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SUPEROVULATION AND EXPRESSION OF FOLLICLE-STIMULATING HORMONE RECEPTOR IN YOUNG RABBIT FEMALES

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Abstract: To optimise the use of juvenile *in vitro* embryo transfer technologies in young rabbit females, superovulation was performed in New Zealand White young rabbit females at different ages and the expression mode of follicle-stimulating hormone receptor (FSHR) was explored using real-time quantitative polymerase chain reaction, and *in vitro* maturation (IVM) together with fertilisation (IVF) was conducted immediately after superovulation. The results showed that (1) the age factor significantly affected superovulation in young rabbit females, with 60 d as an optimal age; (2) the mRNA level of FSHR exhibited a rising trend, though it was lower at 30 to 40 d of age; (3) the maturation rate of the oocytes from 60 d old rabbits was significantly higher than in those from 50 d old rabbits; (4) the fertilisation rate of oocytes was not significantly different among rabbits 50, 60 and 70 d old.

Key Words: rabbit, superovulation, follicle-stimulating hormone receptor, *in vitro* maturation, *in vitro* fertilisation.

INTRODUCTION

Juvenile *in vitro* embryo transfer (JIVET) technology, including superovulation of young animals, *in vitro* maduration (IVM) and *in vitro* maduration (IVF) of the oocytes and embryo transplantation (ET), is a high-tech biological system. JIVET technology can not only improve the animal's breeding potential, through which oocyte resources would be fully utilised under normal idle circumstances, but also shorten the generation interval and speed up the process of genetic improvement. This technology was first proposed by the South Australia Reproduction Institute (SARD) (Zaid *et al.,* 1999). JIVET technology has been successfully applied in many species such as sheep (Guo *et al.,* 2009), goats (Leoni, 2009) and cattle (Yeh *et al.,* 2002) The practice of recovering oocytes from live selected females by laparoscopic ovum pick-up and breeding prepubertal females by JIVET allowed a greater production of valuable goats (Paramio, 2010). But in rabbit, few research works related to JIVET were reported.

Some previous research studied aspects of *in vitro* culture conditions for rabbit oocytes such as the culturing time, medium compositions, gas atmosphere and temperature (Sugimoto *et al.,*2012). Many scholars have studied the age difference and the superovulation method and found that the *in vitro* maturation rates of oocytes from different age groups were different. However, few reports studied the relationship between the age and oocyte condition in young rabbit females.

Superovulation was used to produce the maximum number of oocytes per donor female and follicle-stimulating hormone (FSH) is still used in superovulation treatment in rabbits. The difference in FSH concentration affected follicular growth, differentiation, maturation and ovulation. FSH was essential for primate reproduction and acted via the FSH-receptor (FSHR) (Brune *et al.,* 2010). FSHR expressed in the follicle during follicle development and FSHR existed only in granulosa cells (Charlton *et al.,* 1982; Oxberry and Greenwald, 1982; Shima *et al.,* 1987). The mRNA of FSHR can be found in the primordial follicles with 1 to 2 layers of granulosa cells. Changes in FSHR mRNA level may

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determine the follicular response to gonadotropin, and thereby mature follicles inducing different ovulation response and ovulation (Wilson *et al.,* 2001).

This study exerted superovulation technology in young rabbit females and detected the situation of these superovulated oocytes by quantitative fluorescence techniques to explore the difference in the FSHR gene expression level at different ages in young rabbit females and compare the *in vitro* maturation and fertilisation of oocytes in young rabbit females at different ages, with the aim of optimising JIVET technology.

MATERIALS AND METHODS

Experimental animals

Healthy New Zealand White rabbits were used as the subjects, including females aging from 30 to 365 d old and sexually mature males. Rabbits were caged and fed pellet feed.

Superovulation

Superovulation was conducted in young rabbit females at 30, 40, 50, 60 and 70 d of age (10 in each group), respectively. The young females were injected intramuscularly 30 IU/rabbit of FSH (Bioniche, America) twice daily for 3 consecutive days. Twelve hours after the last injection of FSH, the females were injected intravenously via the marginal ear vein with 30 IU of LH (Bioniche, America).

In vitro maturation (IVM)

We obtained the oocytes via follicle puncture and selected the cumulus-oocyte (COCs) complexes with normal morphology, even cytoplasm and complete cumulus. A hundred and twenty cumulus-oocyte complexes (COCs) obtained respectively from 50, 60 and 70 d old female rabbits were selected for IVM. To observe the IVM of the superovulated oocytes, they were cultured in tissue culture medium TCM-199 (Gibco, USA) containing 10% fetal bovine serum (FBS) (Hyclone, USA), 0.11 g/L sodium pyruvate, 10 μg/mL LH, 10 μg/mL of FSH, 1 μg/mL E_2 (Sigma, USA), 100 IU/mL penicillin (Sigma, America) and 100 IU/mL streptomycin sulphate (Sigma, USA). Each droplet containing 50 μL maturation medium covered with mineral oil (Sigma USA). After equilibrating for 2 h, 10 COCs/droplet were cultured in maturation medium and matured for 24 h at 38.5°C in a humidified atmosphere of 5% CO_2 in air. Oocytes were considered mature when the first polar body (PB I) was expelled.

In vitro fertilisation (IVF)

Sixty mature oocytes obtained respectively from 50, 60, and 70 d old female rabbits were selected for IVF. This procedure was conducted in the medium TCM-199, containing 6 mg/mL of BSA, 0.11 g/L sodium pyruvate (Sigma, USA), 100 IU/mL penicillin and 100 IU/mL streptomycin sulphate. Each droplet containing 50 μL fertilisation medium was covered with mineral oil and equilibrated for 2h at CO_2 incubator box. Semen was collected manually and fresh semen was mixed with capacitating medium by a ratio of 1:2~5. Fresh sperm were washed by centrifugation at 1000 r for 5 min. The lower sperm precipitated by adding 1 mL capacitating medium and placed in CO_2 incubator for 20 to 30 min. The supernatant was then extracted to another sterile centrifuge tube for 1000 r/min centrifugation for 5 min. After appropriate dilution, the fertilisation drops containing live sperm reached a final concentration of 1×10^6 sperm per mL. To fertilise, every 10 mature oocytes were transferred into one droplet and combined with a 7.5 μL droplet of *in vitro* capacitate of semen at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

In vitro development

Oocytes were co-incubated with spermatozoa for 6 h. The oocytes were then washed 3 times in TCM-199 and transferred into development medium for an additional culture. The medium used for *in vitro* development was TCM-199 with 6 mg/mL of BSA, 0.11 g/L sodium pyruvate, 2 mM/L L-glutamine (Sigma, USA),100 IU/mL penicillin and 100 IU/mL streptomycin sulphate. The cleavage of putative zygotes was calculated every 12 h under the stereomicroscope observation. Normal cleavage was used as the standard for successful fertilisation.

Table 1: Primer sequences.

Gene	primer	sequence	Product size (bp)	GenBank accession number
FSHR	upstream	5'-TGCCAAGATAGCAAGGTGAC-3'	140	TF316814
	downstream	5'-ACTGGGAAGATTCTGGAAGG-3'		
β-actin	upstream	5'-TGACCCAGATCATGTTTGAG-3'	308	XM_002722894
	downstream	5'-CATGAGCAACATAGCACAGC-3'		

Detection of the mRNA level of FSHR

Total RNA from ovaries was isolated using Trizol reagent (Tiangen Biochemical Technology Co. LTD) following the manufacturer's protocol. The final RNA pellet was washed with 70% ethanol, air dried and re-suspended in diethyl pyrocarbonate (DEPC) water.

PCR tubes in the ice bath were added to the total RNA of ovaries 6 μL, the Oligo-dT 1 μL, and DEPC-treated H_2O complement to 12 μL, mixed gently, centrifuged for 3~5 s, then immediately ice bathed after denaturation at 70°C or 5 min. Next, 5×RT Buffer 4 μL, dNTP (10 mM) 2 μL, 20 U/μL RNase inhibitor 1 μL was added and mixed gently, centrifuging for 3~5 s, and at 37°C for 5 min. Then, we added M-MLV 1 μL, which was extended at 42°C for 60 min, heated at 70°C for 10 min, and then placed in the ice bath for 5 min, finally obtaining the cDNA, which was stored at –20°C.

The GenBank accession numbers, primer sequences and amplification fragment sizes are shown in Table 1. The gene was detected using the SYBR Green I Real Time PCR young rabbit female. The real-time qPCR was performed using a 20 μL system, including 2.5×real Master SYBR/20×SYBR solution 9 μL, each primer 1 uL, cDNA 2 μL, and pure water 7 μL. Forty cycles of PCR amplification were performed as follows: denaturation at 95°C for 20 s, annealing at 55°C for 30 s, and extension at 68°C for 45 s. Calculating the mRNA levels was performed using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

All the data in this study were processed using the SPSS 11.5 for statistical analysis, with the difference significance test using the Tukey method.

RESULTS

The impact of different age on superovulation in young rabbit females

Superovulation treatment with FSH in combination with LH induced a super-ovulatory response only in young rabbit females with an age equal to or more than 50 d old rabbits, whereas 30 or 40 d old young rabbit females did not respond to treatment (Figure 1). The average ovulation rates at 60 d (43.0±6.85) or 70 d of

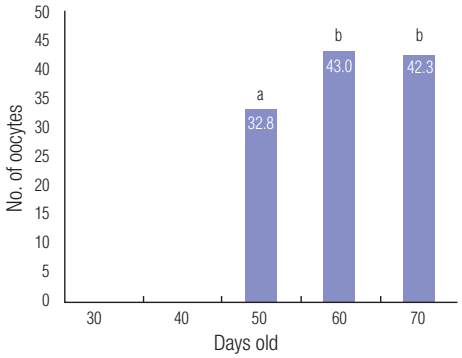

Figure 1: The impact of different age on superovulation in young rabbits. ■ The mean No. of oocytes. [ab]Values in the same column with different superscripts are statistically different ($P<0.05$).

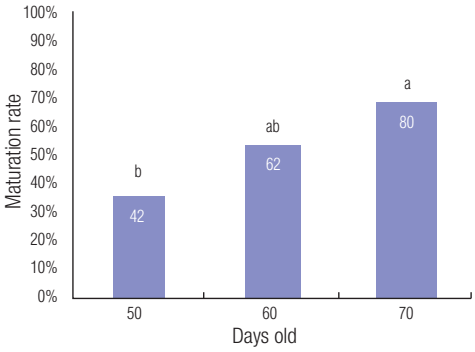

Figure 2: The impact of age on the *in vitro* maturation (IVM) of young rabbits. No. oocytes: 120. ■ No. of maduration oocytes. [ab]Values in the same column with different superscripts are statistically different ($P<0.05$).

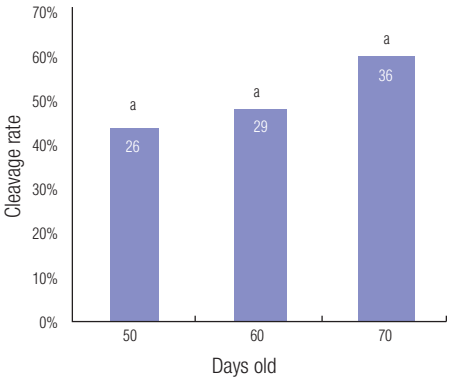

Figure 3: The impact of age on the *in vitro* maturation (IVM) of young rabbits. No. of maduration oocytes: 60. ■ No. of cleavage. [ab]Values in the same column with different superscripts are statistically different ($P<0.05$).

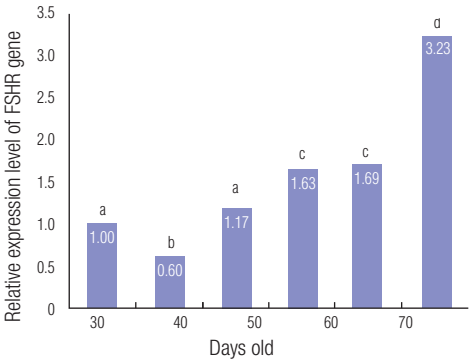

Figure 4: Relative expression of the FSHR gene in the ovaries of different ages in rabbits. [abcd]Values with different superscripts are statistically different ($P<0.05$).

age (42.3±5.50) were significantly higher than at 50 d (32.8±3.91) ($P<0.05$).

The impact of age on the in vitro *maturation (IVM) of young rabbit females*

Thirty or forty-day-old young rabbit females did not respond to treatment, so the oocytes of 50, 60, and 70 d old young rabbit females were used for *in vitro* maturation and *in vitro* fertilisation. The maturation rate at 70 d (66.67%) was significantly higher than at 50 d of age (35%) ($P<0.05$). The maturation rate at 60 d old (51.67%) was slightly higher than at 50 d old, but lower than at 70 d old, and the difference was not significant (Figure 2). It can be seen that age exerted certain effects on the IVM.

The impact of different age on the in vitro *fertilisation (IVF) of young rabbit females*

The cleavage rates of oocyte at 60 d old (48.33%) and 70 d (60.00%) were higher than at 50 d old (43.33%), but the differences were not significant ($P>0.05$). The cleavage rate at 70 d old was higher than at 60 d old, but the difference was not significant ($P>0.05$) (Figure 3). It can be seen that age had a certain impact on the IVF.

Relative expression of FSHR gene in the oocytes of different ages in rabbits

The FSHR mRNA in all of the ovaries of different ages in rabbits had expressions and present dynamic changes (Figure 4). With 30 d of age as a reference, the expression level was 1. The relative expression level of 40, 50, 60, 70, and 365 d old were 0.6, 1.17, 1.63, 1.69, and 3.23, respectively. The expression of 40 d old (0.6) was lower than 30 (1), 50 (1.17), 60 (1.63), 70 (1.69) and 365 d old (3.23), and the differences were significant ($P<0.05$). The expression of 50 d old (1.17) was higher than 30 d old (1), but the difference was not significant ($P>0.05$). The expression of 60 (1.63),70 (1.69) and 365 d old (3.23) were higher than 30 d old (1), and the differences were significant ($P<0.05$). The expression of 60 d old (1.63) was lower than 70 d old (1.69), but the difference was not significant ($P>0.05$). The expression of 365 d old (3.23) was higher than 60 (1.63) and 70 d old (1.69), and the differences were significant ($P<0.05$).

DISCUSSION

Different age and ovulation in young rabbit females

Several studies have reported on the technology using the oocytes of prepubertal livestock (Izquierdo *et al.,* 2002; Koeman *et al.,* 2003). Kalita *et al.* (2000, 2001) proposed that antral follicle size increased from birth to 6 mo of age in goats. The effect of superovulation also increased with age gradually. However, with the growth of young rabbit

females, the ovarian response to hormonal stimulation may tend to reach a peak. A peak in numbers of antral follicles was seen at 4 wk of age in ewe lambs (Tassel *et al.,* 1978). Kelly *et al.* (2005) found that 4- to 8 wk old lambs were particularly sensitive to gonadotropin administration. This study indicated that 50 d old rabbits exhibited a first response to superovulation.

Different ages on IVM or IVF

Increase in follicle and oocyte diameters could improve embryo development (Gandolfi. *et al.,* 2005). The capacity of oocyte maturation was closely related to follicular maturation (Ali *et al.,* 2006). Tsuji *et al.* (1985) found that there was a decreased maturation rate in oocytes from small follicles (3-4 mm) when compared with those from larger follicles (9-15 mm) in the human. Moor and Trounson (1977) observed in sheep that as the follicle size increased, the frequency of oocytes progressing to the second meiotic metaphase increased as well. This experiment indicated that age exerted a significant effect on IVM. This may be due to that small day- old infant rabbits had a smaller follicle and oocyte diameters. The experiment on IVM and IVF showed that 60 d old can be used as an ideal minimal age for JIVET.

FSHR gene expression

FSHR belongs to a subfamily of G protein-coupled receptors. Binding of FSH to its receptor leads to conformational changes in the protein, resulting in activation of the Gs protein and cyclic AMP (cAMP) production (Zhang *et al.,* 1991). Dierich *et al.* (1998) found that FSH-R-deficient females display thin uteri and small ovaries and were sterile due to a block in folliculogenesis before antral follicle formation. Small follicles during development need more stimulation of FSH, and the granulose cell FSHR mRNA content was high. With the further development of the follicle, the dependence on the hormone decreased (Calder *et al.,* 2003). This study initially confirmed the relationship between the mRNA expression of FSHR gene in the young rabbit female ovarian of different ages and superovulation. During superovulation, the expression levels should reach a certain value in order to induce the reaction of young rabbit female ovarian to the hormones. With the increase in FSHR, the effects of superovulation began to decline. This suggested that the FSHR gene may play an important role in young rabbit female superovulation, IVM and IVF.

CONCLUSIONS

In this study, our research showed that the age factor significantly affected superovulation, including IVM and IVF in young rabbit females, with 60 d old as an optimal age. The mRNA level of FSHR exhibited a rising trend with the age, though it was higher at 30 d than at 40 d of age.

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