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APOPTOSIS RATE IN CUMULUS CELLS AS POSSIBLE MOLECULAR BIOMARKER FOR OOCYTE COMPETENCE.

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ABSTRACT

Several lines of evidence showed that apoptosis rate of cumulus cells in oocytes derived by assisted reproductive technologies could be used as an indicator of fertilizing gamete quality. Aim of the study was to investigate the effects of three different ovarian stimulation protocols on the biological and clinical outcome in hyporesponder patients. Collected data showed a higher significant rate of DNA fragmentation index (DFI) in U group (patients treated with Highly Purified human Menopausal Gonadotrophin) than in P group (treated with recombinant human Follicle Stimulating Hormone (r-hFSH) combined with recombinant human Luteinizing Hormone (r-hLH)). Both groups R (treated with r-hFSH alone) and P showed a significant increase in collected and fertilized oocytes number, embryo quality number. This study showed that combined r-hFSH/r-hLH therapy could represent the best pharmacological strategy for controlled ovarian stimulation and suggests to use DFI as a biomarker of ovarian function in hyporesponder patients.

1. Introduction

Ovarian follicle development is a complex process; granulosa and cumulus cells could play a pivotal role during the preovulatory phase, synchronizing nuclear and cytoplasmic maturation through the complete suppression of oocyte transcription until nuclear maturity. Paracrine interactions between somatic and germ cells are critical for normal follicular development [1]. Somatic cells in ovaries are known to participate in regulating oocyte growth and development, meiosis, and global transcriptional activity [2]. On the other hand, oocytes promote granulosa cell proliferation and differentiation [3]. In the assisted reproductive technologies (ARTs) clinical routine, the oocyte selection is based on the morphological parameters of the cytoplasm, polar body and cumulus cells [4]. However, all the morphological criteria for grading and screening of oocytes are subjective and controversial, and are not related to the intrinsic competence of the oocyte [5]. Research is designed to define objective and non-invasive molecular markers predictive of oocyte competence. It has been demonstrated that the apoptosis rate of cumulus cells in women who achieved pregnancy was considerably lower than in women who did not become pregnant. In animal studies, both oocyte DNA fragmentation and their apoptosis might account for poor oocyte competence and lower fertility [6-7]. Cumulus cell apoptosis rate has been evaluated to verify its relationship with pregnancy and implantation rate in different studies. With this in mind, cumulus cell apoptosis rate was suggested as molecular marker in selecting oocytes with higher implantation potentiality [8-10].

In our previous study [11], we have demonstrated that r-hLH supplementation during ovarian stimulation, significantly reduced apoptosis in the cumulus cells, improving oocyte competence, which is necessary for adequate fertilization and a consequent embryo implantation.

HP-hMG, r-hFSH and a combination of r-hFSH/r-hLH, are commonly used for for controlled ovarian stimulation (COS) in infertile women undergoing IVF/ICSI treatments. Several publications have compared the

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effectiveness of these compounds [12]. Most studies have been performed in women undergoing pituitary down-regulation with a GnRH agonist long protocol, focusing on the outcome after r-hFSH and HP-hMG ovarian stimulation [13]. Two meta-analyses showed that HP-hMG ovarian stimulation accounts for an increased live birth rate compared to r-hFSH in the GnRH agonist long protocol [14].

In clinical practice, HP-hMG is the treatment of choice when LH activity is required to achieve successful ovarian stimulation; this is due to the LH activity present in hCG added to this formulation.

Several studies have suggested to add LH activity to FSH to stimulate ovarian function in IVF/ICSI-embryo transfer to improve clinical outcomes [15], but they have not been able to address the role that LH administration plays during the follicular phase of a stimulation cycle for IVF-ET, under pituitary suppression. When r-hFSH is administered to normo-gonadotrophic patients, low levels of endogenous LH can still be present, following pituitary down-regulation with GnRH analogues [16]. It is known that only 1% of LH receptors need to be occupied to drive adequate ovarian steroidogenesis for reproduction [17], but the potential benefit of additional exogenous LH supplementation in ARTs is still controversial [18]. Different meta-analyses did not demonstrate any clinical benefit from r-hLH supplementation [19]. However, in older patients undergoing ARTs, the addition of r-hLH seems to be beneficial [20].

The first aim of this study was to determine oocyte competence. We further compared the effects of stimulation therapy with r-hFSH, r-hFSH/r-hLH or HP-hMG (containing hCG as LH-like activity) in patients undergoing ICSI.

2. Material and methods

Study design

This prospective observational study was performed on 61 patients, being treated at the "Centro di Biologia della Riproduzione", Palermo, Italy. Patients were included in the observation after signing the Informed Consent which included both the agreement for the study participation and for the possible use of any discarded cumulus cells for apoptosis rate assessment. The patients involved in the study were classified hypo-responders given that they presented a normal basic level of FSH < 12 IU/mL and had undergone previous r-hFSH stimulation cycles with more than 3.000 IU to have a reasonable clinical outcomes. These patients were aged 30-40 years and normal body mass index (BMI = kg/m2<25) (table 1).

All patients received a GnRH agonist (Buserelin, Suprefact , Sanofi-Aventis, Italy, 0.2 mL/d), starting on day 21 of the previous cycle, long protocol; 21 patients were stimulated with r-hFSH alone (R group); 18 patients, were treated with r-hFSH combined with r-hLH (150 IU r-hFSH / 75 IU r-hLH, 2:1 ratio) (P group); and 22 patients were treated with HP-hMG (U group) (table 2).

Administration of 150 IU r-hFSH / 75 IU r-hLH (Pergoveris®; Merck Serono, Italy) started from day 3 of the cycle in P group. The stimulation therapy for R group consisted of a fixed initial dose of r-hFSH (Gonal-F; Merck Serono, Italy, 150 IU), while in U group the treatment consisted of HP-hMG (Meropur®, Ferring, Italy, 150 IU), starting from day 3 of the cycle. Follicular growth was monitored on a daily basis using ultrasound and serum estradiol (E2) levels, starting on day 6 of stimulation and modifying the dose of gonadotropins as needed. The ovulatory dose of hCG (Gonasi®; AMSA SRL, Italy, 10,000 IU) was administered when at least 3 follicles presented a diameter of 18 mm.

	TREATMENT GROUPS			p-value
	R Group (r-hFSH)	P Group (r-hFSH/r-hLH)	U Group (HP-hMG)	
N° subject (mean±SD)	21	18	22	
Age (mean±SD)	35.1± 3.6	37.7±1.8	38.6±3.4	ns
BMI (Kg/m2±SD)	23.4±3	21.6±3	21.3±2.8	ns
Basal E2 (pg/mL±SD)	49.0±10.1	54.3±11.3	48.0±13.2	ns
Basal FSH (IU/ml±SD)	6.9±2.3	5.3±1.6	8.6±2.2	ns

Table 1 - Characteristics of patients receiving study treatment. Values are expressed as mean ± SD; ns = not significant; p- value reported when significant.

	R Group	P Group	U Group	p-value
Cycles	22	18	21	ns
Gonadotropins administered (IU±SD)	r-hFSH (1891±982.1)	r-hFSH/r-hLH (1952.2 ± 438.8 / 922.3± 222)	HP-hMG (2782.8 ± 1354.9)	<0.05*

Table 2 - Ovarian stimulation protocols in the treatment groups. alues are expressed as IU ± SD; ns = not significant; *p- value measured P & R vs. U

As a routine practice at our Centre, we evaluated cumulus cells apoptosis rate as a molecular biomarker for oocyte competence in all patients agreeing to donate discarded cumulus cells for research purposes. The collection of cumulus cells material was approved by the Local Ethics Committee of "Centro di Biologia della Riproduzione", Palermo, Italy. As a medical research involving human subjects, this study was conducted according to the Declaration of Helsinki 2008 (Ref.: NCT01872247). The quality embryo was evaluated in according to ASEBIR embryo assessment criteria: A = top quality; B = good quality (not for elective single embryo transfer); C = impaired embryo quality; D = do not recommend to transfer (includes all multinucleated embryos) [21].

Apoptosis in cumulus cells was examined with the use of a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assays [11, 22], as described below, that allowed us to highlight DNA fragmentation (last step of apoptosis pathway). The pool of cumulus cells was collected after incubating the cumulus-oocyte complex in a solution of hyaluronidase (80 IU/mL, Medicult, Jyllinge, Denmark). The released

cells were collected in a test tube (Falcon, Franklin Lakes, NJ) containing 2 ml of basic culture medium (Quinn's advantage medium with HEPES; SAGE IVF, Trumbull, CT) and centrifuged twice at 300 g for 10 min. Oocytes were transferred to a culture medium (fertilization medium, SAGE IVF) and incubated at 37°C, 5% CO2, until intracytoplasmic sperm injection (ICSI) was performed. Cumulus cells were then fixed in 3.7% paraformaldehyde for 60 min. After centrifugation at 300 g for 5 min, the supernatant was removed and phosphate-buffered saline (PBS)-glycine was added (0.1 M glycine in PBS and 0.3 mg/mL bovine serum albumin [BSA]). Finally, the cells were mounted on polylysine-coated glass slides by cyto-centrifugation [23-25].

Fluorescent TUNEL assay, or TdT in situ

Cumulus cells were washed for 5 min in PBS and permeated for 10 min on ice in 0.1% Triton X100 and 0.1% sodium-citrate in PBS, and then washed three times in PBS at room temperature for 5 min each time. Cumulus cells were then incubated for 60 min at 37°C in a humidified chamber in 50 µL of a mixture containing 5 µl of nucleotide mix, 1 µl of TdT enzyme, and 45 µl of equilibration buffer (DeadEnd Fluorometric TUNEL System, Promega Italia SRL, Milano, Italy).

An additional slide was incubated with the same mixture without the TdT enzyme (negative control), while another slide was pretreated for 5 min with 100 µL of DNase buffer, and then treated for 10 min with a DNase buffer solution containing 10 unit/ml of DNase I (positive control). The reaction was blocked with SSC for 15 min, then followed by three 5 min-washes in PBS. Cumulus cells were stained with propidium iodide (1 µg/ml) for 10 min at room temperature and observed under a fluorescent microscope equipped with a x20 0.40 objective.

Apoptosis rate or DNA fragmentation index (DFI) indicates the percent value of apoptotic cells observed; total apoptotic nuclei labelled in green (TUNEL assay) / total nuclei labelled in red (Propidium iodide) x 100.

Statistical analysis

Data were analyzed with the statistical software package SPSS version 17. All the analyses presented were assessed using a two-sided significance level, α, of 0.05.

To verify the normal distribution of the population the Kolmogorov – Smirnov test was used and the Levene test was performed to analyze the homogeneity of variance.

To compare significant differences between the mean values two tests were used, Welch and Brown-Forsythe. Analysis of variance (ANOVA) models were used to compare treatment groups, except for the LH variables, where a T-test for independent variables was used.

The η squared was used to calculate the effect size. Non parametric analysis was performed with Kruskal-Wallis test. Data are presented as mean ± standard deviation (SD), unless otherwise specified.

3. Results

The study of apoptosis on discarded cumulus cells performed on individual groups with Fluorescent TUNEL assay allowed us to quantify the DNA fragmentation index (DFI), expression of apoptosis (Figure 1).

As can you see the fig.1 shows DNA fragmentation, expressed by green fluorescent chromatin for each treatment group (A1, B1, C1), the second column (A2, B2, C2) shows the total nuclei labelled in red (Propidium iodide) (A2, B2, C2) and the third column shows a merge of green and red (A3, B3, C3). (see in orange the cell with DNA fragmentation).

Collected data showed a relatively high DNA Fragmentation Index, measured as total apoptotic nuclei labelled in green (TUNEL assay) / total nuclei labelled in red (Propidium iodide) x 100, in U group. Comparing the results obtained with different ovarian stimulation protocols, the DFI significantly higher in U group was than in the other two, especially compared to the P group (Table 3, Figure 1 and Figure 2).

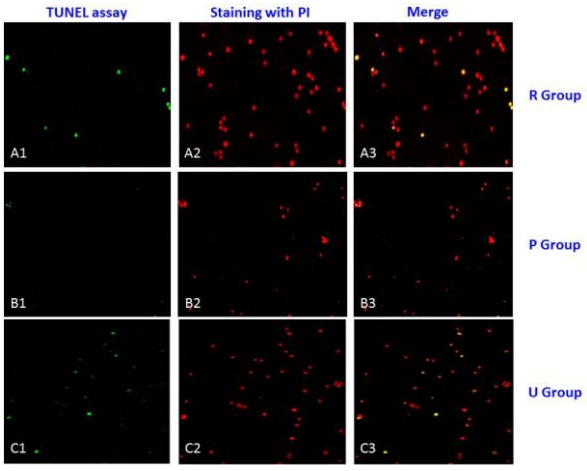

Figure 1 - TUNEL assay in situ. A = R Group; B = U Group; C = P Group. Green: DNA fragmentation (A1, B1, C1). Red: nucleic acids stained with Propidium Iodide (A2, B2, C2). Merge of green and red (A3, B3, C3). Fields observed under fluorescent microscope.

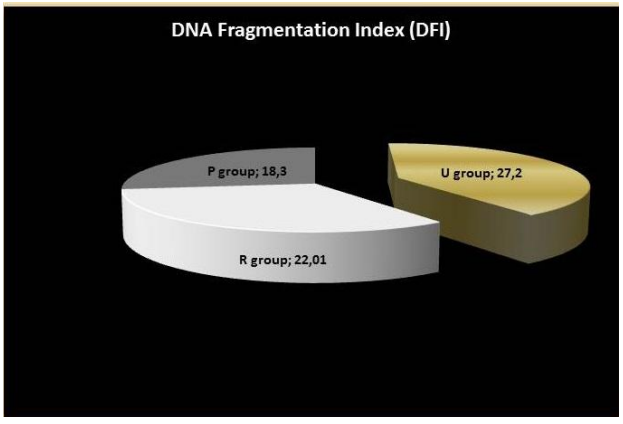

Figure 2 - Apoptosis in cumulus cells: DFI in three treatment group

The respective numbers of collected oocytes, fertilized oocytes and good quality embryo (a/b) were significantly higher in either P or R groups than in U group (see table 4). Collected data were related to only embryos of category A and B (top and good quality) in according to ASEBIR embryo assessment criteria [21].

Furthermore U group patients needed to be treated with higher significant amount of FSH (Table 2). At last, group U showed lower but not statistically significant implantation and pregnancy rates (Table 4).

	R Group	P Group	U Group	p-value
Cycles (n)	21	18	22	
Cells (n)	623 ± 57	582± 64	615 ± 87	
DFI	22.01±11.2	18.3±4.5	27.2±12.5	p<0.05*

Table 3 - Apoptosis in cumulus cells measured TUNEL assay. HP-hMG (U Group), r-hFSH (R Group) and r-hFSH/r-hLH (P Group). DFI = DNA fragmentation index. Values are expressed as mean ± SD; ns = not significant; p- value reported when significant. *(P vs. U and U vs. P)

	R Group	P Group	U Group	p-value
Cycles	22	18	21	
N° Collected oocytes	7.81 ± 4.8	6.1 ± 3	4.4 ± 2.3	R vs. U <0.05
N°GV-MI oocytes	1.6 ± 1.9	0.7 ± 1	0.3 ± 0.5	R vs. U <0.05
N° MII oocytes	6.27±3.4	5.33±2.3	4.26±2.6	ns
N° fertilized oocytes	3.7 ± 1.9	4.6 ± 1.4	3 ± 1	P vs. U <0.05
N° Embryo a/b	3 ± 1.7	3.5 ± 1.1	2.4 ± 1	P & R vs. U <0.05
E2 on day of hCG (pg/mL)	1700.3± 1001	1831 ± 580	1395 ± 614	ns
% Pregnancy	27.2	27.7	19	ns
% Implantation	14	10.3	7.9	ns

Table 4 - Clinical outcome of COS. HP-hMG (U Group), r-hFSH (R Group) and r-hFSH/r-hLH (P Group). GV-MI oocytes = germinal vescicle and metaphase I oocyte MII oocytes = metaphase II oocyte embryo a/b = good quality embryo E2 = 17 beta estradiol. Values are expressed as mean ± SD; ns = not significant; p- value reported when significant.

4. Discussion

In this study we evaluated the effects of different ovarian stimulation protocols on oocyte competence and secondly their clinical outcome, in patients hyporesponders to FSH stimulation. Results obtained showed high efficacy, in term of either DFI or clinical outcome, of recombinant gonadotropins treatments compared to HP-hMG treatment.

Historically in the treatment of infertility and in ARTs, urinary human menopausal gonadotropin (u-hMG), from post-menopausal women, has been used to stimulate folliculogenesis. Over the years, technological advances have led to the ability to produce recombinant forms of human FSH (r-hFSH) and LH (r-hLH).

These are now available as potential alternatives to u-hMG and may be more suitable in those sub populations of patients requiring the addition of LH in their stimulation protocols.

During the follicular phase of the ovarian cycle, growth and follicular recruitment are stimulated mainly by FSH while during the late phase of follicular growth is supported mainly by the LH. The induction of cell differentiation and the support of the final stages of follicle maturation depend on the action of LH. To get more mature follicles, and thus increase the yield of oocytes from patients for ARTs pharmacological strategies rely on the modulation of the administration of exogenous gonadotropins with COS protocols tailored. LH supplementation can be achieved through the administration of r-hLH or hCG (more than 80% of homology with LH). The similarity of action of LH and hCG may depend on the fact that both gonadotropins bind the same LH/CG receptor. For this reason, hCG has become an alternative source of exogenous LH activity during COS for ART cycles. Our results may suggest HP-hMG treatment is less effective than r-hFSH/r-hLH treatment. It is conceivable that, while interacting with the same receptor, r-LH and hCG may differently support follicle maturation and development. These data are consistent with our previous results [11], showing that supplementation with r-hLH related with the reduction of apoptosis rate in COC and production embryos with a higher implantation potential.

Moreover, both in vitro and clinical studies have recently challenged the hypothesis of LH and hCG bioequivalence [26]. Differences were observed when the recombinant forms of the two gonadotropins were used in vitro to stimulate primary and immortalized granulose cells. For example, hCG, was 5-times more potent than LH for cAMP accumulation, while maximal LH stimulation was 6-fold faster. Conversely, LH was a stronger activator of the ERK1/2 and AKT pathways. Inhibition of ERK1/2 enhanced aromatase gene expression mediated by LH. On the contrary this effect was not observed in patient treated with hCG, suggesting that, downstream LHCG-R, intracellular signaling activation may diverge and lead to different targets [27].

In our model, we could speculate that extractive HCG, contained in HP-hMG, was not able to reduce apoptosis in cumulus cells due to the high dosage exposure of LH/CGR to hCG that down-regulated the receptor. In addition, hCG is not acting on the granulose cell with the same pathways involved by LH, and is not effective in activating the AKT pathway which can protect follicle somatic cells from apoptosis.

Our results showed that use of r-hFSH significantly improved the number of collected oocytes and at the same time reduced the dose of exogenous gonadotropins administration. This result is in line with previous literature data [13, 28]. A recently published study demonstrated that the expression level of LH/hCG receptor gene was reduced in hMG-treated granulosa cells compared with recombinant FSH–treated granulosa cells [29].

This could explain why ovarian stimulation with recombinant FSH (with or without r-hLH) induces significantly more follicles and more oocytes retrieved.

We further demonstrated that increased pregnancy and implantation rates were obtained in patients who were treated with recombinant gonadotropins than in patients treated with HP-hMG. These results were not statistically significant probably for the limited number of study patients.

Our data seem to confirm the important role of native LH in reducing apoptosis in the cumulus cells, probably supporting the actions of a series of paracrine factors. Kinase Akt, members of bcl-2 family, KIT-ligand and c-KIT receptors, stem cell factor (SCF), members of TGF-beta family (activing, factors BMP-4 and BMP-7, GDF9), estrogens, insulin and IGFs, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), TGF-α, interleukin 1b (IL-1b), growth hormone (GH) and the member of apoptosis inhibitors, surviving could promote survival during the growth and differentiation of follicles [27].

LH could induce the expression and the synthesis of EGF-like growth factors such as amphiregulin, epiregulin, and betacellulin and activate the EGF receptor pathway in the granulosa cells of preovulatory follicles, thereby impacting on ovulation [26, 27, 29, 30].

Results of this study are in favour of the use of a combination treatment r-hFSH and r-hLH to stimulate ovarian functions. It is tempting to speculate that this strategy may contribute to create a better microenvironment in the follicle than that observed in patient treated with HP-hMG. Recombinant combination treatment was also able to produce better oocyte competence, embryo viability and less FSH administration. A larger, prospective, randomized study is needed to confirm the data of this observational prospective pilot study.

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Competing interests

Monica Lispi is employed at Merck Serono company; all others authors declare that they have no competing interests.

References

1. Eppig JJ, Wigglesworth K, Pendola FL: The mammalian oocyte orchestrates the rate of ovarian follicular development. Proc Natl Acad Sci U S A 2002; 99:2890-2894.
2. Thomas FH, Vanderhyden BC: Oocyte-granulosa cell interactions during mouse follicular development: regulation of kit ligand expression and its role in oocyte growth. Reprod Biol Endocrinol 2006; 4:19.
3. Matzuk MM, Burns KH, Viveiros MM, Eppig JJ: Intercellular communication in the mammalian ovary: oocytes carry the conversation. Science 2002; 296:2178-2180.
4. Wang Q, Sun QY. Evaluation of oocyte quality: morphological, cellular and molecular predictors. Reprod Fertil Dev 2007; 19:1–12.
5. Balaban B, Urman B, Sertac A, Alatas C, Aksoy S, Mercan: Oocyte morphology does not affect fertilization rate, embryo quality and implantation rate after intracytoplasmic sperm injection. Hum Reprod 1998;13:3431–3433.
6. Das K, Stout LE, Hensleigh HC, Tagatz GE, Phipps WR, Leung BS: Direct positive effect of epidermal growth factor on the cytoplasmic maturation of mouse and human oocytes. Fertil Steril 1991; 55:1000-1004.
7. Bosco L, Ruvolo G, Chiarelli R, Agnello M, Roccheri MC: Selection of the best oocytes for intracytoplasmic sperm injection (ICSI) using apoptotic analysis of cumulus cells. Journal of Biological Research 2015; 88: 31-32.
8. Ruvolo G, Fattouh RR, Bosco L, Brucculeri AM, Cittadini E: New molecular markers for the evaluation of gamete quality. J of Assist Reproduct and Genet 2013; 30:207-212.
9. Chiarelli R, Martino C, Agnello M, Bosco L, Roccheri MC: Autophagy as a defense strategy against stress: focus on *Paracentrotus lividus* sea urchin embryos exposed to cadmium. Cell Stress and Chaperones 2016; 21:19-27.
10. Agnello M, Chiarelli R, Martino C, Bosco L, Roccheri MC: Autophagy is required for sea urchin oogenesis and early development. Zygote 2016; 24:918-926.
11. Ruvolo G, Bosco L, Pane A, Morici G, Cittadini E, Roccheri MC: Lower apoptosis rate in human cumulus cells after administration of recombinant luteinizing hormone to women undergoing ovarian stimulation for in vitro fertilization procedures. Fertil Steril 2007; 87:542-546.
12. Carone D, Caropreso C, Vitti A, Chiappetta R: Efficacy of different gonadotropin combinations to support ovulation induction in WHO type I anovulation infertility: clinical evidences of human recombinant FSH/human recombinant LH in a 2:1 ratio and highly purified human menopausal gonadotropin stimulation protocols. J Endocrinol Invest 2012; 35:996-1002.
13. Andersen AN, Devroey P, Arce JC: Clinical outcome following stimulation with highly purified hMG or recombinant FSH in patients undergoing IVF: a randomized assessor-blind controlled trial. Hum Reprod 2006; 21:3217-3227.
14. Coomarasamy A, Afnan M, Cheema D, van der Veen F, Bossuyt PM, van Wely M: Urinary hMG versus recombinant FSH for controlled ovarian hyperstimulation following an agonist long down-regulation protocol in IVF or ICSI treatment: a systematic review and meta-analysis. Hum Reprod 2008; 23:310-315.
15. Griesinger G, Schultze-Mosgau A, Dafopoulos K, Schroeder A, Schroer A, von Otte S et al.: Recombinant luteinizing hormone supplementation to recombinant follicle-stimulating hormone induced ovarian hyperstimulation in the GnRH-antagonist multiple-dose protocol. Hum Reprod 2005; 20:1200-1206.

16. Doody K, Devroey P, Gordon K, Witjes H, Mannaerts B: LH concentrations do not correlate with pregnancy in rFSH/GnRH antagonist cycles. Reprod Biomed Online 201; 20:565-567.
17. Chappel SC, Howles C: Reevaluation of the roles of luteinizing hormone and follicle-stimulating hormone in the ovulatory process. Hum Reprod 1991; 6:1206-1212.
18. Buhler KF, Fischer R: Recombinant human LH supplementation versus supplementation with urinary hCG-based LH activity during controlled ovarian stimulation in the long GnRH-agonist protocol: a matched case-control study. Gynecol Endocrinol 2011; 28:345-350.
19. Baruffi RL, Mauri AL, Petersen CG, Felipe V, Martins AM, Cornicelli J et al.: Recombinant LH supplementation to recombinant FSH during induced ovarian stimulation in the GnRH-antagonist protocol: a meta-analysis. Reprod Biomed Online 2007; 14:14-25.
20. Hill MJ, Levens ED, Levy G, Ryan ME, Csokmay JM, DeCherney AH et al.: The use of recombinant luteinizing hormone in patients undergoing assisted reproductive techniques with advanced reproductive age: a systematic review and meta-analysis. Fertil Steril 2012; 97:1108-1114.
21. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. Hum Reprod 2011; 26:1270-1283.
22. Ruvolo G, Roccheri MC, Brucculeri AM, Longobardi S, Cittadini E, Bosco L: Lower sperm DNA fragmentation after r-FSH administration in functional hypogonadotropic hypogonadism. J Assist Reprod Genet 2013; 30:497-503.
23. Chiarelli R, Agnello M, Bosco L, Roccheri MC: Sea urchin embryos exposed to cadmium as an experimental model for studying the relationship between autophagy and apoptosis. Mar Environ Res 2014;93:47-55.
24. Librizzi M, Chiarelli R, Bosco L, Sansook S, Gascon, JM , Spencer J, Caradonna F, Luparello C: The histone deacetylase inhibitor JAHA down-regulates pERK and global DNA methylation in MDA-MB231 breast cancer cells. Materials 2015; 8: 7041-7047.
25. Casano C, Savona R, Ragusa MA, Bosco L, Gianguzza F: p38 MAPK activation is required for Paracentrotus lividus skeletogenesis. Caryologia 2008; 61:74-81.
26. Troppmann B, Kleinau G, Krause G, Gromoll J: Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor. Hum Reprod Update 2013; 9:583-602.
27. Quirk SM, Cowan RG, Harman RM, Hu CL, Porter DA: Ovarian follicular growth and atresia: the relationship between cell proliferation and survival. J Anim Sci 2004; 82:40-52.
28. Devroey P, Pellicer A, Nyboe Andersen A, Arce JC: A randomized assessor-blind trial comparing highly purified hMG and recombinant FSH in a GnRH antagonist cycle with compulsory single-blastocyst transfer. Fertil Steril 2012; 97:561-571.
29. Grøndahl ML, Borup R, Lee YB, Myrhøj V, Meinertz H, Sørensen S: Differences in gene expression of granulosa cells from women undergoing controlled ovarian hyperstimulation with either recombinant follicle-stimulating hormone or highly purified human menopausal gonadotropin. Fertil Steril 2009; 91:1820-1830.
30. Noma N, Kawashima I, Fan HY, Fujita Y, Kawai T, Tomoda Y et al.: LH-induced neuregulin 1 (NRG1) type III transcripts control granulosa cell differentiation and oocyte maturation. Mol Endocrinol 2011; 25:104-116.