J. Mamm. Ova Res. Vol. 33 (1), 63–68, 2016

63

—Original—

Risk of premature chromatid separation is increased by poor cumulus cell layers and inappropriate culture media for *in vitro* maturation of mouse oocytes

Hiroyuki Kikuchi[1, 2]*, Midori Yoshizawa[3], Kentaro Tanemura[2], Eimei Sato[4] and Hiroaki Yoshida[1]

[1] *Yoshida Ladies Clinic, Sendai 981-1105, Japan*
[2] *Laboratory of Animal Reproduction, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan*
[3] *Laboratory of Animal Breeding and Reproduction, Faculty of Agriculture, Utsunomiya University, Utsunomiya 321-8505, Japan*
[4] *National Livestock Breeding Center, Incorporated Administrative Agency, Fukushima 961-8511, Japan*

Abstract: Recently, the quality of *in vitro* matured (IVM) oocytes has been improved by numerous studies using animal models. However, the implantation rates of IVM oocytes remain lower than those of *in vitro* fertilization (IVF). Chromosome abnormalities, which mainly occur during the first meiotic division, are often observed in IVM oocytes. To clarify how the properties of the cumulus cell layers relate to chromosome abnormalities of oocytes during IVM, we investigated the occurrence of aneuploidy and developmental competence using two different media in a mouse model. Maturation rates were significantly lower in denuded oocytes at oocyte collection in both types of maturation media as compared with those of oocytes mechanically denuded just after collection of cumulus-oocyte complex. Fertilization rates and blastocyst rates were lower in Waymouth's MB752/1 medium than in HTF medium. The occurrence of premature chromatid separation (PCS) remarkably increased in Waymouth's MB752/1 medium compared to HTF medium. This demonstrates that denuded oocytes at oocyte collection have low ability to progress to nuclear maturation under IVM conditions. However, the fertilization ability and developmental competence to the blastocyst stage of denuded oocytes were fulfilled under a suitable culture condition. We suggest that oocytes that are already denuded, before or during oocyte collection, have the potential to be matured *in vitro* if the denuded oocytes are cultured in a suitable culture media.

Key words: *In vitro* maturation, Denuded oocytes, Maturation media, Premature chromatid separation

Introduction

Assisted reproductive technology has remarkably developed and is greatly beneficial to infertile patients. However, some patients still suffer from the adverse effects of gonadotropin stimulation of the ovaries for the collection of mature oocytes *in vivo*. In particular, patients with polycystic ovary syndrome, who are stimulated for oocyte retrieval, frequently develop ovarian hyperstimulation syndrome. One solution to this problem is immature oocyte collection from unstimulated or minimally stimulated ovaries, followed by maturation of the oocytes *in vitro* until metaphase II (MII), a procedure known as *in vitro* maturation (IVM).

Many studies examining IVM of mammalian oocytes have been reported to date. Clinical pregnancy rates with embryos derived from IVM oocytes have gradually increased; however, implantation rates are still lower than those embryos created by conventional *in vitro* fertilization (IVF) [1–4]. Synchronization of nuclear and cytoplasmic maturation is necessary to obtain adequate oocyte developmental competence [5, 6]. However, nuclear and cytoplasmic maturation are not synchronized in IVM oocytes [1, 7, 8]. Additionally, chromosomal abnormalities contribute to the low rate of implantation of embryos de-

rived from IVM oocytes [9].

Chromosomal abnormalities primarily occur during the first meiotic division [10, 11] and more frequently occur in IVM oocytes than in mature oocytes *in vivo* [12]. Nearly all embryos with chromosomal abnormalities result in developmental arrest, abortion, and stillbirth; however, some result in delivery [13]. The occurrence of chromosomal abnormalities in human embryos is higher than that in mouse and hamster embryos [14]. Moreover, it is possible that offspring with chromosomal abnormalities can have intellectual disabilities, malformations, or inheritable conditions that can be passed on to future generations. Therefore, to avoid the risk of chromosomal abnormalities, IVM culture media and techniques need to be improved.

Chromosomal abnormalities during IVM have been reported to be associated with culture media, addition of follicle stimulating hormone (FSH), culture time, and other factors in many species, including humans [15–19]. Shioya *et al.* reported that poor cumulus cell layers decrease oocyte maturation and embryo development rates [20]. Furthermore, the surrounding cumulus cells have been shown to influence oocyte respiration activity [21]. Therefore, oocytes with poor cumulus cell layers are not generally used in IVM. However, in human IVM, spontaneous loss or mechanical loss of cumulus cells is observed during oocyte collection. To determine whether the surrounding cumulus cells influence oocyte chromosomal abnormalities during IVM, we investigated the occurrence of aneuploidy and premature chromatid separation (PCS) in matured oocytes using two different media in a mouse model.

Materials and Methods

Immature female imprinting control region (ICR) mice aged 3–4 weeks were used in the experiments. The present study was approved by the Ethics Committee for the Care and Use of Laboratory Animals of Tohoku University. The mice were intraperitoneally injected with 5 IU of pregnant mare serum gonadotropin (PMSG) (Teikokuzohki, Tokyo, Japan). Forty-eight hours later, the mice were euthanized, and their ovaries were removed and transferred into Leibovitz's L-15 medium (Invitrogen, Carlsbad, CA) supplemented with 5% fetal bovine serum (FBS)(Invitrogen). Germinal vesicle (GV) oocytes were obtained as cumulus–oocyte complexes (COCs) by puncturing the antral follicles from the ovaries using a 29-G needle. They were then washed 3 times in Waymouth's MB752/1 medium (Invitrogen) supplemented with 5% FBS and 0.23 mM sodium pyruvate (Sigma, St.

Louis, MO), or human tubal fluid medium (HTF)(Irvine Scientific, Santa Ana, CA) supplemented with 5% FBS. Denuded GV oocytes were collected using a stereomicroscope. Additionally, artificially denuded oocytes were created from oocytes with compact and dense cumulus cells by removing cumulus cells by pipetting for comparison with denuded oocytes. After washing and classification, 10–20 GV oocytes were cultured for 12 h in 100-μL droplets of each culture medium overlaid with paraffin liquid (Nacalai Tesque, Kyoto, Japan) in a humidified atmosphere of 5% CO_2 in air at 37 °C. The mature oocytes were then identified, based on the presence of the first polar body extrusion, under a stereomicroscope and then prepared for use in the experiments.

For *in vitro* fertilization, male ICR mice aged 8–12 weeks were euthanized, and their caudal epididymal spermatozoa were released by puncturing in HTF medium supplemented with 4 mg/mL bovine serum albumin (BSA) (Sigma). The samples were incubated for 1.5–2 h at 37 °C to allow for capacitation. After IVM, MII oocytes were inseminated with approximately 2 × 10^5 motile sperm per mL for 6 h in 200-μL droplets of HTF medium overlaid with paraffin liquid in a humidified atmosphere of 5% CO_2 in air at 37 °C. After 6 h of insemination, the oocytes were determined to be fertilized and cultured in KSOM-aa medium (Zenith Biotech, Guilford, CT) supplemented with 4 mg/mL BSA. The number of embryos at the 2-cell cleavage and blastocyst stages was recorded at 24 and 120 h after IVF.

To investigate aneuploidy and the occurrence of PCS, chromosome preparations were produced as described by Yoshizawa *et al.* [22]. MII oocytes were first treated with 1% hypotonic sodium citrate for 15 min at room temperature. The oocytes were then briefly fixed in fixative (acetic acid:methanol [1:3]), dropped onto a slide, and air-dried. The entire process was monitored under a stereomicroscope. Chromosome preparations were stained with Giemsa, and the chromosome numbers were counted at 1,000×final magnification. Only preparations in which chromosomal numbers could be unambiguously counted were included in the analysis. MII oocytes were classified as normal if they contained 20 dyads. Oocytes classified as 20.5 contained 20 dyads and one chromatid. The numbers of hypoploid (n=15–19.5), haploid (n=20), hyperploid (n=20.5–25), and diploid (n=40) MII oocytes were recorded (Fig. 1). Chi-square analysis and the Bonferroni adjustments were used to determine the statistical significance of the outcome measures between the groups. Statistical significance was accepted for values of *P* < 0.05.
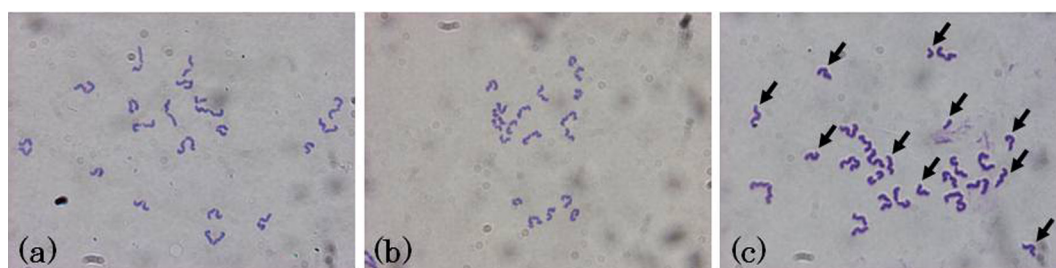
Fig. 1. Representative mouse metaphase II oocytes after Giemsa staining of chromosomes. (a) Euploid (n = 20). (b) Hypoploid (n = 19). (c) Premature chromatid separation. Arrows indicate the chromatids.

Table 1. Results of *in vitro* maturation–*in vitro* fertilization using two different media

Medium	Cumulus cell condition	No. of GV oocytes	Matured (%)	Fertilized (%)	2-Cell cleavage (%)	Blastocysts (%)
Waymouth's MB752/1	Denuded	102	52 (51.0)[c]	25 (48.1)[b]	19 (76.0)[b]	7 (28.0)[b]
	Artificially denuded	96	94 (97.9)[a]	57 (60.6)[b]	55 (96.5)[a]	25 (43.9)[ab]
HTF	Denuded	109	75 (68.8)[b]	60 (80.0)[a]	55 (91.7)[ab]	32 (53.3)[a]
	Artificially denuded	110	108 (98.2)[a]	97 (89.8)[a]	93 (95.9)[a]	58 (59.8)[a]

[a-c] Values with different superscripts within each column are significantly different (P<0.05). GV, germinal vesicle; HTF, human tubal fluid.

Table 2. Chromosomal analysis of metaphase II oocytes matured using two different media

Medium	Cumulus cell condition	No. of oocytes analyzed	Ploidy (%)				PCS (%)
			Euploid (n = 20)	Hypoploid (n = 15–19.5)	Hyperploid (n = 20.5–25)	Diploid (n = 40)	
Waymouth's MB752/1	Denuded	53	46 (86.8)	1 (1.9)	3 (5.7)	3 (5.7)	25 (47.2)[b]
	Artificially denuded	83	78 (94.0)	4 (4.8)	0 (0.0)	1 (1.2)	42 (50.6)[b]
HTF	Denuded	53	48 (90.6)	2 (3.8)	1 (1.9)	2 (3.8)	3 (5.7)[a]
	Artificially denuded	90	87 (96.7)	2 (2.2)	0 (0.0)	1 (1.1)	3 (3.3)[a]

[a-b] Values with different superscripts within each column are significantly different (P<0.005). PCS, premature chromatid separation; HTF, human tubal fluid.

Results

Maturation rates of denuded oocytes and artificially denuded oocytes were 51.0% and 97.9%, respectively, in Waymouth's MB752/1 medium and 68.8% and 98.2%, respectively, in HTF medium (Table 1). Significant decreases were found in denuded oocytes compared to artificially denuded oocytes in both maturation media. The fertilization rates of denuded oocytes and artificially denuded oocytes were 48.1% and 60.6%, respectively, in Waymouth's MB752/1 medium and 80.0% and 89.8%, respectively, in HTF medium (Table 1). The rates of blastocysts derived from denuded oocytes and artificially denuded oocytes were 28.0% and 43.9%, respectively,

in Waymouth's MB752/1 medium and 53.3% and 59.8%, respectively, in HTF medium (Table 1). As significant decrease in the blastocyst rate was observed in denuded oocytes in Waymouth's MB752/1 medium compared to denuded oocytes and artificially denuded oocytes in HTF medium. Compared to denuded oocytes, artificially denuded oocytes showed similar rates of fertilization and blastocysts, despite the high maturation rates observed in both maturation media.

The occurrence rates of PCS from denuded oocytes and artificially denuded oocytes were 47.2% and 50.6%, respectively, in Waymouth's MB752/1 medium and 5.7% and 3.3%, respectively, in HTF medium (Table 2). Compared to HTF medium, the occurrence rates of PCS in

denuded oocytes and artificially denuded oocytes were significantly increased in Waymouth's MB752/1 medium. Compared to denuded oocytes, artificially denuded oocytes showed similar occurrence rates of PCS in both maturation media.

Discussion

In oocyte collection from humans, oocytes with poor cumulus cell layers are likely to be produced by aspiration pressure, or by collection from atretic follicles. Although it is known that the maturation competence of oocytes that are already denuded in the follicular atretic process is lower than that of oocytes surrounded by cumulus cells [20], whether the denuded oocytes are derived from atretic follicles or by the removal of cumulus cells during aspiration cannot be determined. Thus, the development of a maturation technique that is suitable for both types of denuded oocytes is essential. In this study, we demonstrated that the maturation rates of oocytes artificially denuded after COC collection was more than 90% when the denuded oocytes were cultured in Waymouth's MB752/1 medium and HTF medium (Table 1). However, the fertilization rates and blastocyst rates of the artificially denuded oocytes in Waymouth's MB752/1 medium were significantly lower than those of the oocytes matured in HTF medium (Table 1). Interestingly, HTF medium significantly improved not only the oocyte maturation rate, but also the fertilization rate and developmental competence of the spontaneously denuded oocytes. The positive effect of HTF was also observed in the rate of PCS (Table 2). Thus, if HTF medium were used as the IVM medium for denuded oocytes, the matured oocytes would gain developmental competence.

The morphology and distribution of microtubules in IVM oocytes differs from those in mature oocytes *in vivo* [8, 23]. Chromosomal abnormalities result from spindle and chromosome configuration abnormalities during meiosis [3, 24]. In fact, IVM oocytes exhibit higher chromosomal abnormality rates than mature oocytes *in vivo* [12]. Under the *in vivo* follicular condition, FSH stimulates both granulosa cells and cumulus cells to induce follicular development [25]. During this step, metabolic activity in cumulus cells increases to transfer the energy source to the oocyte by gap junctional communication [26, 27]. Support from cumulus cells induces oocyte growth leading to the resumption meiosis and progression to the metaphase II stage with fertilization and developmental competence [28]. The LH surge acts on granulosa cells to induce EGF-like factor expression. The secondary factors stimulate cumulus cells to induce oocyte maturation by disrupting the gap junctional communication [29]. In the IVM technique, the cumulus oocyte complex is used for culture because it is very difficult to keep granulosa cell function under the *in vitro* culture condition. Moreover, in this study, we used denuded oocytes that were not supported by cumulus cells, assuming that their maturation competence would be lower than that of IVM oocytes with COC.

Waymouth's MB752/1 medium contains high concentrations of amino acids and vitamins, whereas HTF medium does not. HTF medium only contains glucose, lactate and pyruvate as energy sources. It has been reported that glucose does not support meiotic maturation in denuded oocytes [30, 31], and the oocyte has a TCA cycle, but not a glycolytic pathway, for producing ATP [32, 33]. Therefore, we initially hypothesized that Waymouth's MB752/1 medium would be the better medium. However, the present results show that the minimum energy condition improved oocyte maturation competence, suggesting that too much energy activates the mitochondria function increasing the risk of oxidative stress leading to reduced oocyte quality [6]. The present data indicates the need for care in the selection of the maturation medium.

In human infertility care, low responders show low responses to FSH stimulation, and ovarian hyperstimulation syndrome is easily induced in high responders. Treatment with FSH plays an important role in follicular development, and FSH is also involved in the survival of granulosa and cumulus cells by preventing follicle atresia [34]. Thus, in both cases, the majority of oocytes are collected from atretic follicles. In this study, spontaneously denuded oocytes showed an ability to progress to meiosis with developmental competence when HTF medium was used as the culture medium. These results indicate that low quality oocytes collected from atretic follicles are available for IVM. Thus, increased numbers of oocytes are available for IVM culture, which will increase the chance of successful pregnancy in human infertility care.

References

1) Piquette, G.N. (2006): The in vitro maturation (IVM) of human oocytes for in vitro fertilization (IVF): is it time yet to switch to IVM-IVF? Fertil. Steril., 85, 833–835, discussion 841. [Medline] [CrossRef]

2) Lanzendorf, S.E. (2006): Developmental potential of in vitro- and in vivo-matured human oocytes collected from stimulated and unstimulated ovaries. Fertil. Steril., 85, 836–837, discussion 841. [Medline] [CrossRef]

3) Li, Y., Feng, H.L., Cao, Y.J., Zheng, G.J., Yang, Y., Mullen, S., Critser, J.K. and Chen, Z.J. (2006): Confocal microscop-

ic analysis of the spindle and chromosome configurations of human oocytes matured in vitro. Fertil. Steril., 85, 827–832. [Medline] [CrossRef]

4) Combelles, C.M., Cekleniak, N.A., Racowsky, C. and Albertini, D.F. (2002): Assessment of nuclear and cytoplasmic maturation in in-vitro matured human oocytes. Hum. Reprod., 17, 1006–1016. [Medline] [CrossRef]

5) Sun, Q.Y., and Nagai, T. (2003): Molecular mechanisms underlying pig oocyte maturation and fertilization. J. Reprod. Dev., 49, 347–359. [Medline] [CrossRef]

6) Krisher, R.L. (2004): The effect of oocyte quality on development. J Anim Sci., 82 E-Suppl, E14–23.

7) Roberts, R., Franks, S. and Hardy, K. (2002): Culture environment modulates maturation and metabolism of human oocytes. Hum. Reprod., 17, 2950–2956. [Medline] [CrossRef]

8) Sanfins, A., Plancha, C.E., Overstrom, E.W. and Albertini, D.F. (2004): Meiotic spindle morphogenesis in in vivo and in vitro matured mouse oocytes: insights into the relationship between nuclear and cytoplasmic quality. Hum. Reprod., 19, 2889–2899. [Medline] [CrossRef]

9) Requena, A., Bronet, F., Guillén, A., Agudo, D., Bou, C. and García-Velasco, J.A. (2009): The impact of in-vitro maturation of oocytes on aneuploidy rate. Reprod. Biomed. Online, 18, 777–783. [Medline] [CrossRef]

10) Angell, R.R., Xian, J., Keith, J., Ledger, W. and Baird, D.T. (1994): First meiotic division abnormalities in human oocytes: mechanism of trisomy formation. Cytogenet. Cell Genet., 65, 194–202. [Medline] [CrossRef]

11) Hassold, T., and Hunt, P. (2001): To err (meiotically) is human: the genesis of human aneuploidy. Nat. Rev. Genet., 2, 280–291. [Medline] [CrossRef]

12) Emery, B.R., Wilcox, A.L., Aoki, V.W., Peterson, C.M. and Carrell, D.T. (2005): In vitro oocyte maturation and subsequent delayed fertilization is associated with increased embryo aneuploidy. Fertil. Steril., 84, 1027–1029. [Medline] [CrossRef]

13) Yamanaka, M., Setoyama, T., Igarashi, Y., Kurosawa, K., Itani, Y., Hashimoto, S., Saitoh, K., Takei, M. and Hirabuki, T. (2006): Pregnancy outcome of fetuses with trisomy 18 identified by prenatal sonography and chromosomal analysis in a perinatal center. Am. J. Med. Genet. A., 140, 1177–1182. [Medline] [CrossRef]

14) Zenzes, M.T., and Casper, R.F. (1992): Cytogenetics of human oocytes, zygotes, and embryos after in vitro fertilization. Hum. Genet., 88, 367–375. [Medline] [CrossRef]

15) Ocaña-Quero, J.M., Pinedo-Merlín, M. and Moreno-Millán, M. (1999): Influence of follicle size, medium, temperature and time on the incidence of diploid bovine oocytes matured in vitro. Theriogenology, 51, 667–672. [Medline] [CrossRef]

16) Sosnowski, J., Waroczyk, M. and Switonski, M. (2003): Chromosome abnormalities in secondary pig oocytes matured in vitro. Theriogenology, 60, 571–581. [Medline] [CrossRef]

17) A'arabi, S.Y., Roussel, J.D. and Chandler, J.E. (1997): Chromosomal analysis of mammalian oocytes matured in vitro with various culture systems. Theriogenology, 48, 1173–1183. [Medline] [CrossRef]

18) Roberts, R., Iatropoulou, A., Ciantar, D., Stark, J., Becker, D.L., Franks, S. and Hardy, K. (2005): Follicle-stimulating hormone affects metaphase I chromosome alignment and increases aneuploidy in mouse oocytes matured in vitro. Biol. Reprod., 72, 107–118. [Medline] [CrossRef]

19) Xu, Y.W., Peng, Y.T., Wang, B., Zeng, Y.H., Zhuang, G.L. and Zhou, C.Q. (2011): High follicle-stimulating hormone increases aneuploidy in human oocytes matured in vitro. Fertil. Steril., 95, 99–104. [Medline] [CrossRef]

20) Shioya, Y., Kuwayama, M., Fukushima, M., Iwasaki, S. and Hanada, A. (1988): In vitro fertilization and cleavage capability of bovine follicular oocytes classified by cumulus cells and matured in vitro. Theriogenology, 30, 489–496. [Medline] [CrossRef]

21) Murakawa, H., Aono, N., Tanaka, T., Kikuchi, H., Yoshida, H., Yoshida, H., Yokoo, M. and Abe, H. (2009): Morphological Evaluation and Measurement of the Respiration Activity of Cumulus-oocyte Complexes to Assess Oocyte Quality. J Mamm Ova Res., 26, 32–41. [CrossRef]

22) Yoshizawa, M., Takada, M. and Muramatsu, T. (1989): Incidence of chromosomal aberrations and primary sex ratio in first-cleavage mouse eggs. J Mamm Ova Res., 6, 119–125.

23) Moon, J.H., Jee, B.C., Ku, S.Y., Suh, C.S., Kim, S.H., Choi, Y.M., Kim, J.G. and Moon, S.Y. (2005): Spindle positions and their distributions in in vivo and in vitro matured mouse oocytes. Hum. Reprod., 20, 2207–2210. [Medline] [CrossRef]

24) Wang, W.H., and Keefe, D.L. (2002): Prediction of chromosome misalignment among in vitro matured human oocytes by spindle imaging with the PolScope. Fertil. Steril., 78, 1077–1081. [Medline] [CrossRef]

25) Zelinski-Wooten, M.B., Hutchison, J.S., Hess, D.L., Wolf, D.P. and Stouffer, R.L. (1995): Follicle stimulating hormone alone supports follicle growth and oocyte development in gonadotrophin-releasing hormone antagonist-treated monkeys. Hum. Reprod., 10, 1658–1666. [Medline]

26) Brower, P.T., and Schultz, R.M. (1982): Intercellular communication between granulosa cells and mouse oocytes: existence and possible nutritional role during oocyte growth. Dev. Biol., 90, 144–153. [Medline] [CrossRef]

27) Sutton, M.L., Gilchrist, R.B. and Thompson, J.G. (2003): Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. Hum. Reprod. Update, 9, 35–48. [Medline] [CrossRef]

28) Albertini, D.F., Combelles, C.M., Benecchi, E. and Carabatsos, M.J. (2001): Cellular basis for paracrine regulation of ovarian follicle development. Reproduction, 121, 647–653. [Medline] [CrossRef]

29) Park, J.Y., Su, Y.Q., Ariga, M., Law, E., Jin, S.L. and Conti, M. (2004): EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science, 303, 682–684. [Medline] [CrossRef]

30) Biggers, J.D., Whittingham, D.G. and Donahue, R.P. (1967): The pattern of energy metabolism in the mouse oöcyte and zygote. Proc. Natl. Acad. Sci. USA, 58, 560–567. [Medline] [CrossRef]

31) Cekleniak, N.A., Combelles, C.M., Ganz, D.A., Fung, J., Albertini, D.F. and Racowsky, C. (2001): A novel system for in vitro maturation of human oocytes. Fertil. Steril., 75, 1185–1193. [Medline] [CrossRef]

32) Rieger, D., and Loskutoff, N.M. (1994): Changes in the metabolism of glucose, pyruvate, glutamine and glycine during maturation of cattle oocytes in vitro. J. Reprod. Fertil., 100, 257–262. [Medline] [CrossRef]

33) Saito, T., Hiroi, M. and Kato, T. (1994): Development of glucose utilization studied in single oocytes and preimplantation embryos from mice. Biol. Reprod., 50, 266–270. [Medline] [CrossRef]

34) Zhou, X.L., Teng, Y., Cao, R., Fu, H., Xiong, K., Sun, W.X., Zhu, C.C., Huang, X.J., Xiao, P. and Liu, H.L. (2013): Rescue from dominant follicle atresia by follicle-stimulating hormone in mice. Genet. Mol. Res., 12, 2945–2952. [Medline] [CrossRef]