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Influence of gonadotropins on ovarian follicle growth and development *in vivo* and *in vitro*

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Summary

Gonadotropins are the key regulators of ovarian follicles development. They are applied in therapeutic practice in assisted reproductive technology clinics. In the present review we discuss the basic gonadotropic hormones – recombinant human follicle-stimulating hormone, its derivatives, luteinizing hormone and gonadotropin serum of pregnant mares, their origin, and application in ovarian follicle systems in *in vitro* culture systems.

Keywords: Follicle, FSH, Gonadotropin, LH, Ovary, PMSG

Introduction

All follicles at different stages in the ovary can be united by the term 'ovarian reserve', depending on a variety of physiological factors. Reduction in the follicular reserve can be related to a decrease in follicle number or to a significant decline in their quality (Meczekalski *et al*., 2016). Absence of a germ cell store can be evoked by ovary surgery, chemotherapy or radiation exposure during cancer treatment (Dunn & Fox, 2009). There are two basic approaches to solving this problem: cryopreservation of ovarian tissue with subsequent retransplantation or cultivation of ovarian follicles *in vitro* (Filatov *et al*., 2016).

Ovarian tissue cryopreservation with subsequent autotransplantation is a promising method for women with impaired fertility after cancer treatment (chemotherapy or radiotherapy), however this method is associated with several problems. The quality of the cryopreserved/thawed tissue is reduced (Fabbri, 2006), moreover hormonal stimulation can lead to relapse.

Ovarian follicles in *in vitro* cultivation could become an alternative method and the only possible way to obtain mature oocytes if the patient suffers from hormone-dependent cancer or autoimmune diseases (Filatov *et al*., 2016). Oocytes obtained from follicles that were cultivated *in vitro* can be fertilized by *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) and embryos can be cryopreserved and stored until needed by the patient.

Gonadotropic preparations are used to stimulate follicle growth. The two most commonly applied gonadotropins are pregnant mare serum gonadotropin (PMSG) and recombinant human follicle-stimulating hormone (rhFSH). PMSG has been used mainly to stimulate folliculogenesis *in vivo* in laboratory studies on mice (Edwards *et al*., 2004; Kelley *et al*., 2006), while rhFSH is used preferentially in ovarian follicle culture systems (West *et al*., 2007; Dunning *et al*., 2011). Luteinizing hormone (LH) plays an important role in the maturation of the follicle. It supports normal follicle development and induces ovulation. LH is always present in the body, and by stimulating folliculogenesis *in vivo* it is possible to obtain concentrations that are sufficient for follicle maturation (Gal *et al*., 2014). LH has not always been considered a necessary component of the culture medium, but currently the question of its positive influence on follicular growth *in vitro* is being actively discussed (Segers *et al*., 2012; Park *et al*., 2013).

Hormonal regulation of folliculogenesis

Gonadotropins are the main regulators of follicular development and steroid hormone production in the ovary. Follicle-stimulating hormone (FSH) and LH

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are gonadotropic hormones secreted by the pituitary gland that coordinate antral folliculogenesis and ovulation. They are heterodimeric glycoproteins that consist of an α-subunit and a unique β-subunit. The α-subunit is a component of FSH, LH, thyroid stimulating hormone (TSH) and human chorionic gonadotropin (hCG) (Ruman *et al*., 2005). Positive and negative feedback loops in the hypothalamic–pituitary–gonadal axis coordinate follicle maturation with sexual behaviour and preparation for pregnancy. The ovary produces growth factors such as activin, inhibin and follistatin for modulation of FSH secretion and local regulation of follicle development (Edson *et al*., 2009).

The main signalling pathway is activated by FSH binding to specific membrane receptors located on granulosa cells. This interaction alters G-protein complex conformation, which is followed by activation of adenylate cyclase (the enzyme that catalyzes cAMP synthesis from ATP). In the presence of cAMP, protein kinase A (PKA) phosphorylates and activates transcription factors that regulate gene activity, including aromatase, α- and β-subunits of inhibin, the receptor for luteinizing hormone and human chorionic gonadotropic (LHCGR), and many other molecules (Richards *et al*., 2002).

LH stimulates androgen synthesis by theca cells (Liu *et al*., 2015). LHCGR is expressed at high levels in the granulosa cells of preovulatory follicles, this expression allows cells to respond to a surge of LH, which initiates a cascade of events leading to the resumption of meiosis in oocytes, an increase in the number of cumulus cells, rupture of the follicle and the formation of the corpus luteum (luteinization) (Edson *et al*., 2009).

The LH surge activates genes that are responsible for the formation and stabilization of the extracellular matrix of the cumulus oophorus, i.e. the region of the antral follicle wall that carries the oocyte. The LH surge causes a rapid increase in the expression of epidermal growth factor (EGF) family genes (*Areg*, *Ereg* and *Btc*) in cumulus cells. These ligands are integral membrane proteins and are released from the cell surface by ectodomain cleavage. These ligands then bind to EGF receptors (EGFRs) and activate them. In addition, this binding leads to the activation of mitogen-activated protein kinase (MAPK)3 and MAPK1, also known as extracellular signal-regulated kinases 1 and 2 (ERK1/2), respectively. ERK1/2 can stimulate oocyte maturation. However, the specific roles of the EGF network and ERK1/2 in ovulation regulation, oocytes maturation and further follicular luteinization are not yet sufficiently clear (Fan *et al*., 2009; Zhang *et al*., 2015).

As with many other protein hormones, FSH consists of a family of isoforms that differs in size, charge and elimination rate (Roche, 1996). FSH is encoded by a single gene, but the pituitary gland is able to produce more than 17 different FSH isoforms due to post-translational modifications. These isoforms differ in their oligosaccharide structure and in the degree of terminal sialylation and sulfonation. A change in the carbohydrate patterns due to sulfonation (by addition of sulfonic acid groups – SO_3H) and sialylation (by attachment of sialic acid residues) can reduce the pH of the protein and increase the half-life of the isoforms. Remodelling of carbohydrate complexity can significantly affect the biological activity of hormones (Trousdale *et al*., 2009). A change in FSH isoform expression occurs during the ovarian menstrual cycle. The least acidic FSH isoforms were observed during the late follicular phase, and in the mid-cycle. These isoforms have a smaller half-life period, indicating the existence of regulatory mechanisms that control the intensity and duration of FSH signal exposure (Macklon *et al*., 2006). Less sialylated forms of FSH more actively stimulate estradiol and progesterone production by granulosa cells than the acidic isoforms (Loreti *et al*., 2013b).

The normal response of the follicles to gonadotropins requires the presence of both these hormones and their receptors. It has been shown that mice that lack follicle-stimulating hormone receptor (FSHR) are infertile (Dierich *et al*., 1998). Moreover, many isoforms of human FSHR are known. About 2000 single nucleotide polymorphism (SNPs) in the *FSHR* have been reported, however only few of these affect fertility (Casarini *et al*., 2015) because of impaired binding with FSH and FSHR or a decrease in FSHR production. Genetic analysis performed to detect *FSHR* isoforms may be used as a predictive marker of diminished ovarian reserve and infertility (Ilgaz *et al*., 2015).

Gonadotropins are the key regulators of follicular development and steroid hormone production in the ovary. Large numbers of internal ovarian regulators, however, especially insulin-like growth factor (IGF), EGF, transforming growth factor (TGF)-α and TGF-β, can modulate the cellular response to gonadotropic stimulation. Inhibins and activins also have a local effect in the ovaries, in addition to their endocrine and paracrine functions. They suppress FSH secretion by the pituitary gland. IGF-I stimulates the proliferation of granulosa cells and increases their aromatase activity. In theca cells, IGF-I has a positive influence on 17α-hydroxyprogesterone production; IGF-I and IGF-II together with LH stimulate androgen synthesis. EGF and TGF-α are structurally similar and can bind to one receptor. These factors promote granulosa cell proliferation, but inhibit FSH-induced expression of aromatase and estradiol synthesis. TGF-β also stimulates the proliferation of granulosa cells. In

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pre-antral follicles, activin increases the number of granulosa cells and elevates FSH receptor and aromatase expression levels that in turn lead to increased estradiol production (Macklon *et al*., 2006). Inhibins stimulate LH-induced androgen synthesis by theca cells (Roche, 1996).

Another important molecule involved in hormonal regulation of folliculogenesis is anti-Müllerian hormone (AMH). AMH is a dimeric glycoprotein and a member of the TGF-β gene family (Knight & Glister, 2006). AMH is commonly used as a diagnostic and prognostic clinical marker of fertility that can indicate the size of the ovarian reserve (Bhide & Homburg, 2016; Łebkowska & Kowalska, 2017). It has been shown that *AMH* is expressed in the granulosa cells of primary, secondary, pre-antral and early antral follicles but not in primordial follicles, late antral follicles and oocytes (Lee *et al*., 1996; Rajpert-De Meyts *et al*., 1999; Weenen *et al*., 2004). AMH inhibits the initiation of primordial follicle growth (Durlinger *et al*., 1999), and premature ovarian failure (POF) was found in AMH-null mice (Visser & Themmen, 2005). Moreover, AMH suppresses FSH-induced follicle growth. This effect is probably mediated through the induction of *miR-181b* miRNA that, in turn, blocks adenylate cyclase 9, thus decreasing FSH-induced cAMP levels and leading to suppression of FSH signalling. AMH, through the induction of another miRNA (*miR-181a*), targets activin receptor 2A, thus leading to impairment of the effects of activin on folliculogenesis, and preventing follicular growth and proliferation of granulosa cells (Hayes *et al*., 2016). Interestingly, AMH receptors are expressed not only in the ovary but also in the pituitary gland (Bedecarrats *et al*., 2003). It has been shown that LH secretion is induced by AMH through stimulation of hypothalamic gonadotropin-releasing hormone (GnRH) neurons, which express AMH receptors (Cimino *et al*., 2016). Thus, mutations in the gene coding of AMH receptors can lead to infertility (Li *et al*., 2016). Additionally, it has been shown that AMH receptor II is overexpressed in granulosa cells of oligo/anovulatory, but not in normo-ovulatory, women with polycystic ovary syndrome (PCOS; Pierre *et al*., 2013). Therefore, AMH/AMHR signalling is crucial for regulation of folliculogenesis at both the pituitary and ovarian levels.

After implantation, the embryo produces hCG that influences the maternal organism, for example it regulates progesterone production by the corpus luteum. When the placenta becomes capable of progesterone production, hCG starts to control and maintain the fetal blood supply. Human chorionic gonadotropin stimulates angiogenesis by binding to LHCGRs located on uterus spiral arteries. In addition, hCG is involved in placental cell differentiation and stimulation of uterine growth together with growth of the fetus (Ezcurra & Humaidan, 2014). When LHCGR binds to its ligand its conformation changes and activates G-protein, which in turn activates adenylate cyclase (AC). Adenylate cyclase increases the intracellular cAMP pool. Induction of the cyclic AMP/protein kinase A (PKA) pathway eventually results in ovulation and steroid biosynthesis. LHCGR stimulation also activates the phospholipase C/inositol phosphate pathway. This signalling pathway is activated only during the preovulatory LH surge and during pregnancy. Phospholipase C acts as a mediator of terminal granulosa cell differentiation (Choi & Smitz, 2014).

Human chorionic gonadotropin has a greater affinity for this receptor and has a greater effect on follicular development than does LH (Ezcurra & Humaidan, 2014). LH and hCG bind to the same receptor but activate different signalling pathways (Fig. 1). LH has a greater influence on the phospholipase C/inositol 1,4,5-trisphosphate (PLC/IP3) pathway (Choi & Smitz, 2014), leading to the phosphorylation and inactivation of forkhead box O3 (FOXO3), which is an inhibitor of primordial follicle activation. Phosphoinositide-3 kinase (PI3K) catalyzes the conversion of membrane phosphatidylinositol 4,5-bisphosphate (PIP2) to a second messenger – phosphatidylinositol 3,4,5-trisphosphate (PIP3). IP3 activates protein kinase B (PKB) that phosphorylates FOXO3 (Edson *et al*., 2009). hCG promotes the accumulation of cAMP, which activates the PKA signalling pathway (Choi & Smitz, 2014). After the LH peak, the level of cAMP reaches a plateau after 10 min, by comparison in response to hCG the plateau is reached after 1 h (Ezcurra & Humaidan, 2014).

The application of gonadotrophic hormones in assisted reproductive technology

Hormonal stimulation is an essential part of modern assisted reproductive technology (ART). Gonadotropins are applied to stimulate folliculogenesis in humans and animals. This kind of treatment leads to an increase in the number of oocytes or embryos that can be used for ART (Edwards *et al*., 2004).

Human chorionic gonadotropin (hCG) was first isolated from the blood and urine of pregnant women in 1927 and is one of the most commonly used hormones in clinical practice. The action of hCG is very similar to that of Prolan B (LH) and, in 1932, it was released under the commercial name Pregnil (Lunenfeld, 2004).

At the start of the introduction of ART, PMSG was used for ovarian follicle growth stimulation. PMSG is

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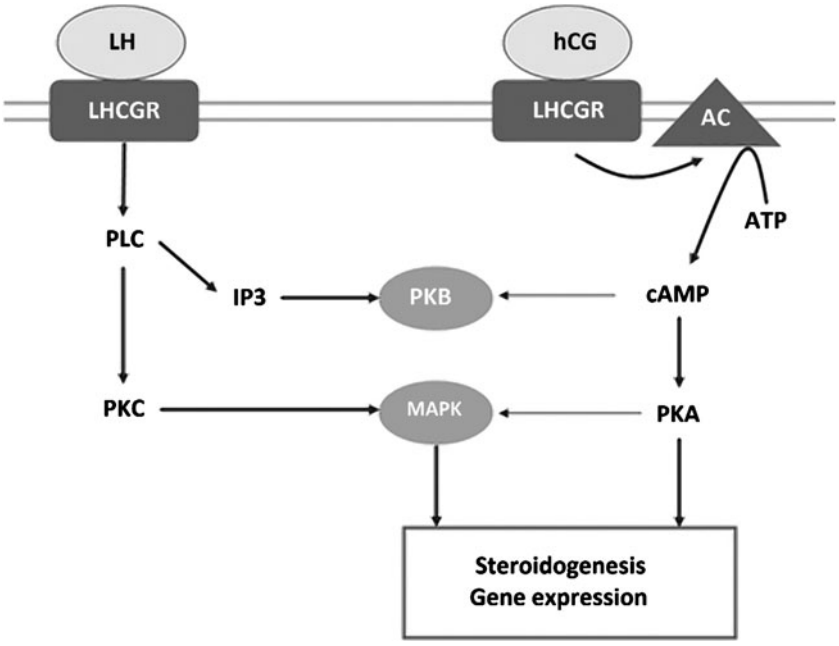

Figure 1 Theoretical pathway of divergence in receptor-mediated signal transduction of LH and hCG (according to Choi & Smitz, 2014). Abbreviations: AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; hCG, human chorionic gonadotropin; IP3, inositol 1,4,5-trisphosphate; LH, luteinizing hormone; LHCGR, receptor for luteinizing hormone and chorionic gonadotropin hormone; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C.

secreted by the endometrial cups (ring-like structures on the surface of the endometrium around the fetus's attachment point) of pregnant mares. PMSG can be detected initially at 37–42 days of pregnancy; it reaches its highest concentration between 50 and 70 days, and almost completely disappears after the fourth month of pregnancy. At present, PMSG is not administered to humans, as this drug can cause an immune response.

In 1941, a 'two-step protocol' was proposed. The main idea of the new protocol was to stimulate follicle growth and ovulation sequentially. In the first step, follicles are stimulated by gonadotropins (PMSG or pituitary gonadotropins from pigs or sheep) and, in the second step, ovulation is induced by human chorionic gonadotrophin (HCG). At this time, a hyperstimulation syndrome was described in which ovaries were dotted with numerous haemorrhagic follicles that lacerated and bled at the slightest touch (Lunenfeld, 2004).

Gonadotropin, isolated from the human pituitary gland in 1958, has been the most suitably used hormone for oogenesis stimulation over time. From 1958 to 1988, it has been used successfully in several medical centres around the world to induce ovulation in the treatment of ovulation disorders. The source of this drug, however, has been too limited. In addition, some patients have shown symptoms of Creutzfeldt–Jakob disease (the primary manifestation of spongiform encephalopathy, or prion disease) more than 20 years after the initiation of pituitary gonadotropin treatment (Lunenfeld, 2004).

In the early 1970s, the idea of individual treatment protocols for FSH and LH administration for each patient became paramount. Attempts to separate these two hormones were not successful as the proposed methods were too cumbersome, complicated or expensive, or were not sufficiently precise or effective. With the advent of genetic engineering, these problems have been overcome. In 1995, the commercial drug Gonal-F was licensed for use (Lunenfeld, 2004).

FSH in its recombinant and purified form is currently used to support ovarian follicle growth in ART. Recombinant human FSH has a short half-life (approximately 24 h after injection), therefore it needs to be injected daily (subcutaneously or intramuscularly) over an average period of 8–12 days. The manufacture of rhFSH-carboxy-terminal peptide (CTP), which has a two to three times greater half-life than rhFSH, made it possible to reduce the number of injections needed, compared with the standard rhFSH protocol (Ruman *et al.*, 2005). Moreover, in the mouse model, it has been shown that the efficiency (the quantity of oocytes after stimulation) of rhFSH-CTP treatment was significantly higher than that of rhFSH application (Trousdale *et al.*, 2009).

More sialylated rhFSH stimulates inhibin B synthesis; less sialylated analogues are not capable of

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stimulating inhibin B synthesis, but have a significant stimulatory effect on the inhibin A synthesis. An increase in inhibin serum concentration was observed in the early follicular phase of the menstrual cycle that coincided with the presence of more acidic forms of FSH. In contrast, the less acidic forms detected in the late follicular phase were associated with the appearance of inhibin A (Loreti *et al*., 2013a).

The improved forms of recombinant FSH (with a long half-life), however, were still less effective than PMSG. PMSG is the most glycosylated pituitary glycoprotein of placental mammals: both subunits of this hormone contain *N*-linked and *O*-linked glycosylation sites. The high sialic acid content greatly increases its half-life compared with other glycoprotein hormones. In mice, only multiple doses of purified or recombinant FSH are equal to a single dose of PMSG for inducing ovulation. PMSG also has luteinizing activity. It is important for the optimal development of follicles in the preovulatory phase (Ruman *et al*., 2005).

A significant increase in progesterone blood concentration was observed after stimulation with PMSG (5 IU) in comparison with rhFSH application (2.5 IU), but the estradiol concentration did not change. In mammals, the progesterone/estradiol ratio is more important for implantation than the concentration of each hormone. Estrogen and progesterone have an antagonistic effect on the endometrium. Progesterone is important during preparation of the endometrium for implantation. Gonadotropic stimulation causes changes in the spatial distribution of the progesterone receptor that accelerate the maturation of the endometrium (Kelley *et al*., 2006); a high level of FSH or estrogen may increase the susceptibility of granulosa cells to LH. This change allows luteinization to be induced even at low LH levels. Luteinization leads to an early increase in progesterone levels, and causes early maturation of the endometrium (Kelley *et al*., 2006).

Estradiol and progesterone regulate the expression of five genes that have a function during implantation: *Lif*, *Vegf*, *Hoxa10*, *Esr1* and *Pgr*. In mice, oogenesis stimulation by gonadotropin reduces *Lif* expression levels in the uterus on the fourth day post coitum (dpc). Leukemia-inhibiting factor (LIF) is essential for implantation. In the absence of *Lif* expression, the mouse endometrium does not respond to blastocyst attachment. LIF is an interleukin-6 family cytokine, and its expression in the mouse endometrium is induced by estrogen. Typically, expression of this gene peaks on the fourth day of pregnancy, and decreases on the fifth day of gestation. Thus, reduced *Lif* expression indicates that exogenous gonadotropins accelerate endometrial maturation. Expression of other key implantation genes in stimulated mice did not differ from that of control mice because expression of these genes does not normally differ at days 4 or 5 of gestation (Kelley *et al*., 2006).

At present, it is commonly assumed that the use of recombinant human gonadotropins is the most effective method of ART. However, current methods of purification have allow high quality fractions of gonadotropins from human menopausal gonadotropin (HMG) to be obtained. HMG is derived from the urine of postmenopausal women (Wolfenson *et al*., 2005). The drugs Bravelle and Menopur are the two most commonly used examples of this class. The urine fraction that contains FSH activity is used for Bravelle preparation, whereas the fractions that have FSH and LH activity are used for Menopur. The ratio of FSH and LH activity in Menopur is 1:1. The LH activity of Menopur is mainly due to hCG because most LH molecules are lost during purification (Wolfenson *et al*., 2005).

Application of exogenous gonadotropins has a negative effect on early embryonic development and implantation. Ovarian stimulation leads to a decrease in the number of microvilli on the surface of the blastocyst in comparison with unstimulated control murine blastocysts and leads to delay in implantation, caused by a reduction in vascular endothelial growth factor (VEGF) expression levels. Furthermore, the duration of ovarian stimulation reduces pregnancy duration in mice (Edwards *et al*., 2004). The frequency of pregnancy in stimulated mice decreased because of asynchrony between embryo development and endometrial maturation (Kelley *et al*., 2006). There is an evidence that the number of abnormal embryos increases with rising doses of gonadotropins. The largest percentage of defective embryos was observed after stimulation with PMSG, mainly due to its long half-life and high levels of luteinizing activity (Edwards *et al*., 2004).

Hormonal stimulation of folliculogenesis *in vitro*

Ovarian follicles in *in vitro* culture require optimum conditions, particularly regarding the composition of the culture medium. The most promising results for follicles cultured *in vitro* were obtained by constant addition of hormones to the culture medium (Hu *et al*., 2011).

FSH is the main hormone that determines the survival and growth of a follicle in culture. Moreover, there is a time dependence between survival rate and hormone addition. Late addition of FSH to the culture medium leads to a decrease in the quantity of live follicles. The rate of granulosa cell proliferation depends on the presence of FSH. The antral cavity

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Table 1 Types of medium for ovarian follicles *in vitro* culture

Culture object	Medium composition	Author, year
Primordial follicles of 5-day mice	α-MEM, 1 IU/ml rhFSH, 5 mg/ml ascorbic acid, 5% serum from adult female mice	Morgan *et al.*, 2015
Pre-antral follicles of 13-day mice	α-MEM, 5% FCS, ITS, 0.1 mIU/ml rhFSH, 0.01 IU/ml of recombinant LH	Sun *et al.*, 2004
Primordial follicles of 3-day mice	α-MEM, 0.1% BSA, 0.05 mg/ml L-ascorbic acid, 0.23 mM sodium pyruvate, 50 μg/ml insulin, 5.27 μg/ml transferrin, 5 IU/ml penicillin, 5 μg/ml streptomycin	Wang *et al.*, 2013
Early secondary follicles of 13-day mice	α-MEM, 5% FCS, ITS, 0.01 mIU/ml FSH, 0.01 IU/ml of recombinant LH	Sanchez *et al.*, 2012
Secondary follicles of 14-day mice	α-MEM, 3 mg/ml of BSA, 0,01 mIU/ml rhFSH, 1 mg/ml bovine fetuin, ITS	Hornick *et al.*, 2013; Shikanov *et al.*, 2009; Xu *et al.*, 2009
Secondary follicles of 12-day mice	α-MEM, 5% FCS, 0.01 mIU/ml of LH, 0.1 IU/ml FSH	Dunning *et al.*, 2011
Secondary follicles of 9-day mouse	α-MEM, 1% FCS	Jin *et al.*, 2010
Primary and secondary follicles of rhesus monkeys	300 ml α-MEM, 3 ng/ml rhFSH, 0.3% human serum albumin, 5 mg/ml insulin, 0.5 mg/ml bovine fetuin, 5 mg/ml transferrin, 5 ng/ml sodium selenite	Xu *et al.*, 2013

α-MEM, α-Minimal Essential Medium; BSA, bovine serum albumin; FCS, fetal calf serum; ITS, insulin–transferrin–selenium; IU, International Units; LH, luteinizing hormone; rhFSH, recombinant human follicle-stimulating hormone.

starts to form not earlier than 4 days after hormone addition to the medium (Adriaens *et al.*, 2004). As mentioned above, FSH has several different isoforms; those that have a lower acidity are more active *in vitro*, but have a short half-life (Roche, 1996).

During *in vitro* culture, follicular survival rate does not depend significantly on medium FSH concentration, but experimental data demonstrate that follicular growth is more intensive in medium with high hormone content. In the Kreeger experiments, FSH effects were already observed on the second day of culture. The diameter of follicles cultured in medium that contained 10 and 25 mIU/ml FSH was markedly larger than follicles in medium that contained 0 or 5 mIU/ml FSH. FSH dose escalation also increased the accumulation of lactate, which indicates activated granulosa cell metabolism. Very high doses of FSH (50 mIU/ml), however, led to a decrease in follicle survival, because products of metabolism accumulated too quickly. A positive correlation between FSH dose and estradiol level was observed in the *in vitro* culture system. Estradiol accumulation was observed only in follicles cultured with added FSH, but not in follicles cultured without this supplement (Kreeger *et al.*, 2005).

Investigation of the influence of LH on follicle maturation *in vitro* showed that follicle survival rate was significantly higher in medium supplemented with LH than without (Park *et al.*, 2013). Moreover, LH bioactivity had an effect on antral cavity formation (Cortvrindt *et al.*, 1998). Follicles cultured in medium with low doses of LH had larger diameters than those cultured in medium with high LH concentrations (Park *et al.*, 2013). Granulosa cell proliferation is inhibited by LH concentrations in the medium of more than 5 IU/L, and the follicle undergoes atresia or luteinization. In addition, high levels of LH led to a decrease in production and sensitivity of LHCGR due to accelerated degradation of *Lhcgr* mRNA (Ezcurra & Humaidan, 2014). Researchers have used various approaches for ovarian follicle *in vitro* culture. A list of the main types of medium applied in *in vitro* culture systems is presented in Table 1.

Medium supplemented with hCG is used to stimulate oocyte *in vitro* maturation, because hCG has a more significant luteinizing effect than LH (Skory *et al.*, 2015).

Conclusion

The use of ovarian stimulation by gonadotropins to obtain mature and compatible oocytes for fertilization started to develop a few decades ago. At present, modified forms of gonadotropins with increased half-lifes are used. The application of sialated and glycosylated isoforms of FSH allows the use of a simplified procedure of ovarian stimulation, via minimizing the number of injections. Moreover, the application of modified isoforms of FSH with prolonged half-lives

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has been investigated using ovarian follicles and ovarian tissue in *in vitro* culture. The use of such isoforms allows a reduction in the dose of hormone for supplementation in the medium and an increase in the time interval between medium changes.

The use of ovarian follicles in *in vitro* culture seems to be a very promising technology, however at present it is not sufficiently developed for modern ART practice. The selection of the optimal follicular culture conditions needed to obtain mature oocytes *in vitro* requires a complex approach. It is necessary to take into account the mechanical and chemical surroundings of the cells, metabolism and paracrine interactions (Filatov *et al*., 2015; Kosheleva *et al*., 2016). To obtain a greater number of mature oocytes is necessary to apply all the factors (including gonadotropin treatment) that are essential for normal follicle growth and development. In most research studies, FSH was added to the medium over the whole period of culture, whereas drugs with LH activity were supplemented only at the end. In only a few studies, researchers have added LH to the culture medium not only at the end of culture to stimulate ovulation, but also in the very early stages because of the need to maintain normal follicle growth and development. Therefore the correct application of gonadotropin preparations allows us to mimic *in vivo* conditions *in vitro*. The study of the effects of different gonadotropin preparations on follicle growth and development remains a major challenge for many groups of researchers, with the continuing appearance of large numbers of publications on this subject.

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Conflict of interest

There are no conflicts of interest.

References

Adriaens, I., Cortvrindt, R. & Smitz, J. (2004). Differential FSH exposure in preantral follicle culture has marked effects on folliculogenesis and oocyte developmental competence. *Hum. Reprod.* **19**, 398–408.

Bedecarrats, G.Y., O'Neill, F.H., Norwitz, E.R., Kaiser, U.B., Teixeira, J. (2003). Regulation of gonadotropin gene expression by Müllerian inhibiting substance. *Proc. Natl. Acad. Sci. USA* **100**, 9348–53.

Bhide, P. & Homburg, R. (2016). Anti-Müllerian hormone and polycystic ovary syndrome. *Best. Pract. Res. Clin. Obstet. Gynaecol.* **37**, 38–45.

Casarini, L., Santi, D., Marino, M. (2015). Impact of gene polymorphisms of gonadotropins and their receptors on human reproductive success. *Reproduction* **150**, 175–84.

Choi, J. & Smitz, J. (2014). Luteinizing hormone and human chorionic gonadotropin: origins of difference. *Mol. Cel. Endocrinol.* **383**, 203–13.

Cimino, I., Casoni, F., Liu, X., Messina, A., Parkash, J., Jamin, S.P., Catteau-Jonard, S., Collier, F., Baroncini, M., Dewailly, D., Pigny, P., Prescott, M., Campbell, R., Herbison, A.E., Prevot, V. & Giacobini, P. (2016). Novel role for anti-Müllerian hormone in the regulation of GnRH neuron excitability and hormone secretion. *Nat. Commun.* **7**, 10055–66.

Cortvrindt, R.G., Hu, Y., Liu, J. & Smitz, J.E. (1998). Timed analysis of the nuclear maturation of oocytes in early preantral mouse follicle culture supplemented with recombinant gonadotropin. *Fertil. Steril.* **70**, 1114–25.

Dierich, A., Sairam, M.R., Monaco, L., Fimia, G.M., Gansmuller, A., LeMeur, M. & Sassone-Corsi, P. (1998). Impairing follicle-stimulating hormone (FSH) signalling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. *Proc. Natl. Acad. Sci. USA* **95**, 13612–7.

Dunn, L. & Fox, K.R. (2009). Techniques for fertility preservation in patients with breast cancer. *Curr. Opin. Obstet. Gynecol.* **21**, 68–73.

Dunning, K.R., Akison, L.K., Russell, D.L., Norman, R.J. & Robker, R.L. (2011). Increased beta-oxidation and improved oocyte developmental competence in response to L-carnitine during ovarian *in vitro* follicle development in mice. *Biol. Reprod.* **85**, 548–55.

Durlinger, A.L., Kramer, P., Karels, B., de Jong, F.H., Uilenbroek, J.T., Grootegoed, J.A. & Themmen, A.P. (1999). Control of primordial follicle recruitment by anti-Müllerian hormone in the mouse ovary. *Endocrinology* **140**, 5789–96.

Edson, M.A., Nagaraja, A.K. & Matzuk, M.M. (2009). the mammalian ovary from genesis to revelation. *Endocr. Rev.* **30**, 624–712.

Edwards, L.J., Kind, K.L., Armstrong, D.T. & Thompson, J.G. (2004). The effects of recombinant human follicle stimulating hormone (rhFSH) on embryo development in mice. *Physiol. Endocrinol. Metab.* **288**, 845–51.

Ezcurra, D. & Humaidan, P. (2014). A review of luteinising hormone and human chorionic gonadotropin when used in assisted reproductive technology. *Reprod. Biol. Endocrinol.* **12**, 1–12.

Fabbri, R. (2006). Cryopreservation of human oocytes and ovarian tissue. *Cell Tissue Bank* **7**, 113–22.

Fan, H.Y., Liu, Z., Shimada, M., Sterneck, E., Johnson, P.F., Hedrick, S.M. & Richards, J.S. (2009). MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science* **324**, 938–41.

Filatov, M.A., Khramova, Y.V., Kiseleva, M.V., Malinova, I.V., Komarova, E.V. & Semenova, M.L. (2016). Female fertility preservation strategies: cryopreservation and ovarian tissue *in vitro* culture, current state of the art and future perspectives. *Zygote* **4**, 1–19.

Filatov, M.A., Khramova, Y.V. & Semenova, M.L. (2015). *In vitro* mouse ovarian follicle growth and maturation in

alginate hydrogel: current state of the art. *Acta Naturae* **7**, 48–56.

Gal, A., Lin, P., Barger, A.M, MacNeill, A.L. & Ko, C. (2014). Vaginal fold histology reduces the variability introduced by vaginal exfoliative cytology in the classification of mouse estrous cycle stages. *Toxicol. Pathol.* **42**, 1212–20.

Hayes, E., Kushnir, V., Ma, X., Biswas, A., Prizant, H., Gleicher, N. & Sen, A. (2016). Intra-cellular mechanism of anti-Müllerian hormone (AMH) in regulation of follicular development. *Mol. Cell. Endocrinol.* **433**, 56–65.

Hornick, J.E., Duncan, F.E., Shea, L.D. & Woodruff, T.K. (2013). Multiple follicle culture supports primary follicle growth through paracrine-acting signals. *Reproduction* **145**, 1–26.

Hu, J., Ma, X., Bao, J.C., Li, W., Chen, D., Gao, Z., Lei, A., Yang, C. & Wang, H. (2011). Insulin–transferrin–selenium (ITS) improves maturation of porcine oocytes *in vitro*. *Zygote* **19**, 191–7.

Ilgaz, N.S., Aydos, O.S., Karadag, A., Taspinar, M., Eryilmaz, O.G. & Sunguroglu, A. (2015). Impact of follicle-stimulating hormone receptor variants in female infertility. *J. Assist. Reprod. Genet.* **32**, 1659–68.

Jin, S.Y., Lei, L., Shikanov, A., Shea, L.D. & Woodruff, T.K. (2010). A novel two-step strategy for *in vitro* culture of early-stage ovarian follicles in the mouse. *Fertil. Steril.* **93**, 2633–9.

Kelley, R.L., Kind, K.L., Lane, M., Robker, R.L., Thompson, J.G. & Edwards, L.J. (2006). Recombinant human follicle-stimulating hormone alters maternal ovarian hormone concentrations and the uterus and perturbs fetal development in mice. *Physiol. Endocrinol. Metab.* **291**, 761–70.

Knight, P.G. & Glister, C. (2006). TGF-beta superfamily members and ovarian follicle development. *Reproduction* **132**, 191–206.

Kosheleva, N.V., Ilina, I.V., Zurina, I.M., Roskova, A.E., Gorkun, A.A., Ovchinnikov, A.V., Agranat, M.B. & Saburina, I.N. (2016). Laser-based technique for controlled damage of mesenchymal cell spheroids: a first step in studying reparation *in vitro*. *Biol. Open.* **7**, 993–1000.

Kreeger, P.K., Fernandes, N.N., Woodruff, T.K. & Shea, L.D. (2005). Regulation of mouse follicle development by follicle-stimulating hormone in three-dimensional *in vitro* culture system is dependent on follicle stage and dose. *Biol. Reprod.* **73**, 942–50.

Lee, M.M., Donahoe, P.K., Hasegawa, T., Silverman, B., Crist, G.B., Best, S., Hasegawa, Y., Noto, R.A., Schoenfeld, D. & MacLaughlin, D.T. (1996). Müllerian inhibiting substance in humans: normal levels from infancy to adulthood. *J. Clin. Endocrinol. Metab.* **81**, 571–6.

Li, L., Zhou, X., Wang, X., Wang, J., Zhang, W., Wang, B., Cao, Y. & Kee, K. (2016). A dominant negative mutation at the ATP binding domain of AMHR2 is associated with a defective anti-Müllerian hormone signaling pathway. *Mol. Hum. Reprod.* **22**, 669–78.

Liu, X., Qiao, P., Jiang, A., Jiang, J., Han, H., Wang, L. & Ren, C. (2015). Paracrine regulation of steroidogenesis in theca cells by granulosa cells derived from mouse preantral follicles. *BioMed. Res. Int*. **2015**, 1–8.

Loreti, N., Ambao, V., Andreone, L., Trigo, R., Bussmann, U. & Campo, S. (2013b). Effect of sialylation and complexity of FSH oligosaccharides on inhibin production by granulosa cells. *Reproduction* **145**, 127–35.

Loreti, N., Fresno, C., Barrera, D., Andreone, L., Albarran, S.L., Fernandez, E.A., Larrea, F. & Campo, S. (2013a). The glycan structure in recombinant human FSH affects endocrine activity and global gene expression in human granulosa cells. *Mol. Cel. Endocrinol.* **366**, 68–80.

Lunenfeld, B. (2004). Historical perspectives in gonadotrophin therapy. *Hum. Reprod. Update* **10**, 453–67.

Łebkowska, A. & Kowalska, I. (2017). Anti-Müllerian hormone and polycystic ovary syndrome. *Endokrynol. Pol.* **68**, 74–8.

Macklon, N.S., Stouffer, R.L., Giudice, L.C. & Fauser, B.C. (2006). the science behind 25 years of ovarian stimulation for *in vitro* fertilization. *Endocr. Rev.* **27**, 170–207.

Meczekalski, B., Czyzyk, A., Kunicki, M., Podfigurna-Stopa, A., Plociennik, L., Jakiel, G., Maciejewska-Jeske, M. & Lukaszuk, K. (2016). Fertility in women of late reproductive age: the role of serum anti-Müllerian hormone (AMH) levels in its assessment. *J. Endocrinol. Invest.* **39**, 1259–65.

Morgan, S., Campbell, L., Allison, V., Murray, A. & Spears, N. (2015). Culture and co-culture of mouse ovaries and ovarian follicles. *J. Vis. Exp.* **97**, 1–10.

Park, K.E., Ku, S.U., Jung, K.C., Liu, H.C., Kim, Y.Y., Kim, Y.J., Kim, S.H., Choi, Y.M., Kim, J.G. & Moon, S.Y. (2013). Effects of urinary and recombinant gonadotropins on *in vitro* maturation outcomes of mouse preantral follicles. *Reprod. Sci.* **20**, 909–16.

Pierre, A., Peigne, M., Grynberg, M., Arouche, N., Taieb, J., Hesters, L., Gonzales, J., Picard, J.Y., Dewailly, D., Fanchin, R., Catteau-Jonard, S. & di Clemente, N. (2013). Loss of LH-induced down-regulation of anti-müllerian hormone receptor expression may contribute to anovulation in women with polycystic ovary syndrome. *Hum. Reprod.* **28**, 762–9.

Rajpert-De Meyts, E., Jorgensen, N., Graem, N., Muller, J., Cate, R.L. & Skakkebaek, N.E. (1999). Expression of anti-Müllerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and granulosa cells. *J. Clin. Endocr. Metab.* **84**, 3836–44.

Richards, J.S., Russell, D.L., Ochsner, S., Hsieh, M., Doyle, K.H., Falender, A.E., Lo, Y.K. & Sharma, S.C. (2002). Novel signaling pathways that control ovarian follicular development, ovulation, and luteinization. *Recent. Prog. Horm. Res.* **57**, 195–220.

Roche, J.F. (1996). Control and regulation of folliculogenesis – a symposium in perspective. *Rev. Reprod.* **1**, 19–27.

Ruman, J.I., Pollak, S., Trousdale, R.K., Klein, J. & Lustbader, J.W. (2005). Effects of long-acting recombinant human follicle-stimulating hormone analogs containing *N*-linked glycosylation on murine folliculogenesis. *Fertil. Steril.* **83**, 1303–9.

Sanchez, F., Romero, S., Albuz, F.K. & Smitz, J. (2012). *In vitro* follicle growth under non-attachment conditions and decreased FSH levels reduces *Lhcgr*expression in cumulus cells and promotes oocyte developmental competence. *Assist. Reprod. Genet.* **29**, 141–52.

Segers, I., Adriaenssens, T., Wathlet, S. & Smitz, J. (2012). Gene expression differences induced by equimolar low

doses of LH or hCG in combination with FSH in cultured mouse antral follicles. *J. Endocrinol.* **215**. 269–80.

Shikanov, A., Xu, M., Woodruff, T.K. & Shea, L.D. (2009). interpenetrating fibrin-alginate matrices for *in vitro* ovarian follicle development. *Biomaterials* **30**, 5476–85.

Skory, R.M., Xu, Y., Shea, L.D. & Woodruff, T.K. (2015). Engineering the ovarian cycle using *in vitro* follicle culture. *Hum. Reprod.* **30**, 1386–95.

Sun, F., Betzendahl, I., Shen, Y., Cortvrindt, R., Smitz, J. & Eichenlaub-Ritter, U. (2004). Preantral follicle culture as a novel *in vitro* assay in reproductive toxicology testing in mammalian oocytes. *Mutagenesis* **19**, 13–25.

Trousdale, R.K., Yu, B., Pollak, S.V., Husami, N., Vidali, A. & Lustbader, J.V. (2009). Efficacy of native and hyperglycosylated follicle stimulating hormone analogues for promoting fertility in female mice. *Fertil. Steril.* **91**, 265–70.

Visser, J.A. & Themmen, A.P. (2005). Anti-Müllerian hormone and folliculogenesis. *Mol. Cell. Endocrinol.* **234**, 81–6.

Wang, S., Yang, S., Lai, Z., Ding, T., Shen, W., Shi, L., Jiang, J., Ma, L., Tian, Y., Du, X., Luo, A. & Wang, S. (2013). Effects of culture and transplantation on follicle activation and early follicular growth in neonatal mouse ovaries. *Cell Tissue Res*. **354**, 609–21.

Weenen, C., Laven, J.S., Von Bergh, A.R., Cranfield, M., Groome, N.P., Visser, J.A., Kramer, P., Fauser, B.C. & Themmen, A.P. (2004). Anti-Müllerian hormone expression pattern in the human ovary: potential implications for initial and cyclic follicle recruitment. *Mol. Hum. Reprod.* **10**, 77–83.

West, E.R., Xu, M., Woodruff, T.K. & Shea, L.D. (2007). Physical properties of alginate hydrogels and their effects on *in vitro* follicle development. *Biomaterials* **28**, 4439–48.

Wolfenson, C., Groisman, J., Couto, A.S., Hedenfalk, M., Cortvrindt, R.G., Smitz, J.E. & Jespersen, S. (2005). Batch-to-batch consistency of human-derived gonadotrophin preparations compared with recombinant preparations. *Reprod. Biomed. Online* **10**, 442–54.

Xu, J., Lawson, M.S., Yeoman, R.R., Molskness, T.A., Ting, A.Y., Stouffer, R.L. & Zelinski, M.B. (2013). Fibrin promotes development and function of macaque primary follicles during encapsulated three-dimensional culture. *Hum. Reprod.* **28**, 2187–200.

Xu, M., Banc, A., Woodruff, T.K. & Shea, L.D. (2009). Secondary follicle growth and oocyte maturation by culture in alginate hydrogel following cryopreservation of the ovary or individual follicles. *Biotechnol. Bioeng.* **103**, 378–86.

Zhang, Y.L., Liu, X.M., Ji, S.Y., Sha, Q.Q., Zhang, J. & Fan, H.Y. (2015). ERK1/2 activities are dispensable for oocyte growth but are required for meiotic maturation and pronuclear formation in mouse. *J. Genet. Genom.* **42**, 477–85.