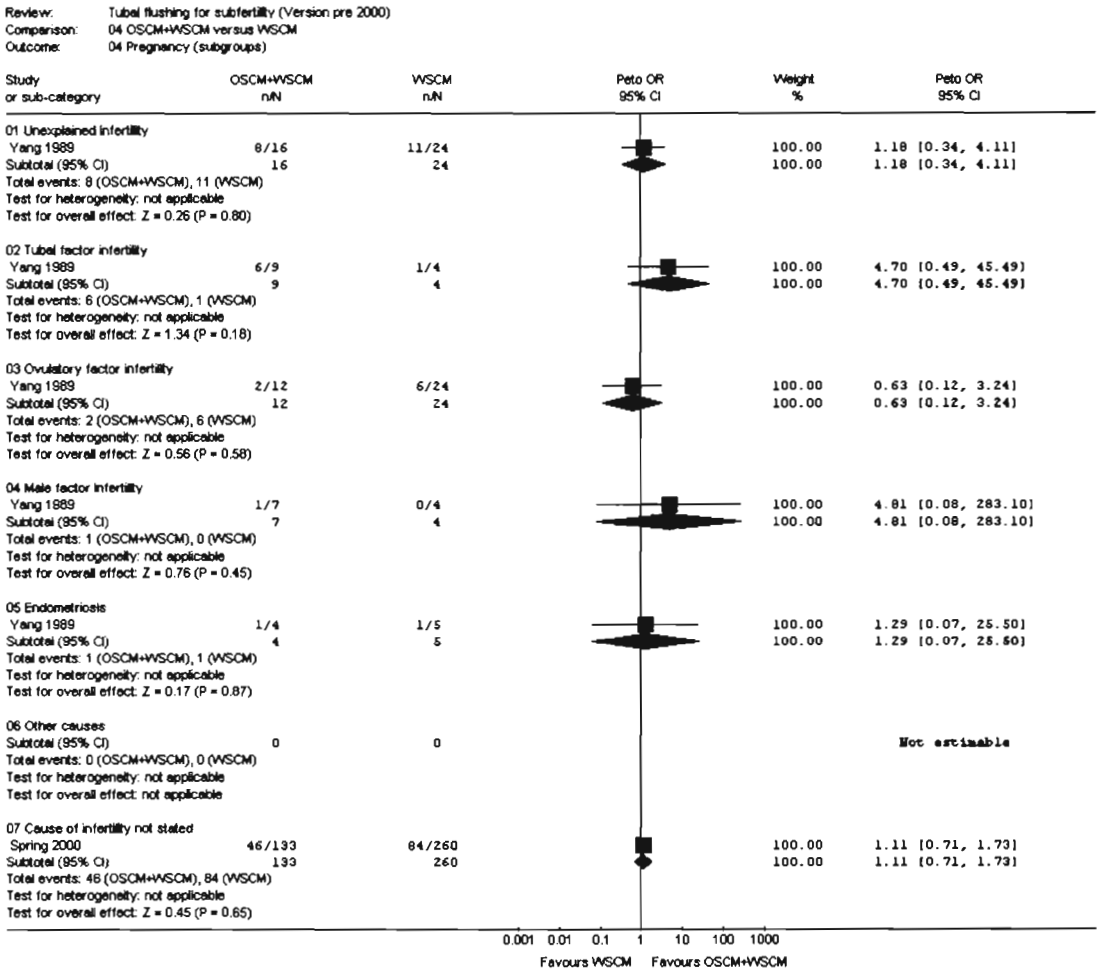


Figure 5.17: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Miscarriage

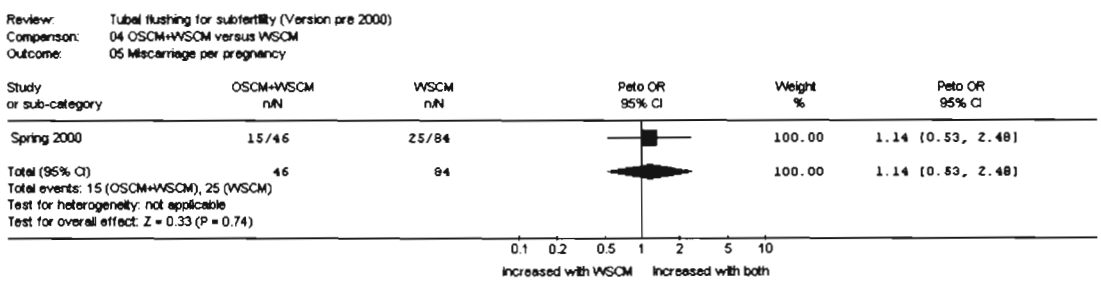
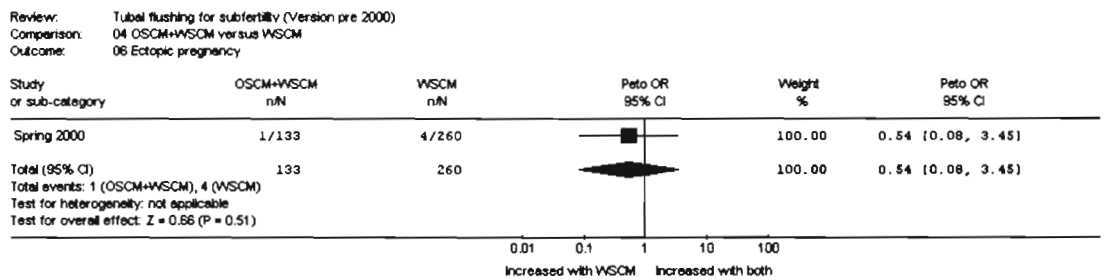


Figure 5.18: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Ectopic Pregnancy



SECTION III: CURRENT PRACTICE AND PRIOR BELIEFS

CHAPTER 6: BAYESIAN STUDY OF LIPIODOL FLUSHING – SURVEY OF AUSTRALASIAN FERTILITY SPECIALISTS

6.1 Introduction

Whether clinicians adopt a new treatment or technique depends not only on the strength or statistical significance of data, but also on what seems rational. If a statistically significant result is not consistent with our prior belief, we will often rationalise this by saying, "Ah, but the numbers were too small" (although the statistics takes account of this), or, "But the methodology was flawed" (then proceed to look for flaws)! Consciously or unconsciously, our prior beliefs dictate how evidence influences our practice.

It is ethical to commence a clinical trial when there is collective equipoise amongst clinicians about the effectiveness of available treatments. Prior beliefs originate from direct evidence (clinical trials) or indirect evidence (laboratory studies, epidemiological plausibility, extrapolation from similar treatments). Surveys eliciting dichotomous "yes" and "no" responses to a question about effectiveness of a treatment are limited because clinicians' beliefs usually amount to more than simply "I believe it is effective (or not)". They might believe the treatment to be highly or marginally beneficial (or harmful). Clinicians will express varying degrees of uncertainty – some might be more certain than the evidence apparently warrants; others may be so uncertain that they believe the treatment may, in due course, turn out to be either greatly beneficial or harmful. Thus formal measures of prior beliefs (that allow respondents the opportunity to signal the magnitude of expected effects and the relative probabilities of different effect sizes) can provide a more accurate picture than dichotomous responses. Published examples of collecting such information are sparse in medicine generally,[133] including gynaecology and obstetrics.[134]

Study data and anecdotal evidence to suggest that a diagnostic test for tubal patency could in itself enhance fertility have existed for over half a century (see Chapter 4). In 2001, some data from randomised trials were available to

suggest that women having their fallopian tubes flushed with oil soluble contrast media (OSCM), such as lipiodol, experienced a fertility benefit, that OSCM might have more of a fertility enhancing effect than water soluble contrast media (WSCM), and that women with unexplained infertility might benefit most from flushing with OSCM.[117]

6.2 Objectives

Lipiodol flushing has not been a widely accepted infertility treatment in current routine practice. The aim of this study was to assess the prior beliefs of clinicians concerning lipiodol flushing at the end of 2001, and to integrate these beliefs with evidence available at that time and with evidence accrued subsequently.

6.3 Methodology

Questionnaires were sent to the clinical directors of 60 Australasian fertility clinics (53 in Australia and 7 in New Zealand) in December 2001, a similar distribution to that used successfully by our group in a previous fertility questionnaire survey.[135] These clinics included 37 primary units recognised by the Reproductive Technology Accreditation Committee (RTAC) and 23 other units which were either associate, satellite or transport units. The clinical directors of these units were asked to distribute the surveys to all the fertility specialists working at the clinic, thus it was unknown how many surveys were actually distributed and which clinicians did not respond. Each clinic was sent one of two types of survey, allocated by non-blocked randomisation (the flip of a coin). The short form (Survey 1) took a simple form, asking clinicians to estimate four values (Appendix 6.1), and this survey was sent to 26 clinics. The long form (Survey 2) was more complex, involving a graphical representation of the clinicians' beliefs, and a feedback form on how easy this questionnaire was to understand and use. This survey type was sent to 34 clinics.

The long form (Survey 2) questionnaire was as follows:

- Clinicians were asked to 'plot' their beliefs on the true effect of lipiodol flushing on the relative probability (RP) of conception over 6 months. The vertical axis was RP of conception – possibilities for the true RP at 6 months after a lipiodol flushing procedure, with values ranging from 5.5 to 0.35, 1.0 representing no treatment benefit or harm. The horizontal axis was an unmarked scale from impossible on the left side to increasingly likely to the right of the page. For each possible RP, the clinician was asked to mark a line on the horizontal scale, representing how relatively likely this possibility is to be 'the truth'. Thus clinicians drew a 'shape' of their beliefs.
- Clinicians were asked to consider the potential side-effects and disadvantages of lipiodol flushing for unexplained infertility, and to decide what increase in the true probability of conception within 6 months would 'balance out' these risks or inconveniences. Clinicians were asked to mark this point on a line labelled Ratio of true chance of conception with lipiodol to baseline chance, with values ranging from 1.0 to 3.0.
- Clinicians were given an example using 'tomorrow's weather' to demonstrate how to plot their beliefs about tubal flushing. There were numbered bullet points which explained that each of the four horizontal lines in the example represented a possibility for tomorrow's true weather (for example mainly rain, showers, dry/cloudy or dry/sunny), and asked clinicians to draw a vertical line through each option to indicate how relatively likely to be true that weather condition seems. Clinicians were told to start with the possibility they considered most likely, making this mark further to the right, and to mark the other possibilities relative to it. The following page gave a similar example for lipiodol flushing (Figure 6.1).

The second part of the long form survey was a feedback sheet (Appendix 6.2).

6.4 Results

Data were extracted from the returned surveys in March 2002. Nineteen specialists (14 Australian and 5 New Zealand) from 12 fertility clinics (10 in Australia, 2 in New Zealand) returned complete or incomplete surveys. Eleven of the returned questionnaires were the short forms (from 7 specialists in Australian primary units, 1 specialist in an Australian associate unit, 3 specialists in a New Zealand primary unit), and the remaining eight were long forms (from 4 specialists in Australian primary units, 2 specialists in an Australian satellite unit, 2 specialists in a New Zealand primary unit).

The responses to the short forms (Survey 1) are summarised in Table 6.1. Eleven short forms (from 6 of the 26 centres to which the forms were sent for distribution amongst fertility specialists) were returned. The best estimates of the RP of pregnancy with lipiodol flushing over 6 months ranged from 1.0 (no increase in the probability of pregnancy with the treatment) to 1.5, with a median value of 1.2. The 5% credible limit of this estimate had a median of 0.9 (range 0.8 to 1.1), and the 95% credible limit had a median of 1.5 (range 1.1 to 2.5). Clinicians believed that the RP of pregnancy which would justify lipiodol flushing as a standard treatment for unexplained infertility (the 'worthwhile value') lay in the range of 1.1 to 2.0, median 1.2. For 7 out of the 11 respondents, their worthwhile value was greater than their best estimate of the RP of pregnancy, suggesting that 7 respondents would not give the treatment, and 4 of these would be confident this is the correct choice. The best estimate of effect and the worthwhile value were equal in 2 out of 11 surveys. The best estimate of effect was greater than the worthwhile value in 2 surveys, although only one respondent would logically have been confident in giving the treatment, while the other respondent was approximately in equipoise.

Eight long forms (Survey 2) were returned (from 6 of the 34 centres to which the forms were sent for distribution amongst fertility specialists). No attempt was made to provide a prior on one form, and on two forms the information provided was inadequate to form a prior (probably because the respondents

did not fully understand the task – one respondent partially filled in an example, but placed only one mark on the actual elicitation form; the other respondent apparently found the RP range provided on the elicitation form did not extend sufficiently high to fully capture the prior – although to most experts a RP of pregnancy of 5.5 or higher from lipiodol flushing seemed completely implausible). Figure 6.2 shows the 'shapes' of the clinicians' beliefs about the true effect of tubal flushing with lipiodol on the relative probability of conception. Of the five distributions elicited, calculating the mean and the 2.5% and 97.5% centiles suggested that one respondent was in equipoise (Doctor 1), two would not give the treatment (Doctors 2 and 3), and two would give the treatment (Doctors 4 and 5) – but no-one would be confident in their choice. For 3 of these 5 respondents the verbal description of their beliefs did not fit well with the elicited prior – all were similar, the respondents used the option "Probably slightly beneficial but could be harmful or moderately beneficial" to describe their beliefs, but the elicited distribution would be better described by another, more optimistic (but similarly uncertain) option "Almost certainly beneficial, but not sure how much", or "Unsure whether beneficial or not, but not harmful".

Table 6.2 summarises the responses from the rest of the survey. When asked to mark a point on the line "Ratio of true chance of conception with lipiodol to baseline chance", where they believed would counteract the potential side effects and disadvantages of the treatment, clinicians marked values between 1.2 and 2.3, with a median value of 1.4. (The similar question in Survey 1 identifying the 'worthwhile value', the RP which would justify lipiodol flushing as routine treatment for unexplained infertility, gave a median response 1.2 and range 1.1 to 2.0.) Regarding the true mean effect of lipiodol flushing on the RP of conception over six months, 3 out of the 8 respondents marked the response "Probably slightly beneficial but could be harmful or moderately beneficial", and 2 out of the 8 identified the statement "Almost certainly moderately beneficial" as that closest to their own beliefs. One thought that the effect was "Almost certainly slightly beneficial", and another believed that the effect was "Almost certainly small, could be harmful or

beneficial". The sources of these beliefs were identified as being personal clinical experience, and other studies or literature.

The other part of Survey 2 was the feedback form, and the results of this are detailed in Table 6.3. In general, clinicians indicated that they found the form of Survey 2 easy to understand and use, although the small number of respondents makes it difficult to generalise the results. Most clinicians found the examples given to be "helpful" or "just worth having", and most found the explanatory bullet points to be helpful. Five out of 8 respondents said that they were not troubled by the lack of a scale on the lines they were asked to mark, and the response to the question "Would you have found it helpful if we had explained that we will calculate a scale that makes the total probability add up to one?" was equivocal.

6.5 Discussion

This was the first published report of formal measures of experts' prior beliefs about a fertility treatment.[136] Amongst the 16 (from total 19) respondents for whom a Bayesian prior could be calculated, only one fertility specialist was confident to offer lipiodol flushing to women with unexplained infertility, 4 were confident not to offer lipiodol and the remaining 11 were approximately in equipoise. This finding, of collective and reasonably balanced uncertainty, further justified my randomised trial (Chapter 8).

There were obvious weaknesses in this study. Although the precise response rate was unknown owing to an uncertain number of specialists receiving the survey, the number of specialists returning forms was very low, meaning that firm conclusions about the population of experts' beliefs from this sample would be inappropriate. There is helpful information for the methodological challenges of future similar studies. Twice as many usable short forms were returned, which is suggestive (but the numbers were too small to conclude) that experts are more likely to return a usable short form. Three of the 8 long forms returned were unusable, which suggests there is a considerable

problem with understanding (or following) the instructions, even when experts are willing to spend the required time. In 3 out of 5 usable long forms, the verbal description did not match the elicited belief, which strongly suggests that respondents have problems in translating their beliefs onto the form. Finally, responses on the short form seem to indicate higher levels of certainty (especially about the upper limit of effectiveness) than responses on the long form, which seemed to be a result of the directly elicited upper 95% credible limits being in general much closer to the best guess than were the calculated upper limits from the long form. There was no indication that the long form-elicited responses were under-confident, and the data now suggests quite high effectiveness (see Chapter 8) – higher than the upper limits given on the short forms. Thus it seems likely that the short form responses are too confident.

At the time of the survey, the available RCT data gave a best estimate RP of 2.9 for pregnancy following lipiodol flushing versus no treatment (where the causes for infertility were not specified)[118] – a higher RP than any of the best estimates of respondents, suggesting their best estimates were conservative. Since the survey, inclusion of more recent RCT data gives a best estimate the RP of 2.1 for unexplained infertility and RP 2.7 for all causes of infertility pooled.[119] It will be interesting to see, over the next few years, since the worthwhile value stated by almost every fertility specialist surveyed is now exceeded by the current best available evidence for unexplained infertility (and far exceeded in every case for mild endometriosis with otherwise unexplained infertility[119]), whether lipiodol flushing does gain widespread acceptance as a routine fertility treatment.

Implementation of a new technique in clinical practice is a complex process, depending on numerous factors both favourable and unfavourable. It remains to be seen not only whether beliefs will be changed as new evidence emerges, but whether this will then lead to a change in practice. The reality is that implementation of a new technique into clinical practice depends on factors other than proof of effectiveness and safety. Other factors that could influence the widespread uptake of lipiodol flushing include:

- the availability of clinicians with the expertise to offer lipiodol flushing, as it requires more specialised techniques than standard hysterosalpingograms with WSCM;
- the perception amongst patients that lipiodol treatment is inexpensive, less invasive, carries a very low risk of complications and no significant increased chance of pregnancy compared to other fertility treatments,[137] indeed uptake has been brisk in New Zealand once lipiodol became available as a treatment option;[138]
- that the availability of an inexpensive and effective treatment could pose a financial threat to IVF clinics, which are often run along business lines.

Our beliefs may be changed by new evidence and the two updates of the Cochrane Review of tubal flushing for infertility since this survey was conducted are highly relevant.[119, 139]

Appendix 6.1: The Short Form (Survey 1)

Survey of Prior Beliefs Concerning Lipiodol Flushing

Please give your best estimate of the relative probability (RP) of pregnancy in the 6 months following a lipiodol hysterosalpingogram, compared to the 'no intervention' probability of pregnancy being 1.0 (for a woman you believe to have average chance of conceiving amongst all couples with unexplained infertility who you see in your practice).

RP =

- Please give confidence limits to this estimate (your 95% confidence interval).

Lower limit =

Upper limit =

- What is the minimum relative probability of pregnancy following a lipiodol hysterosalpingogram that would justify, in your opinion, this being used as a standard treatment for some women with unexplained infertility?

RP =

Appendix 6.2: The Long Form (Survey 2 Feedback Form)

Feedback Form

1. Please mark the single response closest to your beliefs about the true mean effect on Relative Probability on conception over 6 months, of tubal flushing with lipiodol.
   a) Almost certainly harmful or of no benefit
   b) Almost certainly small (up to 25% decrease or increase), could be harmful or beneficial
   c) Almost certainly small or moderate (up to 50% decrease or increase), could be harmful or beneficial
   d) Almost certainly slightly beneficial (around 25% increase)
   e) Almost certainly moderately beneficial (around 50% increase)
   f) Almost certainly highly beneficial (around 100% or greater increase)
   g) Almost certainly beneficial, but not sure how much
   h) Unsure whether beneficial or not, but not harmful (zero or more increase)
   i) Probably slightly beneficial, but could be harmful or moderately beneficial.

   Please say briefly what has influenced these beliefs.

2. Did you find it difficult to use the concept of 'the truth' about the efficacy of treatment? Yes/No
   How might the concept be better explained?

3. How would you describe the 'weather' example?

   Very helpful / helpful / just worth having / not worth having

4. How would you describe the example for tubal flushing?

   Very helpful / helpful / just worth having / not worth having

   How could we improve the example(s)?

5. Please ring any of the numbered 'bullet' points which explained the 'weather' example that you found helpful
   1 2 3 4 5

6. Please ring any of these same numbered 'bullet' points that you didn't find helpful
   1 2 3 4 5

7. Were you troubled by the lack of a scale on the lines you were asked to mark? (*referring to the plot of clinicians' beliefs*)

   Yes / At first, but I soon got the hang of it / Not at all

8. Would you have found it helpful if we had explained that we will calculate a scale that makes the total probability add up to one?

   Yes / No

Table 6.1: Summary of Survey 1 (Short Form) Responses

Clinic location	Best guess of the relative probability of pregnancy	Lower limit	Upper limit	Relative probability which justifies flushing as routine treatment ('worthwhile value')
New Zealand	1.0	0.9	1.5	1.2
New South Wales	1.0	0.9	1.1	2.0
New South Wales	1.0	0.9	1.1	2.0
Tasmania	1.0	0.9	1.1	1.2
New South Wales	1.05	0.85	1.15	1.2
New South Wales	1.2	0.8	1.5	1.2
South Australia	1.3	0.9	1.5	1.2
New South Wales	1.3	0.8	2.0	1.8
New Zealand	1.5	1.0	2.5	2.0
Western Australia	1.5	0.9	1.75	1.5
New South Wales	1.5	1.1	1.9	1.1
Range	1.0 – 1.5	0.8 – 1.1	1.1 – 2.5	1.1 – 2.0
Median	1.2	0.9	1.5	1.2

Table 6.2: Summary of Survey 2 (Long Form) Responses

Clinic location	Ratio of true chance of conception with lipiodol to baseline chance	True mean effect on relative probability of conception over 6 months, of tubal flushing with lipiodol – mark single closest response closest to own beliefs	What has influenced these beliefs?
Queensland	1.2	Almost certainly small (up to 25% incr or decr), could be harmful or beneficial.	Not answered.
South Australia	1.2	Almost certainly slightly beneficial (around 25% increase).	Clinical experience.
New South Wales	1.4	Probably slightly beneficial, but could be harmful or moderately beneficial.	Study/literature.
New Zealand	1.4	Almost certainly moderately beneficial (around 50% increase).	Literature, unspecified.
Queensland	1.8	Probably slightly beneficial, but could be harmful or moderately beneficial.	Not answered.
New Zealand	2.0	Probably slightly beneficial, but could be harmful or moderately beneficial.	Other studies, unspecified.
Queensland	2.3	Almost certainly moderately beneficial (around 50% increase).	Clinical experience.
Victoria	Not answered	Not answered.	Study/literature.
Range	1.2 – 2.3		
Median	1.4		

Table 6.3: Summary of Survey 2 (Long Form) Feedback Responses

Did you find it difficult to use the concept of 'the truth' about the efficacy of treatment?	YES NO	3 5
How would you describe the 'weather' example?	VERY HELPFUL HELPFUL JUST WORTH HAVING NOT WORTH HAVING NOT ANSWERED	0 4 3 0 1
How would you describe the tubal flushing example?	VERY HELPFUL HELPFUL JUST WORTH HAVING NOT WORTH HAVING NOT ANSWERED	0 6 2 0 0
Which of the numbered bullet points that explained the weather example were helpful?	ALL POINTS HELPFUL NO. 1 HELPFUL ONLY NONE HELPFUL NOT ANSWERED	5 1 0 2
Which of these points were not helpful?	ALL POINTS NO's 2, 3, 4,5 NO POINTS NOT ANSWERED	1 1 5 1
Were you troubled by the lack of a scale on the lines you were asked to mark?	YES AT FIRST NOT AT ALL	2 1 5
Would you have found it helpful if we had explained that we will calculate a scale that makes the total probability add up to one?	YES NO	4 4

Figure 6.1: Hypothetical example of how to fill in Survey 2 (long form) to display prior beliefs for the effectiveness of lipiodol flushing for unexplained infertility

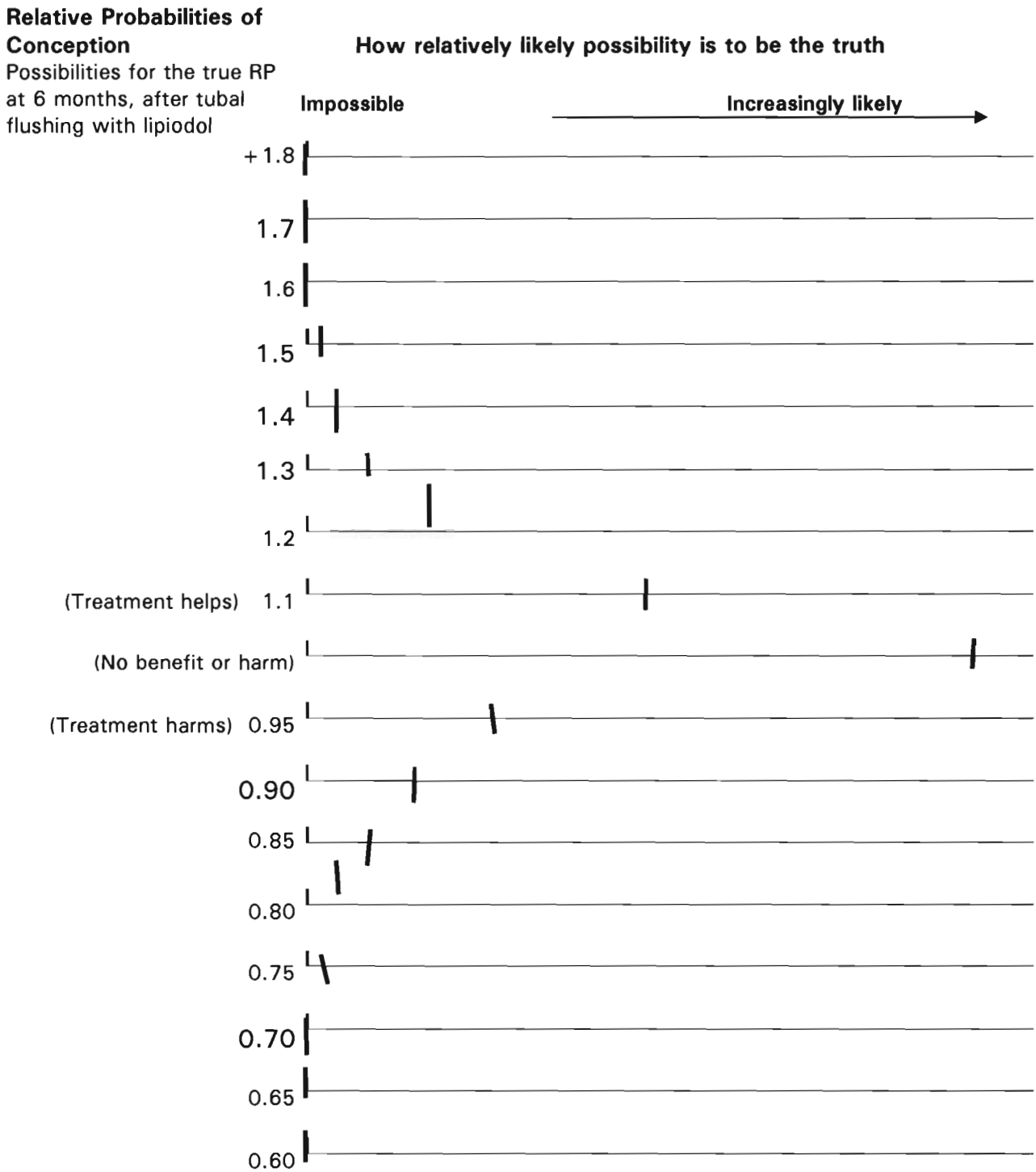
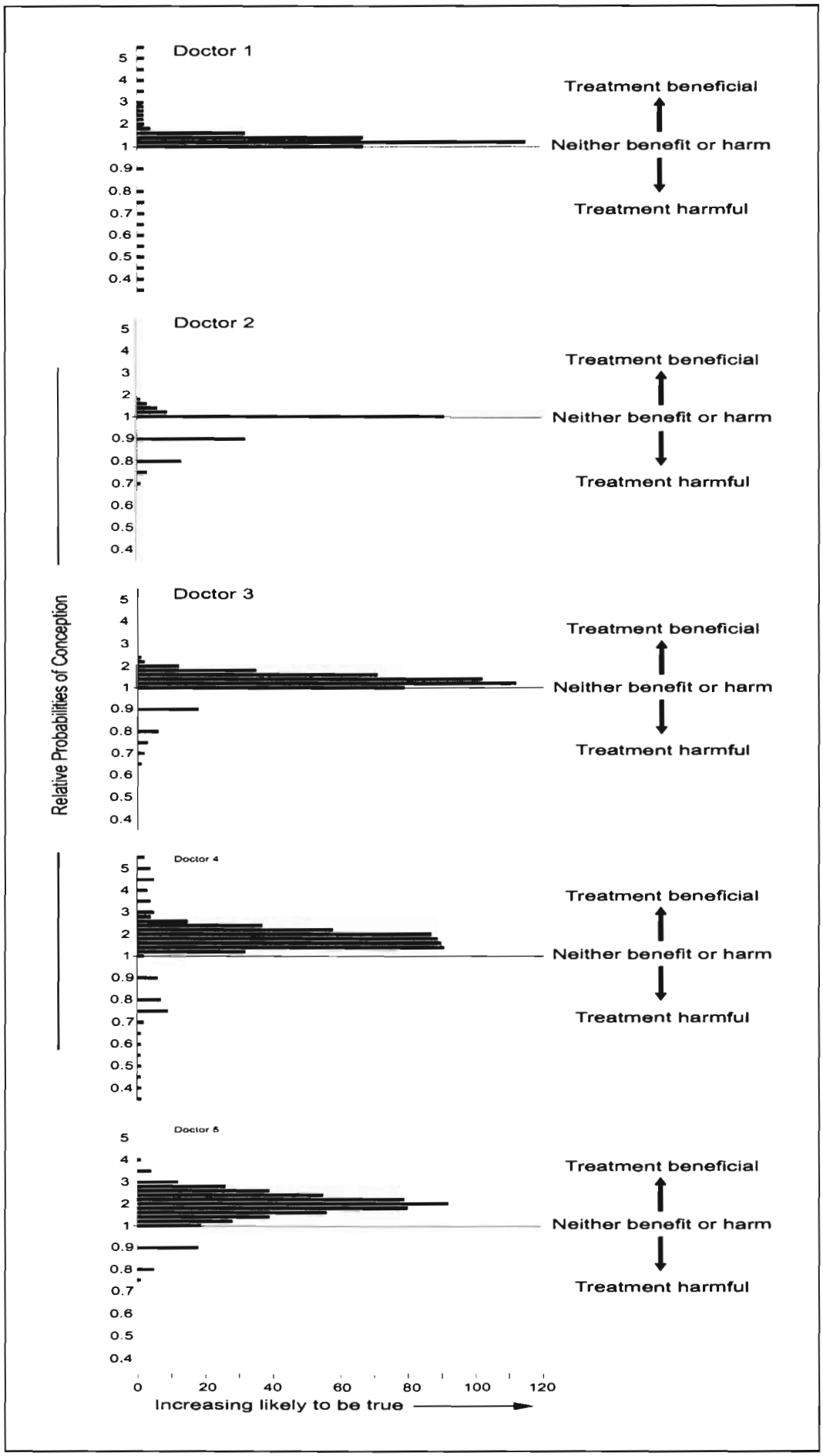


Figure 6.2: Graphical representation of numerical prior beliefs of the effectiveness of lipiodol flushing for unexplained infertility from the long form survey



SECTION IV: MOUSE STUDY

CHAPTER 7: MURINE RANDOMISED TRIAL TO ASSESS THE UTERINE LEUKOCYTE EFFECT OF LIPIODOL

7.1 Introduction

The exact mechanism by which an HSG with an oil soluble contrast medium (OSCM) such as lipiodol enhances fertility is not yet known. Previously the most plausible theories included: (i) dislodging non-occlusive but pregnancy hindering debris from otherwise undamaged tubes;[117] (ii) an effect on the intraperitoneal environment, such as a change in production of cytokines by peritoneal macrophages or an inhibition of sperm phagocytosis by peritoneal mast cells or macrophages.[130]

Although lipiodol improves fertility for women with unexplained infertility,[137] the finding that it is particularly beneficial for women with endometriosis[140] suggests a possible immuno-biological mechanism, rather than simply a tubal flushing phenomenon. Women have had successful pregnancies following lipiodol infusion where the lipiodol has not been seen to infiltrate the fallopian tubes or to reach the peritoneal cavity.[140] Although other possible mechanisms involving the lower genital tract might be contributory, such as improvement of cervical mucous,[117, 141] this raises the possibility of an endometrial implantation-enhancing mechanism.

The female reproductive tract, especially the endometrium and decidua, in humans and mice is rich in leukocytes including natural killer (NK) cells, macrophages, dendritic cells (DCs) and T cells.[142] During pregnancy, the maternal immune system functions to resist microbial and viral infections without eliciting an immune response against the semi-allogeneic fetus. This requires a careful regulation of the endometrial leukocyte numbers and their functions. Macrophages and DCs are phagocytes and antigen presenting cells that are potentially capable of taking up lipiodol and stimulating immune responses.

Macrophages make up approximately 20% of the total leukocyte population of the uterine endometrium and decidua in humans[143] and mice.[144] A reduction in macrophage numbers is associated with reduced fertility in mice:[145] the osteopetrotic mouse, which is homozygous for a null mutation of the macrophage growth factor, colony-stimulating factor (CSF)-1, presents with a deficiency in macrophage numbers in the reproductive tract, as well as reduced fertility.[145]

Dendritic cells are the most potent antigen presenting cells and are essential for the initiation of primary T cell immune responses.[146-148] In the peripheral tissues, immature DCs with high phagocytic capability function as 'sentinels' of the immune system and capture antigens when pathogens or foreign materials are present.[148] The antigen and various cytokines act as maturation signals for the DCs, with mature DCs characterised by increased expression of major histocompatability complex (MHC) molecules[149] and co-stimulatory molecules such as cluster determinant (CD) 40 and CD86.[148] The mature DCs migrate to T cell rich lymph nodes, where they present the antigens to T cells, initiating immune responses.[148] Dendritic cells make up approximately 1-2% of the total cell population in the endometrium of non-pregnant women[150] and mice,[151] as well as during murine gestation.[151] Dendritic cells have been shown to have two contrary roles: to mediate T cell immunostimulatory response or alternatively to induce immunosuppression of T cells.[148]

7.2 Objectives

Lipiodol may alter the cells of the uterine immune system, a mechanism that could influence implantation. This study was performed to investigate possible uterine changes that might underlie the fertility-enhancing action of lipiodol. The hypothesis tested was that infusion of lipiodol into the murine female reproductive tract alters the population of the uterine leukocytes.

7.3 Methodology

This study was performed with the approval of the University of Auckland Animal Ethics Committee.

Study Design

For each experiment 15 female outbred Swiss white mice, aged 7-14 weeks and in pro-estrus were randomised to one of three intervention groups: Group 1 had infusion of the reproductive tract with lipiodol, Group 2 had infusion of the reproductive tract with saline, Group 3 had a sham treatment (so anaesthesia was induced but no flushing procedure was undertaken – no intervention). Four individual repeat experiments were conducted such that a total of 60 mice were studied. The randomisation was computer-generated and allocation concealment was maintained by sequentially numbered opaque envelopes until the mice were anaesthetised immediately prior to the planned intervention, at which time the envelope was opened and treatment assigned.

Treatment of Mice and Tissue Collection

General anaesthesia was administered to each mouse by intraperitoneal injection of Avertin (0.3125g 2,2,2 tribromoethanol, 0.63ml tertiary amyl alcohol, 25ml de-ionised water) at dosage of 32μl per gram of body weight. Half the study population was included in a procedure where the intervention fluid was administered by vaginal instillation and intraperitoneal injection ('vaginal instillation protocol' for 30 mice); the other half was included in a laparotomy procedure where the intervention fluid was injected directly into the left uterine horn at laparotomy and injected intraperitoneally at the same time ('laparotomy instillation protocol' for 30 mice).

The vaginal instillation protocol involved insertion of an 18-gauge blunt needle into the vagina and 0.5ml of lipiodol or saline injected while vaginal closure was

maintained to prevent backflow. The laparotomy instillation protocol involved a small incision at the lower left abdomen and injection of 0.1ml of lipiodol or saline into the exposed left uterine horn via a 27-gauge needle. The laparotomy incision was closed with two silk sutures in the skin. This microsurgical procedure was undertaken using a standard operating microscope. All mice that received saline or lipiodol also had 0.1ml of saline or lipiodol respectively injected intraperitoneally. Mice in the sham treatment group were anaesthetised but given no other treatment (vaginal instillation protocol controls) or underwent a sham laparotomy (laparotomy instillation protocol controls). At time points 24 hours, 48 hours, 72 hours, 96 hours and 168 hours following the treatment (to correspond with different phases of the murine estrous cycle, which is typically 4-5 days long), one mouse from each of the three groups (lipiodol, saline or sham) was sacrificed by cervical dislocation and the reproductive tract tissues harvested.

Freezing and Sectioning of Tissue

The reproductive tract tissues were immersed in cryoembedding compound (BioTek, Auckland, New Zealand) frozen in liquid nitrogen, and stored at -80°C until use. Thin sections (5μm) were cut using a cryostat (Leica CM 1900). The sections were collected on glass slides and air-dried for an hour. The tissues were fixed by immersion in cold acetone (-20°C) for 10 minutes, then air-dried for an hour, wrapped in aluminium foil and parafilm then stored at -20°C.

Immunohistochemistry of the Uterine Sections

The primary antibodies used were F4/80 (Serotec, ALS, Auckland, New Zealand), CD45 (Serotec, ALS, Auckland, NZ), CD205 (Serotec, ALS, Auckland, New Zealand) and CD1 (Santa Cruz Biotechnology, Santa Cruz, USA).

One uterine section was used for each animal. Slides of the reproductive tract tissues were thawed, and blocked with 10% Normal Goat Serum (NGS) in Phosphate Buffered Saline-Tween (PBS-Tween) for 10 minutes. After three

washes with PBS-Tween, the slides were incubated with the primary antibodies for an hour at room temperature. F4/80, CD45, CD205 and CD1 were diluted 1:2, 1:250, 1:320 and 1:25, respectively, in 10% NGS in PBS-Tween. Following three washes with PBS-Tween the slides were incubated with biotinylated mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, ALS, Auckland, New Zealand) diluted 1:1000 in 10% NGS for an hour at room temperature. After three more washes with PBS-Tween, the slides were incubated with alkaline phosphotase conjugated streptavidin (Jackson Laboratories, ALS, Auckland, New Zealand) diluted 1:500 in 10% NGS in PBS-Tween for an hour at room temperature.

Slides were then washed three times in PBS-Tween and incubated with Fast-red chromogen (Zymed, San Francisco, USA) containing Levamisole (Sigma-Aldrich, Sydney, Australia) at 5mg/mL for 15 minutes. Slides were then washed in de-ionised water, counterstained with haematoxylin (Gills no 2, Surgipath, USA) for 30 seconds and washed with tap water. A coverslip was then mounted using Aquamount (BDH, Palmerston North, New Zealand). The stained slides were analysed, by an assessor blinded to treatment allocation of the sections, using a light microscope (Leitz Orthoplan, Wetzlar, Germany) and photographed using a Nikon Coolpix 990 digital camera.

Analysis of F4/80 and CD45 Staining

Analysis was performed only for longitudinal sections through the uterine horn. Using a 0.072 mm^2 counting reticule, the number of stained cells present in 5 high power (x40) fields was counted. The fields were evenly spaced along the longitudinal axis of the uterus. The mean number of macrophages in the 5 counting rectangles was then determined and expressed as the number of macrophages or leukocytes per square millimetre. Data from all the animals in a treatment group were used to calculate the mean number of macrophages or leukocytes per square millimetre for each treatment group.

Analysis of Dendritic Cell Staining

The total numbers of $CD205^+$ and $CD1^+$ DCs in the tissue sections were counted. The slides, as well as a measurement scale were scanned onto a computer using Adobe PhotoShop (Version 5.0). The areas of the tissue samples in square millimeters were then determined by setting the measurement scale, and outlining the scanned images using Image J (Public domain software) program. The number of DCs per square millimetre was then determined. Data from all the animals in a treatment group was used to calculate the mean number of macrophages per square millimetre for each treatment group.

Statistical Analysis

A two sample Student's t-test assuming unequal variances (two-tailed) was used to assess differences in the number of positively stained cells between the groups. A single factor ANOVA was used to test whether there was any variation in the number of positively stained cells over the estrous cycle with time of sacrifice.

7.4 Results

For statistical analysis, data from the mice included in the vaginal instillation protocol were pooled with those involved in the laparotomy instillation protocol. Before pooling, the data from the two instillation-groups were compared using Student's t-test assuming unequal variances (two-tailed), and no statistically significant difference was found. The p values from the t-test were 1.0 for macrophages, 0.65 for $CD205^+$ DCs and 0.10 for $CD1^+$ DCs.

Total Leukocytes and Macrophages

No significant differences were found in the total number of $CD45^+$ endometrial leukocytes between the three treatment groups ($p>0.05$). No significant variation in the number of total endometrial leukocytes was found during the estrous cycle for the three treatment groups ($p>0.05$).

$F4/80^+$ macrophages were found evenly distributed throughout the endometrium in most of the sections. There were no significant differences in macrophage numbers (Figure 7.1) between the mice receiving saline treatment and sham treatment ($p=0.08$), between lipiodol and saline treatment ($p=0.27$), or between lipiodol treatment and sham treatment ($p=0.68$). No significant variation in the number of macrophages was found during the estrous cycle (Table 7.1) for the sham treated and the saline treated groups ($p>0.05$). However, in the lipiodol treated group, there was a significant variation in macrophage numbers during the estrous cycle ($p=0.02$). The number of macrophages at 48 hours was significantly fewer than the number of macrophages at 72 hours and 96 hours.

Dendritic Cells

In general, the majority of the $CD205^+$ DCs were found in the endometrium while $CD1^+$ DCs were found in the endometrium and the myometrium.

The distribution of $CD205^+$ DCs in the three treatment groups is shown in Figure 7.2. The difference between the mean number of $CD205^+$ DCs in the uterus of the sham-treated group (7.9 DCs/mm^2) and the saline treated group (6 DCs/mm^2) was not statistically significant ($p=0.23$). However, the mean number of $CD205^+$ DCs in the uterus of the lipiodol treated group (3.9 DCs/mm^2) was significantly less than both the sham treated ($p=0.007$) and the saline-treated ($p=0.05$) groups.

The significant reduction in the number of $CD205^+$ DCs in the uterus as a whole following lipiodol treatment was also mirrored by a significant reduction in the

number of $CD205^+$ DCs in the endometrium, especially in the stroma and near the glandular and luminal epithelia (Figure 7.3). In the endometrial stroma, there were also significantly fewer $CD205^+$ DCs in the saline treated group compared to the sham treated group ($p=0.01$). There was no significant change in the numbers of $CD205^+$ DCs in the uterine serosa, myometrium or the area surrounding blood vessels following lipiodol treatment. No significant variation in the number of $CD205^+$ DCs was found for the three treatment groups during the estrous cycle ($p>0.05$).

The distribution of $CD1^+$ DCs in the three treatment groups is shown in Figure 7.4. In the sham treated group, 0.2 $CD1^+$ DCs/ mm^2 were found in the uterus compared to 0.3 $CD1^+$ DCs/mm^2 in the uterus of the saline treated group ($p=0.4$). In the lipiodol treated group, the mean number of $CD1^+$ DCs was 0.57/mm^2, which was significantly higher than the sham treated group ($p=0.01$) but was not significantly different from the saline treated group ($p=0.08$). This increase in $CD1^+$ DCs following lipiodol treatment was confined to the endometrial regions of the uterus (Figure 7.5). This distribution is most easily understood by reference to schematic diagram Figure 7.6. No significant variation in the number of $CD1^+$ DCs was found during the estrous cycle for the three treatment groups ($p>0.05$).

7.5 Discussion

In human and murine pregnancy, an antigenically foreign fetus remains in close contact with the maternal immune system for a prolonged period of time. It is possible that in some cases of unexplained infertility, an abnormal maternal immune response to the products of conception may be involved in the pathogenesis of infertility. Dendritic cells are potent antigen presenting cells and essential in stimulation of naive T cells.[147, 148] The significance of uterine DCs is as yet unknown but regulating the uterine DC population may be critical in the establishment and maintenance of pregnancy.

My study suggests that an alteration in dendritic cell phenotype occurs in the murine uterus, particularly the endometrium, after lipiodol infusion. The significant variation in DC numbers was not reflected in changes to the total $CD45^+$ leukocyte population as DCs make up only 1-2% of the leukocyte population.[151] No differences were found in the numbers of endometrial macrophages after lipiodol infusion. This could point to non-involvement or no direct involvement of uterine macrophages in producing the fertility enhancing effect of lipiodol. However the antibody used in this study, F4/80 was a general macrophage marker,[152] giving no indication of the activation status of the macrophage population. There was a significant variation in the endometrial macrophage numbers of the lipiodol treated mice during the estrous cycle. The number of macrophages in the uterus is known to change in response to the changing levels of estrogen and progesterone during the estrous cycle.[153]

In my study, the intervention fluids were administered just prior to estrus (as determined by EC40 Estrus cycle monitor). This time point in the estrus cycle of the mice is analogous to the usual timing of HSG in women, following cessation of menstruation but prior to ovulation.

In my study, the sham treated mice acted as controls for the surgical procedure and the saline treated mice acted as controls for infusion of fluid. The saline control is important, as fluid injection in the appropriately sensitised uterus of mice is known to cause a pseudo-decidualisation reaction,[154, 155] which is associated with an alteration in uterine leukocyte populations.[156] The uterus must be sensitised to this pseudo-decidualisation by appropriate dosing with steroid hormones[154] before introducing the fluid into the uterus. However, in my study, the lack of significant differences between the sham treated and the saline treated control groups suggests that the significant differences in DC numbers in the lipiodol treated mice are due to the presence of lipiodol rather than the fluid instillation procedure. In all of my analyses, the only significant difference

between the two control groups was in the endometrial stroma where a significant reduction in $CD205^+$ DC numbers in the saline treated group compared to sham control was observed. The biological significance of this isolated difference is uncertain and when the data for $CD205^+$ DCs in all of the uterine compartments were pooled, there was no significant difference between the sham and saline control groups.

My study showed a reduction in the number of uterine (particularly endometrial) $CD205^+$ DCs in the lipiodol treated group. CD205 recognises cell surface protein DEC-205, which is involved in receptor mediated endocytosis of antigens and is expressed by most myeloid DCs.[157] Hence, reduction in the number of $CD205^+$ DCs reflects an overall reduction in the antigen sampling and presenting capability of the immune system in the uterus. It is possible to speculate that one of the mechanisms underlying the fertility-enhancing effect of lipiodol might be that a reduction in uterine DC numbers results in reduced sampling of foreign antigens from the semi-allogeneic conceptus and a dampened immune response. However, it should be noted that expression of CD205 alone is not enough to place these DCs into any of the recognised DC subsets. Another important factor is that DCs also express other known receptors involved in antigen uptake, namely CD206 (MMR) and CD209 (DC-SIGN).[158] Still, CD205 is the most commonly expressed endocytic receptor on DCs[159] and the change in my study may have biological importance.

It is not clear why lipiodol reduces the number of $CD205^+$ DCs in the uterus but the possibilities are that lipiodol is toxic to DCs or that lipiodol treatment contributes to the emigration of DCs from the uterus to the lymphoid organs. It is interesting to note that in lipiodol treated mice there appears to be selective reduction in the number of $CD205^+$ DCs at sites close to the intra-uterine lipiodol such as sub-adjacent to the glandular and luminal epithelia.

Major histocompatability complex (MHC) class I and MHC class II molecules have long been known to induce immunity by presenting peptide antigens to T cells, but the MHC proteins do not present lipid antigens. Lipid antigens are presented by the MHC-like proteins of the CD1 family that are expressed by antigen presenting cells such as DCs.[160] Unlike humans, mice express only a single CD1 protein, CD1d.[160] The maturation level of the DCs expressing CD1 molecules is still unclear but $CD1^+$ DCs may be important in mediating the fertility enhancing effect of lipiodol since iodised poppy seed oil consists mainly of lipids. My study showed an increase in number of $CD1^+$ DCs in the lipiodol treated group, which may be involved in inducing tolerance of the maternal uterine immune system in an antigen non-specific manner.

Conclusion

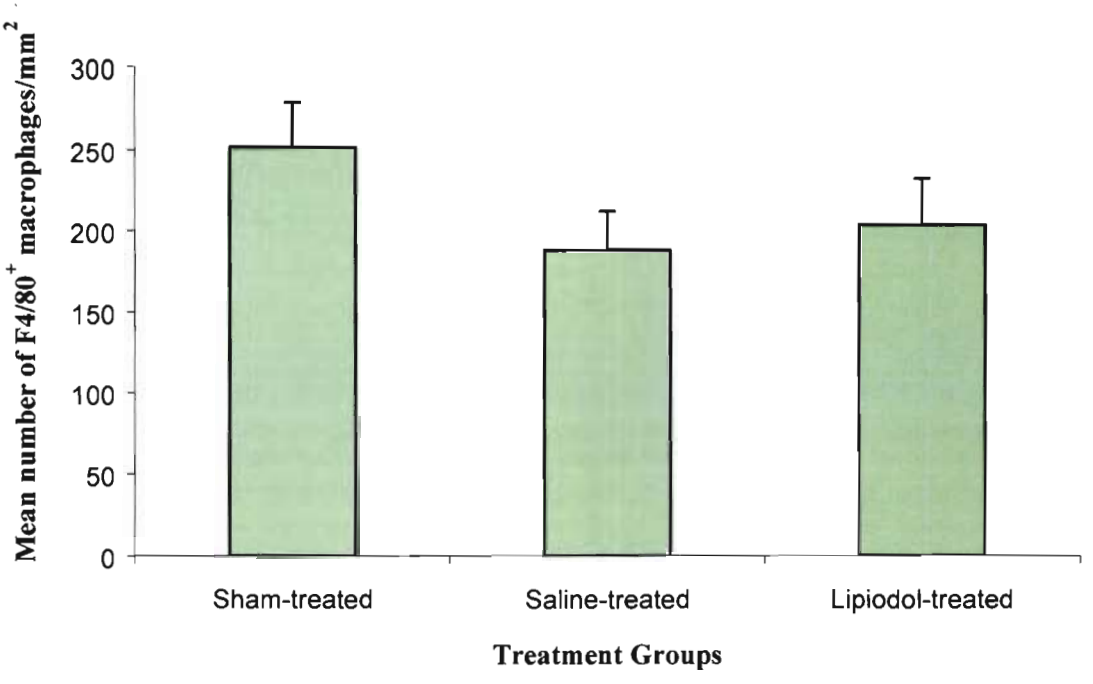
In this study I have made the interesting observation that, in mice, intra-uterine lipiodol infusion reduces the total number of uterine DCs and increases the number of uterine DCs capable of presenting lipid antigens. Such changes are likely to alter the ability of the uterine immune system to respond to antigens, potentially including a semi-allogeneic conceptus. However, additional work is required to demonstrate a causative link between these changes in the uterine immune milieu and the ability of lipiodol to enhance fertility in humans.

Table 7.1: Uterine F4/80, CD205 and CD1 Cells

	Time after treatment (hours)				
	24	48	72	96	168
F4/80+					
Sham	293.75 +/- 191.28	250.00 +/- 85.56	302.78 +/- 114.60	266.67 +/- 102.87	145.14 +/- 38.65
Saline	147.22 +/- 91.65	145.14 +/- 89.28	151.39 +/- 19.04	322.92 +/- 128.75	172.22 +/- 100.87
Lipiodol*	140.28 +/- 44.24	62.50 +/- 50.13	315.97 +/- 136.96	241.67 +/- 102.97	256.25 +/- 130.71
CD205+					
Sham	5.20 +/- 2.51	16.22 +/- 8.00	10.99 +/- 5.95	4.96 +/- 3.96	6.16 +/- 8.19
Saline	4.76 +/- 2.24	3.42 +/- 1.37	7.37 +/- 4.61	6.38 +/- 4.17	8.39 +/- 5.66
Lipiodol	1.29 +/- 1.71	5.43 +/- 3.12	3.78 +/- 3.47	3.31 +/- 1.59	4.04 +/- 2.85
CD1+					
Sham	0.14 +/- 0.20	0.30 +/- 0.20	0.22 +/- 0.22	0.03 +/- 0.04	0.32 +/- 0.52
Saline	0.21 +/- 0.23	0.23 +/- 0.23	0.12 +/- 0.14	0.24 +/- 0.22	0.67 +/- 0.77
Lipiodol	0.37 +/- 0.19	0.76 +/- 0.52	0.33 +/- 0.38	0.35 +/- 0.35	0.92 +/- 0.75

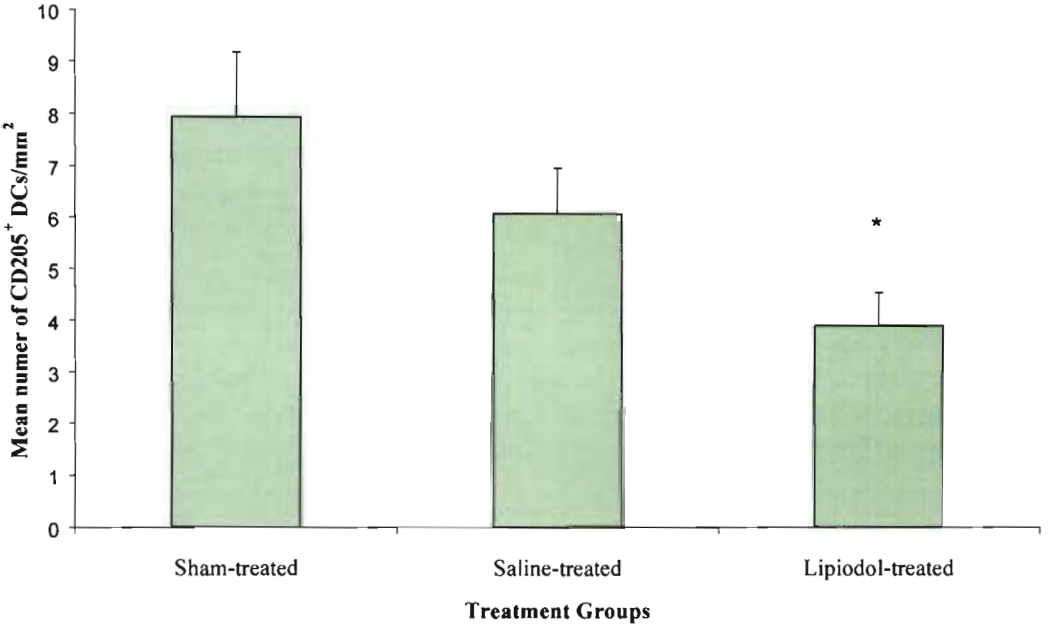
Footnote to Table 7.1: Mean number of positively stained cells (F4/80, CD205 and CD1) per square millimetre of uterus from sham-treated, saline-treated and lipiodol-treated mice at time points following the treatment (corresponding to the estrous cycle). Values are means +/- standard deviation (SD). *Single factor ANOVA showed a significant difference in macrophage number with time after treatment ($p=0.02$).

Figure 7.1: F4/80$^+$ Macrophages



Key to Figure 7.1: Mean number of F4/80$^+$ macrophages per square millimeter of endometrium in the three treatment groups of mice; error bars represent the standard error of the mean

Figure 7.2: CD205$^+$ Dendritic Cells



Key to Figure 7.2: Mean number of CD205$^+$ dendritic cells per square millimeter of uterus from the three treatment groups of mice; error bars represent the standard error of the mean. *Mean number of CD205$^+$ dendritic cells in lipiodol treated mice is significantly lower than sham treated (p=0.007) and saline treated (p=0.05) mice.

Figure 7.3: CD205$^+$ Dendritic Cells associated with Uterine Structures

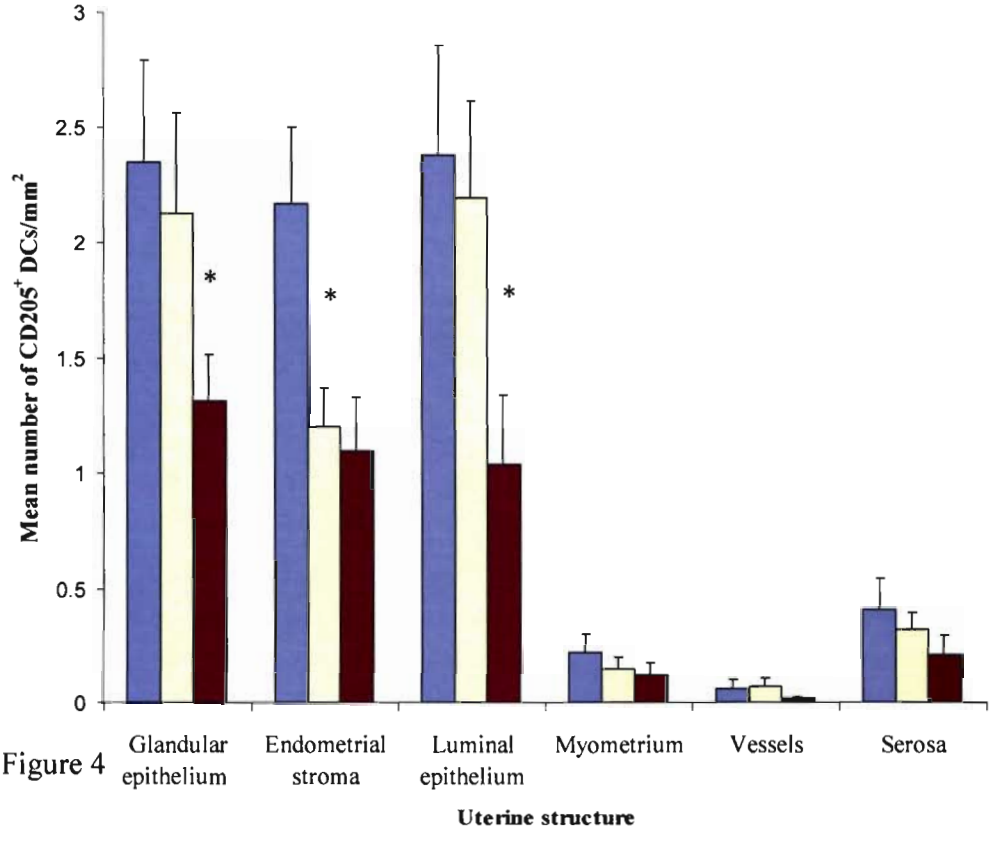
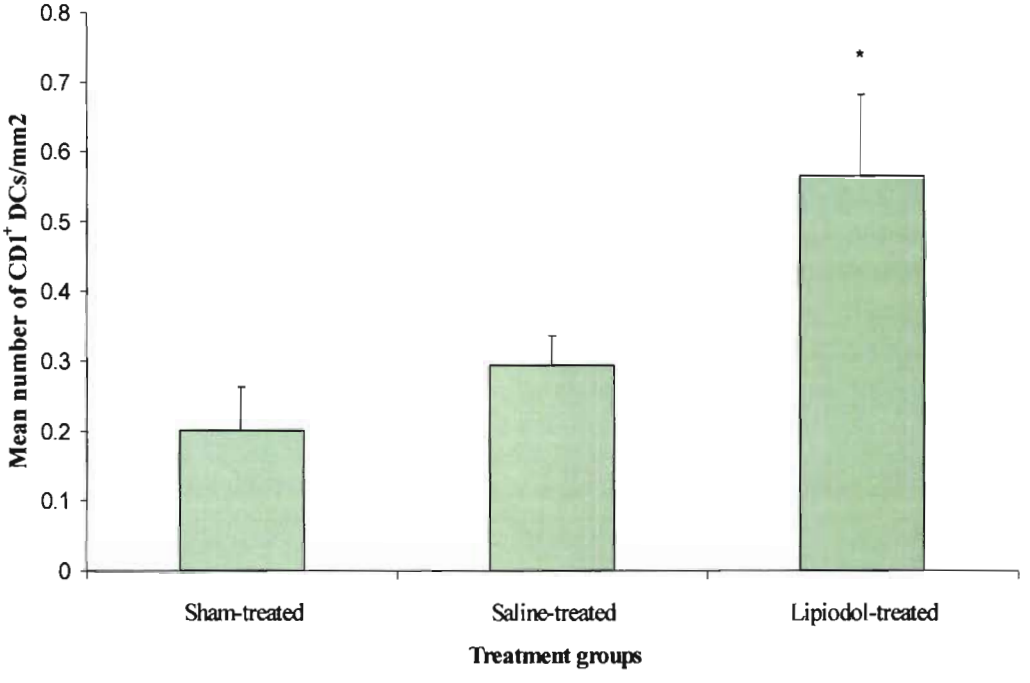


Figure 4

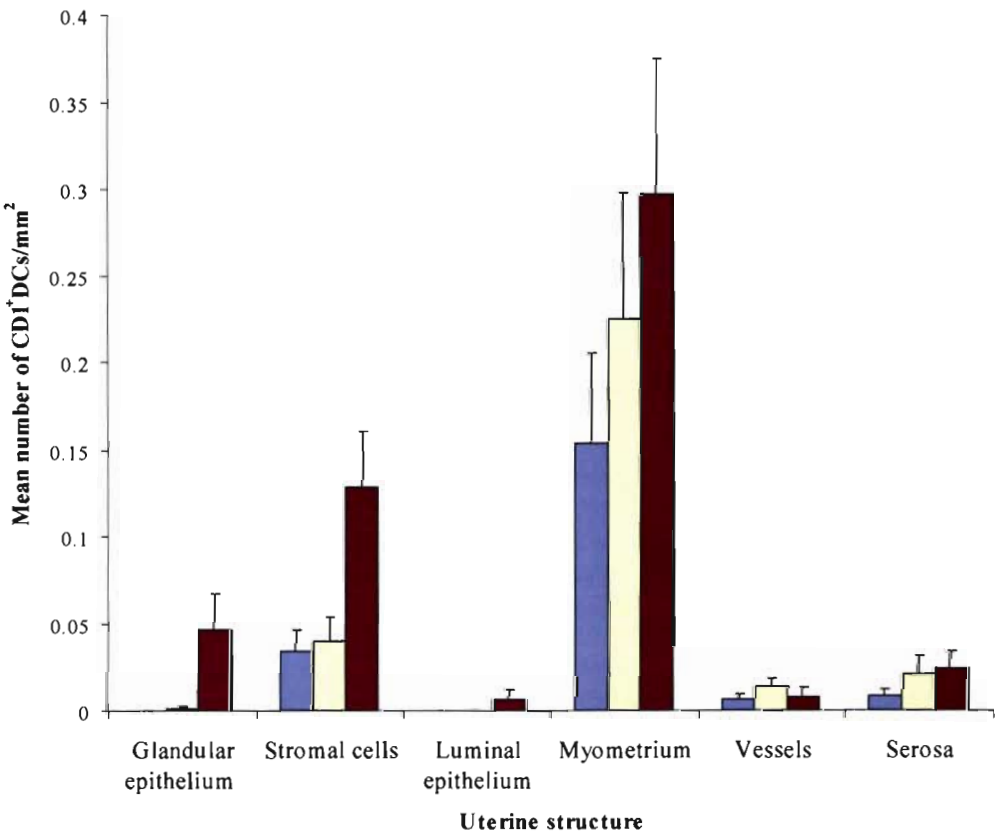
Key to Figure 7.3: Shows the distribution of CD205$^+$ dendritic cells associated with uterine structures from the sham treated (blue), saline treated (white) and lipiodol treated (purple) mice; error bars represent the standard error of the mean. Following lipiodol treatment, there were significantly fewer CD205$^+$ DCs near the glandular epithelium and luminal epithelium in comparison to the sham treated and saline treated groups (*$p<0.05$). There were no significant differences in the distribution of DCs between the three treatment groups in the myometrium, serosa or near the blood vessels ($p>0.05$).

Figure 7.4: CD1$^+$ Dendritic Cells



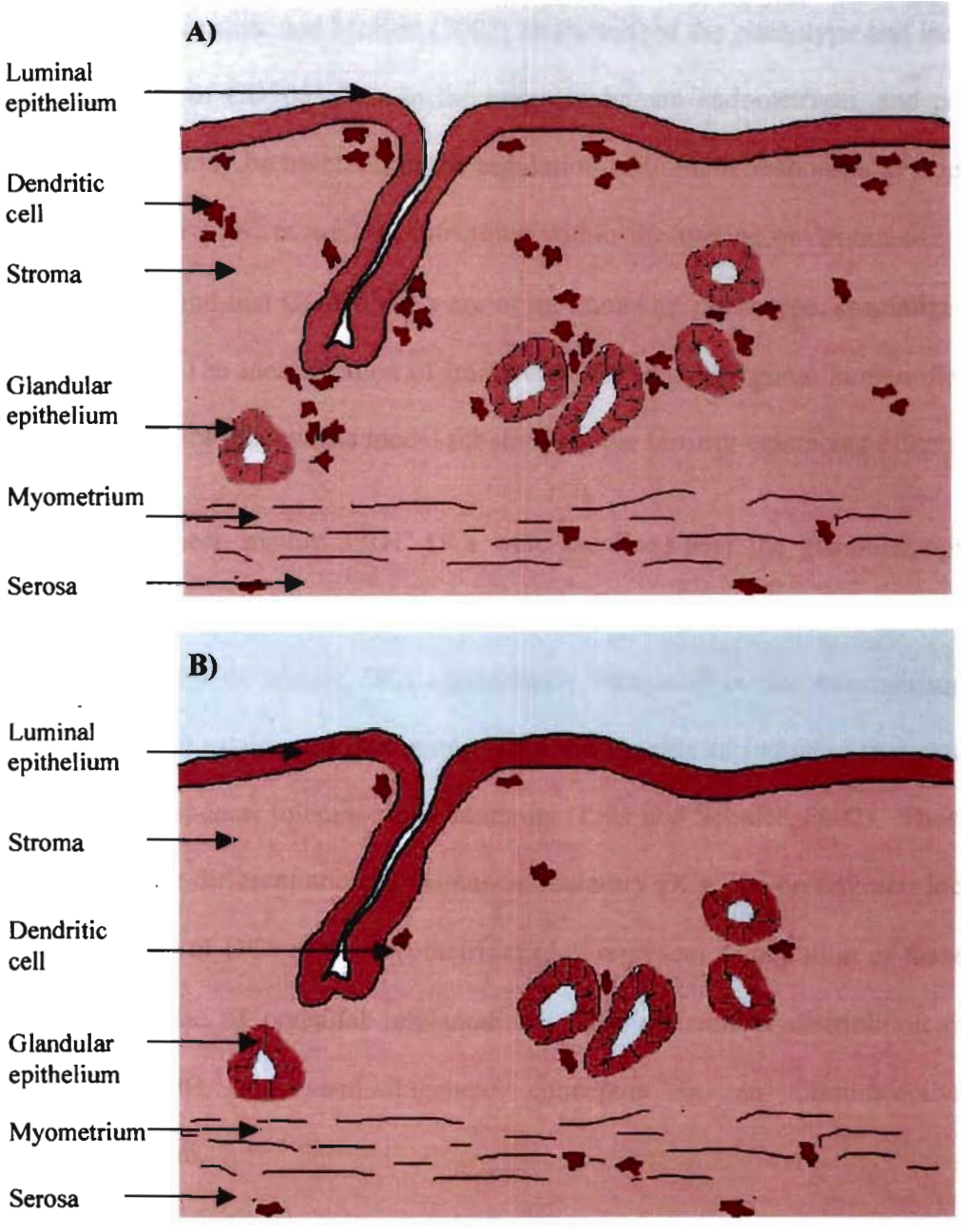
Key to Figure 7.4: Mean number of CD1$^+$ dendritic cells per square millimetre of uterus from sham-treated, saline-treated, and lipiodol-treated mice; error bars represent the standard error of the mean. *Mean number of CD1$^+$ dendritic cells in lipiodol treated mice is significantly higher than sham treated mice (p=0.01).

Figure 7.5: CD1$^+$ Dendritic Cells associated with Uterine Structures



Key to Figure 7.5: Shows the distribution of CD1$^+$ dendritic cells associated with uterine structures from the sham treated (blue), saline treated (white) and lipiodol treated (purple) mice; error bars represent the standard error of the mean. Following lipiodol treatment, there were significantly more CD1$^+$ DCs near the glandular epithelium and in the stroma in comparison to the sham treated and saline treated groups (*$p<0.05$). There were no significant differences in the distribution of CD1$^+$ DCs in the myometrium, serosa or near the blood vessels and in the luminal epithelium between the treatment groups ($p>0.05$).

Figure 7.6: Schematic Diagram Representation of the Distribution of CD1+ Dendritic Cells Following Lipiodol Exposure (A) Compared to the Distribution with No Intervention (B)



SECTION V: RANDOMISED TRIAL OF LIPIODOL IN WOMEN WITH UNEXPLAINED AND ENDOMETRIOSIS-RELATED INFERTILITY

CHAPTER 8: THE FLUSH TRIAL – FLUSHING WITH LIPIODOL FOR UNEXPLAINED (AND ENDOMETRIOSIS-RELATED) SUBFERTILITY BY HYSTEROSALPINGOGRAPHY

8.1 Introduction

The possible therapeutic effect of diagnostic tubal patency testing has been debated in the literature for over half a century (Chapter 4). Historically a variety of agents have been used to 'flush' the fallopian tubes. Some of these agents have been used primarily for diagnostic purposes in assessing tubal patency, such as methylene blue water-soluble dye in conjunction with laparoscopy and the water-soluble contrast media (WSCM) and oil soluble contrast media (OSCM) used for HSGs. Other agents have traditionally been used primarily for therapeutic purposes, such as oil injection and carbon dioxide tubal insufflation,[161] although tubal flushing treatment does not form part of current routine practice.

Diagnostic HSGs were originally performed with OSCM. Their use was gradually replaced by WSCM for a number of reasons: lower cost; better imaging of the tubal mucosal folds and ampullary rugae than OSCM;[106] lower viscosity and more prompt demonstration of tubal patency (reducing the need for a delayed film); less likelihood of persistence of contrast medium within the pelvic cavity and of complications such as intravasation resulting in allergic reactions or anaphylaxis or long-term lipo-granuloma formation. There were several reports of severe reactions, even fatalities, after the use of oily media in radiology (before the use of fluoroscopy screening) but none were reported after 1967.[108] It is reassuring that the advent of fluoroscopy screening appears to have abolished severe adverse reactions following the use of oil based media in radiology[108] and the safety of HSGs with OSCM in this context has been confirmed.[110] Lipo-granuloma formation has not been reported in women with patent fallopian tubes following OSCM studies and is not known to have long-term consequences.[107]

Many fertility centres, internationally, have moved towards the use of laparoscopy with tubal patency testing rather than HSG, owing to the increased information available concerning tubo-peritoneal abnormalities (including endometriosis) from laparoscopy and the possibility of treating endometriosis and adhesions surgically.[4] Currently those centres performing HSGs tend to use WSCM rather than OSCM for the reasons outlined above.

Lipiodol, in the form of Lipiodol Ultra Fluide (Guerbet, France), is an iodised poppy seed oil, obtained by substitution of ethyl esters for the glyceryl esters of lipiodol. One ml of Lipiodol Ultra Fluide contains 0.48g iodine. Lipiodol was one of the commonest OSCM used for diagnostic HSGs with OSCM in previous decades.

Systematic reviews of randomised trials, examining the therapeutic efficacy of tubal flushing, have highlighted a number of problems with these trials (see Chapter 5). Firstly, much of the data were from trials where the interventions were performed as diagnostic tests rather than as therapeutic interventions. Secondly, data on sexual frequency were not available for the 'flushing' versus 'no treatment' trials hence the notion that the increased pregnancy rate might be due simply to an increased sexual frequency in the group who received treatment. Thirdly, although 5 RCTs evaluated the effect of OSCM versus WSCM[120, 123, 126-128] and 3 RCTs evaluated the effect of adding OSCM to WSCM,[120, 122, 162] there were only 2 RCTs which assessed flushing with OSCM versus no intervention, one with small numbers in couples with unexplained infertility,[121] the other in couples for whom the cause for infertility was not specified.[125] Fourthly, the individual RCTs were often underpowered to detect a clinically important treatment effect. Finally, not all trials have reported the most important outcome, live birth. The conclusion of the systematic review prior to this trial was that there were insufficient data to support the routine use of tubal flushing as a fertility treatment.[139]

8.2 Objective

The objective of this trial was to ascertain the effectiveness of lipiodol flushing for enhancing fertility in women with unexplained and endometriosis-related infertility.

8.3 Methodology

A single-centre open parallel randomised trial of lipiodol was undertaken in couples with unexplained infertility. Approval for the trial was granted by the Auckland Ethics Committee prior to commencement and annually thereafter; the ethical standards of the Helsinki Declaration of 1975, as revised in 1983, were met.

Protocol

The inclusion criteria for women in the study population were as follows: unexplained infertility (or endometriosis where the fallopian tubes and ovaries were unaffected by endometriotic disease, in the context of otherwise unexplained infertility) of duration 12 months or more; full investigation for the cause of infertility completed; age 18 to 39 years inclusive; early follicular FSH level of 10 IU/L or less; mid-luteal progesterone level of 25 mmol/L or more in a spontaneous cycle; bilateral tubal patency confirmed either by dye studies at laparoscopy or by HSG. The partner's semen analysis had to be normal by World Health Organisation (1992) criteria.[163] Exclusion criteria for women were: abnormal fallopian tubes or a history of tubal ectopic pregnancy; laparoscopic evidence of endometriosis which had affected either the fallopian tubes or ovaries; iodine allergy. Eligible couples who had given consent were stratified into two populations prior to randomisation: Group A comprised women with unexplained infertility; Group B comprised women with endometriosis in the

context of normal fallopian tubes and ovaries and otherwise unexplained infertility. Women in the endometriosis population had previously had a laparoscopy at which endometriosis was visualised.

Women were randomised to receive lipiodol flushing performed by a hysterosalpingographic technique with fluoroscopic X-ray screening, or to no intervention. Both groups received the same fertility information sheet, which reinforced the principle of the pre-ovulatory fertile phase of the cycle, the timing of sexual intercourse to the fertile phase and the optimal sexual frequency (at least every 48 hours) in the fertile phase. The lipiodol contrast medium was Lipiodol Ultra Fluide (Guerbet, France). Lipiodol flushing was carried out by one of two research clinicians (WH n=45, NJ n=27[140]) in the follicular phase of the cycle between the end of menses and Day 12 of the cycle. With the woman in the left lateral or supine position, using a 'no-touch' technique after antiseptic solution application, the cervix was cannulated by a Leech-Wilkinson cannula (Downs Distributors, New Zealand) under speculum visualisation. In the supine position and with intermittent fluoroscopic X-ray guidance, pre-warmed (37°C) lipiodol was slowly instilled into the uterine cavity. Typically 10mls of lipiodol was instilled with the instillation being stopped once unequivocal bilateral spill of contrast from the fallopian tubes into the peritoneal cavity had been observed. If no peritoneal spill was observed after use of 10mls, further lipiodol was instilled. If intravasation of lipiodol was observed on x-ray (contrast apparent in the venous system), the instillation was immediately stopped.

The primary outcomes were clinical pregnancy (a positive urine or serum pregnancy test in association with an intrauterine gestation sac on ultrasound scan or histological evidence of trophoblastic tissue in the context of a miscarriage or ectopic pregnancy) at 6 months following randomisation, and live birth (once data for pregnancy outcomes became available). Secondary outcomes were miscarriage, ectopic pregnancy, multiple pregnancy, any other complications including a diagnosis of lipo-granuloma, and a comparison of pre-

and post-randomisation sexual frequency and focus to the fertile stage of the cycle. These latter sexual behaviour variables were based on an estimate of sexual frequency before randomisation and at the completion of follow-up. (For couples achieving a pregnancy during the six-month follow-up phase, these estimates of sexual behaviour were based on that occurring prior to pregnancy.) A subjective increase in sexual frequency and a greater focus of sexual activity to the fertile phase of the cycle were both defined as that estimated by the woman at the 6-month follow-up; an objective increase in sexual frequency was defined by a comparison of the woman's estimate of sexual frequency at the 6-month follow-up compared to her estimate prior to randomisation. For those undergoing lipiodol HSG, data were collected for the HSG appearances, procedural pain, intravasation and other lipiodol HSG-related complications.

An a priori power calculation was undertaken within the overall population to determine an appropriate sample size to demonstrate what was deemed to be a clinically important treatment effect of lipiodol flushing. For couples with unexplained infertility (which could include women without confirmed endometriosis and women with confirmed endometriosis in the context of otherwise unexplained infertility), in order to have 80% power at the 95% confidence level to detect an increase in the pregnancy rate at 6 months post-randomisation from 7% with no treatment to 25% with lipiodol flushing, 150 participants would be required for analysis. It was determined to allow a maximum of 3 years to attain this number, but recruitment was stopped once 158 couples had been recruited.

Data were collected from study participants by a telephone consultation with a research nurse; data concerning the lipiodol flushing procedure were collected in the presence of the study participant by the clinician performing the procedure.

Statistical methods

At the primary follow up at 6 months, numbers are presented as counts (percentages) for dichotomous data; continuous data are presented as either median (inter-quartile range) or mean (standard deviation). Pregnancy outcomes for the unexplained infertility population (Group A) and endometriosis population (Group B) are presented separately. The results are also pooled in a 'total population', according to the a priori specification upon which the power calculation was based. Statistical analyses were performed using a chi-square test or Fisher's exact test for dichotomous data and either Student's t-test or Mann-Whitney test for continuous data using SPSS software. The primary analysis was conducted a priori on an intention-to-treat (ITT) basis, but an exploratory analysis of actual treatment received was also carried out.

Assignment

Women were recruited from publicly funded and private secondary level gynecology clinics and a tertiary level fertility clinic in Auckland, New Zealand. Some women from other similar clinics throughout New Zealand contacted the trial co-ordinators to express interest and were evaluated for eligibility. Having given informed consent to participate, women were randomised in the menstrual phase of the cycle in which they had committed to attend for a lipiodol HSG if their randomisation allocated them to receive the treatment procedure.

Randomisation was performed using two computer-generated random number sequences (unknown to the research nurse, the executor of the assignment), known as Group A (women with unexplained infertility without confirmed endometriosis) and Group B (women with endometriosis in the context of otherwise unexplained infertility) at the beginning of the cycle on which it had been determined that a lipiodol flushing procedure would be performed. Allocation concealment was securely maintained by storage in sealed, sequentially-numbered opaque envelopes until the interventions were assigned. The randomisation sequences were unblocked with up to 110 available in Group

A (96 actually randomised) and 90 available in Group B (62 randomised). Allocation was strictly maintained sequentially, all envelopes in the sequence being used, with the allocated groups analysed on an ITT basis.

Blinding

It was not possible to blind participants to treatment allocation since the treatment involved a HSG procedure and the control involved no intervention. There was also no blinding of the executor of the assignment, the clinician performing the lipiodol flushing procedure, nor of the assessor at follow-up.

Follow up

The primary outcomes clinical pregnancy at 6 months following randomisation, and live birth (once data for pregnancy outcomes became available) were assessed at 6 calendar months from the date of randomisation, with a subsequent assessment of any pregnancy outcomes as appropriate at a later date approximately 2 weeks after the expected date of delivery of the baby for each women with an ongoing pregnancy at the 6 month follow up.

8.4 Results

Participant Flow

285 women were evaluated for eligibility, with 221 offered entry to the trial over 35 months between February 2000 and December 2002 inclusive (Figure 8.1). Of women offered the trial, 53 declined, the majority citing the possibility of randomisation to 'no treatment' followed by a 6-month 'stand down' during which they were asked not to undergo any other fertility treatments, in the context of their urgent desire for pregnancy, as the reason for declining the trial. 168 couples gave informed consent. Of these 168 women, 10 elected not to proceed with randomisation. 158 women were randomised; 96 in the population with unexplained infertility without confirmed endometriosis, 62 in the population with

endometriosis. Recruitment was closed after 2 years and 11 months, the number of women required by the power calculation having been surpassed.

Unblocked randomisation led to dissimilar numbers in the endometriosis population undergoing lipiodol flushing and no intervention. At the end of the trial, the randomisation master schedule was checked and it was confirmed that no breach of the randomisation sequence had occurred.

Three women were lost to follow-up. There were 2 known deviations from the protocol post-randomisation, both in women randomised to lipiodol flushing. One woman from Group A underwent a further laparoscopy and chromotubation procedure (which confirmed bilateral tubal patency) after a lipiodol HSG had shown neither fill nor spill of contrast from the fallopian tubes, and then conceived with in-vitro fertilisation (IVF) treatment within the 6-month follow-up phase. One woman from Group B conceived prior to the flushing procedure, the presumed 'menstrual period' at the time of randomisation being an implantation bleed, pregnancy then being confirmed prior to the planned flushing procedure. These two women both had live births.

The baseline characteristics of the women at entry to the trial are presented in Table 8.1. The groups randomised to lipiodol flushing compared to no treatment were similar regarding all factors known to be influential on fertility.

The details of the 72 lipiodol flushing procedures are presented in Table 8.2. Two women had to re-attend for a procedure following an initial unsuccessful cervical cannulation and abandoned procedure on the randomisation cycle. These women both had a successful procedure during the subsequent cycle. One woman did not attend for the lipiodol flushing procedure until the third cycle after randomisation. Outcomes were assessed at 6 calendar months post-randomisation, irrespective of any delays in the lipiodol flushing procedures, for women in the treatment and control groups. There were two cases in whom

intravasation was confirmed at the time of lipiodol HSG, although these intravasations were asymptomatic and without sequelae. There were no other complications and, specifically, no diagnosed cases of lipogranuloma over the formal 6 month follow-up phase and no cases have come to light since the end of follow-up.

Six month follow up and analysis

Six month follow up data are presented in Table 8.3 (ITT analysis) and Table 8.4 (actual treatment analysis). The ITT analysis (Table 8.3) demonstrated a statistically significant increase in the pregnancy rate (relative risk 4.44, 95% confidence interval 1.61-12.21) and live birth rate (relative risk 3.70, 95% confidence interval 1.30-10.50) in favour of lipiodol flushing in women with endometriosis. This effect was also present in the pooled total population (pregnancy rate relative risk 2.33, 95% confidence interval 1.33-4.08; live birth rate relative risk 2.43, 95% confidence interval 1.27-4.65). The difference in pregnancy rate (relative risk 1.60, 95% confidence interval 0.81-3.16) and live birth rate (relative risk 1.86, 95% confidence interval 0.81-4.25) in women with unexplained infertility without confirmed endometriosis was not statistically significant. Analysis of the data on an actual treatment basis (Table 8.4) gave results of similar magnitude and statistical significance.

There was no significant difference in the number of couples reporting increased sexual frequency or an increased focus of sexual activity to the fertile phase of the cycle after trial entry for those randomised to lipiodol flushing versus those randomised to no intervention (Table 8.5).

Most of the excess pregnancies in the treatment group occurred in the early months following the lipiodol procedure, with the cycle of the lipiodol procedure and, particularly, the cycle immediately after the lipiodol procedure being the commonest time to become pregnant, with the next cycle also offering a substantial increased chance of pregnancy (Table 8.6).

Table 8.7 shows the relationship of characteristics of the lipiodol flushing procedures to the subsequent occurrence of pregnancy. Notably amongst eight women in whom neither fallopian tube could be confirmed as patent, three became pregnant.

8.5 Discussion

My trial is the first prospective RCT to report on the effectiveness of lipiodol flushing as a fertility treatment in women with endometriosis. I demonstrated at the 6 month follow up that lipiodol flushing results in an increased pregnancy rate and increased live birth rate in couples where the woman has endometriosis with unaffected fallopian tubes and ovaries in the context of otherwise unexplained infertility. This effect was not demonstrated in couples with unexplained infertility without confirmed endometriosis (although I had insufficient numbers within this sample to detect a relative risk below 2.5, given 80% power and 95% confidence). Furthermore, no adverse events occurred in women who had lipiodol flushing. There were insufficient miscarriages, ectopic pregnancies and multiple pregnancies to draw meaningful conclusions of any effect of lipiodol flushing. There were no multiple pregnancies following lipiodol flushing at 6 months, which distinguishes it from most other fertility treatments

Addition of my FLUSH Trial data and those of Steiner et al (2003),[162] the only other RCT published since the previous update of the systematic review in 2002, was undertaken in 2004. The meta-analysis graphs that have changed from those displayed in Chapter 5 (Figures 5.1 to 5.18) are shown in Figures 8.2 to 8.9. When my data are considered in light of data from previous RCTs,[119] the effectiveness of lipiodol flushing is confirmed not only in the endometriosis population, but also in the population with unexplained infertility in a meta-analysis when appropriate pooling of RCTs is carried out (Figure 8.5). In women

with unexplained infertility, meta-analysis of women with unexplained infertility without confirmed endometriosis from my trial and those with unexplained infertility from the trial of Nugent et al (2002)[121] gives a relative risk for pregnancy of 2.05 (95% confidence interval 1.07-3.93) for lipiodol flushing versus no intervention (compared to relative risk 1.60, 95% confidence interval 0.81-3.16 from this trial alone). The estimate of the number needed to treat (NNT) to achieve one additional pregnancy in women with endometriosis, based on my trial data, is approximately 3 (95% confidence interval 2-6; control pregnancy rate 10.8%); NNT for one additional pregnancy in women with unexplained infertility, based on meta-analysis data, is approximately 6 (95% confidence interval 3-39; control pregnancy rate 15.4%).

The main strengths of this study were the randomised design with secure allocation concealment and the analysis on both intention to treat and per protocol basis. The main weaknesses were that the randomisation was not blocked and the absence of blinding. It was my a priori intention to present pooled data for the total population (Group A pooled with Group B) as the primary analysis and the power calculation for sample size was based on the pooling of these populations, and to present outcomes in Group A and Group B separately as a secondary analysis. However the data suggest that the populations were dissimilar in baseline fertility (the no intervention pregnancy rates were 10.8% for women with endometriosis and 20.8% for women with unexplained infertility without confirmed endometriosis) that is consistent with the reduced fecundity (by a factor of approximately one half) previously reported in women with minimal or mild endometriosis undergoing donor insemination[41, 42] and attempting to become pregnant naturally,[31] and dissimilar in the response to lipiodol treatment. Moreover, owing to unblocked randomisation, there was an over-representation of women with endometriosis in the no intervention group (43.5%) compared to the lipiodol flushing group (34.2%) in the total population that has the potential to bias the pooled results in favour of lipiodol flushing.

Although results for the pooled total population are presented, they must be interpreted cautiously.

It is recognised that lack of understanding of the fertile phase of the cycle and the optimal timing and frequency of intercourse to maximise fertility is widespread even in couples attending for tertiary assisted conception treatment.[164] My policy of providing a fertility information sheet to all trial participants was designed to maximise the number of pregnancies in the treatment and control groups. With non-blinded design, it was possible that women undergoing lipiodol flushing could have increased their sexual frequency compared to the control group. However my data do not support a difference in sexual behaviour (in terms of an increase in sexual frequency or an improved focus of sexual activity to the fertile phase of the cycle) amongst women assigned to lipiodol flushing treatment compared to those assigned to no intervention. Thus the increased pregnancy rate following lipiodol flushing is not explained by changes in sexual behaviour following treatment.

An exploratory actual treatment secondary analysis was deemed appropriate owing to the possibility of bias in favour of lipiodol flushing from the primary ITT analysis, given that 2 pregnancies in women randomised to receive lipiodol flushing could not be attributed to this intervention (one woman conceived prior to lipiodol flushing and another conceived in IVF treatment following lipiodol flushing). The conclusions from the trial were the same whether based on an ITT or actual treatment analysis.

This RCT is one of two RCTs assessing tubal flushing known to have been completed since the previous update of the systematic review of RCTs in 2002 (Chapter 5). The other trial suggested that the beneficial effect of adding OSCM to a WSCM, which shortened the time to pregnancy, attenuated over time and had disappeared by 18 months.[162]

Whilst most RCTs have shown a significant benefit of OSCM over WSCM in terms of the subsequent pregnancy rate,[119] one of the largest and methodologically most robust trials comparing OSCM versus WSCM[120] failed to demonstrate any relative benefit and this question remains unresolved.

There can now be little doubt of the efficacy of lipiodol flushing versus no intervention and the effect is unlikely to be due to changes in sexual behaviour following the procedure. The mechanism of the fertility-enhancing effect of OSCM is unknown. One theory was that OSCM may be highly effective at 'flushing out' debris from otherwise undamaged tubes.[117] Such debris may not necessarily block the fallopian tube, but may hinder conception or embryo transport along the fallopian tube. An alternative, immunological hypothesis was that OSCM may enhance fertility for women with unexplained infertility or mild endometriosis by affecting peritoneal macrophages[129] - OSCM have been shown to alter interleukin and prostaglandin production by peritoneal macrophages[130] and to modulate macrophage activity in phagocytosis of sperm.[131] However the fertility-enhancing effect may simply lie at the level of the endometrium. For most couples having unsuccessful IVF treatment, the outcome hinges on failed implantation: it stands to reason that a treatment which substantially increases the likelihood of conception is likely to have some effect on endometrial receptivity. This theory is supported by two spontaneous pregnancies in my series where lipiodol was not seen to spill from either fallopian tube (although bilateral tubal patency had previously been confirmed). There is growing evidence that the infertility related to mild endometriosis may be, at least partly, related to implantation failure owing to impaired endometrial receptivity.[82] The greater treatment effect in women with endometriosis compared to those with unexplained infertility in my study might result from a mechanism where lipiodol corrects an endometrial implantation dysfunction.

The current options for management of unexplained infertility include expectancy, use of empirical clomiphene citrate (CC), intrauterine insemination (IUI) or IVF.

Data are sparse on the relative advantages of these treatment options.[38] Recent evidence has cast doubt on the value of empirical CC treatment and that of IUI over expectant management.[34] Women with endometriosis have the further option of laparoscopic surgical treatment of endometriosis.[86] Lipiodol flushing now presents an alternative treatment option which may be more appealing to many couples. The advantages of lipiodol flushing are that the technique is less invasive than IVF or laparoscopic surgery for removing endometriosis, is relatively low cost (in New Zealand, comparable to the cost of a single IUI cycle), pregnancy is achieved by sexual intercourse thus it is regarded as more 'natural' and there is no increased risk of multiple pregnancy, a problem which has been associated with many other fertility treatment options.

Conclusion

In conclusion, lipiodol flushing is effective in enhancing fertility for women with unexplained infertility and women with mild endometriosis in the context of otherwise unexplained infertility. The greatest short term benefit is apparent in women with endometriosis. It should be considered as a possible first-line fertility treatment for such women, especially in circumstances where resources for other assisted reproductive technologies are limited. Tubal flushing with oily media could represent a simple, less invasive and economic alternative to IVF for couples with unexplained infertility or couples where the woman has normal patent fallopian tubes.

Table 8.1: Baseline Characteristics of the Population

	Unexplained infertility		Endometriosis-related infertility		Total population	
	Lipiodol flush (n=48)	No flush (n=48)	Lipiodol flush (n=25)	No flush (n=37)	Lipiodol flush (n=73)	No flush (n=85)
Age (years)						
Overall mean (SD)	33.8 (2.9)	33.3 (3.8)	34.1 (3.1)	33.7 (3.9)	33.9 (2.9)	33.5 (3.8)
20-24 (%)	0 (0)	1 (2.1)	0 (0)	0 (0)	0 (0)	1 (1.2)
25-29 (%)	3 (6.3)	6 (12.5)	1 (4.0)	7 (18.9)	4 (5.5)	13 (15.3)
30-34 (%)	21 (43.7)	22 (35.8)	16 (64.0)	13 (35.1)	37 (50.7)	35 (41.2)
35-39 (%)	24 (50)	19 (39.6)	8 (32.0)	17 (45.9)	32 (43.8)	36 (42.4)
Ethnicity. (%)						
Maori	1 (2.1)	3 (6.3)	0 (0)	3 (8.1)	1 (1.4)	6 (7.1)
Pacific	1 (2.1)	1 (2.1)	1 (4.0)	1 (2.7)	2 (2.7)	2 (2.4)
European	41 (85.4)	32 (66.7)	19 (76.0)	25 (67.6)	60 (82.2)	57 (67.1)
Chinese	2 (4.2)	2 (4.2)	3 (12.0)	2 (5.4)	5 (6.8)	4 (4.7)
Other Asian	1 (2.1)	2 (4.2)	1 (4.0)	2 (5.4)	2 (2.7)	4 (4.7)
Indian	2 (4.2)	7 (14.6)	1 (4.0)	2 (5.4)	3 (4.1)	9 (10.6)
Other	0 (0)	1 (2.1)	0 (0)	2 (5.4)	0 (0)	3 (3.5)
Body mass index (kg/m^2)						
Overall mean (SD)	24.0 (4.1)	24.3 (5.5)	21.8 (2.4)	23.6 (3.7)	23.3 (3.8)	24.0 (4.8)
<20 (%)	3 (6.2)	9 (18.8)	8 (32.0)	4 (10.8)	11 (15.0)	13 (15.2)
20-24.9 (%)	32 (66.7)	23 (47.9)	14 (56.0)	21 (56.8)	46 (63.1)	44 (51.8)
25- 29.9 (%)	9 (18.7)	9 (18.7)	3 (12.0)	10 (27.0)	12 (16.4)	19 (22.4)
>/=30 (%)	4 (8.4)	7 (14.6)	0 (0)	2 (5.4)	4 (5.5)	9 (10.6)
Smoking status (%)						
Never	38 (79.2)	33 (68.8)	21 (84.0)	31 (83.8)	59 (80.8)	64 (75.3)
Ex-smoker	8 (16.7)	8 (16.7)	4 (16.0)	4 (10.8)	12 (16.4)	12 (14.1)
Current smoker	2 (4.2)	7 (14.6)	0 (0)	2 (5.4)	2 (2.7)	9 (10.6)
Sexual frequency (%)						
>/=5/week	0 (0)	1 (2.1)	0 (0)	0 (0)	0 (0)	1 (1.2)
2-4/week	41 (85.4)	40 (83.3)	20 (80.0)	32 (86.5)	61 (83.6)	72 (84.7)
1-2/fortnight	7 (14.6)	7 (14.6)	4 (16.0)	4 (10.8)	11 (15.1)	11 (12.9)
1-2/month	0 (0)	0 (0)	1 (4.0)	1 (2.7)	1 (1.4)	1 (1.2)
Mean FSH (SD) (IU/L)	6.4 (1.7)	6.0 (1.8)	5.8 (2.5)	5.4 (2.6)	6.2 (2.0)	5.7 (2.2)
Previous laparoscopy (%)	42 (87.5)	38 (79.2)	25 (100.0)	37 (100.0)	67 (91.8)	75 (88.2)
Partner's mean sperm count (SD) (million/ml)	82.3 (46.8)	108.6 (82.2)	90.6 (121.6)	81.4 (65.4)	85.2 (79.8)	96.6 (76.0)
Gravidity (%)						
0	21 (43.8)	28 (58.3)	19 (76.0)	23 (62.2)	40 (54.8)	51 (60.0)
>/=1	27 (56.2)	20 (41.7)	6 (24.0)	14 (37.8)	33 (45.2)	34 (40.0)
Parity						
0	35 (72.9)	37 (77.1)	21 (84.0)	27 (73.0)	56 (76.7)	64 (75.3)
>/=1	13 (27.1)	11 (22.9)	4 (16.0)	10 (27.0)	17 (23.3)	21 (24.7)
Months infertility						
Overall median (IQR)	55 (38-84)	57 (33-87)	60 (33-84)	48 (32-72)	58 (36-84)	48 (33-84)
12-23 (%)	1 (2.1)	3 (6.3)	0 (0)	4 (10.8)	1 (1.4)	7 (8.2)
24-35 (%)	7 (14.6)	9 (18.7)	7 (28.0)	6 (16.2)	14 (19.1)	15 (17.6)
36-47 (%)	6 (12.5)	7 (14.6)	4 (16.0)	7 (18.9)	10 (13.7)	14 (16.5)
48-59 (%)	12 (25.0)	5 (10.4)	0 (0)	5 (13.5)	12 (16.5)	10 (11.8)
>/=60 (%)	22 (45.8)	24 (50.0)	14 (56.0)	15 (40.5)	36 (49.3)	39 (45.9)
Previous treatment (%)						
IVF	15 (31.3)	14 (29.2)	11 (44.0)	13 (35.1)	26 (35.6)	27 (31.8)
IUI	19 (39.6)	24 (50.0)	14 (56.0)	12 (32.4)	33 (45.2)	36 (42.4)
Clomiphene citrate	28 (58.3)	33 (68.8)	15 (60.0)	19 (51.4)	43 (58.9)	52 (61.2)
Lap endo surgery	0 (0)	0 (0)	12 (48.0)	25 (67.6)	12 (16.4)	25 (29.4)

Table 8.2: Characteristics of Lipiodol Flushing Procedures

	Unexplained n=48	Endometriosis n=24
Mean procedural time in mins – speculum inserted to instillation complete (sd)	16.0 (6.5)	11.2 (4.6)
Median volume (mls) lipiodol used (IQR)	10.0 (10.0-20.0)	10.0 (8.0-12.5)
Mean fluoroscopy screening time in mins (sd)	2.7 (1.8)	2.5 (1.8)
Mean no. X-ray films taken (sd)	4.2 (1.5)	3.7 (1.3)
No. with uterine filling defect (%)	7 (14.6)	6 (25.0)
No. with fallopian tube spill (%)		
Bilateral	37 (77.1)	18 (75.0)
Unilateral	7 (14.6)	2 (8.3)
Neither	4 (8.3)	4 (16.7)
Median VAS for pain (IQR)		
During procedure	3.0 (2.0-5.0)	1.0 (0-4.0)
Same day post-procedure	2.0 (1.0-5.0)	2.0 (1.0-3.3)
24 hrs post-procedure	0 (0)	0 (0)

Abbreviations for Table 8.2 :
VAS = Visual analogue scale
sd = Standard deviation
IQR = Inter-quartile range

Table 8.3: Intention-to-treat Analysis of Follow Up Data at 6 Months

	Unexplained infertility				Endometriosis-related infertility				Total population			
	Lipiodol (n=48)	No flush (n=48)	Relative risk (95% CI)	p	Lipiodol (n=25)	No flush (n=37)	Relative risk (95%CI)	p	Lipiodol (n=73)	No flush (n=85)	Relative risk (95%CI)	p
Pregnancy	16	10	1.60 (0.81-3.16)	0.168	12	4	4.44 (1.61-12.21)	0.001	28	14	2.33 (1.33-4.08)	0.002
Live birth	13	7	1.86 (0.81-4.25)	0.132	10	4	3.70 (1.30-10.50)	0.007	23	11	2.43 (1.27-4.65)	0.005
Miscarriage <20wks	2	2			2	0			4	2		
Ectopic pregnancy	1	0			0	0			1	0		
Termination	0	1			0	0			0	1		
Multiple pregnancy	0	0			0	1			0	1		

Footnote to Table 8.3:
Assumption for intention-to-treat analysis: those lost to follow-up did not conceive.
Clinical pregnancy was assessed at 6 months post-randomisation; pregnancy outcomes were subsequently ascertained for women achieving pregnancy by that time.
The termination of pregnancy was carried out at gestation 20 weeks owing to a fetal trisomy 21.

Table 8.4: Actual Treatment Analysis of Follow Up Data at 6 Months

	Unexplained infertility				Endometriosis-related infertility				Total population			
	Lipiodol (n=46)	No flush (n=47)	Relative risk (95% CI)	p	Lipiodol (n=23)	No flush (n=37)	Relative risk (95%CI)	p	Lipiodol (n=69)	No flush (n=84)	Relative risk (95%CI)	p
Pregnancy	15	10	1.53 (0.77-3.05)	0.218	11	4	4.42 (1.60-12.26)	0.001	26	14	2.26 (1.28-3.98)	0.003
Live birth	12	7	1.75 (0.76-4.05)	0.181	9	4	3.62 (1.26-10.41)	0.010	21	11	2.32 (1.21-4.48)	0.009
Miscarriage <20wks	2	2			2	0			4	2		
Ectopic pregnancy	1	0			0	0			1	0		
Termination	0	1			0	0			0	1		
Multiple pregnancy	0	0			0	1			0	1		

Footnote to Table 8.4:
For actual treatment analysis: (i) protocol breaches excluded from analysis of pregnancy outcomes; (ii) losses to follow-up excluded from analysis.
Clinical pregnancy was assessed at 6 months post-randomisation; pregnancy outcomes were subsequently ascertained for women achieving pregnancy by that time.
The termination of pregnancy was carried out at gestation 20 weeks owing to a fetal trisomy 21.

Table 8.5: Relationship of Sexual Activity and Treatment Assignment

	Lipiodol (n=73)	No flush (n=85)	P
Sexual activity			
Subjective increase	6	4	0.5[f]
Objective increase	7	6	0.6
Subjectively more focused to fertile phase	14	20	0.5

Footnote to Table 8.5:
[f] Denotes Fisher's exact test, otherwise p-values based upon chi-square test

Table 8.6: Time relationship of conception to randomisation

Cycle number following randomisation	Unexplained		Endometriosis		Total population	
	Lipiodol	No flush	Lipiodol	No flush	Lipiodol	No flush
0	2*	3	3	1	5*	4
1	5	1	2	0	7	1
2	3	1	1	1	4	2
3	3	2	3	2	6	4
4	2	3	1	0	3	3
5	1	0	2	0	3	0

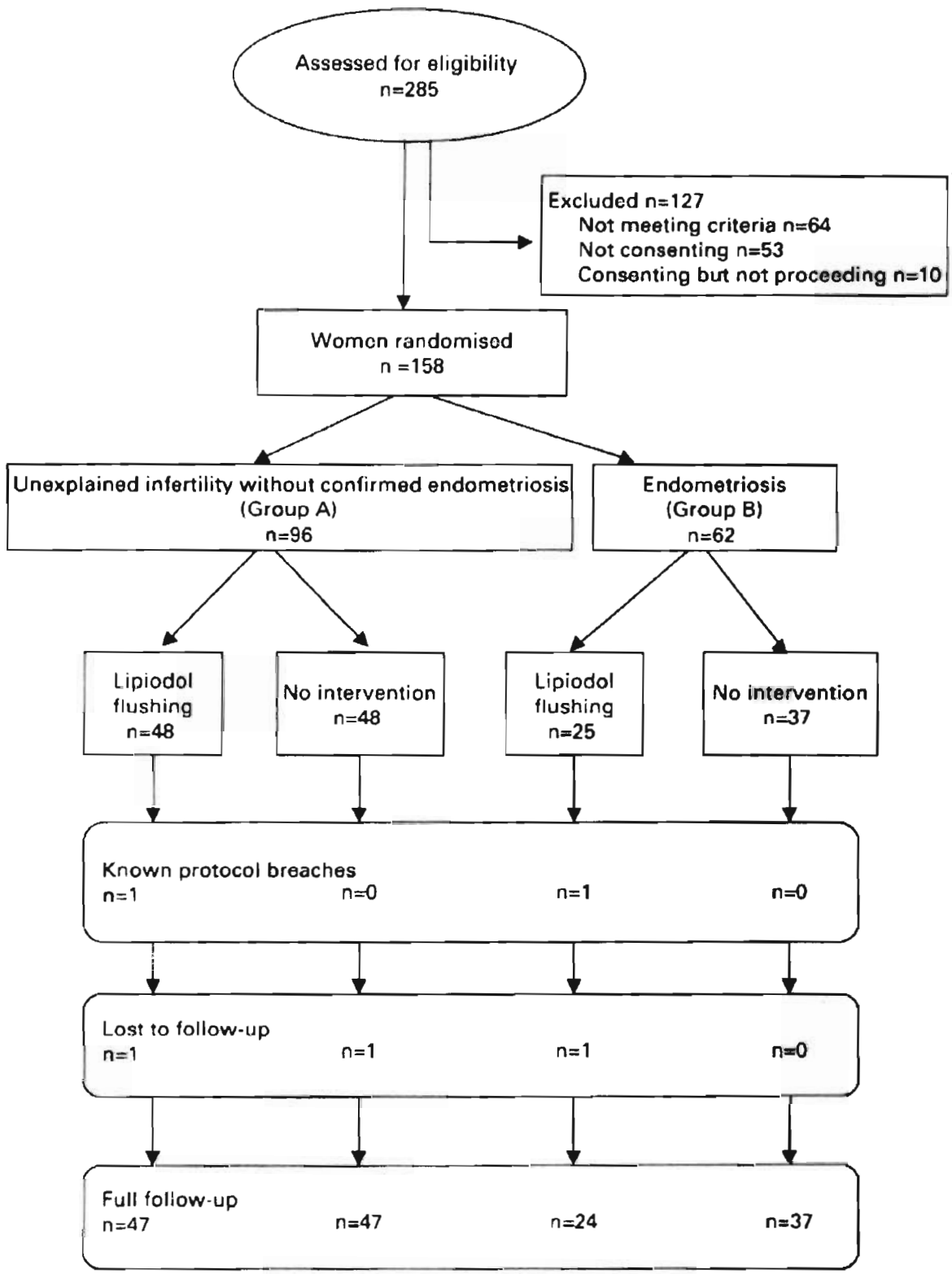
Footnote to Table 8.6:
The cycle number equates to the number of menstrual periods experienced by women prior to pregnancy (cycle 0 being a pregnancy occurring on the same cycle as randomisation and as the lipiodol procedure).
*One of these pregnancies occurred without a prior lipiodol flushing procedure, as one woman was already pregnant at the time of randomisation.

Table 8.7: Relationship of Lipiodol Flushing HSG Characteristics to Pregnancy

	Unexplained (n=48)		Endometriosis (n=24*)	
	Pregnant n=16	Not pregnant n=32	Pregnant n=11	Not pregnant n=13
Mean volume lipiodol in mls (sd)	13.5 (6.6)	12.9 (6.0)	9.1 (1.2)	11.5 (4.7)
Bilateral tubal patency (%)	13 (81.3)	24 (75.0)	8 (72.7)	10 (76.9)
Unilateral tubal patency (%)	2 (12.5)	5 (15.6)	1 (9.1)	1 (7.7)
Neither tube confirmed patent (%)	1 (6.3)	3 (9.4)	2 (18.2)	2 (15.4)
Uterine filling defect	0	7 (21.9)	2 (18.2)	4 (30.8)

*25 women were randomised to lipiodol flushing, but one woman had a pregnancy prior to treatment

Figure 8.1: Flow of Participants Through the Trial



Figures 8.2 to 8.9: Meta-analysis Graphs Following Update of Systematic Review with FLUSH Trial Included
(Adapted from meta-analysis graphs, undertaken using RevMan software, courtesy of the Cochrane Collaboration, adapted from meta-analysis graphs from Johnson et al [2005].[119] Copyright Cochrane Library, reproduced with permission.)

Figure 8.2: Meta-analysis Graph for Effect of OSCM versus No Treatment on Live Birth

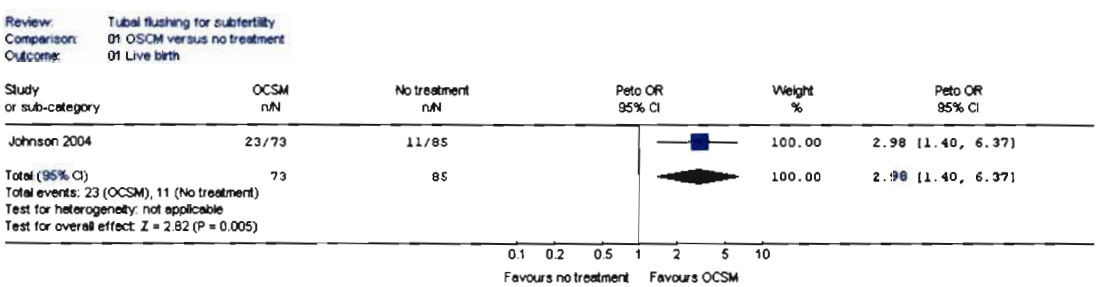


Figure 8.3: Meta-analysis Graph for Effect of OSCM versus No Treatment on Live Birth Amongst Subgroups

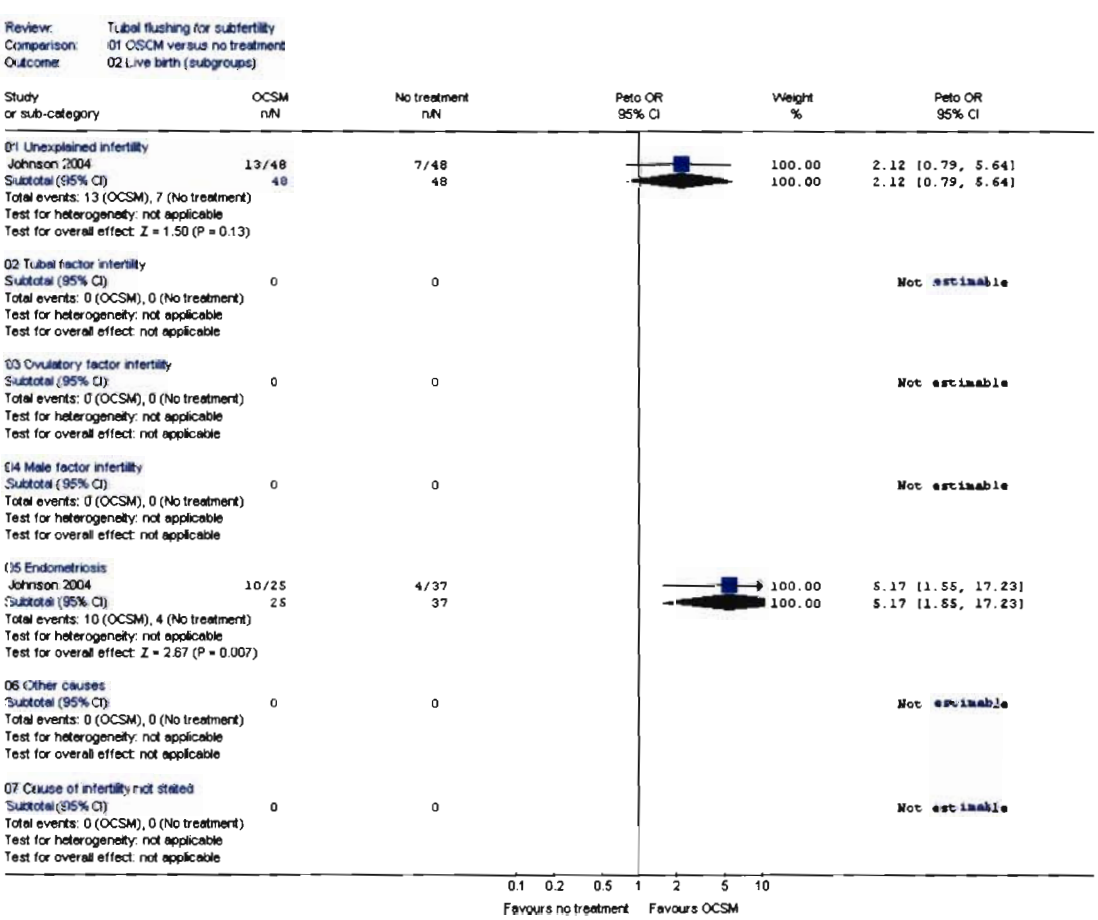


Figure 8.4: Meta-analysis Graph for Effect of OSCM versus No Treatment on Pregnancy

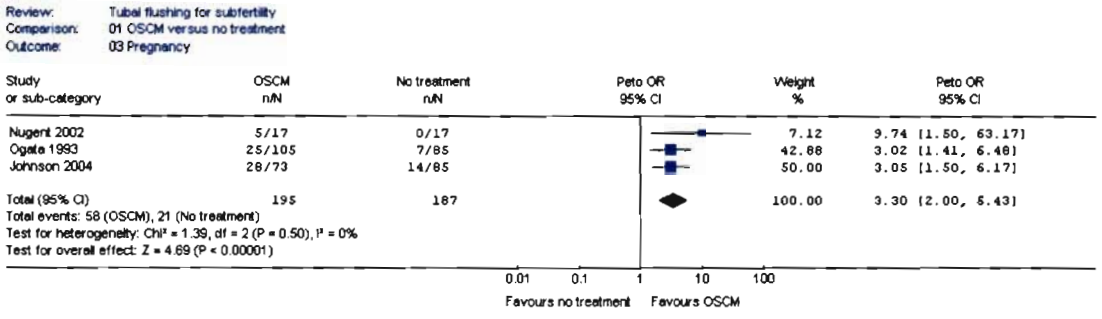


Figure 8.5: Meta-analysis Graph for Effect of OSCM versus No Treatment on Pregnancy Amongst Subgroups

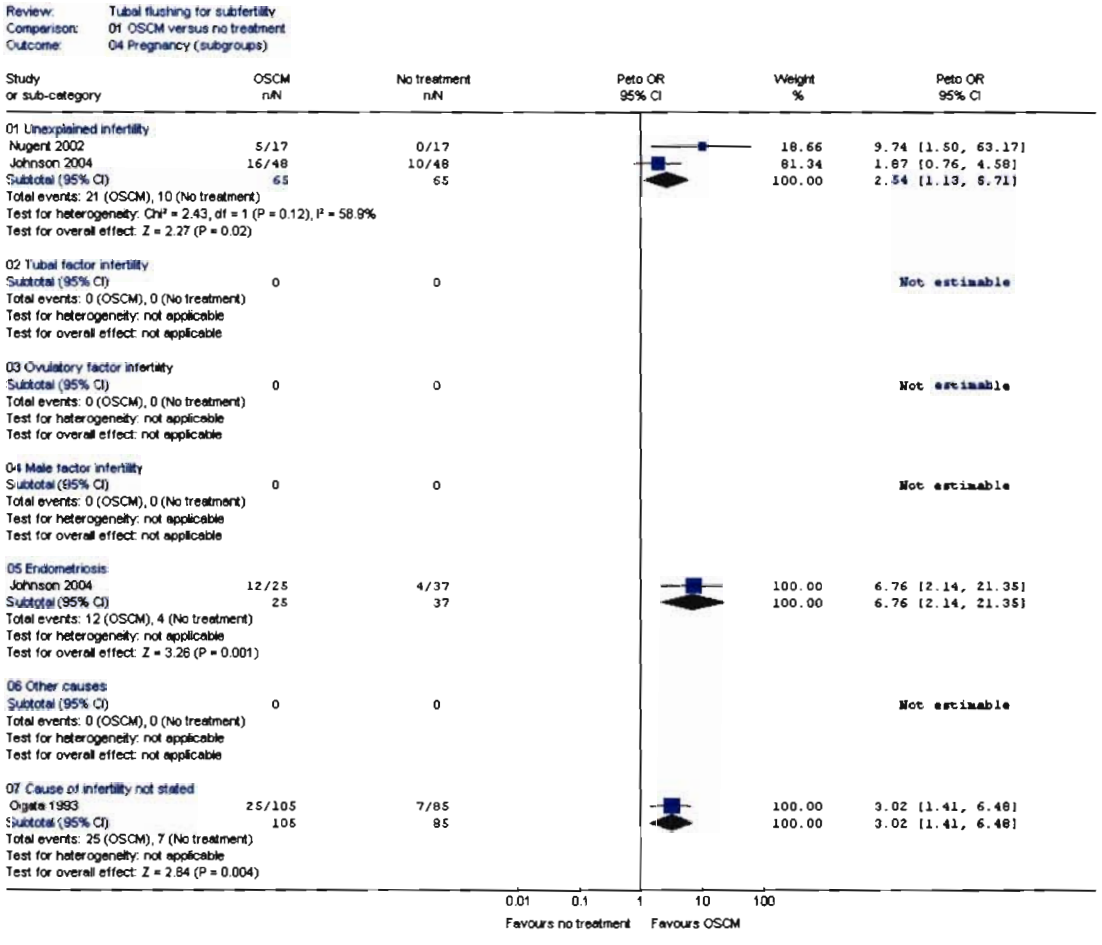


Figure 8.6: Meta-analysis Graph for Effect of OSCM versus No Treatment on Miscarriage

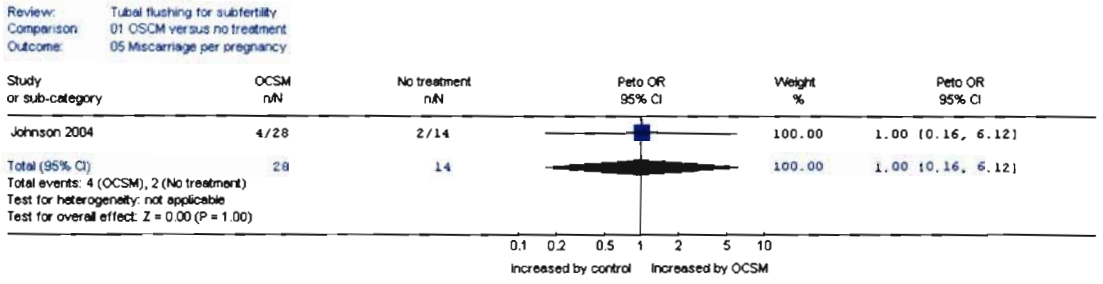


Figure 8.7: Meta-analysis Graph for Effect of OSCM versus No Treatment on Ectopic Pregnancy

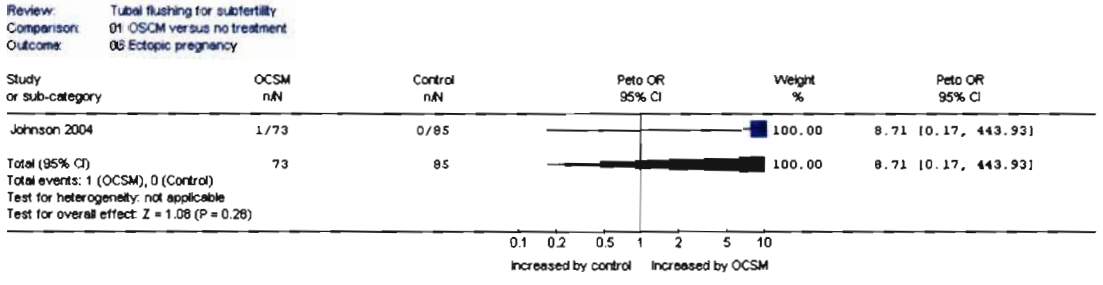


Figure 8.8: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Pregnancy

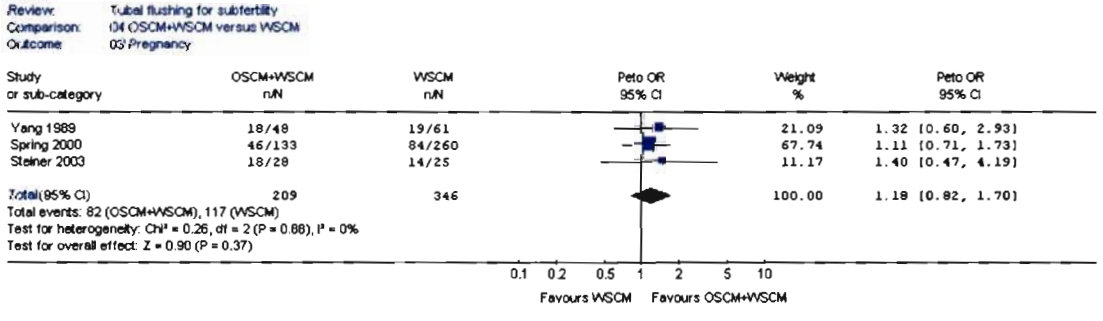
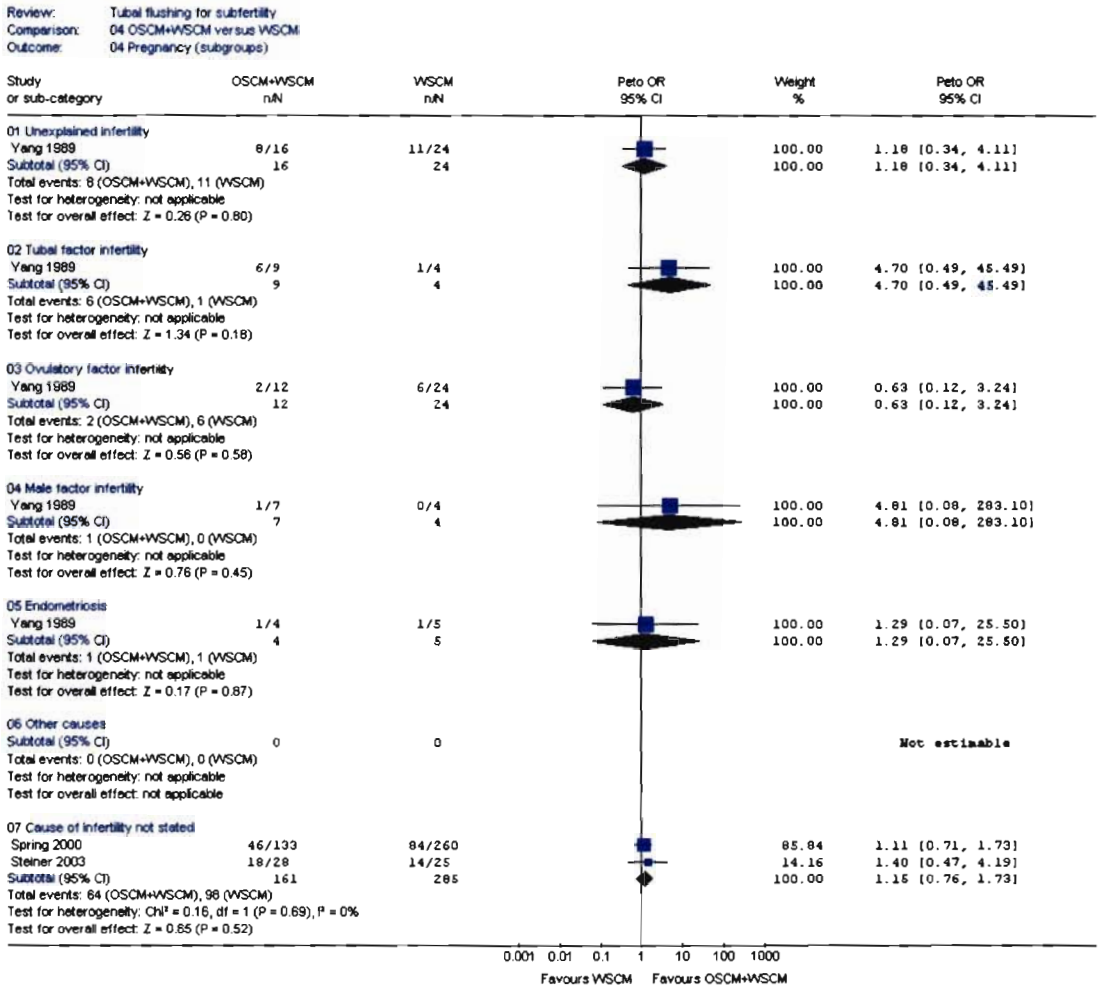


Figure 8.9: Meta-analysis Graph for Effect of OSCM+WSCM versus WSCM on Pregnancy Amongst Subgroups



CHAPTER 9: SURVIVAL ANALYSIS OF 24 MONTH FOLLOW UP OF THE FLUSH TRIAL

9.1 Introduction

Growing evidence has suggested a therapeutic, fertility enhancing effect of a hysterosalpingogram (HSG) with oil soluble contrast media (OSCM).[119] The results of my randomised trial showed a pronounced therapeutic effect of lipiodol at 6 months follow up amongst women with endometriosis[140] and I speculated this might result from an immuno-biological fertility enhancing effect either on the intraperitoneal environment or on the endometrial environment to enhance implantation.[137] Others have suggested that OSCM might simply reduce the latency to pregnancy rather than to increase the overall number of couples who eventually become pregnant.[162]

Of 11 RCTs reporting on the fertility effects of tubal flushing,[119] most have reported only short term outcomes at 4 months,[125] 6 months,[121, 126, 127, 140] 8 months,[122] 9 months,[123] 12 months,[120, 128] with only two RCTs previously reporting outcomes beyond one year – Steiner et al (2003) at 18 months[162] and Lindequist et al (1994) at 20 to 39 months.[108] It does not appear that account has been taken of the possibility that, as time progresses, some participants allocated to no treatment may in fact undergo a flushing procedure, and that the data may be influenced by women undergoing other fertility treatments.

9.2 Objective

The objective of this analysis, on a cumulative basis up to 24 months following entry into my randomised trial (Chapter 8), was to ascertain whether a fertility enhancing effect of lipiodol was present, given that women might have chosen to undergo treatment with lipiodol or other fertility treatments from 6 to 24 months after trial entry.

9.3 Methodology

Study design

A detailed description of my randomised trial protocol, in line with CONSORT criteria, has been presented with the 6 month follow up results of the randomised trial (Chapter 8). The trial was an open parallel RCT of lipiodol flushing versus no intervention in two pre-defined subpopulations of women with unexplained infertility, one group of 62 women with known endometriosis and another group of 96 women with no known endometriosis (pure unexplained infertility).

Follow up at 24 months

Data for the follow up stage 6 to 24 months were collected from study participants by a telephone consultation with a research assistant (between January and June 2005), after completion of 24 months from randomisation and study entry. Dates of further pregnancies and whether further treatments had been undertaken were recorded.

Statistical methods

As women were followed for 24 months from entry to the randomised trial with no control over their treatment after 6 months and some loss to follow up, a Cox proportional hazards regression model was used to assess the effect of lipiodol treatment. This model included the lipiodol treatment as a time dependent factor. The event of interest was pregnancy. As this study was a follow up from a previously published randomised trial, a second Cox proportional hazards regression model was also performed, where time was partitioned into two: firstly up to 6 months (the pre-specified follow up time of the RCT in Chapter 8); secondly from 6 months to 24 months. This model allowed the treatments and the time intervals to be modelled separately or in selected combinations. An intention to treat (ITT) analysis, assuming all women remained in their original treatment group and were followed for 2 years, was used to calculate the relative risk for pregnancy and live birth plus ongoing pregnancy (ongoing pregnancy defined as a viable pregnancy of

gestation 12 weeks or more) in the two original treatment allocation groups. The primary imputation for pregnancy data in the ITT analysis was that all women lost to follow up did not become pregnant, however sensitivity analyses for the imputed pregnancy data (where all those lost to follow up were assumed to be pregnant and where half those lost to follow up were assumed to be pregnant) were also performed.

9.4 Results

Participant Flow

The original trial protocol allowed for completion of 6 months of follow up without other treatment interventions, but participants were able to have further treatment without restrictions between the 6 month and 24 month follow up phase. Figures 9.1 and 9.2 show the flow of participants to the 24 month follow up. Amongst 73 women randomised to lipiodol treatment, 43 were known not to be pregnant at the 6 month follow up, of whom one woman underwent a further lipiodol procedure, 21 underwent other fertility treatments and two women were lost to follow up. Of the 85 women originally randomised to no intervention, 70 were known not to be pregnant at the 6 month follow up, three women underwent a lipiodol procedure, 22 underwent other fertility treatments and eight women were lost to follow up.

Actual treatment analyses

The survival curves showing time to pregnancy for women in the two condition categories by treatment are shown in Figure 9.3, with the survival curves for women with unexplained infertility and endometriosis being shown separately in Figures 9.4 and 9.5 respectively. When modelling over the full 24 months there was no indication that the effect of treatment differed between conditions, endometriosis and unexplained infertility ($\chi^2 = 0.2$, df = 1, p = 0.7), and also there was no indication of a difference in the proportion of pregnancies between the two conditions (hazard ratio 1.1, 95% confidence interval [CI] 0.7 to 1.7, p = 0.7). The model showed an effect of lipiodol

treatment with a hazard ratio of 2.0 (95% CI 1.3 to 3.2). However, as there was an indication of a difference in response to treatment between the two conditions at the end of the RCT (Chapter 8), an assessment of the hazard ratios before and after this time showed an interaction (χ^2 = 5.4, df = 1, p = 0.02). Hence the 24 month follow up data were re-analysed with the two time periods (0 to 6 months and 6 month to 24 months) being assessed separately. This re-analysis showed a strong treatment effect for those with endometriosis during the first six months and no effect after that time (χ^2 = 7.6, df = 1, p = 0.006 for the effects at the two times being the same), while for those with unexplained infertility the treatment effect appeared similar over time (χ^2 = 0.1, df = 1, p = 0.8). The treatment effects at the two time periods were summarised by hazard ratios as follows: hazard ratio 5.4 (95% CI 2.1 to 14.2) for women with endometriosis at 0-6 months; hazard ratio 0.6 (95% CI 0.2 to 2.1) for women with endometriosis at 6-24 months; hazard ratio 1.8 (95% CI 0.8 to 3.9) for women with unexplained infertility at 0-6 months; hazard ratio 2.2 (95% CI 0.9 to 5.4) for women with unexplained infertility at 6-24 months. As the unexplained infertility group had very similar estimates for both time periods a better estimate was for the full 24 months (hazard ratio 2.0, 95% CI 1.1 to 3.5).

Between the 6 and 24 month follow ups, 44 women had at least one further fertility treatment, 56 were recorded as having no further treatment, and information on further treatment was not available for 10 women. To incorporate this into the model the 10 women with no information were censored at 6 months in the survival analysis and assumed to have had no further treatment in the ITT analysis. Also the date of further treatment was unknown, other than it was beyond the end of the RCT, and so it has been assumed that the additional treatment was received half way between the end of the trial and the end of follow up for that woman. With these assumptions the additional fertility treatment had a significant hazard ratio 19 (95% CI 8 to 45). Based on simulations where the timing of the additional treatments was varied, the estimate of the effect of additional treatment changed little, always having a significant association with the onset of pregnancy. The addition of

this variable had no effect on the early endometriosis hazard ratio but moved the later endometriosis hazard ratio and the unexplained infertility hazard ratio towards unity. Using the assumptions above, the hazard ratio for endometriosis after 6 months was 0.8 (95% CI 0.2 to 2.5) while that for unexplained infertility was 1.6 (95% CI 0.9 to 2.9).

Intention to treat analysis

Pregnancy and live birth plus ongoing pregnancy rates at 24 months based on an intention to treat analysis according to group of allocation, and with an assumption that women lost to follow up did not become pregnant, and sensitivity analyses for the imputed pregnancy data, are shown in Table 9.1. These analyses for pregnancy show very similar results to the Cox proportional hazards regression analysis but there are smaller relative risks because of the more conservative approach and the analyses for live births show very similar results to those for the pregnancy outcome.

9.5 Discussion

This 24 month follow up of my randomised trial provides further evidence of the effectiveness of lipiodol flushing for women with unexplained infertility. Whilst there was a positive effect of lipiodol in women with endometriosis at 6 months follow up (Chapter 8), this present analysis shows no evidence that enhanced fertility persists beyond 6 months in women with endometriosis, but suggests a sustained and consistent enhanced fertility up to 24 months in women with pure unexplained infertility.

There are obvious difficulties with the 24 month follow up analysis. The data collection method at for the 24 month follow up was open to possible recall bias, but it was not possible to verify further treatments and further pregnancies from the medical notes, since there was such fragmentation of care between private and public health systems that any particular set of notes would have been even more prone to bias owing to missing information. Further treatment was collected as a dichotomous variable, whereas in

retrospect the study could have been strengthened by ascertainment of the actual timing of further treatment. The ITT analysis at 24 months is complicated by three women allocated to no intervention who have now had lipiodol procedures (all of whom became pregnant) and considerably more women amongst those randomised to both lipiodol and no intervention who have subsequently received other fertility treatments. The similar results of actual treatment and ITT analyses at 24 months, and the fact that analysis taking account of these further treatments did not substantially alter estimates of treatment effect, point to the results as being robust.

It could be argued that the disappearance by 24 months of the significant fertility enhancement from lipiodol in women with endometriosis at 6 months supports the concept suggested by Steiner et al (2003)[162] that only latency to pregnancy and not the eventual pregnancy rate is altered by lipiodol treatment. However the picture is undoubtedly complicated by the number of women becoming pregnant from other fertility treatments between 6 and 24 months.

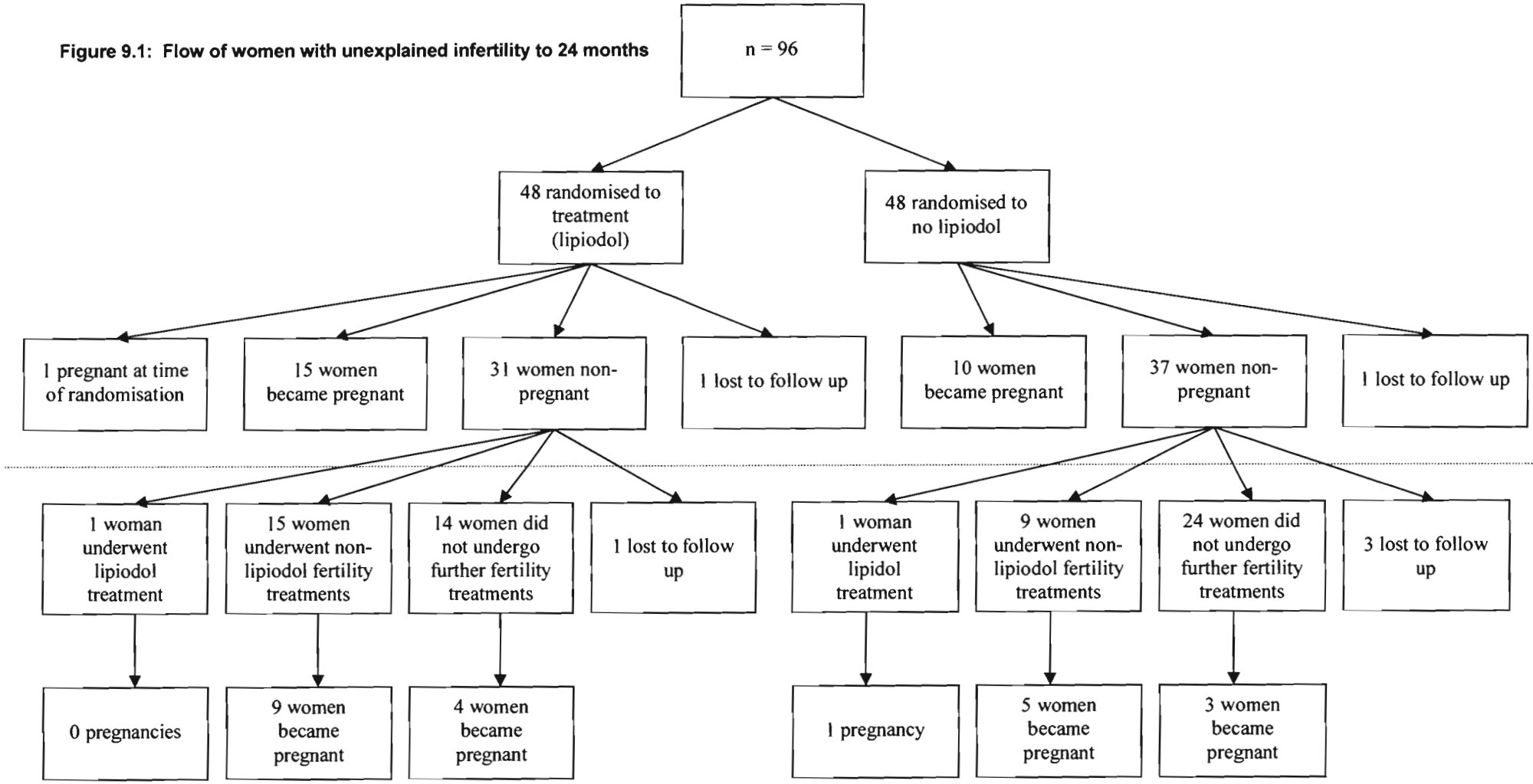
The apparent difference in effect of lipiodol for women with endometriosis compared to women with pure unexplained infertility is consistent with the notion that infertility related to mild endometriosis is a distinct entity and not just another type of unexplained infertility. Indeed women with such endometriosis-related infertility have been shown to have approximately half the fecundity of women with pure unexplained infertility of similar duration,[31, 41, 42, 140] perhaps related to additional mechanisms such as immuno-biological adverse effects on fertility.[137] It is speculative to suggest that more than one mechanism of the fertility enhancing effect of lipiodol might explain these different effects in women with endometriosis compared to women with unexplained infertility. Known immuno-biological dysfunction in women with endometriosis, in conjunction with the pronounced early effect of lipiodol, which later disappears, might suggest an immuno-biological mechanism of lipiodol. An intraperitoneal effect that could influence egg quality or sperm-egg interaction is plausible, but we have increasing evidence of an endometrial effect of lipiodol, which, in a murine model, had an effect on

uterine dendritic cell populations (Chapter 7). It is possible that lipiodol has an implantation enhancing effect on the endometrium, a hypothesis that I am now investigating further. Whether the more sustained effect of lipiodol in women with pure unexplained infertility might be due to another mechanism, such as mechanical flushing of the fallopian tubes, remains unclear.

Conclusion

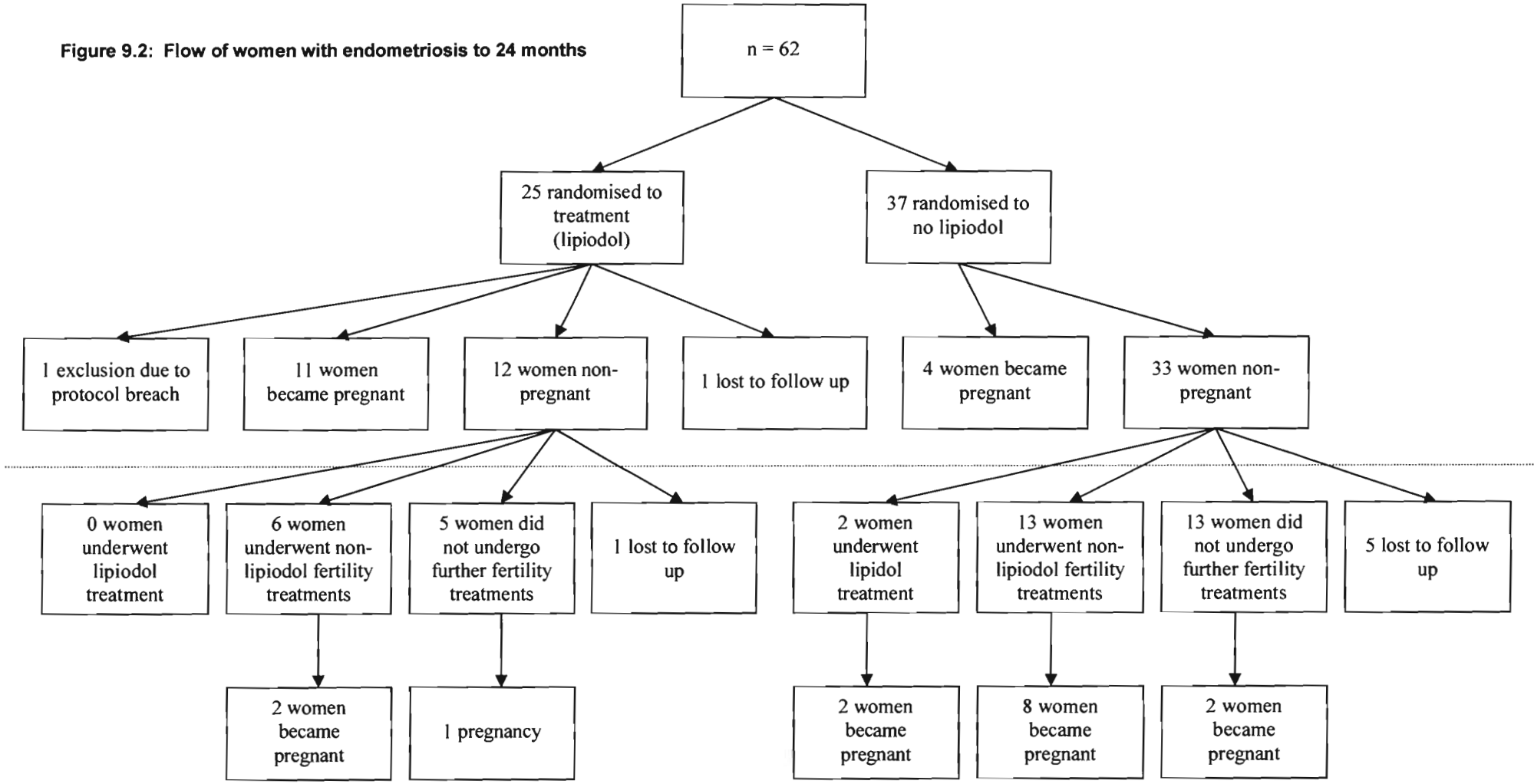
This study lends further support to the effectiveness of lipiodol flushing for treating unexplained infertility. Whilst previous evidence suggested the greatest short term benefit is apparent in women with endometriosis, this study provides compelling evidence of more sustained efficacy for women with pure unexplained infertility.

Figure 9.1: Flow of women with unexplained infertility to 24 months



Key to Figure 9.1: Above the horizontal dotted line represents events from the FLUSH trial until the 6 month follow up, and below the horizontal dotted line relate to events between 6 and 24 months of follow up.

Figure 9.2: Flow of women with endometriosis to 24 months



Key to Figure 9.2: Above the horizontal dotted line represents events from the FLUSH trial until the 6 month follow up, and below the horizontal dotted line relate to events between 6 and 24 months of follow up.

Figure 9.3 Survival Curve of Pregnancy over 24 Months in Overall Population

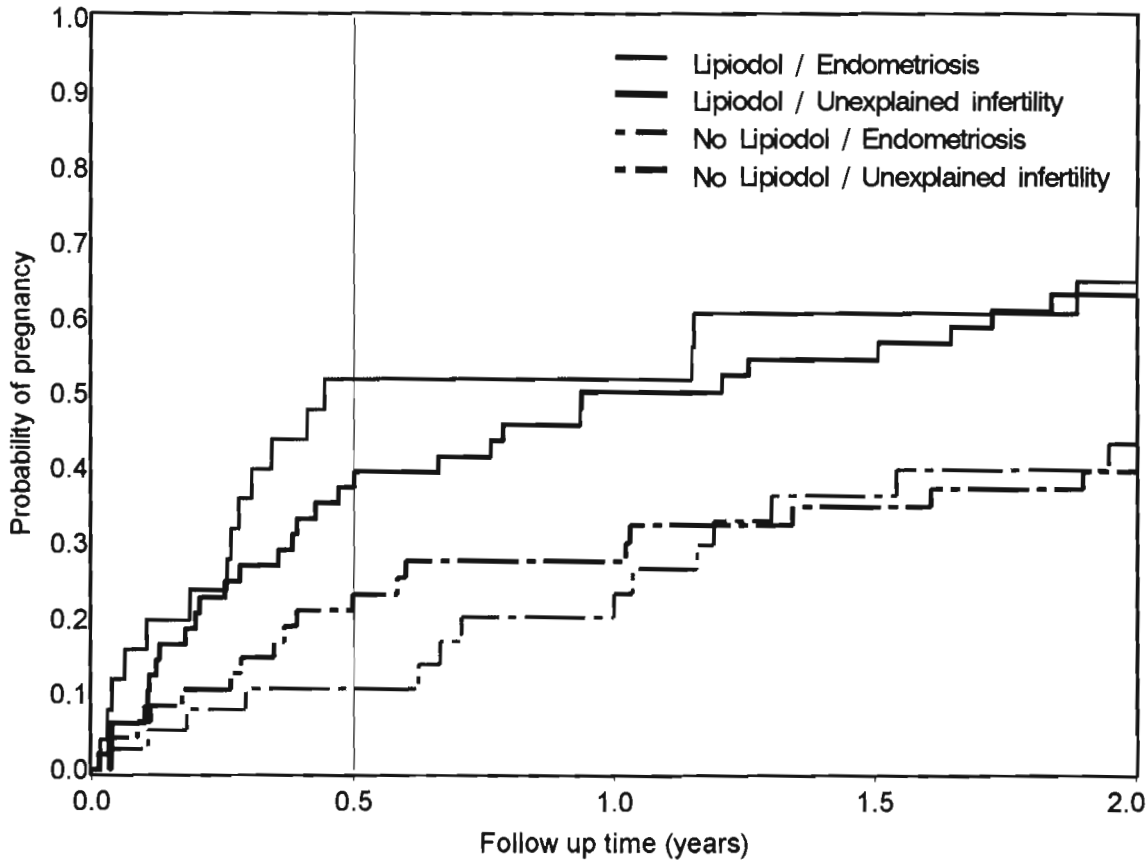


Figure 9.4: Survival Curve of Pregnancy Over 24 Months in Women with Unexplained Infertility

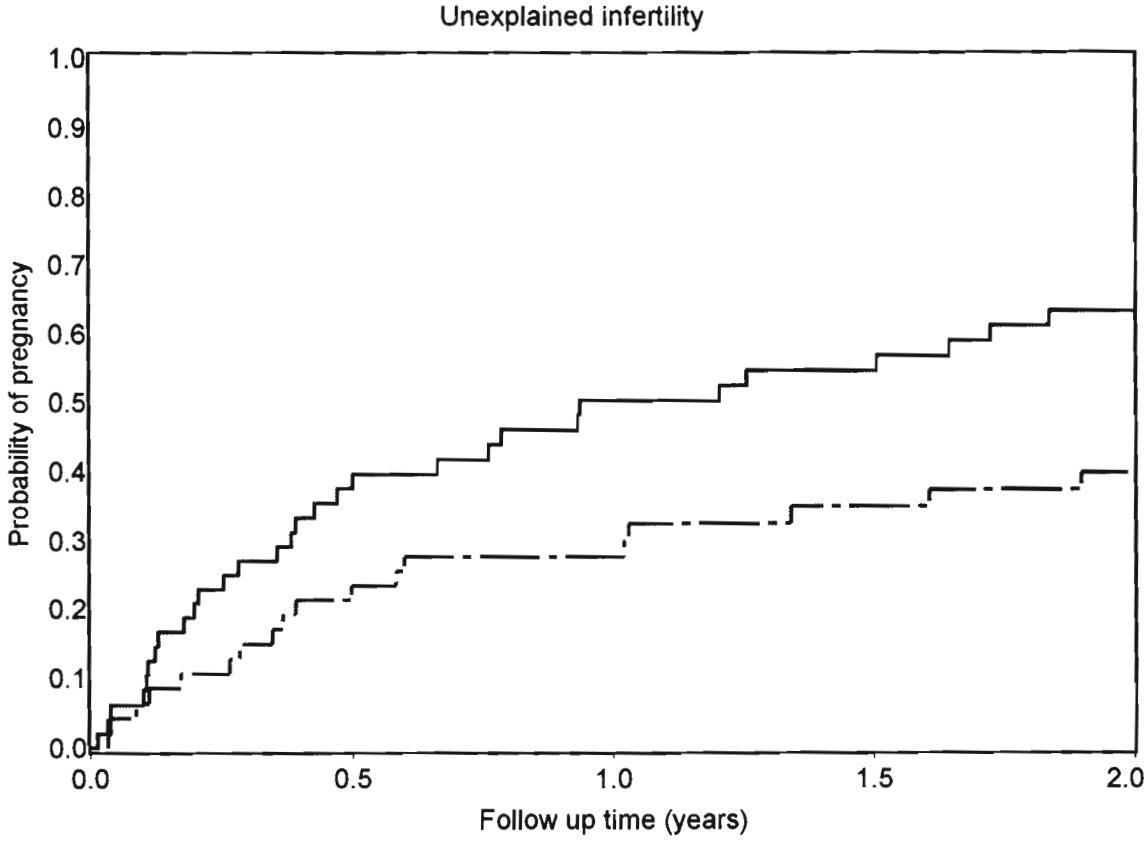


Figure 9.5: Survival Curve of Pregnancy Over 24 Months in Women with Endometriosis

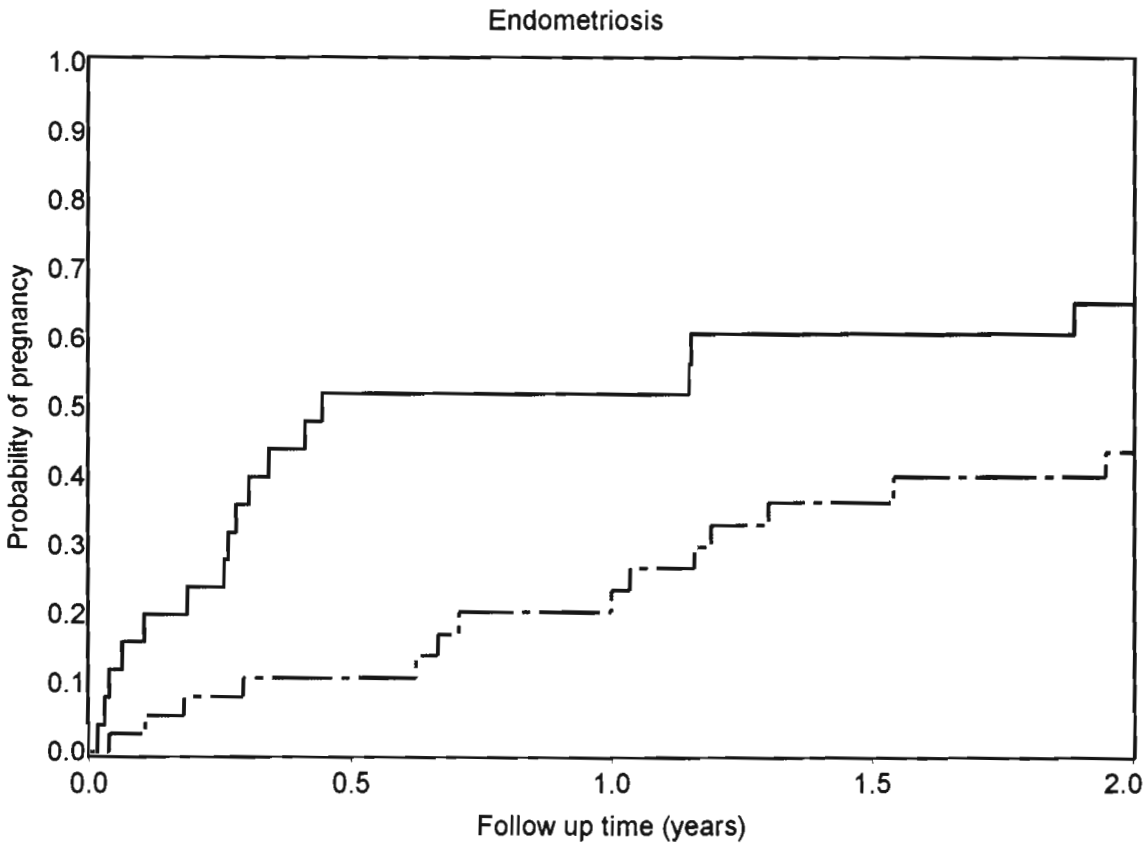


Table 9.1: Intention-to-treat Analysis of Follow Up Data at 24 Months

	Unexplained infertility				Endometriosis-related infertility				Total population			
	Lipiodol (n=48)	No flush (n=48)	Relative risk (95% CI)	P	Lipiodol (n=25)	No flush (n=37)	Relative risk (95% CI)	p	Lipiodol (n=73)	No flush (n=85)	Relative risk (95% CI)	p
Clinical Pregnancy	29	19	1.5 (1.0 to 2.3)	0.04	14	16	1.3 (0.8 to 2.2)	0.32	43	35	1.4 (1.0 to 2.0)	0.03
Sensitivity analyses of imputation for clinical pregnancy: (i)	31	23	1.4 (0.9 to 1.9)	0.11	17	21	1.2 (0.8 to 1.8)	0.36	48	44	1.3 (1.0 to 1.7)	0.08
(ii)	30	21	1.4 (1.0 to 2.1)	0.07	16	19	1.3 (0.8 to 1.9)	0.31	46	40	1.3 (1.0 to 1.8)	0.05
Live birth plus ongoing pregnancy	25	15	1.7 (1.0 to 2.8)	0.05	12	12	1.5 (0.8 to 2.8)	0.21	37	27	1.6 (1.1 to 2.4)	0.02
Ectopic pregnancy	1	1			0	0			1	1		
Multiple pregnancy	2*	0			0	1			2*	1		

Key to Table 8.6:
Assumption for intention-to-treat analysis: those lost to follow up did not become pregnant.
Sensitivity analyses of imputation for clinical pregnancy: assumption (i) that all those lost to follow up became pregnant; assumption (ii) that half of those lost to follow up became pregnant.
Clinical pregnancy was assessed at 24 months post-randomisation; pregnancy outcomes were subsequently ascertained for women achieving pregnancy by that time.
*These two multiple pregnancies were achieved through IVF from 6 to 24 months follow up.

SECTION VI: LIPIODOL AS AN INNOVATIVE TREATMENT

CHAPTER 10: OBSERVATIONAL STUDY OF LIPIODOL AS AN INNOVATIVE FERTILITY TREATMENT

10.1 Introduction

Having confirmed the effectiveness of lipiodol as a fertility treatment in a randomised trial (Chapter 8), this approach became available to New Zealand women in September 2003. There was a strong suggestion that the fertility enhancing affect might be greatest in the short term amongst women with endometriosis, but it is also effective for women with pure unexplained infertility (Chapter 8).

The FLUSH Trial[140] was the first prospective randomised controlled trial (RCT) to report on the effectiveness of lipiodol flushing as a fertility treatment in women with unexplained infertility, with and without endometriosis. The trial was completed in June 2003, demonstrating that lipiodol flushing results in a pregnancy and live birth rate most improved in women with endometriosis but normal patent fallopian tubes. In meta-analysis, the effectiveness of lipiodol flushing in the population with unexplained infertility was also confirmed.[137] Following the success of the FLUSH trial, lipiodol flushing was offered as an innovative treatment in New Zealand from September 2003 onwards.

Randomised trials are the gold standard technique to demonstrate effectiveness of a treatment. However ongoing surveillance in observational studies, in new approaches to treatment, is essential to exclude rarer side effects that the RCT may not have been powered to detect.

10.2 Objectives

The objective of this observational study was to assess the demographics, efficacy and safety of lipiodol flushing in the first cohort of 100 New Zealand

women to undergo the procedure as an innovative treatment, after completion of the Auckland RCT.

10.3 Methodology

Lipiodol flushing was approved as an innovative treatment by the Auckland Ethics Committee in 2003. As part of this approval, informed consent given by women prior to their procedure included an agreement to follow up six months after the treatment.

The study sample comprised the first 100 women with infertility in the context of known bilaterally patent fallopian tubes attending our service consecutively for a therapeutic lipiodol flushing procedure at Auckland Radiology Group between September 2003 and April 2004. Infertility was defined as failure to become pregnant following at least 12 months of unprotected sexual intercourse. Exclusion criteria were a history of ectopic pregnancy, a known iodine allergy, or known tubal occlusion. Routine pre-treatment investigations for couples included an early follicular FSH level, a confirmatory test of ovulation (usually mid-luteal progesterone, but urinary LH detection or ultrasound follicle detection were acceptable) and a tubal patency test (either hysterosalpingogram or laparoscopy and dye studies) for women, with a semen analysis for men. All women were referred after evaluation by a fertility specialist. It was my policy not to perform lipiodol flushing for women within six months of laparoscopic surgical removal of endometriosis.

All women received lipiodol flushing performed by a HSG technique with fluoroscopy X-ray screening, carried out by one of research clinicians (NJ and WH [138]) from September 2003 to April 2004 inclusive. The lipiodol contrast medium was Lipiodol Ultra Fluid (Guerbet, France), iodized poppy seed oil obtained by substitution of ethyl esters for the glycerol esters of lipiodol. One milliliter of Lipiodol Ultra Fluid contains 0.48g iodine. Lipiodol flushing was carried out in the follicular phase of the cycle between the end of menses and day 12 of the cycle, with the woman in the left lateral or supine position, using

a 'no touch' technique after antiseptic solution application to the cervix. One of three delivery systems was used: cervical annulations by a medium or large size Leech-Wilkinson canola (Down's Distributors New Zealand); instillation via a cervical suction cap (Cervix Adaptor, Rocket Medical, United Kingdom) or uterine cannulation using the Cook HSG catheter (Obex, New Zealand). Prewarmed (37°C) lipiodol was slowly instilled, with intermittent fluoroscopic X-ray guidance. The instillation was stopped once unequivocal bilateral spill of contrast from the fallopian tubes into the peritoneal cavity was observed. If no peritoneal spill was observed after use of 10ml, further lipiodol was instilled. If extravasations was observed on X-ray (contrast apparent in the venous system), the policy was to stop instillation immediately.

The primary outcomes were clinical pregnancy (defined as a positive pregnancy test with an intrauterine gestation sac visualized by ultrasound), and live birth or ongoing pregnancy as determined by follow up at six to eleven months post treatment. Secondary outcomes were miscarriage, Ectopic pregnancy, multiple pregnancy and any other complications, including a diagnosis of lipogranuloma. Data for the lipiodol procedures were prospectively recorded at the time of the procedures. Follow-up data were collected from study participants by a telephone consultation. Pregnancy status and the nature of any other complications were verified against the patient's medical records where appropriate.

Results were then analysed for women aged 39 years or under, as it was this group that was studied in the original FLUSH trial (Chapter 8).

There was no external funding for the study.

10.4 Results

The baseline demographics of the first 100 women to undergo the lipiodol flushing procedure as an innovative treatment are presented in Table 10.1. Forty-seven women had unexplained infertility alone, 43 had endometriosis

related infertility alone, and 10 had an additional cause of infertility ('other' category). Women in the 'other' category included those with abnormally high follicular FSH levels (n=3) and women with a partner with additional male infertility (n=7), three of whom had mild endometriosis and four of whom had no other diagnosed infertility factor. The total population had a mean age of 36.8 years (range 27-46 years), although 30 women were aged 40 years or older, and 6 of these were 45 years or older. Ninety-one women were of European ethnicity; the remaining women were of Indian (n=5), Chinese (n=3) and Maori (n=1) ethnicity. One woman (in the endometriosis group) was known to be a current smoker, with smoking status for another 10 women unknown. Five women were lost to follow-up (and were assumed for analysis purposes not to have become pregnant). Three women gave only their status of pregnancy, and no other required information.

Of the 100 procedures performed, 99 were satisfactorily completed. The woman who underwent the one failed procedure (owing to unsuccessful cervical cannulation) decided not to attempt a second procedure, and was subsequently also lost to follow up. Three women had evidence of intravasation, one of whom became pregnant. No women had evidence of lipo-granuloma formation. There were no cases of post-procedural pelvic inflammatory disease reported.

Pregnancy and live birth or ongoing pregnancy rates were collected at six months following the procedure (Table 10.2). The overall pregnancy rate for the 100 women was 30% (18% with lipiodol alone) and the live birth or ongoing pregnancy rate 27%. Of the 30 women who became pregnant, 12 had undergone an additional procedure (in vitro fertilisation [IVF] or intrauterine insemination [IUI]). Table 10.3 shows the outcomes for women under 40 years old. 32% of women under 40 years old with unexplained infertility became pregnant (21% with lipiodol alone), with 25% having a live birth or ongoing pregnancy. Women under 40 years old with mild endometriosis in the context of otherwise unexplained infertility had a 50% pregnancy rate (29% with lipiodol alone) and a 47% live birth or ongoing pregnancy rate.

Of women aged 40 years and older (n=29), the pregnancy rate was 13% and the live birth or ongoing pregnancy rate was 13% at six months (Table 10.4).

80% of women had bilateral tubal patency confirmed by the lipiodol HSG: of these, 33% (26 from 80) became pregnant. 13% of women (2 from 15) in whom lipiodol HSG confirmed only unilateral patency became pregnant; 25% of women (1 from 4) in whom lipiodol HSG failed to confirm patency of either fallopian tube became pregnant (Table 10.5).

10.5 Discussion

The results of this observational study are very promising. To assess the efficacy of the procedure, women under 40 years old were isolated and analysed separately, providing a comparison with the FLUSH trial results (Chapter 8), and showed very similar outcomes. The pregnancy rates of 32% and 50% respectively in women under 40 years with unexplained infertility and endometriosis are similar to those observed in the FLUSH Trial (33% and 48% respectively). These figures included women who chose to undergo additional interventions following their lipiodol procedure, including IUI and IVF – it is uncertain whether these women would have become pregnant without these additional treatments. Live birth or ongoing pregnancy rates of 25% and 47% respectively amongst women under 40 years with endometriosis are also similar to the respective rates of 27% and 40% in the FLUSH Trial (Chapter 8). The observation of no complications underscored the high level of safety of lipiodol flushing.

It is my policy to offer lipiodol treatment only to women who have previously had confirmation of normal patent fallopian tubes, in order to minimise the likelihood of intravasation and the rare complication of lipogranuloma formation.[137] Despite this, I was unable to confirm patency of either fallopian tube at the time of the lipiodol procedure in four women, of whom one subsequently became pregnant. This suggests a possible effect of lipiodol on

the endometrium rather than a mechanical tubal flushing effect or an effect on the intra-peritoneal environment.[137]

Another important feature of the demographics of the women undergoing the procedure was age. Rates of pregnancy were twice as high for women aged 39 or under than for women aged 40 or older (Table 10.4). 30% of women who underwent the procedure were aged 40 or above, an age group that was excluded from the FLUSH Trial. As with natural conception and results from all other fertility treatments, the likelihood of pregnancy following lipiodol flushing declines with advancing age. However the 13% pregnancy rate and 13% live birth or ongoing pregnancy rate in women aged 40 years and older undergoing lipiodol flushing (albeit in the context of a high loss to follow up rate of 13% in this group) compared favourably to the published results for IVF treatment in Australia and New Zealand in 2002 in this age group, which showed a 10.1% pregnancy rate per cycle started and a 5.7% live birth rate per cycle started.[165] For women in this age group, lipiodol flushing seems a more cost effective option than IVF.

There were no reports of multiple births, ectopic pregnancies or other adverse effects (including lipogranuloma formation) following lipiodol flushing in this series. Three women had evidence of intravasation, however none had any adverse effects following this, and one such procedure resulted in pregnancy. Three women complained of pain during ovulation or menstruation for several months following the procedure (none of whom became pregnant). By contrast, two women reported that their usual dysmenorrhoea was reduced following the procedure.

Whilst it has been suggested that the mechanism underlying the fertility enhancing effects of lipiodol may be that OSCM flush non-occlusive but pregnancy-hindering debris from the fallopian tubes,[117] or exert an immuno-biological effect on the intraperitoneal environment, possibly mediated through peritoneal macrophages,[129] my recent data have suggested an additional effect on uterine dendritic cells in mice (Chapter 7). Such a mechanism could

explain an implantation enhancing effect of lipiodol on the endometrium. This is the subject of ongoing research.

Lipiodol flushing has not so far been adopted widely as a routine infertility treatment – reasons for this, based on clinicians' prior beliefs, are explored in Chapter 6.

Conclusion

This prospective observational study of lipiodol fertility treatment has confirmed an efficacy and safety similar to the findings of my randomised trial. It provides further evidence that lipiodol is a simple, effective, minimally invasive treatment that carries a low risk of complications and no increased risk of multiple pregnancy. It continues to provide many New Zealand couples an appealing alternative to the established fertility treatments. Consideration of the adoption of lipiodol as a treatment for well selected couples should be given in other countries.

Table 10.1: Baseline Characteristics of the Population

	Unexplained infertility n=47	Endometriosis-related infertility n=43	Other n=10	Total population n=100
Mean age (SD)	37.6 (4.03)	35.72 (4.11)	37.90 (5.26)	36.83 (4.26)
Body mass index (kg/m2)				
Overall mean (SD)	23.27 (4.19)	22.0 (2.26)	21.18 (1.44)	22.50 (3.35)
<20 (%)	4 (9)	7 (16)	3 (30)	14 (14)
20-24.9 (%)	29 (62)	27 (63)	7 (70)	63 (63)
25-29.9 (%)	8 (17)	4 (9)	0 (0)	12 (12)
>30 (%)	1 (2)	0 (0)	0 (0)	1 (1)
Unknown	5 (11)	5 (12)	0 (0)	10 (10)
Mean FSH (IU/l) (SD)	7.11 (3.04)	7.28 (2.25)	9.98 (4.25)	7.60 (3.04)
Previous pregnancy history				
Primary infertility (%)	27 (57)	26 (60)	7 (70)	60 (60)
Secondary infertility (%)	20 (43)	17 (40)	3 (30)	40 (40)
Months of infertility				
Overall median	40.5	48	54	48
12-23 (%)	0 (0)	3 (7)	1 (10)	4 (4)
24-35 (%)	11 (23)	8 (19)	1 (10)	20 (20)
36-47 (%)	12 (26)	7 (16)	1 (10)	20 (20)
48-59 (%)	6 (13)	7 (16)	2 (20)	15 (15)
>60	13 (28)	17 (40)	5 (50)	35 (35)
Unknown	5 (11)	1 (2)	0 (0)	6 (6)
Previous treatment (%)				
IVF	16 (34)	16 (37)	7 (70)	39 (39)
IUI	23 (49)	16 (37)	5 (50)	44 (44)
Clomiphene citrate	28 (60)	16 (37)	3 (30)	47 (47)
Laparoscopic endometriosis surgery	0 (0)	37 (86)	5 (50)	42 (42)
Unknown	5 (11)	2 (5)	0 (0)	7 (7)

Table 10.2: Main Outcomes

	Unexplained infertility n=47	Endometriosis-related infertility n=43	Other n=10	Total population N=100
Satisfactory completion of procedure (%)	46 (98)	43 (100)	10 (100)	99 (99)
Intravasation (%)	1 (2)	1 (2)	1 (10)	3 (3)
Pregnancy (%)				
Total	11 (23)	19 (44)	0 (0)	30 (30)
Lipiodol only	6 (14)	12 (30)	0 (0)	18 (18)
Additional procedure:	5 (11)	7 (16)	0 (0)	12 (12)
IUI	4 (9)	3 (7)	0 (0)	7 (7)
IVF	1 (2)	4 (9)	0 (0)	5 (5)
Live birth or ongoing pregnancy (%)	9 (19)	18 (42)	0 (0)	27 (27)
Lost to follow up	3 (6)	3 (7)	0 (0)	6 (6)

Table 10.3: Main Outcomes for Women Aged ≤39 Years with Unexplained Infertility or Endometriosis

	Unexplained infertility ≤39 years N=28	Endometriosis-related infertility ≤39 years n=34	Total population ≤39 years n=62
Pregnancy (%)			
Total	9 (32)	17 (50)	26 (42)
Lipiodol only	6 (21)	10 (29)	16 (26)
Additional procedure:			
IUI	2 (7)	3 (9)	5 (8)
IVF	1 (4)	4 (12)	5 (8)
Live birth or ongoing pregnancy	7 (25)	16 (47)	23 (37)

Table 10.4: Comparison of Women Aged ≤39 Years with those Aged ≥40 Years

Age (years)	Total	Pregnancy	Live birth or ongoing pregnancy	Lost to follow up
≤ 39 (%)	70 (100)	25 (36)	22 (31)	2 (3)
≥ 40 (%)	30 (100)	4 (13)	4 (13)	4 (13)

Table 10.5: Characteristics of the Procedure in Relation to the Outcome: Patency of Fallopian Tubes at Lipiodol Hysterosalpingogram

Number of patent tubes	Total	Pregnancy	Lost to follow up
0 (%)	4 (100)	1 (25)	1 (25)
1 (%)	15 (100)	2 (13)	2 (13)
2 (%)	80 (100)	26 (33)	3 (4)

SECTION VII: CONCLUSIONS AND FUTURE DIRECTIONS

CHAPTER 11: CONCLUSIONS

Summary of Conclusions

I have shown that lipiodol is an effective fertility treatment that is particularly effective in the short term up to 6 months for women with infertility related to endometriosis whose fallopian tubes are normal.[140] The effect for women with unexplained infertility is less pronounced but persists longer and is still present at 24 months after a lipiodol procedure (Chapter 9).

Lipiodol is an inexpensive treatment that is administered simply in a HSG procedure (relatively non-invasive compared to established fertility treatments such as IVF), has no increased risk of multiple pregnancy and carries a very low chance of any adverse events.

This research has therefore provided a firm evidence base for lipiodol treatment, thus adding a credible evidence-based alternative to the fertility treatment armamentarium for couples with unexplained and endometriosis-related infertility. Lipiodol flushing has consequently become a routine fertility treatment option for New Zealand for couples.[138] Indeed lipiodol treatment is so effective in the short term for women with endometriosis-related infertility that there is a compelling argument for offering lipiodol as a first line fertility treatment to couples in this situation. Although the vast majority of Australasian fertility specialists surveyed gave an indication that they consider the worthwhile value of lipiodol treatment to have been exceeded by its proven value in unexplained infertility, and all indicated that the actual value exceeds the worthwhile value in endometriosis-related infertility, numerous factors other than strength of supporting data determine the uptake of a new approach to fertility treatment.[136]

Reconciliation with Other Studies

Meta-analysis of treatment success rates with those of similar RCTs has led to more generalisable estimates of the chance of success of lipiodol treatment in the context of an ongoing chance of pregnancy with no treatment. The best estimates of Peto odds ratios for pregnancy following lipiodol versus no

treatment is 2.52 (95%CI 1.13 to 5.17) for unexplained infertility, 6.76 (95%CI 2.14 to 21.35) for endometriosis-related infertility and 3.02 (95%CI 1.41 to 6.48) where the cause of infertility is unspecified; the corresponding odds ratios for live birth are 2.12 (95%CI 0.79 to 5.64) for unexplained infertility and 5.17 (95%CI 1.55 to 17.23) for endometriosis-related infertility.

Strengths and Weaknesses of Studies

I have used different methodologies to investigate the effectiveness and safety of lipiodol as a treatment for infertility. Gold standard methodology was used in the randomised controlled trial of the effectiveness of lipiodol flushing (Chapter 8) that adhered to CONSORT Statement guidelines.

Of course, the studies all had weaknesses. The survey of Australasian fertility specialists had a very low response rate. My murine study investigated only possible actions of lipiodol on the endometrium, not on the pelvic peritoneal layer or in flushing the fallopian tubes. The loss to follow up rate at the 24 month follow up stage was 8.2% in the survival analysis of women involved in the FLUSH Trial RCT. The observational study of 100 women undergoing lipiodol flushing as an innovative treatment in clinical practice is still underpowered to detect rare adverse events and will need to be extended.

Possible Mechanisms

Numerous theories have been proposed in an attempt to explain the fertility enhancing effect of OSCM such as lipiodol. Many of these theories have little supporting evidence, including 'straightening' of fallopian tubes, disruption of peritubular adhesions, stimulation of tubal ciliary action, improving cervical mucus and an iodine-induced bacteriostatic action on mucous membranes.[126]

Mechanical flushing of the fallopian tubes is a more plausible mechanism of effect. It is possible that OSCM may be more effective than WSCM in 'flushing out' debris from otherwise undamaged tubes. Such debris may not necessarily block the fallopian tube, but may hinder conception or embryo transport along the fallopian tube and the observation that lipiodol tubal flushing is effective for women with confirmed tubal patency[121] would support

this. Furthermore there is increasing evidence that some cases of 'blocked' fallopian tubes may have been due simply to tubal plugs, dislodged by OSCM and thus such participants could be classified on the basis of OSCM HSG findings as having unexplained infertility. Histologic examination of resected 'obstructed' tubal segments often fails to confirm luminal occlusion[166] but amorphous matter has been found within tubal sections[167] and its presence confirmed at falloposcopy.[168] Histology of this tissue obtained by hydrotubating the tube at falloposcopy has revealed casts of the tube comprised of aggregates of histiocytic-like cells from the mucosal stroma. Observational studies[169-171] have reported a high tubal patency and pregnancy rate after selective transcervical fallopian tube catheterisation under fluoroscopic or hysteroscopic control in patients with previously diagnosed proximal tubal obstruction on HSG with a WSCM or dye laparoscopy; which might be attributable to the 'flushing out' of isthmic plugs. Thurmond and Rosch (1990)[171] achieved tubal patency of at least one side in 86 of 100 consecutive patients with infertility and proximal tubal obstruction and found that nine of 20 patients who had bilateral cornual blockage and were waiting for tubal surgery or IVF conceived after using the above technique - the majority doing so in the first four cycles after selective tubal catheterisation.

It is likely that fertility enhancement from lipiodol works, at least in part, through an immuno-biological mechanism. OSCM may enhance fertility for women with unexplained infertility or mild endometriosis by affecting peritoneal macrophages[129] - OSCM have been shown to alter interleukin and prostaglandin production by murine peritoneal macrophages[130] and to modulate peritoneal macrophage activity in phagocytosis of sperm in rats.[131]

The fertility enhancing effect might simply lie at the level of the endometrium. For most couples experiencing unsuccessful IVF treatment, the outcome hinges on failed implantation: it stands to reason that a treatment which substantially increases the likelihood of conception is likely to have some effect on endometrial receptivity. Although there is evidence that lipiodol may exert intraperitoneal immuno-biological effects, the fact that four women out of

12 whose fallopian tubes failed to be flushed by lipiodol, which therefore did not bathe the peritoneal cavity, became pregnant within the typical short (6 month) time frame from the lipiodol procedure,[138, 140] suggests a possible uterine bathing effect, giving rise to an endometrium more receptive to implantation. Other studies have shown that uterine natural killer cells play an important role in the successful development of early pregnancy.[172] It is possible that alterations in endometrial leukocyte populations in response to lipiodol exposure could influence endometrial receptivity. My murine study showed that mouse uteri exposed to lipiodol developed a change in uterine dendritic cell populations in close proximity to the uterine lumen.[173] This uterine bathing phenomenon might produce a similar endometrial immuno-biological change in women that could improve endometrial receptivity to the implanting embryo, although this hypothesis remains to be tested.

Unresolved Questions

It remains unclear whether flushing with OSCM offers significant benefit over flushing with WSCM.[119]

Although an immuno-biological mechanism seems likely, the precise mechanism of how lipiodol enhances fertility in women remains unclear. My hypothesis that lipiodol exerts an immuno-biological effect on the endometrium to improve its receptivity to embryo implantation is meritworthy of further investigation.

Many fertility specialist colleagues have queried whether a pre-treatment lipiodol procedure would be expected to enhance then likelihood of IVF treatment being successful. Although there is rationale to believe that this may be so, there is currently no evidence to answer this question.

CHAPTER 12: THE LUBE STUDY PROTOCOL - LIPIODOL UTERINE BATHING EFFECT

12.1. Introduction

Lipiodol is particularly effective in enhancing fertility amongst women with endometriosis (a condition with an immuno-biologic component to the infertility with which it is associated) in the short term (Chapter 8). It is not uncommon for women to become pregnant soon after a lipiodol procedure even when the lipiodol fails to flush the fallopian tubes or bathe the peritoneal cavity: indeed this occurred in 4 out of 12 women in whom patency of neither fallopian tube could be confirmed in the lipiodol procedure.[138, 140] The demonstration of an alteration in the distribution and numbers of uterine dendritic cells in mice exposed to lipiodol raises the question whether a similar mechanism of action of lipiodol in women could be mediated through an endometrial effect with improved receptivity of the endometrium to an implanting embryo.

12.2 Objectives

To determine whether lipiodol uterine bathing is associated with changes in endometrial leukocyte populations or changes in the endometrial expression of key genes believed to be important in implantation. To then determine whether any differences in endometrial gene expression or endometrial leukocyte populations correlate with subsequent pregnancy. It is hoped that this study will:

(a) provide insight into the fertility enhancing effect of lipiodol;
(b) begin to establish the key changes that make a woman's endometrium more receptive to implantation;
(c) begin to address the fundamental question of what makes a woman more fertile;
(d) be the first step to use a model to develop more effective implantation enhancing media.

12.3 Methodology

An open pilot randomised trial of 12 women with infertility and endometriosis (but normal patent fallopian tubes) will be undertaken.

Population

Inclusion criteria will be women aged 18-39 years with a history of 12 months infertility and known endometriosis but with normal fallopian tubes (normal appearance at laparoscopy, bilateral tubal patency demonstrated and no tubal adhesions), with full investigation for infertility by mid-luteal progesterone level, early follicular FSH level within previous 12 months, laparoscopy and tubal patency test and semen analysis for the partner undertaken. Exclusion criteria will be a history of tubal ectopic pregnancy, known iodine allergy, or another cause for infertility – oligo-anovulatory defined by infrequent menstrual bleeding or absence of progesterone levels >25mmol/L, diminished ovarian reserve defined by early follicular FSH levels above 10IU/L, tubal adhesions or absence of demonstrable bilateral tubal patency, or abnormal semen analysis in the male partner.

Interventions, Procedures and Monitoring

Women will be randomised to immediate lipiodol uterine bathing (Group A) versus delayed lipiodol uterine bathing after an interval of 3 menstrual cycles (Group B). For all endometrial sampling cycles, study participants will be advised to use condom barrier contraception to avoid pregnancy on a cycle in which luteal phase endometrial sampling is performed.

Women in Group A (immediate lipiodol uterine bathing group) will undergo a lipiodol procedure in the follicular phase of the cycle on which they are randomised and will undergo pipelle endometrial sampling by curettage under local anaesthetic 5 days after ovulation.

Women in Group B (delayed lipiodol uterine bathing group) will undergo endometrial sampling 5 days after ovulation in the cycle on which they were randomised; they will then undergo a lipiodol procedure after 3 completed menstrual cycles, followed by a similar endometrial sampling procedure 5 days after ovulation in the same cycle as the lipiodol procedure. (The timing of

endometrial sampling for 5 days after ovulation is intended to obtain endometrium at the so called 'implantation window', the time that an embryo would be implanting, standardised to a specific time after ovulation in all women.)

Lipiodol uterine bathing procedures will be performed in the follicular phase of the cycle between the end of menses and Day 12 of the cycle, as previously described in Chapter 8.

The pre-ovulatory hormone surge will be detected by serum luteinising hormone (LH) measurements, commencing 5 days before anticipated ovulation based on a detailed menstrual history, once the plasma estradiol level exceeds 300 pmol/L. The day of ovulation will be defined as the day after LH has reached a level measuring at least three times the basal LH level.

Endometrial sampling procedures will be performed 5 days after ovulation as follows. With the patient fully conscious in the supine position, and using aseptic technique, a pipelle endometrial sampling device will be passed trans-cervically. The endometrial sample will be divided into two, one portion being cryoembedded and cryostored for later immunochemistry analysis, the other portion being stored at 4°C in 'RNA later' for microarray studies.

Allocation

Randomisation will be computer generated in a block of 12 unbeknown to the research nurse, the executor of the assignment. Allocation concealment will be maintained by sequentially numbered sealed opaque envelopes.

Outcome Measures

I will examine differences in:

(a) leukocyte population numbers in endometrial tissue;

(b) endometrial expression of key genes important in implantation.

The comparison groups will be:

(a) women randomised to lipiodol flushing versus no intervention

(b) women as their own controls undergoing, firstly, no intervention, then, after a period of 3 months, lipiodol flushing;
(c) women subsequently becoming pregnant versus those not becoming pregnant.

The primary outcome measures will be:

(i) clinical pregnancy (based on a positive pregnancy test and an intrauterine gestation sac) assessed at the end of 3 completed menstrual cycles (or 3 calendar months for those becoming pregnant) post-randomisation for the immediate lipiodol uterine bathing group (Group A) and assessed at the end of 6 completed menstrual cycles (or 6 calendar months for those becoming pregnant) for the interval lipiodol uterine bathing group (Group B);
(ii) live birth after subsequent assessment of women becoming pregnant.

Sample Size

No power calculation was initially performed since this is a pilot study with no known prior data upon which to base a meaningful power calculation. However concerns have been raised as to whether a sample size of 12 will be sufficient to genuinely test the hypotheses and meet the objectives of the study. I have already shown that the pregnancy and live birth rate following lipiodol flushing is significantly increased for women with endometriosis and this is not one of the objectives of this study. Whilst power calculations are not routinely carried out in genetic microarray studies, there is a wide acceptance that n=12 is a reasonable sample size to detect up- or down-regulated genes that are likely to be clinically important. Undoubtedly a sample size of, say, 20 would increase the power of the study, but this must be carefully evaluated against whether it is reasonable to include a further 8 participants in a pilot study, given the invasiveness of the procedures for the women involved (albeit that I intend to perform these procedures in as minimally invasive a fashion as possible, for example to retrieve sufficient endometrial tissue at endometrial biopsy). Assuming that a similar dendritic cell response to lipiodol will occur in the uterus of women compared to mouse uteri (Chapter 7) following lipiodol exposure (CD1+ counts of 0.2 in those who do not become pregnant and 0.89 in those who do become

pregnant, a difference of 0.69), and that around half of the women in the LUBE Study will become pregnant, the study will have 80% power at the 95% confidence level to detect a 0.69 difference (with similar standard deviations to that seen in the mouse study) between the mean CD1+ cell count in the group who become pregnant versus those not becoming pregnant, with a sample size of 12.

Laboratory Procedures

It is intended to examine the endometrial tissue for concentrations of total leukocytes, dendritic cells, macrophages, natural killer cells and T-cell sub-types, in addition to endothelial cells. Immunochemistry of the endometrium will be performed as follows.

Cryoembedding and cryostorage: The endometrial tissue will be immersed in cryoembedding compound (BioTek, Auckland, New Zealand) frozen in liquid nitrogen, and stored at -80°C until use. Thin sections (5μm) will be cut using a cryostat (Leica CM 1900). The sections will be collected on glass slides and air-dried for an hour. The tissue will be fixed by immersion in cold acetone (-20°C) for 10 minutes, then air-dried for an hour, wrapped in aluminium foil and parafilm then stored at –20°C.

Immunohistochemistry of the endometrium: The primary antibodies will likely be CD14 (L Chamley, University Dept Obstetrics & Gynaecology, Auckland, New Zealand) (macrophages), CD45 (Serotec, ALS, Auckland, NZ) (leukocytes), CD205 (Serotec, ALS, Auckland, New Zealand) (dendritic cells) and CD1 (Santa Cruz Biotechnology, Santa Cruz, USA) (lipid-presenting dendritic cells). Slides of the endometrial tissue will be thawed, and blocked with 10% Normal Goat Serum (NGS) in Phosphate Buffered Saline-Tween (PBS-Tween) for 10 minutes. After three washes with PBS-Tween, the slides will be incubated with the primary antibodies for an hour at room temperature. CD14, CD45, CD205 and CD1 will be diluted 1:2, 1:250, 1:320 and 1:25, respectively, in 10% NGS in PBS-Tween. Following three washes with PBS-Tween the slides will be incubated with a biotinylated anti-mouse IgG diluted 1:1000 in 10% NGS for an hour at room temperature. After three more washes with PBS-Tween, the slides will be incubated with alkaline

phosphotase conjugated streptavidin (Jackson Laboratories, ALS, Auckland, New Zealand) diluted 1:500 in 10% NGS in PBS-Tween for an hour at room temperature. Slides will then be washed three times in PBS-Tween and incubated with AEC chromogen (Zymed, Christchurch, New Zealand) for 15 minutes. Slides will then be washed in de-ionised water, counterstained with haematoxylin (Gills no 2, Surgipath, USA) for 30 seconds and washed with tap water. A coverslip will be mounted using Aquamount (BDH, Palmerston North, New Zealand). The stained slides will be analysed, by an assessor blinded to treatment allocation of the sections, using a light microscope (Leitz Orthoplan, Wetzlar, Germany) and photographed using a Nikon Coolpix 990 digital camera.

Analysis CD14 and CD45 staining:- Analysis will be as performed only for longitudinal sections through the uterine horn. Using a 0.072 mm^2 counting reticule, the number of stained cells present in 5 high power (40x) fields will be counted. The fields will be evenly spaced along the longitudinal axis of the curettings. The mean number of macrophages in the 5 counting rectangles will then be determined and expressed as the number of macrophages or leukocytes per square millimetre. Data from all the women in each comparison group will be used to calculate the mean number of macrophages or leukocytes per square millimetre for each group.

Analysis of Dendritic Cell Staining:- The total numbers of $CD205^+$ and $CD1^+$ dendritic cells (DCs) in the tissue sections will be counted. The slides, as well as a measurement scale will be scanned onto a computer using Adobe PhotoShop (Version 5.0). The areas of the tissue samples in square millimetres will then be determined by setting the measurement scale, and outlining the scanned images using Image J (Public domain software) program. The number of DCs per square millimetre will then be determined. Data from all women in each comparison group will be used to calculate the mean number of DCs per square millimetre for each group.

Immunochemistry Controls:- Positive controls will include uterine tissue sections known to contain the relevant cell types. Negative control sections will also be stained with irrelevant isotype matched monoclonal antibodies.

Endometrial microarray analyses will be performed as follows. The portion of endometrial tissue stored in 'RNA Later' will be transported to the Centre for Genomics and Proteomics, School of Biological Sciences at the University of Auckland, for microarray analyses to be undertaken by Liam Williams. Microarray gene expression analyses will determine which genes are up- and down-regulated in the three comparison groups above. Once the microarray results are available, cryostored endometrium will be assessed via protein lysates – this will determine whether altered expression of key genes in women's endometrial samples following lipiodol that correlates with subsequent pregnancy outcomes is reflected in the presence of key proteins within the endometrium. So doing adds validation to the gene microarray studies.

Statistical methods

Immunochemistry results will be tested for significance between the following groups using either Student's t-test (two-tailed unpaired for differences between groups and two-tailed paired for differences between individuals) or the Mann-Whitney test.

Microarray statistical analyses will be carried out by Associate Professor Cris Print, University of Auckland.

CHAPTER 13: THE IVF-LUBE TRIAL PROTOCOL - LIPIODOL UTERINE BATHING EFFECT PRIOR TO IN VITRO FERTILISATION

13.1 Introduction

The vast majority of couples undergoing in vitro fertilisation (IVF) treatment will reach the stage of embryo transfer. For most couples experiencing uncuccessful IVF treatment, the outcome hinges on failed implantation. Despite much research on the endometrium, there remains no single intervention proven to improve endometrial receptivity to implantation.

If lipiodol is indeed enhancing fertility through a mechanism of improving endometrial receptivity to implantation (Chapter 12), this raises the question of whether administering lipiodol to women prior to an IVF cycle will improve the chance of successful IVF treatment. This would be of particular relevance to couples with a low chance of successful implantation of transferred embryos, such as those who have experienced recurrent IVF implantation failure. If lipiodol does improve the efficacy of IVF treatment, its effect might be expected to also be pronounced in the women with endometriosis, the group with the biggest improvement in their natural fertility following lipiodol treatment (Chapter 8).

Assuming that lipiodol dues work through an immuno-biological mechanism, whether this acts through an endometrial effect described above, or an effect on intraperitoneal immuno-biology (perhaps improving egg quality or sperm-egg interaction), a pre-IVF lipidol procedure might be expected to improve the chance of success of IVF treatment.

13.2 Objectives

To determine whether a pre-IVF lipiodol uterine bathing procedure improves the outcome for women undergoing IVF treatment.

To determine whether a pre-IVF lipiodol uterine bathing procedure improves the outcome for women in predefined sub-populations, namely those with recurrent IVF implantation failure and those with endometriosis.

13.3 Methodology

An open parallel randomised controlled trial will be undertaken. The trial will be of multi-centre design, with Auckland acting as the co-ordinating centre for this Australasian multi-centre trial.

Population

Inclusion Criteria will be:

- Women aged 18-39 years.
- Planning IVF treatment and scheduled to commence a long course GnRH analogue IVF stimulation cycle on the current cycle.
- Bilateral tubal patency confirmed (or single tubal patency demonstrated where a damaged fallopian tube has been surgically removed).
- Normal early follicular FSH level within previous 12 months.
- Otherwise fully investigated for cause of infertility, by having had (a) a mid-luteal progesterone level or other tests to confirm a cause of anovulation; (b) partner semen analysis checked.
- Agreeable and able to attend for a lipiodol uterine bathing procedure after the end of menses but on or before day 12 of the cycle on which down-regulation with GnRH analogue treatment will commence in the mid-luteal phase (as part of a long course agonist stimulation protocol).

The additional inclusion criterion for the recurrent IVF implantation failure sub-population will be:

- Three previous embryo transfers (which may be either fresh embryo transfers, embryo thaw cycles for cryopreserved embryos, or any combination of these, where embryo(s) of good quality on morphological grounds have been transferred in an uncomplicated embryo transfer procedure, none of which have resulted in clinical pregnancy.

The additional inclusion criterion for the endometriosis sub-population will be:

- Laparoscopic diagnosis of endometriosis made previously (whether or not this has been surgically removed).

Exclusion criteria will be:

- Blocked or damaged fallopian tubes (unless the damaged fallopian tube has been surgically removed and the other fallopian tube shown to be patent).
- History of tubal ectopic pregnancy.
- Known iodine allergy.

Allocation and Trial Conduct

Women interested in the trial will be referred to research nurses at each centre, who will ensure eligibility is confirmed and that potential participants are fully informed about the nature of treatments involved in the trial. The research nurse will carry out formal enrolment in the trial through an on-line application, which will supply the treatment allocation by a third party randomisation allocation (randomisation being computer generated).

True third party randomisation will be performed by a computer generated randomisation schedule. Allocation concealment will be maintained by a third party system involving an on-line application once entry criteria are fulfilled – women will be allocated to either lipiodol plus IVF or IVF alone. Three separate randomisation schedules will be used: one for women with recurrent IVF implantation failure; one for women with endometriosis; one for all other women in the IVF population (the three a priori specified sub-populations). Each randomisation schedule will be blocked in units of 10, but the research nurses at the various centres will be unaware of this. Women will be randomised early in the follicular phase of the cycle (as close to the first day as possible) on which they are due to commence GnRH analogue down-regulation treatment in the mid-luteal phase of the cycle, as part of the long course agonist stimulation protocol.

No blinding of participants, assessors, clinicians or those involved in analysis will be employed.

Interventions and Procedures

Three sub-population groups will be identified from the overall population of 600 women. I will randomise at least 250 women with recurrent IVF implantation failure, at least 250 women with endometriosis, and the remainder of the total of 600 women undergoing IVF for any other reason.

Lipiodol procedures will be performed as previously described. The precise IVF protocol will be that of the respective participating fertility centres where women are due to undergo this treatment.

Standard monitoring of an IVF stimulation cycle according to the protocol of the participating fertility centres will be undertaken. All women will be prescribed folic acid supplementation.

Outcome Assessment and Follow-up

The primary outcome measure will be live birth. Secondary outcome measures will be:

- biochemical pregnancy, defined as a positive monoclonal urinary hCG test or a serum beta-hCG greater than 5 IU/L;
- clinical pregnancy, defined as an intrauterine gestation sac in association with a positive pregnancy test;
- viable pregnancy, defined as the presence of intrauterine fetal heart activity;
- multiple pregnancy;
- miscarriage, defined as pregnancy loss before 20 completed gestational weeks
- ectopic pregnancy, defined by positive extrauterine histology following surgery or inadequately rising beta-hCG levels in association with an adnexal mass suggestive of ectopic pregnancy;
- live birth of a healthy singleton baby at term;

- IVF outcomes, including cycle cancellation prior to oocyte retrieval, number of eggs collected, cycle cancellation prior to embryo transfer, number of embryos available for cryopreservation, complications of lipiodol procedures including adverse reaction, intravasation, lipogranuloma formation or pelvic inflammatory disease;
- complications of IVF including hospital inpatient admission for ovarian hyperstimulation syndrome, pelvic inflammatory disease or other procedure-related complications.

The outcomes will be assessed at the end of the index IVF cycle, initially assessed 1 month after oocyte retrieval. For patients having all embryos cryopreserved (a 'freeze all', usually recommended for an over-response following hormonal stimulation and egg collection), the outcome from the first embryo thaw cycle will be the definitive outcome – for such delayed outcomes, further follow-up 6 months after egg collection will be undertaken. Women becoming pregnant will have a further follow-up one month after the expected date of delivery.

Sample Size

600 trial participants will be randomised – at least 250 women with recurrent IVF implantation failure; at least 250 women with endometriosis (in the absence of recurrent IVF implantation failure); the remainder with any cause for infertility as long as the entry criteria are fulfilled.

For women with (a) recurrent IVF implantation failure and (b) endometriosis, 240 analysed participants would be required for 80% power at the 95% confidence interval to demonstrate an increase in live birth rate from 15% in women receiving IVF alone, to 30% in women receiving lipiodol and IVF. Allowing for withdrawals after randomisation, the required number of participants in each of these sub-populations will be 250. For the total population undergoing IVF treatment, 588 analysed participants would be required for 80% power at the 95% confidence interval to demonstrate an increase in live birth rate from 20% in women receiving IVF alone, to 30% in

women receiving lipiodol and IVF. Allowing for withdrawals after randomisation, the required number of participants will be 600.

Statistical Analyses

For dichotomous variables, the test of statistical significance will be the chi-square test; for continuous variables, statistical analysis will be a Student's t-test for normally distributed variables, or a Wilcoxon rank sum test for non-parametric data. 'Intention to treat' analysis (by group of allocation) will be employed, in addition to an exploratory 'actual treatment' analysis.

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