Effect of Recombinant Follicle Stimulating Hormone and Recombinant Luteinizing Hormone on Maturation of Mammalian Oocytes *in Vitro*

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ABSTRACT----- ***In vitro maturation (IVM) of oocytes is an important application in assisted reproductive medicine and reproductive biology research. It can provide high quality oocytes from unstimulated ovaries. However, IVM remains a challenge in mammalian species, and has only been achieved with limited degrees of success. This study was carried out to investigate the effect of adding recombinant Follicle Stimulating Hormone (rFSH), recombinant Luteinizing Hormone (rLH) or the combination of both hormones on the IVM of rabbit oocytes, as rabbit is considered an important model system for human reproduction. Unstimulated ovaries of female rabbits were encountered. Oocytes were collected via tissue slice technique. The Cumulus-Oocyte Complexes (COCs), containing immature oocytes at Germinal Vesicle (GV) stage, were counted, washed, and cultured in different concentrations of rFSH, rLH and a combination of both rFSH and rLH. After cultivation, oocytes were examined for maturation. Our study revealed that the addition of hormone combination (rFSH + rLH) with certain concentrations could significantly improve the results of IVM and increase the percentage of oocytes reaching the second Metaphase (MII) stage.***

Keywords--- *In vitro* maturation, Hormones, Rabbit oocytes

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1. INTRODUCTION

Oocyte maturation is a lengthy process, during which the oocyte attains the competence to be fertilized and undergo embryogenesis. Most oocytes in the ovary are not growing and are small and immature. At regular intervals, a number of oocytes start to grow and mature, but of this cohort only few ones will be ovulated, while the rest will die by atresia (Hardy *et al.*, 2000). In the follicular phase of the human menstrual cycle, a single follicle grows up to the pre-ovulatory stage, and then releases its oocyte for possible fertilization, while the other follicles undergo atresia. In the human ovary, each fully grown oocyte resumes maturation in response to gonadotropins (Chen *et al.*, 2010). In response to the rise of FSH, the antral follicles begin to secrete estrogen and inhibin, which have a negative feedback effect on FSH (de Ziegler *et al.*, 2007). Follicles that have fewer FSH receptors will not be able to develop further; they will show retardation of their growth rate and become atretic.

In vitro maturation of oocytes is one of the promising techniques of the *in vitro* fertilization (IVF) process. Since the studies of Pincus and Enzmann (1935), who succeeded in inducing maturation of rabbit follicular oocytes in culture, several workers have studied different aspects of IVM in mammalian oocytes (Pawshe *et al.*, 1996; Ghasemzade-Nava and Tajik 2000; Wani *et al.*, 2000; Roa *et al.*, 2002; Kharche *et al.*, 2005).

During an IVF cycle, women are subjected to gonadotropin stimulation course via receiving hormonal treatment in order to induce several follicles to grow, so that several fertilizable oocytes are produced. These treatments, often preceded by down-regulation of the pituitary, are long and may cause side effects that are more or less well tolerated. Unfortunately, controlled ovarian stimulation involves various downsides, the most important of which is ovarian hyper-stimulation Syndrome (OHSS). Hyper-stimulation occurs in 2% of ovarian stimulations (Le Du *et al.*, 2005) and may be serious, even life-threatening. 2-5 % of OHSS patients require hospitalization (Gomez *et al.*, 2010). Women with polycystic ovaries (PCO) or polycystic ovarian syndrome (PCOS) are particularly prone to develop OHSS with an incidence of up to 6% (MacDougall *et al.*, 1993) as an effect of the growth of very numerous follicles even after the administration of relatively low doses of gonadotropins.

IVM requires no or small doses of gonadotropins and does not imply the growth of follicles to a large size. Therefore it can totally abolish the risk of OHSS. PCO and PCOS patients have become the first natural candidates for IVM treatment as early as 1994 (Trounson *et al.*, 1994). Other patients, such as poor responders, could also benefit from IVM (Liu *et al.*, 2003).

In addition to OHSS risks, ovarian stimulation protocols are associated with high costs, daily injections of gonadotropins and close monitoring (Brinsden *et al.*, 1995). The cost of these treatments is very high, constituting a sizeable proportion of health care expenditure. An early puncture of immature follicles followed by IVM of oocytes represents a feasible alternative for the management of infertile couples.

Current technology does not allow the direct observation of the oocyte *in vivo*. Manipulation of oogenesis *in vivo* has limitations as well, as an effect of a myriad of systemic influences that can make the interpretation of individual experimental conditions difficult or equivocal. For these reasons, IVM has been conceived and developed as a more accessible investigation strategy to study the conclusive events of oogenesis (Coticchio *et al.*, 2012).

In the last few years, IVM has been also emphasized as an additional opportunity for female germ cell preservation in women suffering from cancer (Gidoni *et al.*, 2008). Technically, immature oocytes collected from antral follicles in the absence of gonadotropin administration may be cryopreserved before or after maturation *in vitro* (Cao *et al.*, 2009). Therefore, IVM represents a suitable opportunity for the recovery of oocytes destined to cryopreservation in cases in which tumor estrogen-sensitivity and/or urgency to start therapy conflict with the implementation of a full controlled ovarian stimulation treatment (Wallace, 2011).

IVM remains a challenge in mammalian species, and has only been achieved with limited degrees of success. For study purposes, IVM was first established and brought to a certain level of success in rodent species (Schroeder and Eppig, 1984). In mice, isolated secondary follicles containing 2 or 3 layers of granulosa cells can be cultured to pre-ovulatory stages (Nayudu and Osborn, 1992); they can be induced to ovulate (Boland *et al.*, 1993) and oocytes from them were fertilized *in vitro*. A few viable offspring have resulted from such procedures after embryo transfer (Spears *et al.*, 1994). A single live mouse has resulted from IVF and embryo transfer after the *in vitro* growth and maturation of a primordial follicle (Eppig and O'Brien, 1996). In domestic species, production of embryos from oocytes matured *in vitro* from antral follicles is in commercial use, but success rates are low. Growth of pre-antral follicles from cow, pig and sheep has been achieved to varying extents in culture, but no oocytes from these follicles have been fertilized (Telfer *et al.*, 1999).

However, very little is known about the suitable conditions, maturation and developmental capacity of immature human oocytes for clinical application. Although our understanding is currently incomplete, it is now clear that addition of gonadotropins [Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH)] to the culture medium is beneficial to cytoplasmic maturation *in vitro* (Cha and Chian, 1998).

Collective evidence suggests that FSH should be included in an IVM culture system. FSH is in fact generally recognized as a hormone that improves follicle cells health status (Hillier *et al.*, 1995). However, no proof is available on the fact that the concentration normally adopted in IVM media, approximately 10^{-1} IU/ml, is appropriate. Recent experiments in the cow suggest that the dose of FSH can profoundly influence COCs function *in vitro*. In particular, it has been observed that a relatively high concentration (10^{-1} IU/ml) of the hormone is unable to assure the maintenance of the cumulus cells-oocyte gap junction communication. Vice versa, a much lower concentration (10^{-4} IU/ml) preserves gap junction coupling. In such a way, at meiotic resumption, chromatin condensation and transcription silencing can occur in a coordinated fashion, a condition required for the generation of a developmentally competent oocyte (Luciano *et al.*, 2011).

Recombinant FSH, produced by inserting the DNA encoding the α and β subunits of FSH into a Chinese hamster ovary cell line containing a higher proportion of less acidic isoforms, have been introduced for the treatment of infertility (Liu *et al.*, 2015). There are two rFSH preparations currently available for clinical use: follitropin alpha, marked as Gonal-F® by Ares-Serono, Geneva, Switzerland, and follitropin beta, marked as Puregon® or Follistim® ny NV Organon, Oss, the Netherlands. Although both preparations have been developed using the same technique, the post-translation glycosylation process and purification procedures are not identical (Olijve *et al.*, 1996). The purification procedure used for follitropin alpha includes the use of immunochromatographic methods, whereas purification of follitropin beta doesn't involve immunological methods (Olijve *et al.*, 1996).

Recombinant FSH displays a large proportion of relatively basic isohormones, known to exhibit a higher *in vitro* bioactivity compared to acidic isoforms (Matikainen *et al.*, 1994). In contrast, uFSH is known to exhibit a more acidic isohormone profile with a lower *in vitro* potency and a longer half life (Lambert *et al.*, 1995; de Leeuw *et al.*, 1996; Lambert *et al.*, 1998). This seems related to the fact that women in their postmenopausal years predominantly produce acidic isoforms of FSH (Anobile *et al.*, 1998). Compared to urinary FSH, highly purified uFSH (HP-uFSH) appears to be even more acidic in nature (Lambert *et al.*, 1995).

Urinary-extracted FSH contains a higher proportion of acidic isoforms, but rFSH contains a higher proportion of less acidic isoforms (Lispi *et al.*, 2006). Less acidic isoforms have a faster clearance and thus a shorter half-life than the acidic FSH isoforms (Vitt *et al.*, 1998; D'Antonio *et al.*, 1999). The slow clearance of the acidic isoforms has a longer half life and stronger stimulation (West *et al.*, 2002). At start of stimulation with acidic FSH (uFSH), there were fewer follicles developing and at a slower growth rate. The follicles stimulated with acidic FSH required 5 days to reach the dimensions recorded at days 3 with least acidic FSH (Vitt *et al.*, 1998). In comparison to uFSH or HP-uFSH, rFSH has an absolute purity (no LH content, no contamination by protein molecules), a higher batch-to-batch consistency, and no risk of transmission of infectious diseases (Howles, 1996).

Numerous studies have compared rFSH and urinary gonadotropins in terms of clinical efficacy and efficiency, but this remains a controversial area as no unequivocal results have been reached (Bergh *et al.*, 1997; Abate *et al.*, 2009; Aboulghar *et al.*, 2010; Al-Inany and Abou-Setta, 2012). A previous meta-analysis (Daya, 2002) showed that rFSH is more effective than uFSH because of the higher rates of clinical pregnancy per cycle started and is more efficient because the total dose of gonadotropin required was lower, but another (van Wely *et al.*, 2003) showed that no significant advantage of either rFSH or HP-uFSH in terms of assisted reproductive technology (ART) outcome.

Many reports have demonstrated the efficacy of rFSH on ovarian stimulation (Recombinant Human FSH Study Group, 1995; Aboulghar *et al.*, 1996; Out *et al.*, 1996). Recombinant FSH and uFSH have been repeatedly compared in trials dealing with super-ovulation induction for IVF. In some of these studies, rFSH was reported to yield a better ovarian response with a higher number of retrieved oocytes (Out *et al.*, 1995; Strehler *et al.*, 2001), a significantly lower total FSH dose (Out *et al.*, 1995; Bergh *et al.*, 1997; Hugues *et al.*, 2001), higher number of embryos obtained (Out *et al.*, 1995), and higher pregnancy rates (Out *et al.*, 1997).

In a study comparing another rFSH and HP-uFSH using a starting dose of 150 IU for the first 6 days, it was also concluded that rFSH is more effective than HP-uFSH (Bergh *et al.*, 1997). It was also demonstrated that treatment outcome of a fixed daily dose of 150 IU of rFSH is comparable to a fixed daily dose of 225 IU of HP-uFSH. The number of ampoules used was equal for both groups, resulting in a significantly lower dose needed (nearly 700 IU less) with rFSH (Hoomans *et al.*, 1999).

Treatment with rFSH resulted in a significantly higher embryo development rate (69.6% versus 56.2%; $P = 0.003$) and more embryos accessible for the embryo freezing program (3.3 versus 2.0; $P = 0.02$) compared to HP-uFSH (Hoomans *et al.*, 1999). The observed difference in embryo development rate and the number of embryos available for the freezing program can be explained by the higher proportion of mature to immature oocytes recovered following rFSH treatment compared to HP-uFSH and suggests a favorable impact of rFSH on oocyte and embryo development.

Consistent with the previous reports (Yarali *et al.*, 1999; Silverberg *et al.*, 2001; Fulghesu *et al.*, 2001; Gerli *et al.*, 2004a, b), the total FSH dose necessary to achieve ovulation was significantly lower using rFSH (Revelli *et al.*, 2006). The reason for this can likely be found in the higher biological potency of rFSH, in turn linked to the more basic spectrum of isoforms that gives more receptor binding affinity to the molecule, as well as to the lower proportion of degraded FSH forms in rFSH (Olijve *et al.*, 1996). An alternative possibility is that rFSH could be more active in inducing the synthesis of intra-ovarian factors (e.g. Inhibin A) able to amplify the effects of FSH at the ovarian level (Balasch *et al.*, 1998).

In a further study (Balen *et al.*, 2007) HP-uFSH was compared with rFSH to evaluate induction ovulation results using a low dose step-up protocol in 151 PCOS patients who were resistant to clomiphene citrate. The ovulation rate was 85.2% with HP-uFSH and 90.9% with rFSH. No differences were noted between groups in number of follicles ≥12mm, ≥15mm or ≥18mm, mono-follicular development, pregnancy rates, endometrial thickness, number of ovarian stimulation syndrome cases. While a relatively recent comparison (Sohrabvand *et al.*, 2012) showed that HP-uFSH and rFSH have similar clinical efficacy regarding the mean number of oocytes, grade A embryos transferred and clinical pregnancy rate in PCOS patients.

Also in a more recent study (Levi Setti *et al.*, 2015), rFSH resulted to be more effective in comparison with human menopausal gonadotropin (hMG). Differences between rFSH and uFSH that might explain the increased effectiveness include the isohormone profile (Matikainen *et al.*, 1994), the pharmaceutical formulation, contaminating proteins with possible FSH-inhibiting activity in uFSH, and small differences in the oligosaccharide structure (Hård *et al.*, 1990). As in previous studies of rFSH, serum immunoactive levels were lower than those of uFSH (Out *et al.*, 1995; Mannaerts *et al.*, 1993).

LH is important in regulating steroidogenesis throughout follicular development and adequate LH is particularly important for oocyte maturation (Hillier, 2009). The pre-ovulatory surge of LH performs physiological stimulation for final oocyte maturation and causes ovulation (Farrag *et al.*, 2008). After LH surges the process of meiosis progress, as the oocyte completes meiosis I and starts meiosis II, the oocyte and cumulus cells separate from the follicle wall and eventually lead to release of the COC (Gougeon, 1996). Throughout assisted reproduction cycles, premature LH surge affects cycle outcomes, leading to premature ovulation and as a result, interfere with oocyte collection during cycles. Premature luteinization also negatively affects egg quality and synchronization between embryos and endometrium (Eftekhar *et al.*, 2012).

Placental human chorionic gonadotropin (HCG) is produced by the trophoblasts as early as 6 days post-conception, and stimulates the corpus luteum and early feto-placental endocrine function (Pierce and Parsons, 1981). It is a glycoprotein from the same family as the pituitary gonadotropins (FSH and LH), and is structurally similar to LH; both hormones have similar effect and bind to the same receptors (Pierce and Parsons, 1981; Lei *et al.*, 2001). Thus, it has been used for many decades as a therapeutic analogue for LH for final oocyte maturation and luteal phase support (Gemzell, 1965; The European Recombinant Human Chorionic Gonadotropin Study Group, 2000; Humaidan *et al.*, 2005; Cole, 2009). The clinical uses of HCG are based on its molecular similarity to LH where the first 114 amino acids of each compound share 80% homology.

Recombinant HCG (rHCG) with high specific activity has become available. It is produced in a Chinese hamster ovary cell line expressing the genes for the alpha and beta subunits of HCG, and the protein is then purified by repeated chromatographic steps to produce a high specific activity outcome (Loumaye *et al.*, 1996; Recombinant Human FSH Product Development Group, 1998; Abdelmassih *et al.*, 2005; Kovacs *et al.*, 2008) and the pharmaceutical product was named Ovidrel®. The pharmacokinetics and pharmacodynamics of rHCG are comparable to that of urinary-extracted HCG (uHCG) with linearity over a dose range of 500-20,000 IU and a terminal elimination half life of approximately 30 hours (Lathi and Milki, 2001; Trinchard-Lugan *et al.*, 2002). A clinical trial comparing two doses of rHCG has shown that 250 μg is at least as effective as 5000 IU of uHCG for final follicular maturation without the higher incidence of adverse effects originally reported for the 500-μg dose (Chang *et al.*, 2001).

In clinical practice, several trials have been performed to compare safety and efficacy of uHCG and rHCG preparations with different points of views. Some randomized trials have found equal efficiency with these two preparations (Driscoll *et al.*, 2000; Sakhel *et al.*, 2007; Kovacs *et al.*, 2008; Madani *et al.*, 2013) whereas some others have observed better outcomes in women who received rHCG (The European Recombinant Human Chorionic Gonadotropin Study Group, 2000; Papanikolaou *et al.*, 2010).

The European rHCG study group in Geneva compared rHCG and uHCG, and demonstrated that despite administration of similar doses, rHCG was associated with higher numbers of mature oocytes per cycles. He suggested that HCG degenerated products in the urinary preparation have slight interference with the active HCG molecules and consequently with the HCG induced oocyte maturation (The European Recombinant Human Chorionic Gonadotropin Study Group, 2000; Chan *et al.*, 2005). Consistent with Geneva, Farrag *et al.* analyzed the effect of rHCG on oocyte nuclear and cytoplasmic maturity compared to uHCG when it is used for inducing ovulation. They showed that rHCG resulted in statistically higher rates of mature oocytes regarding nuclear and cytoplasmic maturity (Farrag *et al.*, 2008).

Therefore, the present study was designed to evaluate the effect of addition of rFSH, rHCG or a combination of them on the IVM of rabbit oocytes.

2. MATERIALS AND METHODS

This study was performed on twenty female rabbits (*Oryctolagus cunuculus*), weighing around 1.6-1.8 Kg.

Chemicals and plastics

Culture media: The basic media used for the manipulation of oocytes were ISM1 culture medium (1050) containing human albumin solution (HAS) , Penicillin 40.000 IU/l and streptomycin 40 mg/l (Medicult media - Denmark) and the Earle's Balance Salt's solution (Sigma E2888) as a washing medium.
Hormones: Recombinant follicular stimulating hormone (rFSH), GONAL-f® 75 IU/ml was provided from Merck Serono, (Ovidrel®) 5000 IU/ml was provided from Merck Serono. rHCG was used due to the fact that it has a biological activity similar to that of rLH.
Polystyrene: Non-pyrogenic plastic culture dishes (60 x 15 mm) were purchased from (Falcon, USA) REF (353004) and Glass Pasteur pipettes, BD syringes, scalpel and dissecting scissors.

Oocyte collection and maturation

Rabbit ovaries were obtained from local slaughter house and transported to the laboratory within 2 hours in Earle's Balanced Salt solution (Sigma-E2888). Immature oocytes at GV stage for culture system of IVM were collected from the rabbit ovaries by tissue slice technique which is currently the most successful method of maintaining follicular integrity. The main advantage is that oocyte granulosa-theca-stroma interactions appear to be maintained.

Then the immature oocytes were divided into 8 groups:

Group 1 (Control group: culture media without hormones):

Unstimulated ovaries of two female rabbits were encountered in this group. The visible follicles (1mm in diameter) on the ovarian surface were counted. COCs were collected using slicing technique and put in tissue culture dishes containing 4 ml Earl's media. We had 90 COCs, containing immature oocytes at GV stage. COCs were then transferred using a Pasteur pipette into culture dishes containing 2 ml of a washing medium. After washing, COCs were transferred into a culture dish, containing 9 drops of ISM1 medium, each drop (20 µl) containing 10 COCs. This culture dish was incubated in an incubator (Forma®) at 37° C, 5.5% CO_2 and pH 7.27. After 24-30 hours, the dish was examined under a stereomicroscope (Nikon®) for maturation of oocytes. The number of mature oocytes was recorded as follows: number of MII oocytes, number of MI oocytes, number of GV oocytes and number of degenerated oocytes. Examination of the oocytes was repeated after 48 hours and results were recorded in the same way.

Group 2 (Culture media with 0.75 IU/ml rFSH):

In this group all the steps of IVM were the same as group 1, but the 90 COCs were transferred into a culture dish containing 9 drops of ISM1 medium, each drop (20 µl), with 0.75 IU/ml rFSH, contains 10 COCs.

Groups 3, 4, 5, 6 and 7 (Culture media with 0.05, 0.1, 0.5, 0.75 and 1.0 IU/ml rLH) respectively:

In the 3rd, 4th, 5th, 6th and 7th groups all steps of IVM were the same as group1, but the 90 COCs were transferred into a culture dish containing 9 drops of ISM1 medium, each drop (20 µl), with 0.05, 0.1, 0.5, 0.75 and 1.0 IU/ml rLH respectively, contains 10 COCs.

Group 8 (Culture media with 1.0 IU/ml rLH + 0.75 I.U/ml FSH):

In this group all steps of IVM were the same as group 1, but the 90 COCs were transferred into a culture dish containing 9 drops of ISM1 medium, each drop (20 µl), with 1.0 IU/ml rLH + 0.75 IU/ml rFSH, contains 10 COCs.

N.B.: It is worth mentioning that the concentration of FSH used was selected according to Le Du *et al.* (2005), while in case of the LH concentration, different concentrations were used and it was found that the rate of maturation of oocytes increases with the increase of LH concentration up till the concentration of 1 IU/ml and afterwards any increase in the concentration will not increase the rate of maturation of oocytes.

3. RESULTS

The number of oocytes reaching MII stage raised by addition of either rFSH (group 2) or rLH (group 3) to the culture media. By increasing the concentration of rLH in the culture media, the number of oocytes reaching MII stage increased gradually (groups 4, 5, 6 and 7). The maximum oocyte maturation rate was reached by addition of a

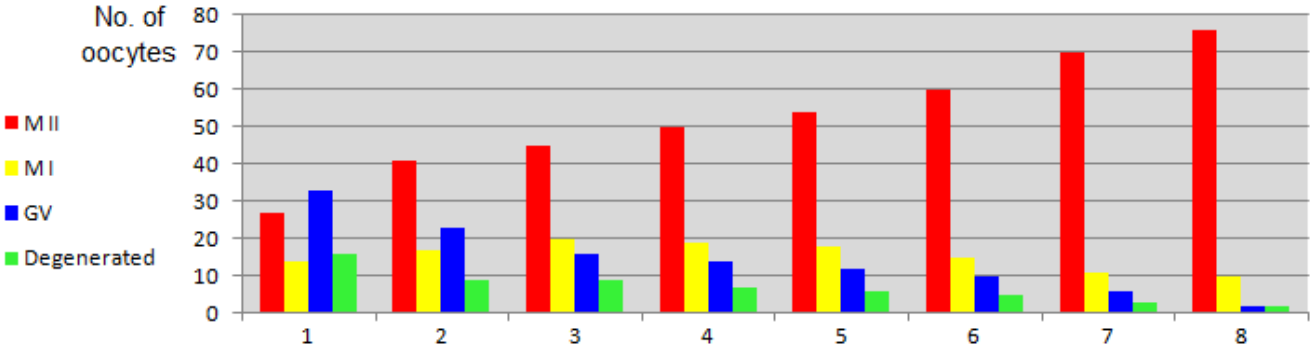
combination of 0.75 IU/ml rFSH and 1.0 IU/ml rLH to the culture media (group 8). The final results of all culture groups after 48 hrs are shown (Fig. 5).

Fig. 1: A photomicrograph of a rabbit oocyte in the GV stage, showing the nucleus inside the cytoplasm.

Fig. 2: A photomicrograph of a rabbit oocyte in the MI stage, showing the disappearance of the nucleus.

Fig. 3: A photomicrograph of a rabbit oocyte in the MII stage, showing the exposure of the first polar body.

Fig. 4: A photomicrograph of a degenerated rabbit oocyte.



Group number	1	2	3	4	5	6	7	8
M II	27	41	45	50	54	60	70	76
M I	14	17	20	19	18	15	11	10
GV	33	23	16	14	12	10	6	2
Degenerated	16	9	9	7	6	5	3	2
Total	90	90	90	90	90	90	90	90

Fig. 5: Showing the final results of all oocyte groups. It is clear that the maximum oocyte maturation rate during the first 48 hrs of culture *in vitro* was seen with the culture medium, supplemented with a combination of 0.75 IU/ml rFSH and 1.0 IU/ml rLH.

4. DISCUSSION

Since gonadotropins have an essential rule in acquisition of developmental capacity of mammalian oocytes, many workers have studied the effects of recombinant gonadotropins in aspects of clinical efficiency during ovarian stimulation and effects on nuclear and cytoplasmic maturation of oocytes (Recombinant Human FSH Study Group, 1995; Aboulghar *et al.*, 1996; Out *et al.*, 1996; Bergh *et al.*, 1997; Abate *et al.*, 2009; Aboulghar *et al.*, 2010; Al-Inany and Abou-Setta, 2012). Some of these studies showed that rFSH and rLH are more effective than uFSH and uLH respectively, while the others showed that there is no significant difference between recombinant and urinary preparations.

The technology used to obtain pharmacologically available human FSH has traditionally been extraction and purification from the urine of postmenopausal women. The purification process has been progressively improved, finally yielding a HP-uFSH with less than 0.001 IU of LH per FSH ampoule and a low amount of protein contamination (Revelli *et al.*, 2006). The increasing spread of ART that has taken place all over the world in the last years has rapidly increased the need of bulk amounts of FSH for therapeutic use, and a recombinant technology to get theoretically unlimited amounts of rFSH from cultured cells has been successfully developed (Howles, 1996). In comparison to uFSH or HP-uFSH, rFSH has an absolute purity (no LH content, no contamination by protein molecules), a higher batch-to-batch consistency, and no risk of transmission of infectious diseases (Howles, 1996).

The safety and tolerability of rFSH have been extensively evaluated since it became available. The most obvious clinical safety advantages arise from the high purity of rFSH; it has been proven to have better overall tolerability than any previous FSH preparation (Devroey *et al.*, 1994). In fact, filled by mass manufacturing process of follitropin alpha eliminates the intrinsic variability of the rat bioassay and ensures high batch-to-batch and vial-to-vial consistency of rFSH content. Furthermore, analytical assessment of commercially available rFSH pharmaceutical products has shown that follitropin alpha filled by mass is the most consistent rFSH in terms of protein content (Bassett *et al.*, 2005).

In contrast, since hMG preparations are directly extracted from human urine, the FSH activity in the preparations is highly variable between batches; the control of raw material of the individual contributors and the variation of purification processes are the major barriers in improving the quality of urinary preparations (Bassett *et al.*, 2009). Systematic literature reviews provide an excellent method to address eventual deficiencies of individual trials by considering several clinical studies. However, differences in results among studies could exist and could depend on clinical trials with different design and clinical practice, rather than differences in participants and clinical settings (Levi Setti *et al.*, 2015).

The present study was carried out to examine the effects of rFSH and rLH on rabbit oocyte maturation. It was designed along with a previous study, by El-Ghareeb *et al.* (2011), which studied the effects of FSH and LH on maturation of rabbit oocytes *in* vitro, using urinary extracted preparations. We have found that the oocyte maturation was significantly affected in a dose dependent manner by concentration of rFSH, rLH, or a combination of both.

Out *et al.* (1997) demonstrated that controlled ovarian hyperstimulation with rFSH (follitropin beta, Puregon®) leads to statistically clinically significantly higher ongoing pregnancies compared with uFSH and hMG. In another prospective, multicenter, assessor-blind, randomized, clinical trial, Coelingh Bennink *et al.* (1998) showed that rFSH (Puregon®) is more efficient than uFSH clomiphene criteria-resistant WHO group II patients, as the total dose needed to reach ovulation was lower.

The European Recombinant Human Chorionic Gonadotrophin Study Group (2000) indicated that rHCG is more effective than uHCG in inducing follicular maturation and early luteinization, and is associated with more mature oocytes, higher progesterone concentration on days 1 and 6-7, and improved tolerance.

In a much recent study, Levi Setti *et al.* (2015) stated that rFSH has resulted to be more effective in comparison with hMG. These findings were in agreement with a previous report comparing the use of recombinant and urinary FSH in IVF (Out *et al.*, 1995).

On the other hand, a meta-analysis by Al-Inany *et al.* (2003) showed that there is no evidence of clinical superiority for rFSH over different urinary gonadotropins. In another prospective, randomized study, Revelli *et al.* (2006) showed that both HP-uFSH and rFSH can be safely and effectively used to induce ovulation induction in both normo-ovulatory patients with unexplained infertility and in clomiphene criteria-resistant PCOS patients.

A more recent comparison, made by Sohrabvand *et al.* (2012), showed that HP-uFSH and rFSH have similar clinical efficacy redarding the mean number of oocytes, grade A embryos and clinical pregnancy rate in PCOS patients.

In 2013, Madani *et al.* demonstrated that rHCG shows equivalent efficacy to uHCG in terms of the number of oocytes per aspirated follicles in selected patients undergoing ICSI.

Different results in various studies are perhaps due to biological differences in patients, dosage of drugs consumed and study designs. Pharmacodynamic and pharmacokinetic studies have also confirmed that a broad diversity exists among individuals in response to urinary and recombinant FSH primarily because of individual ovarian sensitivity to FSH (le Cotonnec *et al.*, 1994).

Differences between recombinant and urinary FSH that might explain the increased effectiveness include the isohormone profile (Matikainen *et al.*, 1994), the pharmaceutical formulation, contaminating proteins with possible FSH-inhibiting activity in uFSH, and small differences in the oligosaccharide structure (Hård *et al.*, 1990). As in previous studies of rFSH, serum immunoactive levels were lower than those of urinary FSH (Mannaerts *et al.*, 1993; Out *et al.*, 1995). This is in agreement with the predominantly basic character of the isohormone profile of rFSH and the finding that basic isoforms are removed more quickly than acidic isoforms from the circulation (Ulloa-Aguirre *et al.*, 1992).

Consistently to previous reports (Coelingh Bennink *et al.*, 1998; Yarali *et al.*, 1999; Matorras *et al.*, 2000; Fulghesu *et al.*, 2001; Silverberg *et al.*, 2001; Gerli *et al.*, 2004a, b), the total FSH dose necessary to achieve ovulation was significantly lower using rFSH. The reason for this can likely be found in the higher biological potency of rFSH, in turn linked to the more basic spectrum of isoforms that gives more receptor binding affinity to the molecule, as well as to the lower proportion of degraded FSH forms in rFSH (Olijve *et al.*, 1996). An alternative possibility is that rFSH could be more active in inducing the synthesis of intra-ovarian factors (e.g. inhibin A) able to amplify the effects of FSH at the ovarian level (Balasch *et al.*, 1998).

In conclusion, our results indicate that the maximum oocyte maturation rate during the first 48 hours of culture *in vitro* was seen with addition of a combination of 0.75 IU/ml rFSH and 1.0 IU/ml rLH to the culture medium. These results suggest that rFSH and rLH have a significant effect on accelerating maturation of rabbit oocytes during the first 48 hours of culture *in vitro*.

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