The use of kisspeptin as a novel physiological oocyte maturation trigger for patients undergoing IVF treatment

MD(Res) Thesis

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Declaration of Originality

This is to certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.
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Abstract

Background: Patients with inactivating mutations in kisspeptin signalling are infertile. Kisspeptin-54, the major circulating isoform of kisspeptin in humans, potently stimulates reproductive hormone secretion in humans. Animal studies suggest that kisspeptin is involved in the generation of the luteinising hormone surge, which is required for ovulation. I hypothesised that kisspeptin-54 could be a novel method used to trigger egg maturation in women undergoing in vitro fertilisation therapy.

Methods: This was a proof of concept study. Following superovulation with follicle stimulating hormone, and gonadotrophin releasing hormone antagonist administration to prevent premature ovulation, 53 women were administered a single subcutaneous injection of kisspeptin-54 (1.6nmol/kg, n=2; 3.2nmol/kg, n=3; 6.4nmol/kg, n=24; 12.8nmol/kg, n=24) to induce a luteinising hormone surge and egg maturation. Eggs were retrieved transvaginally 36h after kisspeptin injection, assessed for maturation (primary outcome), and fertilised by intracytoplasmic sperm injection with subsequent transfer of one or two embryos.

Results: Overall, egg maturation was observed in 96% (51/53) of patients. Fertilisation of eggs and transfer of embryos to the uterus occurred in 92% (49/53) of patients. Biochemical and clinical pregnancy rates were 40% (21/53) and 23% (12/53), respectively.

Conclusion: This study demonstrates that a single injection of kisspeptin-54 can induce high rates of egg maturation in women with sub-fertility undergoing in vitro fertilisation therapy. Subsequent fertilisation of eggs matured following kisspeptin-54 administration and transfer of resulting embryos can lead to successful human pregnancy.
Trial registration: ClinicalTrials.gov NCT01667406

Funding: Medical Research Council, Wellcome Trust & National Institute of Health Research.
Declaration of Contributors

The author of this thesis performed the screening and recruitment of the patients included in the study. The author supervised the phase of ovarian stimulation and transvaginal oocyte retrieval of the included patients and devised the clinical ease of collection grading system.

The author further performed the data collection and data analysis for patients who had a GnRH agonist as oocyte maturation trigger.

The collected oocytes were assessed for maturity and underwent intracytoplasmic sperm injection at the Embryology Laboratory of the IVF Unit at Hammersmith Hospital.

Dr Ali Abbara had a pivotal role in the collection and analysis of data and Professor Waljit Dhillo supervised the progress of the work through structured, regular meetings.

Mr Geoffrey Trew provided senior clinical input regarding the individual response of patients during the phase of ovarian stimulation.
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I would like to thank Dr Channa Jayasena for his advice and guidance.

Lastly, I would like to thank my family and Tina for their support and encouragement, always.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>2PN</td>
<td>Two pronuclei stage</td>
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<tr>
<td>AMH</td>
<td>Anti-mullerian Hormone</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproduction Techniques</td>
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<tr>
<td>ARDS</td>
<td>Adult Respiratory Distress Syndrome</td>
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<tr>
<td>ASRM</td>
<td>American Society of Reproductive Medicine</td>
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<tr>
<td>AVPV</td>
<td>Anteroventral Periventricular nucleus</td>
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<tr>
<td>BMI</td>
<td>Body Mass Index</td>
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<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>FSH</td>
<td>Follicle Stimulating Hormone</td>
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<tr>
<td>Gm</td>
<td>Geometric mean</td>
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<tr>
<td>GnRH</td>
<td>Gonadotrophin Releasing Hormone</td>
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<td>GnRHa</td>
<td>GnRH agonist</td>
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<tr>
<td>hCG</td>
<td>Human Chorionic Gonadotrophin</td>
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<tr>
<td>hMGs</td>
<td>Human Menopausal Gonadotrophins</td>
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<tr>
<td>ICM</td>
<td>Inner Cell Mass</td>
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<td>ICSI</td>
<td>Intracytoplasmic Sperm Injection</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like Growth Factor 1</td>
</tr>
<tr>
<td>IVF</td>
<td>In Vitro Fertilisation</td>
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<tr>
<td>IL-1b</td>
<td>Interleukin 1b</td>
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<td>IL-6</td>
<td>Interleukin 6</td>
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<tr>
<td>IR</td>
<td>Immunoreactivity</td>
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<tr>
<td>Kg</td>
<td>Kilogram</td>
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<td>Kg/m²</td>
<td>Kilogram per square meter</td>
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<tr>
<td>KP</td>
<td>Kisspeptin</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>L</td>
<td>Litre</td>
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<tr>
<td>LH</td>
<td>Luteinising Hormone</td>
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<tr>
<td>LPS</td>
<td>Luteal Phase Support</td>
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<tr>
<td>Lq</td>
<td>Lower quartile</td>
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<tr>
<td>Mm</td>
<td>Millimetre</td>
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<tr>
<td>M2</td>
<td>Metaphase 2</td>
</tr>
<tr>
<td>mIU</td>
<td>milli- International units</td>
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<tr>
<td>ml</td>
<td>millilitre</td>
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<tr>
<td>nmol</td>
<td>nano mole</td>
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<tr>
<td>ng</td>
<td>nano gram</td>
</tr>
<tr>
<td>OHSS</td>
<td>Ovarian Hyperstimulation Syndrome</td>
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<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
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<tr>
<td>Pg</td>
<td>Pico gram</td>
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<tr>
<td>Pmol</td>
<td>Pico mole</td>
</tr>
<tr>
<td>POPV</td>
<td>Preoptic Periventricular Nucleus</td>
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<tr>
<td>rFSH</td>
<td>Recombinant FSH</td>
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<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>TGF</td>
<td>Transforming Growth Factor</td>
</tr>
<tr>
<td>Uq</td>
<td>Upper quartile</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>Vascular Endothelial Growth Factor Receptor-2</td>
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CHAPTER 1

Introduction
Infertility

Infertility is defined by the Human Fertilisation and Embryology Authority (HFEA) as an inability of a couple to conceive after two years of regular sexual intercourse. The National Institute for Clinical Excellence (NICE) estimates that one in six couples in the UK have difficulty conceiving. Infertility constitutes, therefore, a significant health problem, which can have substantial medical, psychological and financial implications for women and couples who find it difficult to conceive. IVF has now become an important aspect of assisted conception treatment for such couples.

IVF treatment

Since Louise Brown, the first IVF baby, was born in 1978, the science of *in vitro* fertilisation (IVF) has changed considerably. IVF treatment was initially indicated for patients with tubal blockage, but it has since been established as the treatment of choice for many couples with unexplained infertility, male infertility, polycystic ovarian syndrome and endometriosis.

IVF treatment consists of seven, sequential steps, which occur continuously during a single menstrual cycle:

1. **Controlled ovarian stimulation.** Exogenous hormones are administered to the patients to stimulate the ovaries such that multiple follicles containing oocytes develop simultaneously. Conversely during a physiological menstrual cycle, the ovaries typically produce only one follicle containing one oocyte.
2. *Preventing a premature LH surge.* A premature surge of luteinising hormone (LH) could lead to premature ovulation and to a decrease in the number of eggs, which could then be retrieved. The use of GnRH analogues during the phase of stimulation of the ovaries can prevent the LH surge through reversible blockade of the pituitary GnRH receptors.

3. *Triggering oocyte maturation.* In order for oocytes to become mature and gain the competence for fertilisation by sperm, LH exposure is required. This can be achieved through the bolus administration of pharmacological agents such as hCG, which simulates the effects of the natural mid-cycle LH surge.

4. *Oocyte retrieval.* The oocytes are usually retrieved from the ovaries in an outpatient setting. The operating doctor passes a needle through the vaginal wall under ultrasound guidance and aspirates the fluid from the follicles to retrieve the egg.

5. *In vitro fertilisation.* The collected oocytes are then placed adjacent to sperm in laboratory petri dish for in vitro fertilisation (IVF). Alternatively the embryologist may perform a procedure called intracytoplasmic sperm injection (ICSI), during which a single spermatozoon is injected directly into the oocyte for fertilisation.

6. *Embryo transfer.* The cultured embryos are subsequently replaced in the uterus using a specially designed catheter under ultrasonographic guidance. This should lead to pregnancy if the embryo successfully implants into the uterus.

7. *Luteal phase support:* After the embryos have been replaced in the uterus, the patients are given exogenous progestogens to enhance the luteal phase and promote the implantation of the embryos.
I will describe each of these sections of IVF treatment below.

1. **Controlled ovarian hyperstimulation.**

   The aim of the initial phase of IVF treatment is to promote multi-follicular growth via pharmacological ovarian stimulation. To achieve this, human menopausal gonadotrophins (hMGs), which are manufactured from urine of menopausal women, or recombinant FSH (rFSH) are used. Their defining difference is that hMG exerts a dual FSH and LH activity, unlike rFSH, which does not exert any LH activity (van Wely et al, 2011).

2. **Preventing a premature LH surge**

   **(a) preventing a premature LH surge using GnRH agonists**

   During in vitro fertilisation (IVF) cycles, the general aim is to improve success rates by producing a sufficient number of embryos. During the initial stage of controlled ovarian stimulation, patients are administered individualised doses of gonadotrophins in order to induce the growth of multiple follicles. However, a premature LH surge constitutes a significant risk, which could lead to premature ovulation and to a decrease in the number of oocytes, which can therefore be retrieved. The use of GnRH agonists during the phase of controlled stimulation has allowed for prevention of a premature LH surge through downregulation of the GnRH receptors of the pituitary gland (Maheshwari 2011). The positive effect of the use of GnRH agonists in IVF cycles was first demonstrated by researchers in Hammersmith Hospital. Namely Rutherford et al (1988) first reported that the use of buserelin to down-regulate pituitary receptors prior to commencing ovarian stimulation with urinary gonadotrophins was associated with significantly improved numbers of recovered oocytes, obtained embryos and clinical pregnancies compared to the usual
time- regime of clomiphene citrate and urinary gonadotrophins.

More specifically, the GnRH agonists bind to their pituitary receptors and lead to an initial release of high amounts of FSH and LH, also known as the flare-up effect. After prolonged administration, the GnRH agonist / receptor complex is internalised in the pituitary gonadotroph cell and a down-regulation of GnRH receptors occurs. This desensitisation of the pituitary gland usually occurs after at least two weeks of GnRH agonist treatment. The return to normal function of the GnRH receptor after cessation of the GnRH agonist administration is gradual, which may also have implications for the luteal phase of an IVF cycle.

During a long GnRH agonist protocol, GnRHa is administered for a minimum of 14 days to achieve pituitary down-regulation before the gonadotrophin is commenced (Maheshwari et al, 2011). Two distinct long protocols exist: in the luteal phase (or day 21) long protocol the GnRHa is commenced from day 21 (mid-luteal phase) of the previous cycle. In comparison during a follicular phase protocol, GnRHa is commenced on day 2 of the menstrual cycle. Concurrent GnRHa and gonadotrophins administration continues until an adequate number of stimulated follicles are present, at which stage a bolus injection of hCG is administered to induce oocyte maturation. When comparing the two long GnRHa protocols, Kondaveeti-Gordon et al (1996) reported no significant difference in pregnancy rates, although Jenkins et al (1996) reported that the follicular phase long protocol was associated with a higher risk of functional cysts, which can cause significant disruption in the planning of an IVF cycle.
(b) Preventing a premature LH surge using GnRH antagonists

The long GnRHa protocol appears to be an effective method of ovarian stimulation in IVF cycles and has played a pivotal part in the reduction of the premature surge of LH, which would lead to IVF cycle cancellations. On the other hand the long protocol requires a minimum of 2 weeks to induce ovarian desensitisation. This can have a significant impact to patients and increase the psychological burden associated with fertility treatment. It may also have a relatively high cost in terms of finances and time, due to an increase in for gonadotrophin requirement and the subsequent need for frequent ultrasound assessments (Oliviennes et al, 1994).

GnRH antagonists have now been developed as the alternative option for the prevention of a premature LH surge during IVF treatment. GnRH antagonists can prevent a premature LH surge during the phase of ovarian stimulation as well as the notable hypo-oestrogenic side effects or long period of administration associated with the use of GnRH agonists (Al Inany et al, 2011). GnRH antagonists prevent endogenous GnRH from stimulating the pituitary gonadotroph cells through the competitive blockade of the GnRH receptors. This blockade of the GnRH receptors of the pituitary gland leads to a cessation of FSH and LH secretion from the pituitary gonadotroph cells. As a result, the main advantage of using GnRH antagonists to prevent premature LH surge is the rapidity of onset of action and the rapidity of offset, due to the fact that they are competitive antagonists and can therefore be displaced from the receptor by a GnRH agonist. The use of GnRH antagonists thus allows for the use of a GnRH agonist to trigger oocyte maturation.
The GnRH antagonist (ganirelix or cetorelix) is administered in daily 0.25mg doses, from the sixth day of controlled ovarian stimulation onwards (Huirne et al, 2007). A further variation was introduced to reduce the required number of GnRH antagonist doses by the use of flexible protocols. In a flexible protocol, the GnRH antagonist is commenced when the largest ovarian follicles reach a size >14-16 mm in mean diameter.

A meta-analysis to compare the fixed and flexible GnRH antagonist protocols, has demonstrated no statistically significant difference in pregnancy rates, although there appeared to be a trend towards higher pregnancy rates when the fixed protocol was used (Al Inany et al, 2005). On the other hand, the flexible protocol was associated with a statistically significant decrease in the number of GnRH antagonist doses and the total amount of required gonadotrophins used.

A prerequisite for the GnRH antagonist protocol to commence is the occurrence of spontaneous menses, as the phase of controlled ovarian stimulation generally commences on day 2 of the menstrual cycle. This may pose various difficulties in the scheduling of the IVF treatment to meet the organisational needs of the patient and the IVF unit with subsequent practical and financial implications. Oral contraceptive pill pretreatment has been used to counter this problem. The timing of the withdrawal bleed after the contraceptive pill pretreatment can be manipulated in GnRH antagonist cycles and can help with the scheduling of the oocyte retrieval to the convenience of both the patient and the physician (Griesinger et al, 2008).

The initial results of randomised studies and meta-analyses (Al Inany et al, 2002) comparing GnRH agonist and antagonist protocols identified a 5% absolute reduction in clinical pregnancies associated with the antagonist protocol. This difference in
pregnancy rates was also demonstrated after the analysis of the results of a large German national IVF database and—as a result, the GnRH antagonist approach became the second choice for a large number of clinicians (Griesinger et al, 2005). These studies comparing the two suppression protocols employed hCG as the oocyte maturation trigger. By contrast the GnRH antagonist protocol was consistently associated with a reduced risk of ovarian hyperstimulation syndrome (OR 0.46, 95% CI 0.26-0.82) (Al Inany et al, 2001; Al Inany et al, 2006).

Conversely the most recent systematic review and meta-analysis by the same authors (Al Inany et al, 2011) failed to identify any significant differences live birth rates when GnRH antagonist and GnRH agonist protocols were compared (OR 0.86, 95% CI 0.72-1.02). With regards to the safety during IVF treatment, GnRH antagonists continued to result in a significant reduction in the incidence of OHSS. These changes in the results of the most recent meta-analysis can potentially be attributed to more appropriate patient selection and IVF protocol allocation. In other words the improved learning curve and further experience with the use of GnRH antagonist protocols may have led to these favourable outcomes.

3. Triggering oocyte maturation

(a) hCG triggering of final oocyte maturation

During a natural cycle, the mid-cycle surge of pituitary LH (and FSH) secretion induces ovulation. hCG shares the same alpha subunit and 81% of the amino acids of the beta subunit of LH. Both LH and hCG bind to the same receptor, which was first described by Kessler et al in 1979. Exogenous urinary hCG (at doses of 5000–10,000
IU) has been successfully introduced to simulate the natural LH surge and lead to oocyte maturation. The use of recombinant hCG has now become routine practice. Indeed in a randomised controlled trial by Papanikolaou et al (2010), patients were randomized to recombinant hCG (250 µg) or urinary-derived hCG (10,000 IU) to induce oocyte maturation. The pregnancy rate was significantly improved with recombinant hCG compared to urinary hCG.

Notably hCG has a significantly longer half-life than LH and will provide support to the multiple corpora lutea for seven to ten days, after which time hCG is cleared from circulation (Damewood et al., 1989; Mannaerts et al., 1998). Importantly the longer half-life of hCG in comparison to endogenous LH also results in a prolonged luteotrophic effect (promotion of multiple corpora lutea) and elevated serum oestradiol and progesterone levels during the luteal phase (Itskovitz et al., 1991), which has been shown associated with improved pregnancy rates, but also an increased risk of ovarian hyperstimulation syndrome (Haning et al., 1985). As a result alternative trigger agents have been investigated and developed because of this complication associated with the use of hCG.

(b) GnRH agonist triggering of final oocyte maturation

GnRH agonists can also promote oocyte maturation by inducing a surge in gonadotrophon secretion (flare-up effect), similar to the surge that occurs in the physiological cycle (Gonen et al., 1990; Itskovitz et al., 1991). However, when a GnRH agonist is used to down-regulate the pituitary GnRH receptors prior to commencing the phase of ovarian stimulation (Porter et al., 1984), the use of GnRHa for triggering oocyte maturation would not be applicable, as the GnRH receptor is still
down-regulated and non-responsive. Since the introduction of GnRH antagonists for the prevention of a premature LH surge during IVF treatment (Albano et al., 1997; Itskovitz-Eldor et al., 1998; Borm and Mannaerts, 2000;), it has become possible to induce oocyte maturation with a bolus dose of a GnRH agonist as an alternative to HCG. The GnRH agonist displaces the antagonist from the pituitary GnRH receptor and thus leads to a surge of endogenous gonadotrophins (LH and FSH).

The GnRH agonist-induced surge of FSH and LH bears differences to the surge in gonadotrophins, which occurs in a physiological menstrual cycle. Specifically the physiological cycle surge of LH appears to have three sequential phases: a phase of rapid ascent with a duration of fourteen hours, a plateau with a duration of fourteen hours and a descending phase lasting twenty hours, such that the total duration is 48 hours (Hoff et al., 1983). In comparison, the GnRH agonist-induced LH surge consists of two phases; a short ascending phase lasting >4 hours and a long descending phase lasting >20 hours and has a total duration between 24 to 36 hours (Itskovitz et al., 1991). This difference results in a significant reduction in the amount of released pituitary gonadotrophins when a GnRH agonist is used as the trigger of oocyte maturation when compared with the natural menstrual cycle (Gonen et al., 1990; Itskovitz et al., 1991). As a result, GnRH agonist trigger has been associated with a deficient luteal phase and thus lower pregnancy rates (Balasch et al., 1995; Segal and Casper, 1992). To overcome the deficient luteal phase in IVF cycles, modified luteal phase support is used in IVF cycles to improve the pregnancy outcomes following GnRH agonist triggers (Humaidan et al., 2010).
Despite this, the use of a GnRH agonist trigger has significant safety advantages compared to hCG triggering, namely a significant reduction in and in some studies the complete prevention of ovarian hyperstimulation syndrome (Kol and Itskovitz-Eldor, 2000; Kol, 2004; Orvieto, 2005). In addition, the use of a GnRH agonist trigger has been shown to result in a larger number of retrieved, mature, metaphase II (MII) oocytes when compared with an hCG trigger (Humaidan et al., 2005; Imoedemhe et al., 1991; Oktay et al., 2010). This particular finding could partly be attributed to the endogenous FSH surge, which occurs concurrently with the LH surge when a GnRH agonist trigger is used (Kol and Humaidan, 2010). In contrast an increase in FSH does not occur when hCG is used as a trigger of oocyte maturation. Theoretically the endogenous, concurrent surge in FSH can lead to oocyte nuclear maturation, through resumption of the second meiotic division and promote the formation of LH receptors in the luteinising, granulosa cells, thus benefiting the function of the multiple corpora lutea (Eppig, 1979; Stickland and Beers, 1976; Yding Andersen, 2002; Yding Andersen et al., 1999; Zelinski-Wooten et al., 1995).

4. Oocyte retrieval

Under transvaginal ultrasound guidance, the operating doctor inserts a long needle through the lateral fornix of the vaginal wall directly into an ovarian follicle, taking care not to injure any structures between the point of insertion of the needle into vaginal wall and the ipsilateral ovary. The proximal end of the needle is attached to a gentle suction apparatus. After each follicle is penetrated, gentle suction of 150mmHg is applied to aspirate the follicular fluid and with it the oocyte surrounded by granulosa cells. The follicular fluid is delivered directly to the embryologist in the laboratory, who will subsequently identify and quantify the collected oocytes.
all the accessible ovarian follicles have been aspirated from the ipsilateral ovary, the needle is withdrawn from the lateral fornix, and the procedure is repeated at the contralateral ovary. The procedure usually lasts from 20–40 minutes.

5. In vitro fertilisation and ICSI

During routine IVF each egg is transferred in a culture droplet containing motile sperm at a concentration of 100 000 per ml. A single sperm will then spontaneously bind to and penetrate the zona pellucida of the egg to achieve fertilisation. In contrast, ICSI involves the injection of one (single) motile spermatozoon directly into the cytoplasm of the oocyte (ooplasm) (Figure 1.1). ICSI is often recommended when the male partner has a reduced sperm count; if sperm demonstrates poor morphology and/or poor motility and/or low concentration; if there was failed fertilisation or a low fertilisation rate at previous in vitro fertilisation (IVF) cycles.

![Figure 1.1](image_url)  
**Figure 1.1.** Intracytoplasmic sperm injection procedure. The polar body is visible at 6 o’clock, the micro-injector is seen at 3 o’clock and the end of the holding pipette at 9 o’clock. (Courtesy of IVF Hammersmith, Imperial College Healthcare NHS Trust)

6. Embryo development and transfer

The oocytes are assessed for fertilisation 16-18 hours after the ICSI procedure has
taken place. The oocytes are considered as fertilised when two clearly identifiable pronuclei are visible in the ooplasm (2-PN stage). The female pronucleus is a vesicular nucleus, which contains the haploid copy of the female chromosomes and forms in the oocyte immediately after entry of the spermatozoon. The spermatozoon forms the male pronucleus; which contains the haploid copy of the male chromosomes, after its tail detaches and degenerates.

The 2-PN oocytes will enter the cleavage stage, which results in embryos with multiple cells (blastomeres). Normal, good-quality embryos enter the four-cell stage on day 2 and the 8-cell stage on day 3 (Figure 1.2). At that point, the number and size of blastomeres along with the presence of anucleate fragments is recorded. Given that the embryonic genome becomes fully activated at the 8-cell stage, it would be preferable to use embryos that have undergone this transition from the maternal to the

![Figure 1.2](image)

**Figure 1.2.** Day 3 embryo (8-cell stage). Blastomeres appear similar in size without fragmented components.
Figure 1.3. Day 4 embryo (morula). The blastomeres have undergone full compaction (16-32-cell stage)

Figure 1.4. Day 5 embryo (blastocyst). The inner cell mass (ICM) is easily discernible at 7 o’clock and the trophoectoderm can be seen as a layer of cells throughout the periphery of the embryo.

(Images are a courtesy of the IVF Hammersmith, Imperial College Healthcare NHS Trust)

embryonic genome (Braude et al, 1988). This would potentially allow the identification of the embryos with the optimal potential for development. As a result, when feasible, embryos are allowed to progress to day 5 before transfer. Following a
full compaction of the embryonic cells on day 4 (morula stage) (Figure 1.3), an embryonic cyst gradually appears (blastocoele) (Figure 1.4). The blastocoele will separate the embryonic components to the inner cell mass (embryoblast) and the trophoectoderm which will subsequently form the placenta.

The decision on which of the embryos are more suitable to return to the uterus for implantation depends on a grading system, which is based on morphological criteria (Table 1.1).

Similar pregnancy outcome have been achieved following the transfer of a single blastocyst compared to two blastocysts (Criniti et al, 2005). A statistically significant increase in biochemical pregnancy (confirmed by the presence of a positive pregnancy test) (OR 1.45, 95% CI 1.03–2.04) and clinical pregnancy rates (defined by the presence of an active fetal heartbeat at the 6th gestational week ultrasound) (OR 1.63, 95% CI 1.12–2.35) was shown a single blastocyst transfer compared with the transfer of a single cleavage stage (day 3) embryo in patients aged less than 36 years old (Papanikolaou et al, 2006; Zech et al, 2007).
Table 1.1. Embryo Morphology Scheme for Day 3 and Day 5 embryos

7. Luteal phase in IVF

During the luteal phase of a natural cycle a corpus luteum is formed. This produces progesterone and oestrogen, which are vital to maintain pregnancy. In a natural menstrual cycle, pituitary LH secretion maintains the corpus luteum. Progesterone is pivotal during the endometrial secretory phase, which is characterised by the thickening of the endometrial glands and increased vascularisation to promote
implantation. Following implantation, the developing embryo forms an inner cell mass, the embryoblast, and an outer cell mass, the trophoblast. The trophoblast, which will eventually form the placenta, produces human chorionic gonadotrophin (hCG) immediately after implantation. hCG is able to bind to and activate the LH receptor and hence maintain the corpus luteum (Penzias et al, 2002, Pabuccu et al, 2005).

The luteal phase of IVF cycles is known to be deficient (Humaidan et al, 2012). The aetiology behind luteal phase deficiency of IVF treatment cycles is attributed to the high, circulating steroid levels (Fatemi et al, 2009) produced from the multiple corpora lutea, compared to the single corpus luteum formed in a natural cycle. The increase in oestrogen and progesterone levels then leads to suppression of the pituitary gland gonadotrophin secretion (negative feedback effect). Pituitary LH secretion is vital to maintain the corpus luteum. Hence during an IVF cycle where pituitary LH is suppressed, this results in premature breakdown of the corpus luteum which is termed luteolysis. Premature luteolysis, is associated with particularly poor pregnancy rates following IVF treatment (Van der Linden et al, 2011). To prevent premature luteolysis, various luteal phase support protocols have been described, but practice still varies considerably throughout the world (Fatemi et al, 2009). Luteal phase support (LPS) methods include the administration of hCG (Nosarska et al, 2005) or progesterone via the oral (Chakravarty et al, 2005), vaginal (Zarutskie et al, 2009), rectal (Simunic et al, 2007) or intramuscular route (Leeton et al, 1985). All meta-analyses and systematic reviews performed to date have demonstrated that progesterone administration via the intramuscular, vaginal or rectal routes would give comparable pregnancy rates in IVF treatment cycles when hCG was used as oocyte maturation trigger (Fatemi et al, 2009, Van der Linden et al, 2011, Tay et al, 2005).
Conversely, natural micronised progesterone administered orally has not be shown to be efficient for luteal phase support due to its reduced bioavailability, since it is naturally subjected to first-pass hepatic metabolism (Fatemi et al, 2009).

The addition of oestrogen to the progesterone for luteal phase support has been suggested as a means of improving implantation and pregnancy rates after IVF (Shahara et al, 1999). This has been hypothesised to be potentially beneficial based on the fact that during a natural cycle, the corpus luteum produces both oestrogen and progesterone. When progesterone-only luteal phase support is used, lower mid-luteal oestradiol concentrations could result in decreased pregnancy rates (Shahara et al, 1999). However systematic reviews and meta-analysis have not demonstrated that the addition of oestrogen to progesterone-only luteal phase support protocols improves live birth rates in GnRH agonist and GnRH antagonist cycles (Van der Linden et al, 2011, Jee et al, 2010, Kolibianakis et al, 2008).

hCG has also been administered as an alternative option to progesterone for luteal phase support (LPS). A systematic review and meta-analysis of eighteen trials (Nosarka et al. 2005) demonstrated that hCG-based LPS appeared to be superior when compared with progesterone-based LPS (Nosarka et al, 2005). On the other hand, hCG-based LPS was also associated with a significant increase in ovarian hyperstimulation syndrome (Fatemi et al, 2007). Therefore, due to this risk, luteal phase support with hCG should be reserved for patients who are not at risk of ovarian hyperstimulation syndrome. hCG for LPS should not be used for patients at high risk of OHSS (e.g. polycystic ovarian syndrome, previous history of OHSS) (Van der Linden et al, 2011).
Ovarian hyperstimulation syndrome

A better understanding of the reproductive endocrinology aspects behind IVF has led to an improvement in pregnancy outcomes and has allowed IVF to become the mainstay of fertility treatment worldwide. The improvement in success rates, however, has come at the cost of new complications such as ovarian hyperstimulation syndrome (OHSS). Ovarian hyperstimulation syndrome can be associated with severe morbidity and even rare cases of mortality have been reported. It constitutes an iatrogenic complication, which arises secondary to ovarian stimulation, and appears to be solely encountered in patients undergoing IVF treatment.

Ovarian hyperstimulation syndrome (OHSS) is characterised by an excessive ovarian response to the administration of exogenous gonadotrophins. OHSS is typically only associated with ovarian stimulation with gonadotrophins in IVF cycles.

OHSS symptomatology resolves spontaneously if conception does not occur, but may persist for many weeks when implantation of an embryo is successful and pregnancy occurs due to the endogenous production of HCG. OHSS encompasses a broad spectrum of symptoms, which range from mild abdominal distension to severe multisystem disease requiring intensive care support.

Pathophysiology

The use of exogenous hCG to trigger oocyte maturation in IVF cycles is pivotal in the pathophysiology of ovarian hyperstimulation syndrome. hCG directly promotes the production of vascular endothelial growth factor (VEGF) by endothelial cells. A variety of cytokines have also been identified as mediators in the pathophysiology
process of OHSS, which include interleukin-1b (IL-1b), interleukin-6 (IL-6) angiotensin II, insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), transforming growth factors (TGF) A and B and platelet-derived growth factor (PDGF), (Geva et al, 2000; Whelan et al, 2000; Warren et al, 1996; Ferrara et al, 1997).

The principal pathophysiology of OHSS is an increase in capillary permeability, which results in a fluid shift from the intravascular compartment to the third space (Tollan et al, 1990, Goldmann et al, 1995). Certain mechanisms have been implicated in this process and include the increased exudation of fluid from the expanded ovarian surface (Bergh et al, 1992, Koninckx et al 1980a, Koninckx et al, 1980b), increased levels of prorenin and renin in follicular fluid (Sealey et al, 1985, Donnez et al, 1982) as well as angiotensin-mediated alterations in capillary permeability (Derkx et al, 1987, Lightman et al, 1987).

A key factor in the pathophysiology of the syndrome is the increased release of VEGF (Geva et al, 2000). VEGF is an angiogenic cytokine, which can potently stimulate the vascular endothelium and appears to play a pivotal part in follicular development and corpus luteum secretion. VEGF levels have been shown to correlate with the severity of the syndrome (Levin et al, 1998) and recombinant VEGF administration can produce similar sequelae to OHSS, which can be reversed with the use of a specific antiserum (McClure et al, 1994; Neulen et al, 1995).
Risk factors

Ovarian hyperstimulation syndrome has been independently linked to certain risk factors which are described in Table 1.2 (Whelan et al, 2000; Navot et al, 1988; Delvigne et al, 1993; McDougall et al, 1992; Buyalos et al, 1996; Forman et al, 1990; Mizunuma et al, 1992; Mordel et al, 1993; Haning et al, 1983).

<table>
<thead>
<tr>
<th>Risk factors for OHSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young maternal age</td>
</tr>
<tr>
<td>Low body mass index (BMI)</td>
</tr>
<tr>
<td>Polycystic ovary syndrome</td>
</tr>
<tr>
<td>High doses of exogenous FSH during ovarian stimulation</td>
</tr>
<tr>
<td>Elevated serum oestradiol during ovarian stimulation</td>
</tr>
<tr>
<td>Previous history of OHSS</td>
</tr>
</tbody>
</table>

Table 1.2. Independent risk factors associated with ovarian hyperstimulation syndrome

The risk of OHSS is also related to the number of developing follicles during the phase of ovarian stimulation (Enskog et al, 1999), and the number of retrieved oocytes at the end of the cycle (McDougall et al, 1992). The risk also appears to increase when higher hCG doses are administered to induce oocyte maturation. OHSS manifestation becomes less likely when progesterone-based luteal phase support is used instead of hCG (Foreman et al 1990). As expected, once implantation has been successful, increasing levels of hCG will also increase the duration and severity of the OHSS symptomatology.
Clinical Features

According to its traditional classification (Golan et al, 1989), OHSS may manifest as mild, moderate, or severe. Similarly the Royal College of Obstetricians and Gynaecologists has employed the following classification:

<table>
<thead>
<tr>
<th>Grade</th>
<th>Symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild OHSS</td>
<td>Abdominal bloating</td>
</tr>
<tr>
<td></td>
<td>Mild abdominal pain</td>
</tr>
<tr>
<td></td>
<td>Ovarian size usually &gt;8 cm*</td>
</tr>
<tr>
<td>Moderate OHSS</td>
<td>Moderate abdominal pain</td>
</tr>
<tr>
<td></td>
<td>Nausea ± vomiting</td>
</tr>
<tr>
<td></td>
<td>Ultrasound evidence of ascites</td>
</tr>
<tr>
<td></td>
<td>Ovarian size usually 8–12 cm*</td>
</tr>
<tr>
<td>Severe OHSS</td>
<td>Clinical ascites (occasionally hydrothorax)</td>
</tr>
<tr>
<td></td>
<td>Oliguria</td>
</tr>
<tr>
<td></td>
<td>Haemoconcentration haematocrit &gt;45%</td>
</tr>
<tr>
<td></td>
<td>Hypoproteinaemia</td>
</tr>
<tr>
<td></td>
<td>Ovarian size usually &gt;12 cm*</td>
</tr>
<tr>
<td>Critical OHSS</td>
<td>Tense ascites or large hydrothorax</td>
</tr>
<tr>
<td></td>
<td>Haematocrit &gt;55%</td>
</tr>
<tr>
<td></td>
<td>White cell count &gt;25,000/ml</td>
</tr>
<tr>
<td></td>
<td>Oligo/anuria</td>
</tr>
<tr>
<td></td>
<td>Thromboembolism</td>
</tr>
<tr>
<td></td>
<td>Acute respiratory distress syndrome</td>
</tr>
</tbody>
</table>

The clinical features of OHSS, however, are expressed in the form of a continuous spectrum, which may render strict classification impossible. The mild form of the syndrome is relatively common and includes abdominal distension, transient abdominal pain and diarrhoea, which are encountered in up to 33% of all patients undergoing IVF treatment. The onset of symptoms typically occurs after oocyte retrieval, but may be delayed and manifest only after the embryo transfer has occurred. The manifestations of moderate OHSS include the presence of ascites, which may be diagnosed clinically by the increase in abdominal girth or after ultrasound examination. The progression to the severe form of the syndrome may be
characterised by the presence of tense ascites, tachycardia, tachypnoea, orthostatic hypotension, hydrothorax and oliguria.

Hypotension results from the fluid shift towards the third space and the contraction of the vascular volume, whilst the subsequent oliguria is a sequel of decreased vascular volume and increase intra-abdominal pressure. The risk of venous thromboembolism increases as a result of haemoconcentration and relevant inactivity due to abdominal distension. Finally the life-threatening or critical form of OHSS includes acute renal failure, adult respiratory distress syndrome (ARDS) and venous thromboembolism (Whelan et al, 2000, Zosmer et al, 1987, Abramov et al, 1999).

Management of OHSS

Outpatient Management

The management of patients with mild or moderate OHSS can be on an outpatient basis. Oral simple analgesia and patient education are the principles of management in such cases. Patients are advised to avoid intercourse as it may increase the risk of ovarian torsion or bleeding. Nausea and vomiting can be treated with anti-emetics, such as cyclizine, metoclopramide or ondansentron. Patients with such symptoms can still be monitored on an outpatient basis, but may require more regular follow-up visits, which would include a clinical and ultrasound assessment to detect any potential increase in ascitic fluid and serial measurements of blood levels of haemoglobin, haematocrit, urea, creatinine and electrolytes. Close monitoring of the patient is important and includes at least daily assessment and clinical examination, to ensure that any progression to the severe form of the syndrome is promptly recognised. Patients are advised to maintain an adequate oral fluid intake of 2-3L/day
and to maintain light physical activity, which may decrease their risk of venous thromboembolism.

**Hospitalisation**

Hospitalisation is indicated in the case of severe OHSS. The Royal College of Obstetricians and Gynaecologists has advised that patients with symptoms and signs of severe or critical OHSS based on their classification, should be hospitalised. Similarly, the American Society of Reproductive Medicine (ASRM) has set the following criteria for inpatient management of OHSS (Table 1.3).

<table>
<thead>
<tr>
<th>Clinical features</th>
<th>Laboratory findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe abdominal pain</td>
<td>haematocrit &gt;45%</td>
</tr>
<tr>
<td>Peritonism</td>
<td>white blood cell count &gt;15,000</td>
</tr>
<tr>
<td>Inability to maintain oral intake</td>
<td>Na⁺ &lt;135 mM</td>
</tr>
<tr>
<td>Oliguria</td>
<td>K⁺ &gt;5.0 mM</td>
</tr>
<tr>
<td>Tense ascites</td>
<td>creatinine clearance &lt;50 mL/min</td>
</tr>
<tr>
<td>Dyspnoea</td>
<td>serum creatinine &gt;120</td>
</tr>
</tbody>
</table>

**Table 1.3.** Hospital admission criteria for severe OHSS (ASRM Guideline on ovarian hyperstimulation syndrome)

**Prevention of ovarian hyperstimulation syndrome**

Many strategies have been employed to decrease the risk of OHSS in women undergoing IVF treatment. The use of lower gonadotrophin doses has been shown to reduce the incidence of OHSS in patients with polycystic ovarian syndrome (Marci et al, 2001). The introduction of GnRH antagonists has also significantly decreased the
incidence of OHSS in high-risk populations. Indeed the meta-analysis by Kolibianakis et al (2006) revealed a significant decrease in hospital admissions due to OHSS when a GnRH antagonist protocol was used instead of the prolonged administration of a GnRH agonist (OR: 0.46, 95% CI 0.26-0.82).

The use of metformin in patients with polycystic ovarian syndrome has also been shown to reduce the incidence of OHSS in women with PCOS, without reducing pregnancy rates (Tso et al, 2009). Similarly dopamine agonists such as cabergoline have been shown to decrease the incidence of OHSS by binding to VEGFR-2 and hence decreasing vascular permeability (Soares et al, 2012).

The use of hCG to trigger oocyte maturation is the major cause of OHSS since it causes excessive stimulation of the ovaries. As such, the development of alternative oocyte maturation triggers, which could result in physiological oocyte maturation without excessive stimulation has gained worldwide interest. To date, the only available alternative to hCG trigger during IVF treatment is the use of a single dose of a GnRH agonist, which leads to the endogenous release of LH. The release of VEGF is significantly reduced at the mRNA and the protein level, when a GnRH agonist is used instead of hCG (Cerillo et al, 2011). Consequently many trials have reported prevention of OHSS through the use of a GnRH agonist to induce oocyte maturation (Kol 2004, Engmann 2008, Griesinger 2008). However the use of a GnRH agonist trigger has been associated with a defective luteal phase and with consistently lower pregnancy rates compared to hCG (Griesinger et al, 2010). This is thought to be due to excessive stimulation of the GnRH receptor resulting in desensitisation and subsequent reduced secretion of LH during the luteal phase, which is required to maintain sex steroid production and endometrial receptivity in the luteal phase of the
cycle. As such there is a need to identify novel physiological triggers, which could result in physiological oocyte maturation without excessive stimulation during an IVF cycle. Kisspeptin is a recently identified hormone, which may have this potential. Kisspeptin is a recently identified hormone which stimulates endogenous GnRH release and subsequently gonadotrophins. Hence kisspeptin may be able to effectively trigger oocyte maturation during an IVF cycle in a more physiological manner than currently used triggers for oocyte maturation.
Chapter 2

Kisspeptin
Kisspeptin

The KISS1 gene

KISS1, which constitutes the gene that encodes kisspeptins, was first identified in 1996 through its action as a metastasis suppressor gene in human malignant melanoma (Lee et al., 1996). Since it was first discovered in Hershey USA, it was named after the famous local chocolate product called ‘Kisses’. The SS in KiSS1 stands for ‘suppressor sequence’. The KISS1 gene is located at chromosome 1q32 and consists of four exons with the first two not translated (West et al., 1998).

The GPR54 receptor and its ligands

G protein-coupled receptors form the largest category of human cell membrane receptors (Bockaert et al, 1999). Lee at al (1999) first described the novel GPR54 receptor in the rat model. GPR54 is a G protein-coupled membrane receptor, which has a distinct distribution in human tissues. It is predominantly expressed in areas of the central nervous system (caudate nucleus, substantia nigra, hippocampus, amygdala and thalamus), endocrine organs (pituitary gland and pancreas) and the placenta. The highest expression of the GPR54 receptor has been identified in the pituitary and the placenta (Kotani et al). In fact, serum kisspeptin levels are 7000 times higher compared to non-pregnant levels during the third trimester of pregnancy in humans (Horikoshi et al, 2003).

The GPR54 gene is located at chromosome 19p13.3 and consists of five exons, which encode a protein of 398 amino acids and seven hydrophobic, transmembranic domains (Muir et al., 2001).
Upon binding to kisspeptin, the GPR54 receptor activates phospholipase C and subsequently recruits intracellular component messengers, such as inositol triphosphate and diacylglycerol. These intracellular mediators will in turn modify calcium release to mediate the function of kisspeptin (Muir et al., 2001; Liu et al., 2008; Constantin et al., 2009). Activation of the GPR54 receptor will result in a biphasic rise of intracellular calcium, with an initial steep increase followed closely by a more sustained phase (Min et al., 2014).

The kisspeptin receptor mediates the action of a family of ligand neuropeptides termed kisspeptins, which are encoded by the KISS-1 gene (Kotani et al., 2001). The KiSS-1 gene initially encodes a long 145-amino acid peptide, which is subsequently cleaved into a 54-amino acid product called kisspeptin-54. Kisspeptin-54 has been described as the endogenous ligand to the GPR-54 receptor (Ohtaky et al., 2001). Shorter peptidic fragments (14, 13 and 10-amino acid long) have been identified as ligands to the GPR54 receptor, through a common C-terminal part, which is responsible for the high affinity binding and subsequent activation of the GPR54 receptor (Kotani et al., 2001).

At the level of the hypothalamus, specialised neurons release kisspeptin, which in turn stimulates the secretion of endogenous GnRH. GnRH stimulates the secretion of endogenous gonadotrophins (LH and FSH) from the pituitary gland (Irwig et al, 2004; Clarkson et al, 2006). Seminara et al (2003) reported that mutations of the GPR54 gene are the cause of autosomal recessive hypogonadotrophic hypogonadism in humans and as a result, the kisspeptin/GPR54 signaling is pivotal in the regulation and function of the hypothalamic-pituitary-gonadal axis.
Animal Studies

Gottsch et al (2004) observed that KiSS-1 mRNA is predominantly expressed in the hypothalamic anteroventral periventricular (AVPV) nucleus and the preoptic periventricular (POPV) nucleus in mice. Furthermore, Messager et al (2005) were able to demonstrate that GPR54 transcripts are co-localised with GnRH neurons in the mouse hypothalamus. Clarkson et al (2006) used antisera against kisspeptin-10 and described three populations of kisspeptin neurons in the mouse model, located in the anteroventral periventricular nucleus (AVPV) and the preoptic periventricular nucleus (POPV) of the hypothalamus, the dorsomedial hypothalamus and the arcuate nucleus. Through dual immunofluorescence, they were able to demonstrate appositions between kisspeptin neuron fibres and GnRH neuron cell bodies. Interestingly they were also able to demonstrate a significant 10-fold sex difference (female-dominant) in the number of hypothalamic kisspeptin neurons. The same researchers (Clarkson et al, 2008) were subsequently able to demonstrate the absence of the LH surge in transgenic mice null for kisspeptin, or its receptor.

Thompson et al (2004) were first able to demonstrate that exogenous administration of kisspeptin can potently stimulate the hypothalamic-pituitary-gonadal axis in the rat model. Through intra-cerebroventricular and intravenous administration of kisspeptin-10 to adult male rats, they described a dose-dependent increase in plasma LH, FSH and testosterone at 20 and 60 minutes following kisspeptin injection. In addition, intravenous infusion of kisspeptin in ovariectomised sheep produced an increase of serum FSH and LH release (Caraty at al. 2007).
To further study the roles of GPR54 signalling in primates, Shahab et al (2005) implanted castrated juvenile male monkeys with a cerebroventricular cannula and a central venous catheter. Both intracerebroventricular and intravenous administration of kisspeptin-10 induced a prominent secretion of LH within 30 minutes from injection.

Inoue et al (2011) were able to abolish the proestrus LH surge in shrews by a specific monoclonal antibody, which blocks the actions of hypothalamic kisspeptin. Conversely, Matsui et al (2004) demonstrated that the peripheral administration of kisspeptin promotes oocyte maturation in adult female rats primed with gonadotrophins.

Kisspeptin neurons show sexual dimorphism

Current evidence has identified the presence of sexual dimorphism in kisspeptin pathways in humans. The female hypothalamus exhibits significantly more kisspeptin fibres compared to those seen in men (Hrabovszky et al., 2010). A similar sex difference exists for the expression of kisspeptin cell bodies, which are observed only in the female periventricular zone (Hrabovszky et al., 2010). Similarly fewer kisspeptin cell bodies are present in the male infundibulum, when compared to the high number of kisspeptin cell bodies in the female (Hrabovszky et al., 2010). Similar sex differences can be seen in the arcuate nucleus of sheep (Cheng et al., 2010). Importantly pre-ovulatory positive feedback by sex steroids appears to be unique to females. The adult female rat hypothalamus contains 10-fold kisspeptin neurons compared to males in the rostral periventricular region of the third ventricle (RP3V) region. Conversely, the arcuate nucleus regulating negative sex steroid feedback does
not display such sexual dimorphism (Clarkson and Herbison, 2006; Kauffman et al., 2007).

*Kisspeptin and metabolism*

Reproductive function in humans may be influenced by extremes of nutrition, whether this refers to under-nutrition or obesity. Kisspeptin could be the link between nutritional status and reproductive function by translating information from energy stores into regulation of pulsatile GnRH secretion. When pubertal rats and monkeys were subject to fasting, the subsequent expression of Kiss1 mRNA and gonadotrophin secretion were reduced (Castellano et al., 2005; Cota et al., 2006; Roa et al., 2009; Wahab et al., 2011). Kisspeptin has been shown to restore delayed vaginal opening and to increase gonadotrophin and sex steroid levels associated with prolonged under-nutrition in rats of pre-pubertal age (Navarro et al., 2004, Castellano et al., 2005).

Humans with leptin / leptin receptor mutations can develop hypogonadism (Farooqi and O'Rahilly, 2009). Although the leptin receptor is not expressed on GnRH neurons, 40% of arcuate nucleus kisspeptin neurons in mice express the leptin receptor (Smith et al., 2006). This finding suggests the presence of a potential role of kisspeptin in mediating leptin signals on the HPG axis. Leptin-deficient mice demonstrate a decrease in the expression of Kiss1 mRNA, which may be partially up-regulated by leptin (Smith et al., 2006). However mice with a selective deletion of the leptin receptor from kisspeptin neurons exhibit a normal pubertal development, and fertility, which demonstrates that the action of leptin on kisspeptin neurons is not mandatory for these reproductive processes to occur (Donato et al., 2011).

Decreased testosterone levels have been observed in obese and type 2 diabetic men. A
decrease in GnRH secretion has been implicated as the aetiology (Dandona et al., 2008). Streptozocin treated rats, which have been rendered diabetic, showed decreased levels of hypothalamic Kiss1 mRNA with resulting low circulating gonadotrophins and sex steroids (Castellano et al., 2006, 2009). This might suggest that decreased secretion of kisspeptin could be the potential, pathophysiological mechanism underlying hypogonadotropic hypogonadism in obese and diabetic patients (George et al., 2010). Indeed George et al (2013) demonstrated that kisspeptin-10 could increase the frequency of LH pulse secretion in hypogonadal, type 2 diabetic men (George et al., 2013). Possible mechanisms for this down-regulation of kisspeptin pathways include increased oestrogen negative feedback, which appears to be significantly elevated in obesity (Schneider et al., 1979). Other candidate mechanisms include resistance to leptin (Finn et al., 1998), hyperglycaemia and insulin resistance (Castellano et al., 2006, 2009).

In conclusion the available data suggest that kisspeptin can convey information from energy stores to GnRH neuron and regulate reproductive function accordingly. This important finding highlights a potential therapeutic role of kisspeptin to restore the reproductive function in conditions such as anorexia nervosa and diabetes.

**Human studies**

Dhillon et al (2005) performed a double-blind, placebo-controlled, crossover study and administered an intravenous infusion of kisspeptin-54 and a control intravenous infusion of normal saline 0.9% three days apart in random order to 6 healthy, adult male volunteers. They used a dose of 4pmol/kg.min for the initial 30 minutes and halved the dose for the remaining 60 minutes of the infusion. The infusion of
kisspeptin-54 was associated with a significant increase in serum levels of LH and testosterone compared with saline infusion (Figure 2.1).

**Figure 2.1.** Mean plasma kisspeptin-IR, LH, FSH, inhibin B and testosterone after kisspeptin-54 and control saline infusions. (Dhillon et al, 2005)

The subjects included in the study were assessed for any blood pressure or pulse changes every 15 minutes for the first 3 hours and every 30 minutes thereafter. No
changes in blood pressure or pulse were recorded. The patients reported no nausea or other adverse symptoms during or after the kisspeptin administration.

Chan et al (2011) confirmed the findings of Dhillo et al (2005). They administered a single intravenous bolus injection of kisspeptin-10 to thirteen healthy male volunteers and reported that kisspeptin-10 resulted in a single, large LH pulse in the patients included.

Dhillo et al (2007) subsequently investigated the effects of kisspeptin-54 on LH secretion during the different phases of the menstrual cycle in healthy female volunteers in a double-blind, placebo-controlled study (Dhillo et al, 2007). Each volunteer received a subcutaneous bolus dose of kisspeptin (0.4nmol/kg) on one day and then a bolus subcutaneous injection of saline on the other day. These injections were performed during the follicular, the pre-ovulatory and the luteal phase of the menstrual cycle and alternated between kisspeptin and placebo over the course of two menstrual cycles. The study days of the different menstrual cycle phases were separated by a minimum period of seven days. This dose of kisspeptin-54 was associated with a significant rise in plasma LH and FSH in all three phases of the menstrual cycle (Figures 2.2 & 2.3).
Figure 2.2. Mean ± SEM increase in plasma LH after saline or kisspeptin injection in the various phases of the female menstrual cycle. (A: follicular, B: pre-ovulatory, C: luteal, D: all three phases). ***, P<0.001. (Dhillo et al, 2007)

Figure 2.3. Mean ± SEM increase in plasma FSH after saline or kisspeptin injection in the various phases of the female menstrual cycle (A: follicular, B: pre-ovulatory, C: luteal, D: all three phases). ***, P<0.001. (Dhillo et al, 2007)
When the effects of kisspeptin administration were compared for the different phases of the menstrual cycle, a significantly greater increment in plasma LH and FSH was observed in the pre-ovulatory phase of the cycle compared to the follicular and luteal phase (Figure 2.4).

![Figure 2.4](image)

**Figure 2.4.** Change in plasma LH (iu/l) (Y-axis) after a sc bolus injection of (0.4 nmol/kg) kisspeptin-54 (KP) or saline injection in the different phases of the menstrual cycle. ***, P<0.001 (Dhillo et al, 2007)

Jayasena et al (2009) performed a prospective, randomised, double-blinded study to investigate the effect of kisspeptin-54 in women with hypothalamic amenorrhoea. Ten patients with hypothalamic amenorrhoea were administered subcutaneous injections of kisspeptin-54 at a dose of 6.4nmol/kg twice a day for a period of two weeks. The response to kisspeptin-54 injections was investigated by assessing the serum gonadotrophin and oestradiol levels on the 1st and 14\textsuperscript{th} day (4 hours post injection). The results indicated a potent increase in serum LH and FSH on the first day of injections. This response appeared to be significantly reduced by day 14, suggesting that chronic administration of kisspeptin may result in desensitisation of its effects on FSH and LH release (Figure 2.5).

Chan et al (2012) administered an intravenous bolus dose of kisspeptin-10 to ten women in the early follicular phase, three in the preovulatory phase and fourteen in
the midluteal phase. They demonstrated a varied gonadotrophin secretory response, which depended on the phase of the menstrual cycle. They also reported that the response to kisspeptin-10 (based on the GnRH-induced LH surge) was particularly prominent in the preovulatory phase of the menstrual cycle.

Based on this evidence, it is possible that kisspeptin could potentially lead to a more physiological release of an endogenous LH surge during a cycle of controlled ovarian stimulation and IVF. In addition, whilst hCG only acts through an LH-like mechanism, kisspeptin has been demonstrated to induce the concomitant release of FSH.

Figure 2.5. Effects of kisspeptin-54 and saline injection on the first day (A,B,C) and the 14th day (D,E,F) to plasma levels of LH, FSH and serum oestradiol. Data are shown at mean ± SEM. *,P<0.05. ***, P<0.001 (Jayasena et al, 2009)
Although the mid-cycle surge of LH has been primarily associated with final oocyte maturation and ovulation, FSH has been shown to increase the formation of LH receptors in granulose cells and promote nuclear maturation and cumulus expansion (Eppig et al, 1979; Yding Andersen et al, 1999). As such kisspeptin may be more beneficial than hCG for triggering oocyte maturation, as it may result in a more physiological LH surge, because it stimulates the release of endogenous GnRH, which should also result in a reduced risk of OHSS in women undergoing IVF treatment.
CHAPTER 3

Kisspeptin as potential novel trigger of oocyte maturation during IVF treatment
Hypothesis
Kisspeptin is a novel physiological trigger for oocyte maturation in IVF treatment.

Aim
The aim of the study was to assess whether the administration of kisspeptin can induce oocyte maturation in women undergoing an IVF cycle. This is the first human study that investigates the use of kisspeptin in an assisted conception cycle.
Methods

Regulatory approval

This study was approved by the Hammersmith and Queen Charlotte’s Research Ethics Committee, London (reference: 10/H0707/2). The study was performed in the IVF Unit at Hammersmith Hospital under a license from the UK Human Fertilisation and Embryology Authority (HFEA). Written informed consent was obtained from all subjects. Approval of this study as a Clinical Trial of an Investigative Medicinal Product was granted by the Medicines and Healthcare Products Regulatory Agency, UK. The study was registered on the National Institutes of Health Clinical Trials database (NCT01667406). The study was performed in accordance with the Declaration of Helsinki.

Peptide

Kissepeptin-54 was synthesised, purified and tested to Good Manufacturing Practice standards by Bachem (Bachem Holding AG, Bubendorf, Switzerland). Vials of freeze-dried kisspeptin-54 were stored at -20°C and reconstituted in 0.5ml of 0.9% saline as described previously (Dhillo et al, 2005).

Subjects

Sixty infertile patients with an indication for in vitro fertilisation treatment were screened for participation at Hammersmith Hospital between August 2012 and September 2013. Eligible patients received one IVF treatment cycle; all treatment costs for this single IVF cycle were covered by study participation since the efficacy of kisspeptin to induce oocyte maturation was unknown prior to commencing the study. The inclusion criteria were: age 18-34 years; early follicular phase serum FSH
≤12mIU/mL; serum anti-Müllerian hormone (AMH) 10-40pmol/L; intact ovaries bilaterally; regular, menstrual cycles (24-35 days long); body mass index (BMI) of 18-29kg/m². Exclusion criteria were: moderate/severe endometriosis (as diagnosed during previous laparoscopy); previous poor response or more than one previous cycle of IVF treatment; evidence of clinical and/or biochemical hyperandrogenaemia or the presence of polycystic ovarian syndrome.

Study outcomes

The primary outcome of the study was oocyte maturation during a single IVF treatment cycle. Oocyte maturation was assessed in two different ways: the absolute number of mature oocytes and the proportion of collected oocytes that were mature. The oocytes were classed as mature by identifying the first polar body and by the round appearance of the ooplasm (Macklon et al, 2006).

The secondary outcomes of the study were the serum levels of reproductive hormones following the administration of kisspeptin-54, fertilisation rate, embryo quality, the rate of biochemical pregnancy at 12 days following embryo transfer, and the clinical pregnancy rate at 6 weeks’ gestation. Fertilisation rate refers to the number of two pronuclear zygotes divided by the total number of mature oocytes injected (Macklon et al, 2006). Biochemical pregnancy was defined as a plasma hCG level >10mIU/mL 12 days after the embryo transfer procedure. Clinical pregnancy was defined as the presence of an intrauterine gestational sac with an active fetal heartbeat at 6 weeks’ gestation. The outcomes of pregnancy and live birth rates were documented in all women with a diagnosed clinical pregnancy.
Dose allocation

The efficacy of kisspeptin to induce oocyte maturation prior to commencing the study was not known, because kisspeptin had not previously been administered to infertile patients undergoing IVF treatment. This obviously posed ethical implications for the present study. Consequently the dose allocation was designed to minimise the number of patients exposed to kisspeptin doses which would not result in clinically effective oocyte maturation. As such a modification of the conventional 3+3 trial design was employed. The first nine patients would be equally randomised between the three lowest kisspeptin doses of 0.4, 0.8 and 1.6nmol/kg. If fewer than 2 women in each group demonstrated proven oocyte maturation, then the following nine women would be randomised between the three higher kisspeptin doses of 3.2, 6.4 and 12.8nmol/kg.

The clinical ease of oocyte retrieval after kisspeptin-54 administration (compared with the trigger most commonly used in the clinical setting – hCG) was also documented. The reason behind this approach was that a clinically effective kisspeptin dose should result in mature oocytes that could be retrieved with comparable clinical ease to that associated with the currently available and widely used oocyte maturation triggers (i.e hCG). As a result the clinical ease of collection was graded from 1 to 4 by experienced IVF clinicians, who were blinded to the kisspeptin dose allocation. The grading system is as follows:

- **1**, not feasible to collect any oocytes;
- **2**, significantly more difficult than what expected during a routine IVF cycle using hCG for oocyte maturation (excessive number of follicular flushing attempts were needed in order to aspirate oocytes and scanty number of granulosa cells present in the obtained aspirates);
• 3, slightly more difficult compared to using hCG as maturation trigger (present but less abundant granulosa cells in the follicular aspirates. Cumulus cells appeared more densely packed than following hCG triggering);

• 4, comparable level of clinical ease of oocyte retrieval when compared with hCG.

When a kisspeptin dose was shown to induce proven oocyte maturation in at least 2 patients in a cohort of 3, then additional patients would be recruited to increase the sample size of that dose group to a minimum of 15. An independent Data Monitoring and Ethics Committee approved the dose allocation. Clinicians, who were blinded to the dose allocation of patients, performed all transvaginal oocyte retrievals. The embryologists and the included patients were also blinded to the kisspeptin dose allocation. During each phase of the study, simple randomisation was used to allocate patients to kisspeptin doses.

Sample size
When oocyte maturation in patients undergoing IVF is the primary endpoint, there is no relevant data on the use of kisspeptin-54 as an oocyte maturation trigger and therefore no data was available on which sample sizes estimates could be based upon prior to commencing this study. However, current evidence (Dubuisson et al, 1991) suggested an association between successful egg maturation and elevation of LH; successful egg maturation did occur after a mean serum LH rise to 106 IU/L 4hours after a GnRH agonist trigger in a standard GnRH antagonist ovarian stimulation protocol. In addition we have previously noted that kisspeptin-54 at a dose of 12.8nmol/kg can increase serum LH up to 148 IU/L in healthy females during the
preovulatory phase of their cycle. Using the 2-group t-test approach, it was estimated that a sample size of 13 patients per effective kisspeptin dose would have a 90% power to detect a difference of 148 IU/L between any two groups (two-sided 5% significance level without correction for multiplicity and common standard deviation of 78 IU/L). We anticipated that the drop-out rate in our sample would be less than 10%, based on similar studies performed in the past, as infertile patients undergoing IVF are highly motivated. As a result a minimal size of 15 women at each clinically effective kisspeptin dose was calculated. The recruitment process ceased once this sample size target had been achieved for the clinically effective kisspeptin doses of 6.4 and 12.8 nmol/kg. For ethical reasons, however, patients who had already been screened and consented at the time of stopping recruitment were allowed complete the study protocol; this resulted in a total recruitment of 24 patients for each of the effective kisspeptin doses of 6.4 and 12.8 nmol/kg.

Protocol

All of the recruited patients underwent controlled ovarian stimulation using a recombinant FSH and GnRH antagonist protocol. A single dose of kisspeptin-54 was used as the oocyte maturation trigger.

Superovulation protocol

Once daily subcutaneous recombinant FSH injections (Gonal F 150IU, Merck Serono, Geneva, Switzerland, administered at 9am daily) were commenced on day 2 of the menstrual cycle. A pelvic ultrasound was performed five days later to assess the individual progress of ovarian follicular development. Daily subcutaneous injections
of a GnRH antagonist (Cetrotide 0.25mg, Merck Serono, injected at 9pm daily) were employed to prevent a premature LH surge. The GnRH antagonist was commenced once the lead follicle had reached a mean diameter >14mm. Additional ultrasound examinations were performed on an individual patient basis according to size of the growing follicles and the response to superovulation (Figure 3.1).

![Diagram showing stimulation protocol]

**Figure 3.1.** The stimulation protocol used in the study. Once daily subcutaneous recombinant FSH injections commenced on day 2 of the menstrual cycle. The GnRH antagonist commenced once the lead follicle had reached a mean diameter >14mm. When at least three ovarian follicles ≥18mm diameter were visible on ultrasound, a subcutaneous bolus injection dose of kisspeptin-54 was administered between 2030 and 2130h. The transvaginal oocyte retrieval was performed 36 hours later.

**Kisspeptin-54 trigger of egg maturation**

When at least three ovarian follicles ≥18mm diameter were visible on ultrasound, a subcutaneous bolus injection dose of kisspeptin-54 was administered by an investigator to trigger egg maturation. Kisspeptin-54 was administered 36h prior to egg retrieval (between 2030 and 2130h). Injections of FSH were stopped 12h prior to kisspeptin-54. In order to minimise any effect that the GnRH antagonist had on kisspeptin-54 response the last injection of GnRH antagonist was administered 24
hours prior to kisspeptin-54 (Damewood et al, 1989; Golan et al, 1989). Serum LH, FSH, oestradiol and progesterone were measured immediately before and 12h after kisspeptin-54 injection, and at the time of egg retrieval in all women.

The transvaginal oocyte retrieval was performed under ultrasound guidance 36 hours after the bolus injection of kisspeptin-54. The procedure was performed under intravenous sedation using propofol given by an anaesthetist and the collected mature oocytes were fertilised by intracytoplasmic sperm injection utilising produced sperm from the male partner.

Reproductive hormone secretion during the 12h following kisspeptin injection

A subgroup of women receiving the two highest doses of kisspeptin-54 (6.4 or 12.8nmol/kg, n=10/dose) underwent overnight measurements of serum LH, FSH, oestradiol and progesterone, and plasma kisspeptin immunoreactivity just prior to and during the 12 hours following kisspeptin-54 injection (t=-30, -15, 0, 30, 60, 90, 120, 150, 180, 240, 360, 480, 600 and 705minutes post-injection). These hormonal measurements allowed us to determine a detailed time course of reproductive hormone release following kisspeptin-54 injection.

Egg retrieval, fertilisation, and re-implantation of embryos

Transvaginal, ultrasound-directed oocyte retrieval was carried out 36h following kisspeptin-54 injection, and intracytoplasmic sperm injection was performed in all study cycles. One or two embryos of highest quality during morphological assessment were transferred to the uterine cavity 3-5 days following egg collection. Progesterone
(400mg twice daily suppository/pessary) (Cyclogest; Actavis UK Limited, UK) and oestradiol valerate (2mg orally three times daily) (Progynova; Bayer Plc, UK) were started after egg collection and continued until 12 weeks gestation for luteal phase supplementation.

Hormonal assay methodology

Blood samples were collected as previously described (Caraty et al, 2007; Dhilllo et al, 2005; Jayasena et al, 2009). Serum LH, FSH, oestradiol, and progesterone were measured using automated chemiluminescent immunoassays (Abbott Diagnostics, Maidenhead, UK). Interassay coefficients of variation were as follows: LH, 3.4%; FSH, 3.5%; oestradiol, 3.4%; progesterone, 1.8%. Limits of detectability for each assay were as follows: LH 0.07mIU/ml; FSH 0.05mIU/ml; oestradiol 70pmol/l (19pg/mL); progesterone 0.3nmol/L (0.1ng/mL).

AMH was measured using an enzyme linked immunosorbent assay (Beckman Coulter Inc, Brea, CA, USA). The reference range was 2.2-48.5pmol/L (0.3-6.8ng/mL). The lower limit of detection was 0.6pmol/l (0.08ng/mL). The interassay coefficient of variation was 4.6%; the intra-assay coefficient of variation was 4.0%.

Measurement of plasma kisspeptin immunoreactivity (IR) was performed using an established RIA (Caraty et al, 2007; Dhilllo et al, 2005; Jayasena et al, 2009). The antibody cross-reacted 100% with human kisspeptin-54, kisspeptin-14, and kisspeptin-10 and less than 0.01% with other related arginine-phenylalanine amide proteins, including prolactin-releasing peptide, arginine-phenylalanine amide-related peptide 1, arginine-phenylalanine amide-related peptide 2, arginine-phenylalanine amide-related peptide 3, QRFP43, neuropeptide FF, and neuropeptide AF. The limit
of detectability was 2pmol/l, and the intra- and interassay coefficients of variation were 8.3 and 10.2%, respectively.

Results

Kisspeptin dose allocation and baseline characteristics. The first two patients were randomised to a kisspeptin-54 dose of 1.6 nmol/kg; the oocyte retrieval was significantly more difficult when compared with a typical, routine, transvaginal oocyte retrieval using human chorionic gonadotropin (hCG) as the oocyte maturation trigger (Figure 3.2). Consequently we decided to proceed to the doses of 3.2, 6.4, and 12.8 nmol/kg. A clinically difficult oocyte retrieval was reported for all 3 patients who received the 3.2nmol/kg kisspeptin-54 dose. Consequently the next patients were randomized 1:1 between 6.4 and 12.8 nmol/kg kisspeptin (n = 24 per group). Two independent clinical trialists approved the dose allocation. No significant differences in baseline characteristics were identified between the dose groups (Table 3.1).

Figure 3.2. Enrolment of patients, who were administered kisspeptin-54 as oocyte maturation trigger in their IVF/ICSI cycle

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Table 3.1. Baseline characteristics of patients administered kisspeptin-54. Continuous variables are presented as medians (lower, upper quartile) and categorical variables are presented as totals (percentages). Other known causes of infertility include endometriosis or ovarian endometriomas. The number of follicles was calculated during the last ultrasound assessment prior to the kisspeptin-54 trigger.

Ease of oocyte collection

The oocyte retrievals were carried out in line with the routine departmental policy regarding oocyte retrieval in our IVF unit using other established triggers of oocyte maturation. The embryologist routinely informs the clinician of the presence of an oocyte or granulosa cells in the follicular aspirate before the clinician progresses to the next follicle. Eggs were collected using a single-lumen, 16-gauge ovum aspiration needle (Cook Medical, Bloomington, USA) for the majority of patients. If there were less than five follicles with a diameter greater than 14mm on the day of the
kisspeptin trigger, or when no oocyte was retrieved after aspiration of the first three follicles, a double-lumen 16-gauge ovum aspiration needle (Cook Medical, Bloomington, USA), which allows for follicular flushing with Hartmann’s solution was employed. We graded the clinical ease of the oocyte retrieval procedure in comparison to what a clinician would expect in a routine, transvaginal oocyte retrieval after the administration of hCG as the oocyte maturation trigger in a normoresponsive patient. The results for the patients of the study are presented below (Table 3.2).

<table>
<thead>
<tr>
<th>Clinical ease of collection</th>
<th>1.6 nmol/kg</th>
<th>3.2 nmol/kg</th>
<th>6.4 nmol/kg</th>
<th>12.8 nmol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>100%</td>
<td>3</td>
<td>100%</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>0%</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Total</td>
<td>2</td>
<td></td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.2. The clinical ease of oocyte retrieval was arbitrarily graded as follows (doses of kisspeptin-54 are in nmol/kg). 1: No eggs collected, 2: Substantially more difficult compared to an oocyte retrieval using hCG, 3: Slightly more difficult compared to an oocyte retrieval using hCG, 4: Comparable compared to an oocyte retrieval using hCG. Percentages in right hand column signify the proportion of patients randomised to each dose with that clinical ease of collection score.
Primary outcome

The use of kisspeptin to induce egg maturation.

The study was designed to assess if oocyte maturation can be achieved in women having IVF treatment following kisspeptin-54 administration. Oocyte maturation was observed with all administered doses of kisspeptin-54 (Table 3.3, Figure 3.3). Similar rates of oocyte maturation (percentage of recovered oocytes that were mature), between 75% and 85%, were observed following all administered kisspeptin-54 doses. However, oocyte yield (the proportion of oocytes recovered when compared with the expected number, i.e., number of follicles > 14 mm diameter) and the absolute number of mature eggs appeared to increase with increasing doses of kisspeptin.

<table>
<thead>
<tr>
<th>Kisspeptin-54 dose (nmol/kg)</th>
<th>1.6 (n=2)</th>
<th>3.2 (n=2)</th>
<th>6.4 (n=24)</th>
<th>12.8 (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2</td>
<td>4.5 (3.5)</td>
<td>4.3 (1.5)</td>
<td>7.5 (3.8)</td>
<td>8.8 (4.0)</td>
</tr>
<tr>
<td>%M2</td>
<td>75 (35)</td>
<td>79 (36)</td>
<td>79 (22)</td>
<td>85 (16)</td>
</tr>
<tr>
<td>Oocyte yield</td>
<td>49 (29)</td>
<td>36 (18)</td>
<td>76 (49)</td>
<td>103 (53)</td>
</tr>
</tbody>
</table>

Table 3.3. Summary of oocyte maturation after the use of kisspeptin-54 as oocyte maturation trigger. M2 refers to the absolute number of mature oocytes collected. %M2 refers to the percentage of collected oocytes that were mature. Oocyte yield refers to the percentage of mature oocytes collected from the number of follicles >14mm in mean diameter at the last ultrasound assessment prior to kisspeptin administration. Results are presented as mean (SD).
Figure 3.3. Mature eggs per patient and mature eggs per eggs collected (%) for the different kisspeptin dose groups.

**Secondary outcomes**

*Circulating levels of hormones following kisspeptin-54 administration.*

Circulating levels of reproductive hormones were measured just before, 12 and 36 hours following kisspeptin-54 injection in all patients (except for a single patient who missed the 12-hour blood test). Serum LH, FSH, and progesterone levels appeared to be elevated 12 hours after the administration of kisspeptin-54 (Tables 3.4; 3.5; 3.6; 3.7).
<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>Median</th>
<th>Lq, Uq</th>
<th>Gm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH (mIU/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>2</td>
<td>0.8</td>
<td>0.1</td>
<td>0.7,0.8</td>
<td>0.8</td>
<td>0.7,0.8</td>
<td>0.7</td>
</tr>
<tr>
<td>12 hours</td>
<td>2</td>
<td>6.6</td>
<td>4.7</td>
<td>3.3,9.9</td>
<td>6.6</td>
<td>4.9,8.2</td>
<td>5.7</td>
</tr>
<tr>
<td>36 hours</td>
<td>2</td>
<td>2.2</td>
<td>0.2</td>
<td>2.1,2.4</td>
<td>2.2</td>
<td>2.2,2.3</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>FSH (mIU/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>2</td>
<td>6.2</td>
<td>1.8</td>
<td>4.9,7.4</td>
<td>6.2</td>
<td>5.5,6.8</td>
<td>6.0</td>
</tr>
<tr>
<td>12 hours</td>
<td>2</td>
<td>6.9</td>
<td>0.7</td>
<td>6.4,7.4</td>
<td>6.9</td>
<td>6.7,7.2</td>
<td>6.9</td>
</tr>
<tr>
<td>36 hours</td>
<td>2</td>
<td>4.9</td>
<td>0.7</td>
<td>4.4,5.4</td>
<td>4.9</td>
<td>4.7,5.2</td>
<td>4.9</td>
</tr>
<tr>
<td><strong>Oestradiol (x10^3 pmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>2</td>
<td>4.0</td>
<td>1.7</td>
<td>2.8,5.2</td>
<td>4.0</td>
<td>3.4,4.6</td>
<td>3.8</td>
</tr>
<tr>
<td>12 hours</td>
<td>2</td>
<td>5.9</td>
<td>0.8</td>
<td>5.3,6.5</td>
<td>5.9</td>
<td>5.6,6.2</td>
<td>5.9</td>
</tr>
<tr>
<td>36 hours</td>
<td>2</td>
<td>2.2</td>
<td>0.1</td>
<td>2.1,2.2</td>
<td>2.2</td>
<td>2.2,2.2</td>
<td>2.2</td>
</tr>
<tr>
<td><strong>Progesterone (nmol/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>2</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0,1.0</td>
<td>1.0</td>
<td>1.0,1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>12 hours</td>
<td>2</td>
<td>7.5</td>
<td>0.7</td>
<td>7.0,8.0</td>
<td>7.5</td>
<td>7.2,7.8</td>
<td>7.5</td>
</tr>
<tr>
<td>36 hours</td>
<td>2</td>
<td>5.5</td>
<td>2.1</td>
<td>4.0,7.0</td>
<td>5.5</td>
<td>4.8,6.2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Table 3.4. Circulating levels of gonadotrophins, oestradiol and progesterone following administration of 1.6 nmol/kg kisspeptin-54 (n=2) as oocyte maturation trigger. Serum LH (mIU/ml), FSH (mIU/ml), oestradiol (x10^3 pmol/l) and progesterone (nmol/l) were measured at 0 hours (just before injection), 12 hours after injection and 36 hours after injection (at the time of the oocyte retrieval).

Lq: lower quartile, Uq: upper quartile, Gm: geometric mean
Table 3.5. Circulating levels of gonadotrophins, oestradiol and progesterone following administration of 3.2 nmol/kg (n=3) kisspeptin-54 as oocyte maturation trigger. Serum LH, FSH, oestradiol and progesterone were measured at 0 hours (just before injection), 12 hours after injection and 36 hours after injection (at the time of the oocyte retrieval).

Lq: lower quartile, Uq: upper quartile, Gm: geometric mean
Table 3.6. Circulating levels of gonadotrophins, oestradiol and progesterone following administration of 6.4 nmol/kg (n=24) kisspeptin-54 as oocyte maturation trigger. Serum LH, FSH, oestradiol and progesterone were measured at 0 hours (just before injection), 12 hours after injection and 36 hours after injection (at the time of the oocyte retrieval).

Lq: lower quartile, Uq: upper quartile, Gm: geometric mean
**Table 3.7.** Circulating levels of gonadotrophins, oestradiol and progesterone following administration of 12.8 nmol/kg kisspeptin-54 as oocyte maturation trigger. Serum LH, FSH, oestradiol and progesterone were measured at 0 hours (just before injection), 12 hours after injection and 36 hours after injection (at the time of the oocyte retrieval).

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>Median</th>
<th>Lq,Uq</th>
<th>Gm</th>
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<tr>
<td><strong>LH (mIU/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>24</td>
<td>1.3</td>
<td>1.4</td>
<td>0.4,5.7</td>
<td>1.0</td>
<td>0.5,1.4</td>
<td>1.0</td>
</tr>
<tr>
<td>12 hours</td>
<td>24</td>
<td>8.8</td>
<td>5.3</td>
<td>1.0,25.4</td>
<td>8.0</td>
<td>5.0,12.3</td>
<td>7.3</td>
</tr>
<tr>
<td>36 hours</td>
<td>24</td>
<td>1.2</td>
<td>0.9</td>
<td>0.4,3.9</td>
<td>0.9</td>
<td>0.6,1.4</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>FSH (mIU/ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>24</td>
<td>8.6</td>
<td>2.2</td>
<td>5.0,13.2</td>
<td>8.9</td>
<td>6.8,10.1</td>
<td>8.3</td>
</tr>
<tr>
<td>12 hours</td>
<td>24</td>
<td>10.8</td>
<td>2.5</td>
<td>6.1,17.9</td>
<td>10.3</td>
<td>9.1,12.7</td>
<td>10.6</td>
</tr>
<tr>
<td>36 hours</td>
<td>24</td>
<td>6.1</td>
<td>1.3</td>
<td>3.8,8.6</td>
<td>6.0</td>
<td>5.2,6.9</td>
<td>5.9</td>
</tr>
<tr>
<td><strong>Oestradiol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(x10^3 pmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>24</td>
<td>7.5</td>
<td>3.8</td>
<td>2.2,17.5</td>
<td>7.3</td>
<td>5.3,9.5</td>
<td>6.6</td>
</tr>
<tr>
<td>12 hours</td>
<td>24</td>
<td>8.4</td>
<td>3.7</td>
<td>2.1,15.8</td>
<td>7.6</td>
<td>5.9,11.2</td>
<td>7.5</td>
</tr>
<tr>
<td>36 hours</td>
<td>24</td>
<td>3.6</td>
<td>2.6</td>
<td>1.0,13.1</td>
<td>3.0</td>
<td>2.2,4.3</td>
<td>2.9</td>
</tr>
<tr>
<td><strong>Progesterone</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(nmol/l)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 hours</td>
<td>24</td>
<td>3.5</td>
<td>2.9</td>
<td>1.0,15.0</td>
<td>3.0</td>
<td>2.0,4.2</td>
<td>2.7</td>
</tr>
<tr>
<td>12 hours</td>
<td>24</td>
<td>20.2</td>
<td>10.1</td>
<td>8.0,45.0</td>
<td>15.8</td>
<td>12.0,28.5</td>
<td>18.1</td>
</tr>
<tr>
<td>36 hours</td>
<td>24</td>
<td>14.5</td>
<td>7.0</td>
<td>4.0,30.0</td>
<td>12.5</td>
<td>9.8,19.0</td>
<td>12.9</td>
</tr>
</tbody>
</table>
A surge in LH (and FSH) was observed during the 12-hour period following kisspeptin administration.

Overnight blood sampling in timed intervals was performed to determine the profile of hormone release in 20 of the subjects at the two highest doses of kisspeptin-54 (6.4 or 12.8 nmol/kg; n = 10 per dose). Peak levels of plasma kisspeptin were observed approximately one hour after injection and subsequently fell to pre-injection levels by 12 hours following injection.

The level of serum LH reached a peak four to six hours following kisspeptin-54 administration and decreased thereafter. Less prominent patterns of elevation were observed for serum FSH and oestradiol following kisspeptin-54 administration. Conversely the level of progesterone appeared to rise continually during the 12-hour period following administration of kisspeptin-54 (Figure 3.4).
Figure 3.4. Serum FSH, LH, oestradiol and progesterone after the administration of kisspeptin by dose (6.4nmol/l and 12.8nmol/l, n=10 per dose) during the 12 hours following kisspeptin-54 trigger administration. Results are presented as median and interquartile ranges of the serum hormone levels at each time interval.

A release of both LH and FSH was documented with all different doses of kisspeptin. For the group of 1.6-3.2 nmol/kg, the mean fold increase in LH ± SEM was 5.2 ±2.4 iu/l. For the groups of 6.4 nmol/kg and 12.8 nmol/kg the mean fold increase in LH ± SEM was 7.1 ±1.2 iu/l and 10.3 ±1.9 iu/l respectively. Performing a one-way ANOVA test to compare multiple groups using the Bonferroni correction, the trend of increasing LH release with higher doses of kisspeptin did not reach statistical significance (p= 0.35) (Figure 3.5).
Figure 3.5. Fold rise of LH (from just prior to trigger to 12hrs following trigger administration) for the different kisspeptin-54 doses. Results are presented as mean (SEM). (1.6nmol/kg, n=5; 6.4nmol/kg, n=24, 12.8 nmol/kg, n=24)

Fertilisation and pregnancy

Although the study design primarily focused on whether egg maturation is achievable in women injected with kisspeptin-54 during IVF treatment, we also collected and analysed data on the rate of egg fertilisation, biochemical pregnancy (hCG>10iu/l at 12 days following embryo transfer), and clinical pregnancy (present fetal heart beat at six weeks gestation). These results are shown in Table 3.8. In total, fertilisation occurred in 92% of patients (defined as at least one fertilised egg), the rate of embryo transfer was 92%, and high-quality embryo(s) were transferred in 58% of included patients. The results of embryo quality are presented in Table 3.9. Furthermore 36 of 49 patients had their embryo transfer on day five following oocyte retrieval. In addition the blastocyst formation rate (defined as the total number of blastocysts divided by the total number of 2 pronuclei zygotes formed) in these patients was 49.4%. 31/36 patients with a day 5 embryo transfer had at least one high-quality embryo transferred. The biochemical pregnancy rate in our population was 40% while
the clinical pregnancy rate was 23% (12 of 53 treated patients). Out of the 12 women who achieved clinical pregnancies, ten women delivered healthy babies (eight had a singleton pregnancy and two women had a twin pregnancy). Two patients had miscarriages (at nine weeks and 12 weeks gestation respectively).

The fertilisation rate can be defined as the percentage of mature (metaphase II) oocytes that fertilised after intracytoplasmic sperm injection. We assessed the fertilisation rates in the different kisspeptin dose groups (Figure 3.6). More specifically the mean fertilisation rates ± SEM for the 1.6-3.2 nmol/kg, 6.4 nmol/kg, 12.8 nmol/kg groups were 40.7 ± 15.0 (%), 74.1 ± 7.4 (%) and 77.4 ± 5.7 (%) respectively (Figure). The one-way ANOVA test with the Bonferroni correction confirmed a statistically significant difference in fertilisation rates amongst the different kisspeptin dose groups (p= 0.032).

Figure 3.6. Fertilisation rate (the percentage of mature oocytes which fertilised after intracytoplasmic sperm injection) for the different kisspeptin-54 dose groups. Results are presented as mean (SEM) (1.6nmol/kg, n=5; 6.4nmol/kg, n=24, 12.8 nmol/kg, n=24),
### Table 3.8. Patient response to IVF treatment with the use of kisspeptin as oocyte maturation trigger.

Biochemical pregnancy has been defined as a serum hCG >10iu/l and a clinical pregnancy as the presence of at least one fetal heart beat on ultrasound at 6 weeks’ gestation.

<table>
<thead>
<tr>
<th>Kisspeptin-54 dose (nmol/kg)</th>
<th>1.6</th>
<th>3.2</th>
<th>6.4</th>
<th>12.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients treated</td>
<td>2</td>
<td>3</td>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>At least one egg collected</td>
<td>2</td>
<td>3</td>
<td>23</td>
<td>24</td>
</tr>
<tr>
<td>At least one M2 egg</td>
<td>2</td>
<td>3</td>
<td>22</td>
<td>24</td>
</tr>
<tr>
<td>At least one fertilised egg</td>
<td>1</td>
<td>3</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td>Embryo transfer</td>
<td>1</td>
<td>3</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>High-quality embryo transfer</td>
<td>0</td>
<td>1</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Biochemical pregnancy at 12 days</td>
<td>1</td>
<td>1</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>Clinical pregnancy at 6 weeks</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>4</td>
</tr>
</tbody>
</table>

### Table 3.9. Fertilisation of collected oocytes and resulting embryo quality. High-quality embryos on day 3 scored as 633 or better based on the NEQAS classification system. High quality embryos on day 5 scored as 3BB or better based on the NEQAS classification system. Raw values are presented for the 1.6 and 3.2 nmol/kg doses. Mean values and SD are presents for the 6.4 and 12.8 nmol/kg doses.

<table>
<thead>
<tr>
<th>Kisspeptin-54 dose (nmol/kg)</th>
<th>1.6 (n=2)</th>
<th>3.2 (n=3)</th>
<th>6.4 (n=24)</th>
<th>12.8 (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fertilised oocytes</td>
<td>2,0</td>
<td>5,1,2</td>
<td>5.1 (2.9)</td>
<td>6.8 (3.8)</td>
</tr>
<tr>
<td>Cleaved embryos (day 3)</td>
<td>2,0</td>
<td>5,1,2</td>
<td>5.0 (2.9)</td>
<td>6.3 (3.4)</td>
</tr>
<tr>
<td>High-quality embryos at day 3</td>
<td>0,0</td>
<td>5,1,1</td>
<td>3.8 (2.7)</td>
<td>4.5 (2.7)</td>
</tr>
<tr>
<td>Patients with day5 transfer</td>
<td>0</td>
<td>1</td>
<td>18</td>
<td>17</td>
</tr>
<tr>
<td>Embryos at day 5</td>
<td></td>
<td></td>
<td>5.2 (2.0)</td>
<td>6.9 (2.3)</td>
</tr>
<tr>
<td>High-quality embryos at day 5</td>
<td>3</td>
<td>1.9 (1.4)</td>
<td>2.4 (1.7)</td>
<td></td>
</tr>
<tr>
<td>High quality embryos transferred at day 5</td>
<td>1</td>
<td>1.3 (0.8)</td>
<td>1.5 (0.7)</td>
<td></td>
</tr>
</tbody>
</table>
Adverse events

The administration of kisspeptin-54 was tolerated well in all patients included in this study. This finding is in keeping with the safety profile reported in previous human studies (Dhillo et al, 2005; Dhillo et al, 2007; Chan et al, 2011; George et al, 2011; Chan et al, 2012; Young et al, 2013; Jayasena et al, 2009). Five adverse events occurred, which are well-recognised complications of fertility treatment and pregnancy (two patients had an ectopic pregnancy, one patient had a heterotopic pregnancy with a remaining viable intrauterine pregnancy and two patients had a miscarriage after confirmation of a fetal heart beat at 6 weeks’ gestation).
Discussion

Kisspeptin signaling is essential for mammalian fertility (de Roux et al, 2003; Seminara et al, 2003; Topaloglu et al, 2012), and exogenous administration of kisspeptin can potently stimulate the release of endogenous reproductive hormones in the mammalian species studied (see previous chapter). Kisspeptin administration may represent a novel method for the treatment of infertile patients. Evidence from animal studies suggests that kisspeptin exerts a physiological role leading to the endogenous LH surge, which is pivotal for ovulation. This study investigated whether a bolus injection of kisspeptin-54 can be used as a new method to induce oocyte maturation in patients undergoing IVF therapy. This study has demonstrated for the first time that a bolus injection of kisspeptin-54 can trigger oocyte maturation and subsequently result in fertilisation, implantation of the embryo at the endometrial level and –importantly– live birth in women with infertility undergoing IVF treatment.

The primary objective of the study was to assess whether oocyte maturation was achievable in women administered kisspeptin-54. Oocyte maturation was assessed in two ways, the absolute number of mature oocytes and the proportion of collected oocytes that were mature (metaphase II). Mature oocytes were observed in 96% of study participants, and the percentage of collected oocytes that were mature, was 75-85% following kisspeptin-54 injection at all doses administered. Circulating kisspeptin levels rose rapidly following kisspeptin-54 injection, and this led to a marked increase in LH secretion and smaller increase in FSH secretion (as expected from previous studies in healthy women). Kisspeptin-54 administration resulted in a rise in LH release sufficient to induce egg maturation in women undergoing IVF therapy; levels of serum LH peaked 4h post-injection, remained elevated 12h post-
injection, and had returned to baseline 36h post-injection. Circulating levels of progesterone increased after kisspeptin-54 injection; a finding, which appears to be consistent with oocyte maturation. Consequently the data demonstrate that kisspeptin-54 may act as a potent oocyte maturation trigger in women undergoing IVF treatment. The progress of patients after administration of kisspeptin-54 was followed prospectively. Successful embryogenesis was demonstrated for nearly all patients, who ovulated after the injection of kisspeptin-54 at the higher doses of 6.4nmol/kg and 12.8nmol/kg.

Importantly twelve clinical pregnancies were documented in the current study. Until today ten women have successfully delivered healthy babies. We need to acknowledge that the number of patients in each group was low, and various factors could affect pregnancy rates after oocyte maturation; it is therefore not meaningful to suggest that differences in pregnancy rates definitely exist between the doses of kisspeptin-54 used. Regardless of that, this study can importantly demonstrate for the first time that a healthy pregnancy in humans may result from kisspeptin-54 administration in women undergoing in vitro fertilisation treatment.

A number of ethical issues in the study design need to be acknowledged. It would obviously be unethical to administer infertile patients undergoing cumbersome IVF cycles a placebo oocyte maturation trigger. It has been well demonstrated that the omission of the oocyte maturation trigger will result in failed oocyte maturation (termed ‘empty follicle syndrome’) (Smith et al, 2006). Furthermore, we need to consider that kisspeptin had never before been administered patients with infertility during IVF therapy prior to the commencement of this study, so the effectiveness of
kisspeptin-54 for this role was in essence unknown. This fact had significant ethical implications the process of dose escalation. As a result, a modified 3+3 trial design was utilised to reduce the number of patients exposed to clinically ineffective kisspeptin-54 doses. Consequently we may have identified kisspeptin-54 doses, which were proven to induce oocyte maturation in infertile women undergoing IVF treatment, but we cannot yet exclude that lower kisspeptin-54 doses might also induce oocyte maturation when used as part of an IVF treatment cycle.

**Kisspeptin vs. GnRH agonist to induce oocyte maturation**

The use of a GnRH agonist can provide a useful alternative treatment approach to hCG for oocyte maturation. GnRH agonists can stimulate the pituitary GnRH receptors and lead to an increased release of LH. The use of GnRH agonists for the purpose of triggering oocyte maturation during IVF has been linked to decreased OHSS rates, but has also been associated with decreased pregnancy rates (Shufaro et al, 2013) in comparison with hCG. Luteal phase insufficiency remains the most plausible mechanism behind this decrease. This insufficiency could be attributed to the fact that GnRH agonists may cause excessive pharmacological stimulation and consequently lead to desensitisation of the pituitary GnRH receptor. Kisspeptin-54 has been implicated in the pathway leading to the endogenous LH surge through the activation of the stimulate endogenous secretion of GnRH (Goodman et al, 2007; Irwig et al, 2004; Gottsch et al, 2004; Messager et al, 2005).

The kisspeptin pathway appears to act as a mechanism, which can be employed to induce oocyte maturation in patients suffering with infertility and undergoing IVF. It is important to stress that further studies directly comparing kisspeptin-54 with hCG
and GnRH agonist triggers are required to determine if the novel application of kisspeptin-54 as an oocyte maturation trigger can potentially result in better and safer outcomes for patients undergoing IVF therapy.

For the purposes of clarity and transparency, the cycle characteristics and outcomes after the use of a bolus GnRH agonist dose as oocyte maturation trigger in the same setting (IVF Unit, Hammersmith Hospital) are presented below (Tables 3.10 & 3.11) to provide contextual information regarding how the GnRHa trigger, as currently practiced in the IVF Unit, has performed. No statistical comparisons were attempted between the two groups.

According to the unit protocol, if ≥18 follicles ≥11mm are present on the day of trigger, a GnRH agonist trigger should be used (2mg buserelin). All patients who underwent a GnRH antagonist fresh IVF/ICSI cycle with the use of GnRH agonist trigger between October 2011 and June 2014 were included. Patients having elective oocyte cryopreservation, egg donors and patients having fertility preservation for medical reasons (e.g. cancer) were excluded.

The intensive luteal phase support for these patients consisted of 100mg intramuscular progesterone once daily and 2mg oestradiol valerate orally twice a day until the day of the pregnancy test. If the pregnancy test was positive, then the intensive luteal phase support continued in the form of vaginal progesterone gel 9% (Crinone) once daily and 2mg oestradiol valerate orally twice a day until 12 weeks’ gestation.
<table>
<thead>
<tr>
<th>Cycle characteristics</th>
<th>GnRHa group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicles ≥ 11mm</td>
<td>23 (20-27)</td>
</tr>
<tr>
<td>Total FSH dose</td>
<td>1500 (1200-2025)</td>
</tr>
<tr>
<td>Days of stimulation</td>
<td>10 (9-12)</td>
</tr>
<tr>
<td>Peak E2 (pmol/l)</td>
<td>9675 (6255-13168)</td>
</tr>
<tr>
<td>Single embryo transfers (N, %)</td>
<td>199/351 (56.6%)</td>
</tr>
<tr>
<td>Day 5 embryo transfers (N, %)</td>
<td>321/350 (91.7%)</td>
</tr>
<tr>
<td>Oocytes collected</td>
<td>18 (14-25)</td>
</tr>
<tr>
<td>Mature (M2) oocytes</td>
<td>15 (11-20)</td>
</tr>
<tr>
<td>2PN zygotes</td>
<td>11 (7-15)</td>
</tr>
</tbody>
</table>

Table 3.10. Cycle characteristics for patients with a GnRH agonist oocyte maturation trigger. Variables that are not normally distributed are presented as median (interquartile range, IQR). (E2:oestradiol, 2PN: two pronuclei zygotes).

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>GnRHa group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive pregnancy rate (N,% )</td>
<td>187/382 (48.9%)</td>
</tr>
<tr>
<td>Positive pregnancy rate per transfer (N,% )</td>
<td>187/351 (53.2%)</td>
</tr>
<tr>
<td>Clinical pregnancy rate (N,% )</td>
<td>119/382 (31.1%)</td>
</tr>
<tr>
<td>Clinical pregnancy rate per transfer (N,% )</td>
<td>119/351 (33.9%)</td>
</tr>
<tr>
<td>Implantation rate (N,% )</td>
<td>159/502 (31.6%)</td>
</tr>
<tr>
<td>Ectopic pregnancies per biochemical pregnancy(N,% )</td>
<td>10/187 (5.3%)</td>
</tr>
<tr>
<td>Miscarriages per transfer (N,% )</td>
<td>54/351 (15.3%)</td>
</tr>
<tr>
<td>Cancellation of embryo transfer++ (N,% )</td>
<td>14/382 (3.9%)</td>
</tr>
<tr>
<td>Hospitalisation for severe OHSS</td>
<td>1/382 (0.26%)</td>
</tr>
</tbody>
</table>

Table 3.11. Cycle outcomes. Outcomes are presented per cycle started unless stated otherwise (i.e. per transfer). (OHSS: ovarian hyperstimulation syndrome)
++ cycles cancelled because of the risk or diagnosis of OHSS

_Kisspeptin and ectopic pregnancy_
Ectopic pregnancies are more common during assisted reproductive technology (ART) when compared with spontaneous pregnancies. This may be partly explained by the selection of women with tubal infertility, and also by alterations in tubal contractility as a consequence of the hormonal milieu during IVF cycles (Shufaro et al, 2013). Interestingly Zhao et al (2015) have recently demonstrated that the use of exogenous progestogens can decrease cilia beat frequency of human fallopian tubes and rat oviducts. In unselected IVF cycles, ectopic pregnancy is thought to occur in 2-8.6% of pregnancies and up to 1% of pregnancies may be heterotopic (Shufaro et al, 2013). Furthermore, ectopic pregnancy has been reported to occur in up to 11% of IVF pregnancies in women with tubal infertility (Chang et al, 2010; Malak et al, 2011). In our current study 10 of the 53 women had a diagnosis of tubal infertility as the indication for ART. Of the three women with ectopic pregnancy, one of the patients may have had a higher personal risk of ectopic pregnancy, as she had been diagnosed with ectopic pregnancy requiring surgery in a previous pregnancy, as well as a pregnancy of unknown location in another pregnancy. The participant who was diagnosed with heterotopic pregnancy had a previous diagnosis of endometriosis, which was deemed mild enough for her inclusion in the study, but may have yet contributed to an increased personal risk of ectopic pregnancy. We would not expect kisspeptin treatment to confer any increase in ectopic pregnancy rate, since only a single injection of kisspeptin was administered and the half-life of exogenously administered kisspeptin is short (28 minutes) (Dhillon et al, 2005).

The overall number of patients in this study is small, so the increased rate of ectopic pregnancy may have occurred due to statistical artefact. However, it is important to recognise that a higher than expected rate of ectopic pregnancy was observed, based
on the total number of patients in this study and the published literature. It is therefore possible that kisspeptin triggering has resulted in an increased rate of ectopic pregnancy by an as of yet unidentified mechanism; for example some dysfunctionality in the triggering of ovulation may have led to dyskinetic alterations in the changes in uterine or tubal contractility following induction of ovulation and this could have resulted in increased ectopic pregnancy rate.
Conclusion and Future Work

This is the first human study to assess the use of kisspeptin as an oocyte maturation trigger in an assisted conception cycle. We have shown for the first time that kisspeptin can be used effectively in women undergoing IVF treatment to induce an endogenous LH surge, which subsequently leads to oocyte maturation, fertilisation and can result in a healthy baby. Kisspeptin seems to be a novel factor in assisted conception that could greatly influence future treatment protocols.

The detailed analysis of the FSH, LH, oestradiol and progesterone levels during the luteal phase will allow us to gain further insight into the reproductive hormone milieu after the administration of kisspeptin. Future work will focus on the use of kisspeptin in high-risk populations for OHSS, such as women with polycystic ovarian syndrome. Long-term plans would also involve a direct comparison of kisspeptin with hCG or even more interestingly kisspeptin and GnRH agonist trigger, to assess the clinical and live birth rates as well as the incidence and severity of ovarian hyperstimulation syndrome.
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