Vrije Universiteit Brussel

Faculty of Medicine & Pharmacy

ENDOCRINOLOGY OF OVARIAN STIMULATION

Is there a genetic predisposition of the ovarian hyperstimulation syndrome?

Graduation thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in de geneeskunde

Sari Daelemans

Promotor: Prof. Dr. C. Blockeel
Co-promotor: Dr. K. Stouffs

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Introduction

1. What is Assisted Reproductive Technology (ART)?

About 17% of the couples at reproductive age are not able to conceive a child in 12 months or more with regular unprotected intercourse (Zegers-Hochschild *et al.*, 2009). Fifty six percent of these couples seek medical care to remedy this problem (Boivin *et al.*, 2007). Assisted reproductive technology (ART) can be one of the medical possibilities. This covers all procedures and treatments that include the in vitro handling of human oocytes, sperm or embryos with the aim of establishing a pregnancy. It does not include assisted insemination using sperm from a sperm donor or a woman's partner (Zegers-Hochschild *et al.*, 2009).
In vitro fertilisation (IVF) is the first and most common procedure of ART. The first child conceived by IVF, was born in 1978 (Hillier, 2013). Since then, there has been a great improvement of all reproductive techniques. Nowadays, 1,3 to 3% of the newborns in Europe are conceived by ART (Ferraretti *et al.*, 2012).

2. Ovarian Stimulation

2.1 Physiology of the menstrual cycle

Ovarian stimulation is a crucial aspect of IVF. This stimulation leads to the development of multiple follicles, which can be aspirated, fertilized and transferred, increasing the pregnancy rates (Oehninger & Hodgen, 1990).
To understand the different protocols, it is necessary to comprehend all physiologic mechanisms of the normal menstrual cycle. There are 3 phases in the ovarian cycle: the follicular phase, the ovulation and the luteal phase. These phases are driven by the hypothalamus-pituitary-gonadal axis (Boron & Boulpaep, 2009).
The hypothalamus secretes gonadotropin-releasing hormone (GnRH) that stimulates the anterior pituitary gland to secrete luteinising hormone (LH) and follicle stimulating hormone (FSH) by binding on the GnRH receptor. LH binds on the LH receptors localised on the granulosa cells and FSH binds on the FSH receptors on both the granulosa- and thecacells. This binding leads to the stimulation of producing and secreting estrogens and progestins. There are two other peptides produced by the ovaries: activins and inhibins. These steroids and peptides exert a negative and positive feedback on the hypothalamus and anterior pituitary gland (Blockeel & Devroey, 2012).

2.1.1 The follicular phase

The follicular phase starts when menstruation is initiated and lasts about 14 days. This period is the most variable of the menstrual cycle. In the luteal phase of the preceding cycle, FSH levels start to rise. This increase in FSH levels stimulates the recruitment and development of multiple follicles. In the early follicular phase, LH levels also start to rise. LH is responsible for the uptake of LDL by the theca cells. This contains cholesterol, which is converted to androgens, which in turn diffuses to the granulosa cells. There they are converted to estrogens. Together with FSH, estrogens are responsible for the proliferation of the granulosa cells and so the FSH receptors. They

also induce the proliferation of the LH receptors on the granulosa cells. Estrogens also cause the thickening of the endometrium.
FSH starts to decrease with the rise of oestradiol because of the negative feedback on the anterior pituitary gland. The follicle with the most FSH receptors is the most sensitive to FSH and has enough stimulation of the low levels of FSH to convert the androgens, produced by the theca cells, to estrogens. The other follicles are not stimulated enough and cannot convert the androgens, which leads to atresia of these follicles. This process is responsible for the development of a dominant follicle.

2.1.2 The ovulation

In the late follicular phase, oestradiol levels rise and have a positive feedback on the secretion of LH resulting in the LH surge 24-36h later. During this peak, the oocyte maturation is finalized and twelve hours after the LH surge, ovulation occurs.

2.1.3 The luteal phase

After ovulation the remained theca- and granulosa cells form the highly vascularized corpus luteum. This is the start of the luteal phase. The corpus luteum produces both and progesterone. The rise of progesterone is more prominent then the rise of estrogen, which leads to a negative feedback and thus a decline of GnRH, LH and FSH. Progesterone is also responsible for the further secretory development of the endometrium to prepare it for implantation. Both estrogen and progesterone inhibit folliculogenesis. At the end of the luteal phase, when a fertilisation does not occur, the levels of estrogen and progesterone decrease because the corpus luteum degenerates and forms a fibrotic corpus albicans. This decrease is called luteolysis and is responsible for menstruation, 14 days after ovulation. If fertilisation does occur, the corpus luteum is saved by the production of hCG by the placenta and menstruation does not occur.

2.2 Protocols for ovarian stimulation

The stimulation is performed with exogenous gonadotropins in the early follicular phase before dominance of a follicle is achieved. Because of this stimulation, there will be a selection of more than one follicle (Oehninger & Hodgen, 1990).

Different stimulation protocols have been developed over the years. There is a protocol with no stimulation, the natural cycle, one with minimal stimulation, using clomiphene citrate, and a more aggressive stimulation with exogenous gonadotropins. The latter has been optimised with the use of GnRH analogues.

2.2.1 Clomiphene citrate

Clomiphene citrate is an anti-estrogen, which lowers the negative feedback on the anterior pituitary gland by binding on the estrogen receptors and so causing a rise in FSH and LH levels. Because the function of estrogen is supressed, an LH surge is prevented and premature ovulation does not occur (Oehninger & Hodgen, 1990). This protocol has a pregnancy rate of 6%, which is significantly lower than those observed

with human gonadotropins (20 to 36%). Clomiphene stimulation does not produce enough mature follicles to maximize the pregnancy rate (Cramer *et al.*, 1999).

2.2.2 Exogenous gonadotropins

The two most commonly used exogenous gonadotropins are hMG (human menopausal gonadotropin), which consists of both FSH and LH activity (which is hCG driven), and recombinant FSH, which consists only of FSH. HMG was discovered and purified from postmenopausal urine in the late 1940s. Because there were a lot of co-purified proteins, the injections led to hypersensitivity and discomfort. In the 1960s the purification process became more sophisticated and the side effects were reduced. In the 1980s, FSH was purified from hMG by using monoclonal antibodies. This led to a drastic decrease of hypersensitivity and it could be administered subcutaneously. After the development of urinary FSH, recombinant FSH was produced by recombinant DNA technology (Fatemi, Blockeel & Devroey, 2012).

The gonadotropin stimulation is started in the midfollicular phase, defined as day 1 of the stimulation cycle, and is continued until 3 follicles have a diameter of 17mm. This is a period of approximately 12 days (Macklon *et al.*, 2006).
When this criterion has been reached, the stimulation is discontinued and 50 – 52 h later human chorionic gonadotropin (hCG) is administered (Oehninger & Hodgen, 1990). HCG is structurally very alike LH and therefore it can be used to finalize the follicular development. After this administration, the oocytes can be aspirated.

2.2.3 The GnRH agonist protocol

GnRH agonists were first thought to be used as a treatment for anovulation because they have the same function as endogenous GnRH: stimulating the GnRH receptors, which leads to a secretion of FSH and LH. Administering this agonist initially led to a rise of FSH and LH, but when it was used over a longer period, FSH and LH started to decrease. It seemed the agonist had caused a desensitisation of the GnRH receptors, so lowering the secretion of FSH and LH and leading to less stimulation of the ovaries instead of more (Rabin & Mcneil, 1980). This was very interesting to use in IVF. With this desensitisation of GnRH receptors, endogenous LH and FSH levels can be decreased and the LH surge can be prevented.

There are two protocols with GnRH agonists, a long and a short protocol. In the long protocol, GnRH agonist is usually administered in the luteal phase of the preceding cycle and is continued until hCG administration. Because pituitary quiescence is necessary before starting the ovarian stimulation, GnRH agonists are administered for two weeks. The short protocol starts during the follicular phase and uses the initial stimulatory effect of the agonists to recruit the follicles. Only one day later the stimulation commences (Macklon *et al.*, 2006).
A meta-analysis showed that the number of oocytes retrieved and the pregnancy rates were higher in the long protocol compared to the short protocol (Daya, 2000).

2.2.4 The GnRH antagonist protocol

In 2001, GnRH antagonists were registered for use in IVF. These antagonists are responsible for the immediate suppression of the pituitary function. Stopping those antagonists lead to an immediate recovery of this function. This protocol can be started at any day in the follicular phase after commencing the ovarian stimulation (Macklon *et al.*, 2006). It is clear that the GnRH antagonist protocol is shorter than the long GnRH agonist protocol. There is no significant difference in implantation, pregnancy and implantation rates between the long GnRH agonist protocol and the GnRH antagonist protocol (Al-Inany *et al.*, 2011).

The two most important complications occurring in IVF are the development of multiple pregnancies and the ovarian hyperstimulation syndrome (OHSS) (Fatemi, Blockeel & Devroey, 2012).

3. Ovarian hyperstimulation syndrome (OHSS)

3.1 Clinical presentation of OHSS

OHSS is an exaggerated response to ovarian stimulation, characterised by cyst enlargement of the ovaries, abdominal distention and pain, fluid shift from the intravascular space to the third space, which can result in ascites, pericardial and pleural infusions, and even a generalised oedema. This leads to hypovolaemia, haemoconcentration, electrolyte imbalances and coagulation disorders (Vloeberghs *et al.*, 2009). Life-threatening complications of OHSS include haemorrhage from ovarian rupture, adult respiratory distress syndrome, thromboembolism, and renal failure (Practice committee of American Society of reproductive medicine, 2008).

3.2 Pathophysiology of OHSS

At the basis of OHSS lies an exaggerated response to the stimulation of the ovaries with exogenous gonadotropins. In some patients, this stimulation leads to a large number of growing follicles and high estrogen levels. When these patients are then exposed to a bolus of hCG, this leads to a high vascular permeability, an extravasation of fluid to the third space and so causing the clinical presentation of OHSS (Soares, 2012).

The underlying cause of this syndrome is a mutation in the FSH receptors. These have a constitutive activity and are more sensitive to FSH and hCG, causing the stimulation with exogenous FSH to have a stronger effect leading to the large number of growing follicles and high estrogen levels. This is also partially responsible for the cyst enlargement of the ovaries (Montanelli *et al.*, 2004). The key molecule that is responsible for the high vascular permeability is vascular endothelial growth factor (VEGF). VEGF is produced by the granulosa cells after stimulation with gonadotropins and increases strongly after the administration of hCG due to a hypersensitivity to hCG (Papanikolaou *et al.*, 2006; Soares, 2012).

Recently, Orvieto et al. suggested that interleukine-2 (IL-2) could be a mediator of vascular leakage in OHSS because of the similarity of OHSS with vascular leakage syndrome, caused by high IL-2 levels. IL-2 is produced by T-helper cells and a suppressor of cytokine signalling (SOCS) inhibits its effect. HCG can function as a

direct immune modulator because of the expression of hCG receptors on the peripheral blood mononuclear cells (PBMCs). Normally, binding of hCG to these receptors lead to a rise of SOCS responsible for a negative effect of IL-2 production. This can be confirmed by the improvement of Th1-dependent autoimmune diseases during pregnancy. But in OHSS patients IL-2 levels were significantly higher, which suggests OHSS patients have inherited a paradoxical immune response to hCG with IL-2 dominance instead of SOCS (Orvieto *et al.*, 2014).

Until now two factors can be used as a predictor for patients at risk to develop severe OHSS, namely a follicle count of more than 18 follicles with a diameter of 11 mm and/or estrogen levels higher than 5000 mg/L (Papanikolaou *et al.*, 2006; Tarlatzis *et al.*, 2012).

OHSS can occur in two forms. The early-onset pattern of OHSS is associated with gonadotropin stimulation and is seen within nine days after hCG administration and oocyte retrieval. The late-onset pattern occurs after 10 days and is caused by the production of endogenous hCG by the implanted embryo (Papanikolaou *et al.*, 2006).

3.3 Prevalence of OHSS

The prevalence of early OHSS in a GnRH agonist or GnRH antagonist hCG triggered protocol varies a lot from 20-23% for the mild form, 2-6% for the moderate form and 0.1-2% for the severe form (Vloeberghs *et al*., 2009). Cases of death are under reported, but in spite of this low reporting mortality related directly to the IVF treatment is very low (Braat *et al.*, 2010).

3.4 Prevention of OHSS

The risk of OHSS can be reduced by eliminating the use of hCG as trigger. This became possible by the introduction of GnRH antagonists. Because they inhibit the pituitary function directly, the receptors can recover very fast in contrast to the effect of desensitisation by GnRH agonists. This allows us to use GnRH agonists as a trigger instead of hCG in the antagonist protocol leading to an endogenous LH surge. Although this agonist trigger leads to a flare-up of LH, the levels are not high enough to sustain a normal function of the corpus luteum leading to drastic luteolysis, decreased progesterone levels and therefore an underdeveloped endometrium. This leads to menstruation after 5 or 6 days in contrast to 14 days when hCG is used as a trigger. Because the endometrium does not develop completely, implantation rates are significantly lower when the embryo is implanted in the fresh cycle (Humaidan *et al.*, 2005).

To rescue the luteal phase and thus to make a fresh embryo transfer possible Humaidan et al. suggested to administer hCG after oocyte retrieval, supporting the corpus luteum, increasing the progesterone levels and leading to a maturation of the endometrium (Humaidan *et al.*, 2012). But in this protocol the risk of OHSS still exists. Seyhan et al. suggested cryopreserving all the embryos and implanting them in a next natural or artificial cycle (Seyhan *et al.*, 2013). This eliminates the use of hCG completely and further reduces the risk of OHSS (Devroey, Polyzos & Blockeel, 2011).

3.5 OHSS in an hCG-free protocol

Unfortunately, the risk is not reduced to 0%. Until now there are five cases reported of severe OHSS in a GnRH antagonist protocol with GnRH agonist triggering and freeze-all approach (Fatemi *et al.*, 2014; Gurbuz *et al.*, 2014).

Probably another crucial component in the development of OHSS other than hCG, is involved. The two cases reported by Fatemi et al. showed a curious characteristic. In both cases, menstruation occurred after 14 days instead of 5 or 6 days when using the GnRH antagonist protocol followed by a GnRH agonist trigger. This suggests that there could be a genetic deviation on a higher level in the hormone feedback resulting in a higher quantity of FSH and LH.

Hypothesis

In spite of all the measures taken in the IVF protocol and reducing the risk factors for developing OHSS to a minimum, the complication could not be avoided completely. After doing a literature study on the different IVF procedures and OHSS, following hypothesis was formulated:

There is a genetic predisposition for developing OHSS.

To test this hypothesis, the exome of women who developed OHSS after a GnRH antagonist protocol with a GnRH agonist trigger and a freeze-all approach will be sequenced. Next, the exomes will be analyzed and first there will be looked at variants in specific genes. Afterwards, variants in non-specific genes that are potentially involved in the endocrinology of the menstrual cycle will be analyzed.

Materials and methods

1. Study design

To study this hypothesis we will use a case-control design in 210 women, 10 cases and 200 controls. The exome of the patient group will be sequenced and then analyzed for mutations in the specific genes coding for the GnRH receptor, the FSH receptor, the LH receptor, VEGF, FSH and LH. We will also analyze the exome for other genes, coding for components (possibly) involved in the endocrinology of the menstrual cycle. All subjects will have signed a written informed consent. Approval for the study will be requested to the Ethics Committee of the UZ Brussel. Variants that are potentially related to OHSS will be checked in the control group.

2. Subjects

The subjects are divided into two groups, the cases and the control group. The cases consist of women who developed OHSS after a GnRH antagonist protocol with a GnRH agonist trigger and freeze-all approach. The controls are 200 women who did not develop OHSS after a GnRH antagonist protocol with a GnRH agonist trigger and freeze-all approach. We choose these women for the control group, because they are controlled for both infertility problems and the sensitivity to develop OHSS.

3. Exome sequencing

Whole exome sequencing (WES) will be performed at the Brussels Interuniversity Genomics High Throughput core (BRIGHT core) according to the standard procedures. First, DNA will be fragmented to fragments of on average 250 bp by sonification (using the Covaris M220 device). The KAPA Hyper Prep Kit will be used to create DNA libraries. Afterwards, the Roche SeqCap EZ v3.0 kit will be employed to perform the target enrichement (of the exome), followed by clonal amplification on the Illumina cBot using the TruSeq PE Cluster Kit v4-cBot-HS kit. On the Illumina HiSeq 1500, paired-end sequencing will be performed using the TruSeq SBS kit v4-HS (250 cycles) to obtain a 75x minimum average coverage.

4. Data analysis

Raw WES data will be mapped to the human reference genome (by BWA). The mapped reads will be processed using the GATK (Genome Analysis Toolkit) pipeline and Alamut Batch will annotate the detected variants. This part is performed by a bio-informatician of the BRIGHT core.

All detected variants in the exomes will then be analysed. First, we will look specifically at variants/mutations in the genes coding for the GnRH receptor, FSH receptor, LH/hCG receptor, VEGF, FSH and LH. The frequency of these variants will be looked up in the 1000 Genome project to exclude frequent polymorphisms. Because we look at a rare disorder, we will only include variants in genes up to just 1% of the population. Next, we will eliminate variants that are (most probably) not affecting the protein sequence. For the remaining variants, several prediction tools can be applied (SIFT, PolyPhen2, MutationTaster) to select for potential pathogenic variants.
Afterwards, for a selected group of potentially interesting variants, we will perform Sanger sequencing (according to standard procedures) to confirm the presence of the variant in the patient. Moreover, we will analyse this variant in our control group. The 1000 genomes project gives an idea of the frequency of the observed alteration in the general population, but we have no idea about the fertility status of these men and women. Therefore, we will analyse our own well-selected control group in a second phase.
If no mutations are detected in these genes, we can look to the rest of the exome as it is also sequenced.

Discussion

After running this analysis, different outcomes can be detected between the cases and the control group. We foresee 8 possible results.

The gene coding for the GnRH receptor has a mutation.

If the mutation leads to a high functioning GnRH receptor, this could lead to a high production of FSH and LH, which in turn leads to high quantities of estrogen and especially progesterone. This supports the endometrium and could explain the onset of menstruation at day 14 instead of 5. The higher stimulation of the granulosa cells and theca cells by the high levels of FSH and LH could lead to a higher quantity of VEGF, which is responsible for the vascular permeability and the fluid shift to the third space.

The gene coding for the FSH receptor has a mutation or a mutation in the LH receptor gene is detected.

If there is a mutation in the gene coding for the FSH receptor, it could mean that there is another polymorphism than described by Montanelli et al., 2004 that also could be related to the hypersensitivity to exogenous and endogenous FSH. The same could occur when there is a mutation at the level of the LH receptor, which makes it more sensitive to endogenous LH. Both mutations lead to a higher production of the steroids and VEGF, explaining the development of OHSS without hCG.

A mutation in the gene coding for FSH is detected or LH has a different structure because of a genetic deviation in the LH gene.

If FSH has a slightly different structure it could lead to a different response when it binds to the FSH receptor and be the cause of OHSS. A mutation in the gene coding for LH that is responsible for a different structure or function of LH could also be a good explanation why these women developed OHSS.
The GnRH agonist trigger provokes a certain level of LH and FSH. When FSH or LH for example binds longer to respectively the FSH receptor or LH receptor, this could lead to a stronger stimulation of these receptors and therefor a higher response, being a higher production of VEGF and the steroids.

A mutation in the gene of VEGF is discovered.

If this mutation is detected in the case group and not in the control group, this would not explain the late menstruation. But it could explain the OHSS. Even with the GnRH agonist as a trigger leading to lower levels of LH than normally produced, this leads to a stimulation of the corpus luteum, where the granulosa and theca cells produce VEGF. Because of this stimulation, VEGF is produced but this VEGF has a stronger function. This leads to the high permeability and fluid shift.

Another genetic deviation related in the endocrinology of the menstrual cycle is detected.

If the only mutation is found in a gene that codes for another component involved in the menstrual cycle, but is not as obvious as the previous, we have to examine the link of this abnormality and the role it plays in the development of OHSS.

No genetic deviation related in the endocrinology of the menstrual cycle is found.

Another outcome could be that there is no genetic difference in the case or control group. Or the genetic difference is not significant. This means there is no genetic predisposition for developing OHSS and another factor could be the cause.

Conclusion

The ovarian hyperstimulation syndrome is a severe complication that occurs after ovarian stimulation. Different measures in protocols for ovarian stimulation were taken to minimise the risk of developing OHSS. After identifying the main cause of OHSS, hCG, it was completely eliminated in the ovarian stimulation by using a GnRH antagonist protocol with a GnRH agonist trigger and freeze-all approach. Even after this change, some cases of severe OHSS were reported. This could suggest a genetic predisposition to develop OHSS. After establishing this by doing a literature study, the following hypothesis was formulated: “There is a genetic predisposition for developing OHSS.” Subsequently, the materials and methods is discussed that can be used to test this hypothesis. First, the study design is determined. In this case, a case-control study. Then, the cases are defined as the patients that developed OHSS after a GnRH-antagonist protocol with a GnRH agonist trigger and freeze-all approach, and the controls as the patients that did not develop OHSS after the same protocol. Next, the exome sequencing would be performed on the cases and afterwards the sequence is detected for variants. The hypothesis is investigated by first comparing specific genes that are involved in the menstrual cycle, being the genes coding for the GnRH receptor, FSH receptor, LH receptor, FSH, LH and VEGF. If there are no differences detected between the case and control group we look at other deviations of genes involved in the menstrual cycle.

If a genetic deviation would be detected, this could be used to predict the development of OHSS. Now, in order to prevent OHSS for good, should we include the genome in the whole IVF protocol? With the prevalence of OHSS after a GnRH antagonist protocol with a GnRH agonist trigger and freeze-all approach being extremely low, the detection will be more negative than positive when it is included as a standard procedure in the IVF protocol. But will the costs of performing the screening be less than the treatment of OHSS? This is another question for another kind of investigation.

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