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Effect of different superovulation stimulation protocols on adenosine triphosphate concentration in rabbit oocytes

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Summary

Ovarian stimulation protocols are used usually to increase the number of oocytes collected. The determination of how oocyte quality may be affected by these superovulation procedures, therefore, would be very useful. There is a high correlation between oocyte ATP concentration and developmental competence of the resulting embryo. The aim of this study was to evaluate the effect of follicle stimulating hormone (FSH) origin and administration protocols on oocyte ATP content. Rabbit does were distributed randomly into four groups: (i) a control group; (ii) the rhFSH3 group: females were injected, every 24 h over 3 days, with 0.6 μl of rhFSH diluted in polyvinylpyrrolidone (PVP); (iii) the pFSH3 group: females were injected every 24 h over 3 days with 11.4 μg of pFSH diluted in PVP; and (iv) the pFSH5 group: females were injected twice a day for 5 days with 11.4 μg of pFSH diluted in saline serum. Secondly, the effect of pFSH5 protocol on developmental potential was evaluated. Developmental competence of oocytes from the control and pFSH5 groups was examined. Differences in superovulation treatments were found for ATP levels. In the pFSH5 group, the ATP level was significantly lower than that of the other groups (5.63 ± 0.14 for pFSH group versus 6.42 ± 0.13 and 6.19 ± 0.15 for rhFSH3 and pFSH3, respectively; $P < 0.05$). In a second phase, only 24.28% of pFSH5 ova developed into hatched blastocysts compared with 80.39% for the control group. A negative effect on oocyte quality was observed in the pFSH5 group in ATP production, it is possible that, after this superovulation treatment, oocyte metabolism would be affected.

Keywords: ATP content, Developmental competence, Oocyte, Rabbit, Superovulation

Introduction

Rabbits are used frequently for studies in reproductive biotechnology and genetic modelling. Thus, oocyte recovery is essential for a wide variety of assisted reproductive technologies and associated ovarian stimulation protocols are usually used to increase the number of oocytes collected. In rabbits, superovulation protocols have been performed widely with equine chorionic gonadotropin (eCG) or follicle stimulating hormone (FSH) (Kauffman *et al.*, 1998; Mehaisen *et al.*, 2005; Salvetti *et al.*, 2007; Cortell *et al.*, 2008; Viudes-de-Castro *et al.*, 2009). Nevertheless, the recovery efficiency and oocyte quality of these superovulation treatments are both highly variable. One of the causes of the variability in the response after gonadotrophin stimulation may be related to the administration protocol: whereas a single eCG injection is enough to induce superovulation, FSH, due to it shorter lifespan, must be administered at least twice daily for 3 days (Rose *et al.* 2000). However, in order to limit animal manipulation, superovulation treatments

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with FSH diluted in polyvinylpyrrolidone (PVP) solutions have been tested and have resulted in a good superovulation response in distinct species, including rabbits (Kanayama *et al.*, 1994; Hashimoto *et al.* 2004; Mehaisen *et al.*, 2006; Cortell *et al.*, 2008; Viudes-de-Castro *et al.*, 2009).

In addition, when using FSH isolated from the pituitary gland, the ratio between the luteinising hormone (LH) and FSH is unknown, which may results in a variable response to superovulation treatments. In rabbits, the effect of LH on superovulation has been studied using both porcine-purified FSH (Hashimoto *et al.*, 2004; Salvetti *et al.*, 2007) and human recombinant FSH (Cortell *et al.*, 2008; Viudes-de-Castro *et al.*, 2009). In all cases, a superovulatory response has been achieved, however it would be very useful to determine how oocyte quality may be affected by these superovulation procedures.

Oocyte quality determination is critical for most human and animal assisted reproductive technologies. Detrimental effects of superovulation on oocyte quality are often observed (Moor *et al.*, 1985; Blondin *et al.*, 1996; Ertzeid and Storeng, 2001; Van der Auwera and D'Hooghe, 2001). Developmental competence is acquired during folliculogenesis as the oocyte grows and during the period of oocyte maturation (Rodrigues *et al.*, 2008). It is probable that alterations in oocyte quality could be related to a failure in its maturation process. It is well known that organelle and chromosome redistribution are necessary steps in oocyte maturation and meiosis resumption (Ferreira *et al.*, 2009), indeed metabolic, structural and numerical defects have been associated with pre-ovulatory maturational failure for the oocyte and premature arrest or abnormal development for the embryo (Van Blerkom *et al.*, 1995). While it seems that oxidative metabolism is the primary source of energy production during oocytes maturation (Krisher, 2004), mitochondria, by means of their ability to generate ATP, have a central role in the normality of early mammalian development. In order to evaluate embryo and oocytes competence, the ATP concentration and mitochondrial membrane potential in oocytes has been widely studied in different species, showing a high correlation between oocyte ATP concentration and developmental competence of the resulting embryo (Van Blerkom *et al.*, 1995; Stojkovic *et al.*, 2001; Cummins, 2002; Tamassia *et al.*, 2004; Van Blerkom, 2004).

The aim of this study was to assess oocyte ATP content to evaluate the effect of FSH origin and administration protocols on oocyte quality.

Materials and methods

The research was carried out at the experimental farm of the Centro de Investigación y Tecnología Animal (Segorbe, Castellón, Spain). The experiments were approved by the Ethics Committee of the Instituto Valenciano de Investigaciones Agrarias.

All the chemicals unless otherwise stated were reagent grade and purchased from Sigma-Aldrich Química S.A. (Alcobendas, Madrid, Spain). Other experiments were carried out in the Animal Technology Center (CITA-IVIA, Spain).

Animals

Sexually mature White New Zealand rabbit does and males were housed in flat deck cages with light alternating on a cycle of 16 h light and 8 h dark; they were fed with the same commercial diet (17.5% crude protein, 2.3% ether extract, 16.8% crude fibre, 2600 kcal DE/kg) and had free access to water.

Superovulation treatment and ovulation induction

In the first phase of this study, 73 rabbit does were randomly distributed into four groups:

- control group: non-superovulated, females were injected every 24 h for 3 days, with 0.4 ml of 30% polyvinylpyrrolidone (PVP; molecular weight, 40000) in distilled water;
- rhFSH3 group: females were injected, every 24 h for 3 days, with 0.6 μg of recombinant human FSH (Gonal-F® 75, Serono, Italy) diluted in 0.4 ml of 30% PVP;
- pFSH3 group: females were injected every 24 h for 3 days with 11.4 μg of porcine FSH (Stimufol®, Reprobiol, Belgium) diluted in 0.4 ml of 30% PVP;
- pFSH5 group: after dissolving the hormones in physiological saline to a final concentration of 18 μg/ml of porcine FSH (Stimufol®, Reprobiol, Belgium), females were injected twice (12 h intervals) 0.25 ml on day 1, twice 0.5 ml on day 2 and only one injection of 0.25 ml on the morning of day 3.

All females were induced to ovulate, 24 h after the last injection, with 2 μg of Buserelin acetate (Suprefact, Hoechst).

Oocyte recovery

Does were euthanized 14 h after ovulation induction and the reproductive tract was immediately removed. Oocytes were recovered by perfusion of each oviduct with Dulbecco's phosphate-buffered saline (DPBS). Collected liquid was poured into Petri dishes and scored under a stereomicroscope. Cumulus–oocytes complexes (COCs) were localized and only those with homogeneous cytoplasm and compacted cumulus were selected to perform the ATP analysis.

The number of follicles with scare ovulation was recorded to estimated the ovulation rate and number

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of recovered and normal oocytes were recorded by female.

ATP measurement

Selected COCs were suspended in 0.5 % of hyaluronidase in DPBS in order to remove cumulus cells. After mechanical removal, the oocytes from each females were distributed randomly into three groups: (i) in the first group (control group), ATP was measured immediately after recovering fresh oocytes at room temperature (25ºC); (ii) in the second group, ATP was measured after culture of oocytes for 2 h at 25ºC (25ºC – 2 h group); and (iii) finally, in the third group, ATP measurements were done after culture of oocytes for 2 h at 38.5ºC and in 5% CO_2 in air (38.5ºC – 2 h group).

Concentration of ATP was measured using a commercial assay kit based on the luciferin-luciferase reaction (Bioluminescent Somatic Cell Assay Kit, FL-ASC; Sigma) following the technique described by Rieger (1997) and the manufacturer's recommendations. Briefly, two oocytes in 50 µl of PBS were transferred into 5 ml plastic tubes equilibrated on ice (0ºC). One hundred microlitres of ice-cold somatic cell reagent (FL-SAR reagent) were then added to the oocytes and incubated for 5 min on ice. Next, 100 µl of diluted ice-cold assay mix (FL-AAM reagent; 1:25 with ATP assay mix dilution buffer, FL-AAB reagent) was added, and the tubes were kept for an additional 5 min at room temperature in the dark. The solution was then transferred into appropriate plastic tubes fitted for the high-sensitivity (0.01 pmol) luminometer (Bioluminat Junior; Berthold, Wildbad, Germany) and luminescence was measured. To obtain simultaneous measurements of samples, a set of tubes with oocytes was measured once and again in reverse order. The mean of these two values expressed in Relative Light Unit was calculated to establish our final value. A seven-point standard curve (0–6 pmol/tube) was determined and, after every 20 oocytes, a negative control was run. The ATP content was calculated using the formula derived from the linear regression of the standard curve.

Evaluation of developmental competence

As the pFSH5 group was the only one that showed significant differences in ATP content relative to the superovulation group, in the second phase of the study developmental competence of oocytes from the control and pFSH5 superovulation groups was examined.

Twenty-four does were inseminated and ovulation was induced with 2 µg buserelin acetate (Suprefact; Hoechst Marion Roussel, S.A., Madrid, Spain) given intramuscularly 24 h after the last injection.

Presumptive zygotes were collected 14 to 16 h after artificial insemination. In total, 351 presumptive zygotes (155 control and 196 superovulated) were washed twice in DPBS with 2 g BSA/l and placed into culture dishes in 1 ml of HAMS F12 supplemented with 20% fetal calf serum. The cultures were maintained for 78 h at 38.5ºC in humidified 5% CO_2 in air and the development to blastocyst was recorded.

Statistical analyses

The effect of superovulation treatment on the ovulation rate and recovered oocytes were analysed by a General Linear Model Statgraphics®Plus5.1 (Statistical Graphics Corp., Rockville, MO, USA). Females that did not ovulate were eliminated from study.

The effect of superovulation treatment (control, rhFSH3, pFSH3 or pFSH5), the culture, the temperature and its interaction (0 h, 2 h – 25ºC and 2 h – 38.5ºC groups) were used as fixed effects on the oocytes ATP content. These effects were analysed by a General Linear Model Statgraphics®Plus5.1 (Statistical Graphics Corp., Rockville, MO, USA).

The effect of superovulation (control and pFSH5 groups) on the developmental competence was compared using the General Linear Mixed Model (GLIMMIX) procedure of the Statistical Analysis System (SAS Inc. Cary, NC, USA). Treatments (pFSH5 superovulation or control groups) were considered fixed effects in the model, while superovulation session and culture session were regarded as random effects. A binomial distribution with a logit-link function was used. Means were separated using Fisher's protected least significant difference (LSD), with treatment effect declared significant at $P < 0.05$. For ease of interpretation, means and standard errors shown in Table 3 are presented on the scale of the data (logit scale).

Results

In the first phase of the study 73 does were treated, five females were rejected for failure in ovulation induction. The ovulation rate and the number of recovered oocytes in the pFSH5 group were significantly higher than the other groups ($P < 0.05$, Table 1); while the rhFSH3 group showed ovulation rate and number of recovered oocytes significantly higher than the control group, the pFSH3 group results were similar to the control and rhFSH3 groups.

Conversely, as shown in Table 2, differences among superovulation treatments were found in ATP levels. In the pFSH5 group, the ATP level was significantly lower than in other groups (5.63 ± 0.14 pmol of ATP for the pFSH group versus 6.42 ± 0.13 and

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Table 1 Effect of superovulation treatment on ovulation rate and number of oocytes recovered (least square means ± standard error).

Treatment	Females	Ovulation rate	Recovered oocytes
Control	22	10.7 ± 2.0^{a}	8.6 ± 1.9^{a}
pFSH3	18	$11.1 \pm 2.2^{a,b}$	$9.5 \pm 2.1^{a,b}$
rhFSH3	18	16.6 ± 2.2^{b}	15.3 ± 2.1^{b}
pFSH5	10	34.4 ± 2.9^{c}	25.1 ± 2.9^{c}

Control: females treated every 24 h for 3 days, with 0.4 ml of 30% polyvinylpyrrolidone (PVP).
pFSH3 group: females treated every 24 h for 3 days with 11.4 μg of porcine follicle stimulating hormone (FSH) diluted in 0.4 ml of 30% PVP.
rhFSH3: females treated, every 24 h for 3 days, with 0.6 μg of human recombinant FSH diluted in 0.4 ml of 30% PVP.
pFSH5 group: females treated twice (12 h intervals) with 4.5 μg on day 1, twice 9.0 μg on day 2 and only one injection of 4.5 μg on the morning of day 3 of porcine FSH dissolved in physiological saline.
[a,b,c]Values with different superscripts in the same column are statistically different ($P < 0.05$).

Table 2 Effect of superovulation treatment and culture temperature on ATP content (least square means ± standard error).

	Group	*n*	ATP content (pmol)
Superovulation treatment	Control	146	$6.03 \pm 0.17^{a,b}$
	rhFSH3	245	6.42 ± 0.13^{b}
	pFSH3	161	6.19 ± 0.15^{b}
	pFSH5	242	5.63 ± 0.14^{a}
Culture and temperature conditions	0 h 25ºC	393	4.95 ± 0.10^{a}
	2 h at 25ºC	153	6.17 ± 0.16^{b}
	2 h at 38ºC	248	7.08 ± 0.13^{c}

n: number of oocytes.
Control: females treated every 24 h for 3 days, with 0.4 ml of 30% polyvinylpyrrolidone (PVP).
pFSH3 group: females treated every 24 h for 3 days with 11.4 μg of porcine follicle stimulating hormone (FSH) diluted in 0.4 ml of 30% PVP.
rhFSH3: females treated, every 24 h for 3 days, with 0.6 μg of human recombinant FSH diluted in 0.4 ml of 30% PVP.
pFSH5 group: females treated twice (12 h intervals) with 4.5 μg on day 1, twice 9.0 μg on day 2 and only one injection of 4.5 μg on the morning of day 3 of porcine FSH dissolved in physiological saline.
[a,b,c]Values with different superscripts in the same column are statistically different ($P < 0.05$).
Interaction was not significant.

6.19 ± 0.15 pmol of ATP for rhFSH3 and pFSH3, respectively; $P < 0.05$) but similar to the control group.

Regarding the culture effect on ATP contents, oocytes cultured for 2 h at 25ºC or 38.5ºC presented higher ATP levels than those measured immediately after recovery (6.17 ± 0.16 and 7.08 ± 0.13 pmol of ATP for 25ºC – 2 h and 38.5ºC – 2 h groups respectively, versus 4.95 ± 0.10 pmol of ATP for the control group; $P < 0.05$, Table 2). In addition, ATP levels in the 38.5ºC cultured oocytes group were significantly higher than in the 25ºC cultured oocytes group.

For the second phase of the study, the developmental competence of pFSH5 superovulated oocytes and control groups is shown in Table 3. The pFSH5 superovulation treatment has a substantial influence on the developmental ability of oocytes, with 24.28% of ova developing into hatched blastocysts after *in vivo* fertilization versus a figure of 80.39% for the control group ($P < 0.0001$). Considering only the fertilized ova, the developing rate for the pFSH5 group was significantly lower than that of the control group (79.04% versus 95.16% for the pFSH5 and control groups, respectively).

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Table 3 Effect of pFSH5 superovulation treatment on developmental competence (mean ± standard error).

Group	Nº of does	*n*	HR (%)	DR (%)
Control	16	155	80.39 ± 6.97^{a}	95.16 ± 2.48^{a}
pFSH5	8	196	24.28 ± 7.86^{b}	79.04 ± 7.92^{b}

n: number of presumptive zygotes.
HR: hatching rate = (hatched blastocyst/cultured presumptive zygotes) × 100.
DR: development Rate = ((hatched blastocyst – oocytes)/cultured embryos) × 100.
[a,b]Values with different superscripts in the same column are statistically different ($P < 0.05$).

Discussion

Several studies have revealed that administration of exogenous gonadotropin yields high numbers of oocytes and embryos but may result in a decrease in its developmental competence (Fujimoto *et al.*, 1974; Moor *et al.*, 1985; Blondin *et al.*, 1996; Kauffman *et al.*, 1998; Ertzeid & Storeng, 2001; Mehaisen *et al.*, 2005). Nevertheless, the way in which gametes are altered by gonadotropins could be a response to different factors, including an alteration in oocyte maturation or in its energy metabolism. Mitochondria, by means of their ability to generate ATP by oxidative phosphorylation, are the main organelle involved in energy regulation (Ramalho-Santos *et al.*, 2009).

Given that it has been observed that FSH in the absence of LH could induce a superovulatory response in rabbit does using both purified porcine FSH or recombinant human FSH (Salvetti *et al.*, 2007; Cortell *et al.*, 2008; Viudes-de-Castro *et al.*, 2009), in the present study the effect of these hormonal preparations on ovulation rate and oocyte quality was compared. Regarding the superovulatory response, both purified pFSH administered in five injections, twice a day (pFSH5), and rhFSH dissolved in PVP administered in three injections, once a day for 3 days (rhFSH3), resulted in a higher ovulation rate than the control group. However, in spite of their ability to induce a superovulation response, while rhFSH3 reached similar results to those reported previously with oFSH, pFSH and rhFSH, increasing to twice the ovulation rate and the number of oocytes or embryos recovered in superovulated does (Kauffman *et al.*, 1998; Mehaisen *et al.*, 2006; Salvetti *et al.*, 2007; Viudes-de-Castro *et al.*, 2009), the pFSH5 treatment showed a ovulation rate 3.5 times higher than that of the control group. Despite an increase in the number of oocytes produced per female, when animals were treated with the pFSH5 protocol the blastocyst developmental rate per oocyte was significantly decreased compared with control females (15% less development rate). Superovulation treatments with FSH diluted in PVP permits slower absorption of FSH and more prolonged blood concentration levels. Conversely, when pFSH was administered once a day for 3 days, no superovulatory response was observed. The poorer ovarian response in the pFSH3 group at the concentration used in the present work may be affected by the treatment schedule or by the hormone vehicle. This idea would be supported by our previous studies (data not shown) in which rhFSH applied in saline serum twice a day for 5 days resulted in a higher ovulation rate than if applied in PVP once a day for 3 days. Thus, these results may indicate that the amount of FSH reaching the ovary with the dose of pFSH used in the present work is less when the hormone is administered diluted in PVP than when it is administered diluted in saline serum. Although PVP has been used as a hormone vehicle previously successfully in rabbit does (Kanayama *et al.*, 1992; Hashimoto *et al.*, 2004; Cortell *et al.*, 2008; Viudes-de-Castro *et al.*, 2009), to our knowledge this regime has not been compared with the same hormonal dose administrated in saline serum. However, it has been noted that the reduction of injection number using PVP as vehicle may affect the ovarian response after superovulation (D'Alessandro *et al.*, 2001; Lopes da Costa *et al.*, 2001). In order to determine the effect of these superovulation treatments on oocyte quality, the amount of ATP in recovered oocytes was determined. It has been described previously that mitochondrial dysfunction and ATP content may be related to developmental competence in oocytes and embryos (Van Blerkom *et al.*, 1995; Thouas *et al.*, 2004; Nagano *et al.*, 2006). A significant difference in ATP content between different superovulatory treatments was found. Our data suggest that the reduced level of ATP in pFSH5-produced oocytes may reflect a minor mitochondrial activity, which plays a critical role in the acquisition of developmental competence in the oocyte. It is well known that the oocyte maturation process depends on FSH and LH stimulation and oocyte–follicle interaction (Hillier, 2009) and superovulation treatments could affect granulosa cell activity or accelerate follicular growth (Blondin *et al.*, 1996). In fact, an effect on ATP content by superovulation treatment has been described previously both in mouse (Combelles and Albertini, 2003) and hamster (Lee *et al.*, 2006).

However, how ATP levels are affected during oocyte maturation is still unclear and seems to be very species specific; it has been observed that *in vitro* maturation and maturation conditions may effect mitochondrial activity in rabbits (Kanaya *et al.*, 2007), mouse (Combelles and Albertini, 2003), cows (Stojkovic *et al.*, 2001; Nagano *et al.*, 2006), gilts (Brevini *et al.*, 2005) or cats (Freistedt *et al.*, 2001).

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Conversely, oocyte ATP levels increased significantly after culturing for 2 h, however results were higher in those oocytes cultured at 38.5ºC than in those cultured at 25ºC. This ATP variation with the incubation temperature suggests that incubation at 38.5ºC might allow the oocytes to recover ATP synthesis more quickly than at 25ºC. The low ATP contents when oocytes were recovered may due to ATP release under stress conditions and its degradation by ecto-ATPases sited in cell surface (Joseph *et al.*, 2003; Schwiebert and Zsembery, 2003). In fact, this scenario would be in accordance with observations by Tamassia *et al.* (2004) in cows, who detected lower ATP levels in oocytes obtained from the slaughterhouse than in oocytes obtained from ovum pick-up (OPU), probably due to the more stressful conditions and transport when they were recovered from a sacrificed female. Moreover, these oocytes achieved ATP contents similar to those of oocytes from OPU after culture.

Finally, our results show that porcine FSH administered in five doses, every 12 h, may induce a greater response in ovulation rate than recombinant human FSH dissolved in PVP and administered in three doses, every 24 h. Conversely, a negative effect on oocyte quality has been observed in the pFSH5 group in ATP production when compared with other superovulation treatments. Possibly, the alteration in oocyte quality affected the competence of oocyte to be fertilized and could explain the lower development rate observed. This finding could be related to a failure in its maturation process probably due to the long period between the last FSH dose and ovulation induction. This situation suggests that, in the case of FSH in saline adjuvant, ovulation induction time may affect the developmental competence of oocytes.

Findings from the current study suggest that rhFSH dissolved in PVP administered once a day for 3 days allows the achievement of adequate follicular maturation during ovarian stimulation. By contrast, in the case of pFSH5, the long period between the last FSH dose and ovulation induction could affect the developmental competence of oocytes.

In conclusion, the reduced level of ATP may reflect reduced activity of mitochondria, which play a critical role for acquisition of developmental competence.

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