IBBJ

Autumn 2021, Vol 7, No 4

Original Article

The Effect of Retinoic Acid on in Vitro Maturation of Mouse Immature Oocytes: An Experimental Study

Seyede Zahra Talebian Moghadam[1], Seyed Gholam Ali Jorsaraei[2]*, Ramzan Khanbabaei[1]*, Maryam Gholamitabar Tabari[3], Ali Asghar Ahmadi[2, 4]

1. *Department of Biology, Qaemshahr, Branch, Islamic Azad University, Qaemshahr, Iran.*
2. *Infertility and Reproductive Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran.*
3. *Health Reproductive Research Center, Sari Branch, Islamic Azad University, Sari, Iran.*
4. *North Research Center, Pasteur Institute of Iran, Amol, Iran.*

Submitted 23 Sep 2021; Accepted 27 Oct 2021; Published 14 Dec 2021

Various culture media along with some supplements and enrichment materials have been suggested for in vitro maturation. This study aims to investigate the effect of retinoic acid on nuclear maturation of immature mouse oocytes in vitro. Immature oocytes were obtained from 6-8 weeks old NMRI mice, 48 hours after intraperitoneal injection of 7.5 units of PMSG. The collected oocytes were divided into two groups: 1. the control group including the base medium 2. The experimental group including base medium supplemented with 2 µM retinoic acid. 100 oocytes (50 GV oocytes and 50 MI oocytes) were considered for each group. The oocytes of each group were cultured in incubator with 5% CO2 at 37 ºC for 24 hours. The percentage of immature GV and MI oocytes that matured into MII oocytes in the experimental and control groups was (50 & 38) and (38 & 28) respectively. Regarding the maturity of GV and MI oocytes in the experimental group, although the percentage of GV oocytes transformed into MII oocytes was higher than MI oocytes 50% versus 38%, this difference was not statistically significant (PV=0. 1). Results of the present study demonstrated that a higher percentage of immature oocytes in the experimental group matured into MII oocytes. Moreover, GV oocytes are more capable to reach in MII stage compared with MI oocytes in vitro.

Keywords: In vitro maturation, Retinoic acid, Germinal Vesicle,Metaphase I

Infertility is a common medical problem in today's world. Infertility rate has increased by 50% worldwide from 1955 until now and currently 20% of couples suffer from this problem (1). There are diagnostic and therapeutic methods that tend to solve this problem and give infertile couples the hope for having children. Polycystic ovarian syndrome is one of the female infertility factors and several studies have been done to solve that. Polycystic ovarian syndrome is a heterogeneous disease and is the most common cause of decreased ovulation in women. It is identified as hyper androgenism, chronic unovulation, oligomenorrhea, amenorrhea, high body mass index, increased LH level and store of small follicles as cysts in the ovaries. Women with this disease including a considerable percentage of infertile women (2, 3).

Sometimes Assistant Reproductive Technologies require increased ovarian stimulation, which causes release of premature oocyte. Because of their

*Correspondence: Ramzan Khanbabaei: Department of Biology, QaemShahr Branch, Islamic Azad University, km-7 Allameh Tavaresi St. (Nezami road), QaemShahr, Mazandaran, Iran. Mobile: +989111133513 E-mail: khanbabaee@gmail.com
Seyed Gholam Ali Jorsaraei: Infertility and Reproductive Health Research Center, Health Research Institute, Babol University of Medical Sciences, Babol, Iran. Mobile: +989113142743 E-mail: alijorsara@yahoo.com

high value for patients, it is need to mature them in the laboratory through maturation process, it may led to createspoor quality oocytesin these patients (4, 5). Therefore, it is necessary to use different in vitro maturation methods that have ability to enhance of high quality oocytes for fertilization and embryogenesis (6). Nowadays, induction of oocyte maturation in vitro is one of the methods used in the assisted reproductive technology (7). In vitro maturation (IVM) is an advanced in vitro technique in which immature oocytes are retrieved from ovarian antral follicles before their growth is completed in vivo from unstimulated or slightly stimulated ovaries. Then, they are cultured for 24-48 hours from germinal vesicle (GV) stage to completion of Metaphase of Meiosis II with polar body formation. Such a development is followed by numerous scientific and clinical advantages (8). In vitro maturation may be beneficial for infertile people undergoing in vitro fertilization (IVF). Currently, IVF patients are treated by high doses of gonadotropins to obtain more oocytes. However, this method may be exposing them in the risk of ovarian hyper stimulation syndrome (OHSS). In vitro maturation of oocytes reduces the gonadotropin injection in these people and prevents such harmful effects. It also leads to less damage, easier treatment and cost effective for patients (9).

Choosing the appropriate essential medium for IVM is highly important and very difficult. Therefore, some researchers compared the potentials of various culture media (10). Culture media are required to support a long period of in vitro oocyte maturation.Due to the weakness of simple culture media, co-culture is one of the methods used to support embryonic development in cleavage and implantation stage for in vitro production of animal and even human. Some of the advantages of the co-culture system include secretion of growth-stimulating factors such as nutrients and various substrates, growth factors and cytokines as well as removal of toxins from the culture medium by cells present in the co-culture system (11). According to the results of various studies, we can affect immature oocytes by some certain factors to induce them into maturation. This can be used to increase the chance of success for assisted reproductive techniques. The co-culture factors were usedin several studies to mature the immature oocytes (12). Modifications used in the medium to increase IVM Success, such as the use of retinoids, which is a large family of natural compounds, are very close to vitamin A (All-Trans Retinoid) (13). The all-trans type retinoic acid is the most important retinoid in vertebrate embryogenesis (14). This retinoic and 9-cis are the important regulators of embryonic development and cellular activity (15). Mohan et al. reported that most nuclear retinoic acid receptors including Retinoic Acid Receptors (RAR [α, β]) and Retinoic X Receptors (RXR [α, β]), retin aldehyde dehydrogenase and peroxisome proliferator were detected in bovine oocytes, cumulus cells and embryos (16, 17). The all-trans retinoic acid increases the activity of RAR receptor. This receptor has the ability to bind to a specific sequence of DNA called ‘factors of retinoic acid receptor’ it is need to increase or decrease gene expression and induce differentiation in many cell systems (14). Since RA acts to establish or change the pattern of gene expression oncells and because of having antioxidant properties, it can affect cytoplasmic maturation and ovulation capacity for growth and development. Therefore, this research studies the effect of 2 μM retinoic acid in meiotic maturation on immature oocytes in vitro.

Materials and methods

Animals

This study was conducted using 20 NMRI mice with 6-8 weeks old (25-30 g). These animals were kept and treated under controlled conditions (12 hours of light and 12 hours of dark) with adequate food ad libitum in the animal house of Babol University of Medical Sciences. The mice were stimulated for more ovulation through intraperitoneal injection of 7.5 units of PMSG

(Pregnant Mare's Serum Gonadotropin). 48 hours later, the animals were scarified using cervical dislocation. The ovaries were removed from the body under sterile conditions and were placed in a previously incubated culture medium (including α-MEM and penicillin + streptomycin + 5% FBS). Then, the additional fat around the ovary was removed and the ovary was transferred to a clean drop.The cumulus-oocyte complexes (COCs) of germinal vesicle (GV) and MI were taken using insulin needles. The denuded oocytes were obtained from COCs through rapid pipetting technique and the surrounding cumulus cells were removed using a small pipette (slightly larger than the diameter of the egg).

The study groups

The immature oocytes (GV and MI) collected from several female mice were randomly divided into two the study groups. MI and GV oocytes were collected and cultured for at least 10 times in each group.

1-**The experimental group:** The experimental group included the minimum essential medium alpha (α-MEM, 100 mIU/ml FSH, 7.5 IU/ml hCG and 100 µg/ml penicillin + 50 µg/ml streptomycin + 5% FBS) enriched with 2 µM retinoic acid. The all-trans retinoic acid was solved in 0.2% ethanol (v/v) and was kept in Eppendorf covered with aluminum foil in a freezer at -20 ºC. (18).

2- **The control group:** The control group included the minimum essential medium alpha (α-MEM, 100 mIU/ml FSH, 7.5 IU/ml hCG and 100 µg/ml penicillin + 50 µg/ml streptomycin + 5% FBS). We use 100 immature oocyte (50 GV and 50 MI) per group.

In vitro maturation of oocytes

After separation of oocytes from cumulus cells, denuded oocytes were transferred to small drops (10 µL) to be differentiated from MI and GV stage. The MI and GV oocytes were detected and separated using an inverted microscope. Then, the clean and healthy oocytes were transferred to the culture medium. About 5 oocytes were placed in a 30 µL droplet for in vitro maturation. GV oocytes with spherical shape, clear cytoplasm and transparent layer of zonapellucida with the perivitelline space and MI oocytes with broken nucleus with the beginning of meiosis were selected and cultured in two groups as healthy oocytes. They were cultured in incubator with 5% CO2 at 37 ºC for 24 hours. Morphologic changes in the nucleus were evaluated with the release of the first polar body

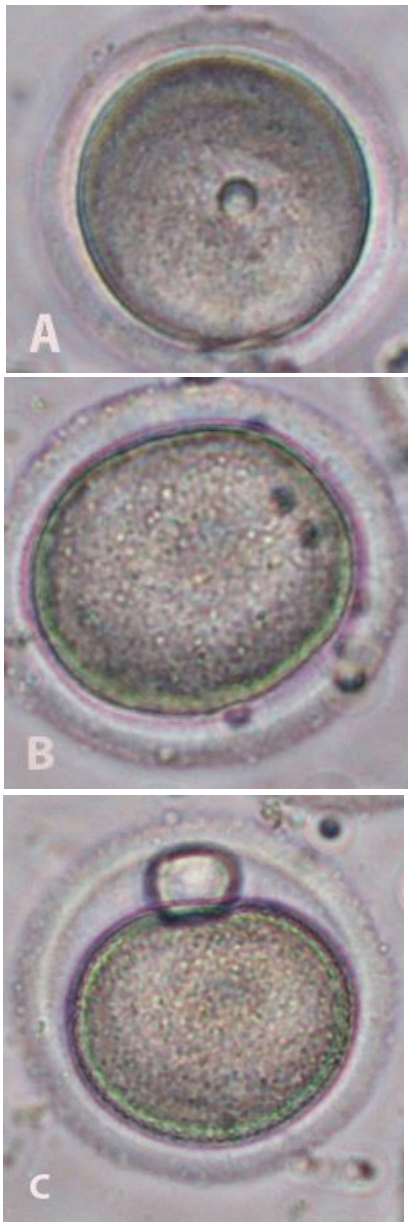

Figure 1. Mouse oocyte maturation stages: **A:** Germinal Vesicle ‹**B:** Metaphase I orGerminal Vesicle Break Down, **C** : Metaphase II.

metaphase II (MII) as a measure of nuclear maturation of immature mouse oocytes (Figure1).

Statistical analysis

The proportion of immature oocytes reaching MII stage was compared among the two groups using t-test. Correlation test were also used to evaluate the successful rate of IVM oocytes. The statistical analysis was accomplished using the Statistical Package for the Social Sciences version 22 (SPSS). A value of P<0.05 was considered statistically significant.

Results

To study the maturation of immature oocytes, they were divided into two groups: 1. GV and 2. MI and were then compared. The investigations in the experimental and control groups revealed the following results. In the immature GV oocytes group, the percentage of immature GV oocytes that matured into MII oocytes in theexperimental and control groups was 50% and 38%, respectively. Although the percentage of GV oocytes matured in the experimental group was higher, the difference was not statistically significant (PV=0. 5). The resumption of meiosis or oocyte maturation from GV stage to MI stage was 12 and 24% in the experimental and control groups, respectively and the difference between the two groups was not significant (PV=0.09). This result shows that higher percentage of oocytes in the experimental group that passed this stage and rich to MII oocytes. Moreover, the ratio of oocytes that stayed in GV stage was 18 and 14% in the experimental and control groups,

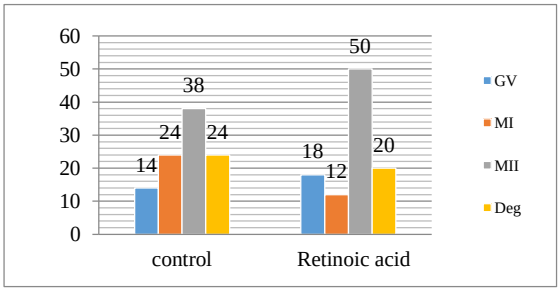

Figure 2. Comparing the maturation of immature oocytes in the group of immature GV oocytes based on percentage.

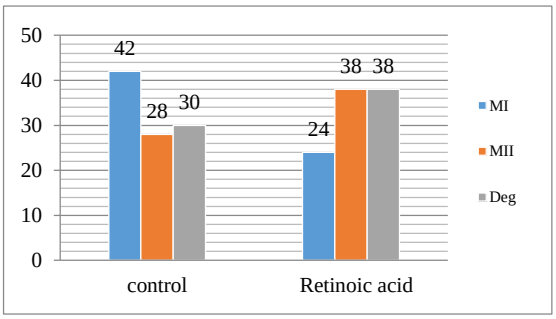

Figure 3. Comparing the maturation of immature oocytes in the group of immature MI oocytes based on percentage.

respectively. This difference was not statistically significant (PV=0.5). In addition, the degeneration of immature GV oocytes in the experimental and control group was 20% and 24%, respectively. This result shows that higher percentage of oocytes was degenerated in control group. However, the difference was not statistically significant (PV=0.4) (Figure 2).

In the immature MI oocytes group, the percentage of immature MI oocytes that matured and transformed into MII oocytes in the experimental and control groups was 38% and 28%, respectively. Although the percentage of MI oocytes matured in the experimental group was higher, the difference was not statistically significant (PV=0.6). Moreover, the ratio of oocytes that stayed in MI stage was 24% and 42% in the experimental and control groups, respectively. This results shows that higher percentage of oocytes stayed in MI stage in control group, However, this difference was not statistically significant (PV=0.2). In addition, the degeneration of immature MI oocytes in the experimental and control group was 38 and 30%, respectively. This difference was not statistically significant (PV=0.5) (Figure 3).

Discussion

Results of this study demonstrated that retinoic acid is competent to improve oocyte maturation, growth and development of mouse immature oocytes. The number of matured oocytes increased in the experimental group compared with control group. Therefore, this study aims to present an

appropriate mouse model with the capacity of in vitro meiosis maturation of oocytes, so that the results of this study can be generalized to fertility and infertility centers. Retinoids, including retinoic acid (RA), are metabolites of vitamin A and are among the essential factors in the fertility of men and women (18). It was previously demonstrated that vitamin A participates in regulating the growth and development of vertebrates, cell differentiation and tissue function (19). The all-trans type is the most important retinoid in vertebrate embryogenesis. The differentiation caused by retinoid is associated with specific changes in the expression of homeobox genes, growth factors and their receptors (20). The effect of retinoids on the bovine immature oocytes to improve the cytoplasmic and nuclear maturation of oocytes has been investigated in recent years. Retinoic acid enrichment before oocyte maturation, caused embryo development and growth (18). Investigating the effects of retinoic acid on in vitro bovine oocyte maturation, Vahidi et al. demonstrated that 1 µM retinoic acid increases bovine oocyte maturation in TCM199 culture medium (21). Nasiri et al. investigated the effect of retinoic acid on in vitro maturation and fertilization at GV stage and demonstrated that all-trans retinoic acid increases in vitro maturation of mouse oocytes and improves fertility in a dose-dependent way (22). Shuangliang et al. showed that the effects of retinoid metabolites in nuclear maturation, cytoplasmic maturation and gene expression of canine oocytes during in vitro maturation. He demonstrated that 5nM 9-cis all-trans retinoic acid has positive effects on nuclear and cytoplasmic maturation of canine oocytes and has significant effect on in vitro maturation of oocytes (23). Adding antioxidant during IVM to improve oocyte maturation rate has been reported in a wide range of species. In vitro culture system produces more oxygen concentration compared with in vivo culture. Reactive oxygen species (ROS) including superoxide (O2-), hydroxyl radical (OH) and hydrogen peroxide (H2O2) anions are created in cell through normal chemical pathways and all of them have the ability to react with membranes, nucleic acids, enzymes and other small cells (24). Excessive amounts of ROS create oxidative stress and may damage molecular structure of oocyte and granulosa cells. ROS accelerates oocyte aging and decreases oocyte quality (25). Manipulation of oocytes and embryos during in vitro maturation and in vitro fertilization are with the risk of exposure to high levels of activated ROS samples such that aspiration of oocytes, fertilization and embryo culture in high-density oxygen can produce higher levels of free radicals (26). Since during in vitro oocyte and embryo culture, the level of antioxidant is lower than in vivo culture, the defense mechanisms of oocyte may not be enough to protect its delicate structure in such condition (26). To protect the oocyte and embryo from oxidative stress, the use of antioxidants in culture medium can prevent the overproduction of ROS (27). Adding antioxidants alone is not enough to prevent the destructive effects of ROS. Selecting the right antioxidant and its concentration is also important (25). Retinoids, as antioxidant agents, increases the antioxidant activity in the cell (28). Guerin et al. reported that retinoids are necessary for oocyte maturation, since they maintain the intrinsic growth of oocytes and the level of antioxidants (29).

According to the results of Ahlemeyer et al. retinoic acid plays a role in preventing oxidative stress in cell. In living cells, the all-trans retinoic acid is attached to retinoic acid receptors and its isomer, 9-cis retinoic acid, is specifically attached to retinoid X receptors. However, 9-cis retinoic acid activates both RXR and RAR under in vitro conditions. The beneficial effects of RA in bovine oocytes, particularly in cytoplasmic competence after IVM and embryo quality and development are proved. As important components of IVM medium, retinol metabolites are suggested to improve cytoplasmic maturation and embryo development (30).

Tahaei et al demonstrated that co-culture of

retinoic acid and granulosa cells during in vitro maturation increases oocyte meiosis resumption and MII formation and also increases the blastocyst stage from 2 cells mouse embryo (31). The oviduct granulosa cells are the appropriate physiological instance for co-culture, since they are naturally exist in developing follicles and because of their regulatory effect on oocyte surrounding area, play an important role in the process of oocyte maturation (32). Steroidogenesis is the main role of granulosa cell. Cumulus cells contain retinoic acid receptors and therefore, all-trans retinoic acid is able to regulate transcription in granulosa cells (33). The role of retinoic acid in maturation of immature oocytes is not clearly specified yet. However, we know that it directly or indirectly improves the ability of nucleus and cytoplasm and thus increases the growth and development ability of oocyte to reach the next stage of growth (34).

Conclusion

This research demonstrated that adding 2 µM retinoic acid to culture medium created more optimal conditions for the growth of immature mouse oocytes. Therefore, it is possible that retinoic acid can secrete some of the factors that support oocyte maturation in the culture medium and improve oocyte maturation. Moreover, according to the results of this study regarding GV and MI immature oocytes, the development rate of GV oocytes to MII oocytes was more than MI oocytes. Therefore, GV oocytes are more capable to develop and transform into MII oocytes in laboratory conditions.

Acknowledgement

This article is the result of a Master's thesis from Islamic Azad University, Ghaemshahr, Iran. Hereby, we express our deepest sense of gratitude and indebtedness to Officials of Fertility & Infertility Research Center, Health Research Institute and Babol University of Medical Sciences.

Conflict of interest

The authors declared no conflict of interest.

References

1. Oliva A, Spira A, Multigner L. Contribution of environmental factors to the risk of male infertility. Hum Reprod. 2001;16(8):1768-76.

2. Franks S. Medical progress polycystic ovary syndrome. New Eng J Med. 1995;333(21):1435-.

3. Futterweit W. Pathophysiology of polycystic ovarian syndrome. Androgenic disord. 1995:77-166.

4. Krisher R. The effect of oocyte quality on development. J Anim Sci. 2004;82(13_suppl):E14-E23.

5. Trounson A, Wood C, Kausche A. In vitro maturation and the fertilization and developmental competence of oocytes recovered from untreated polycystic ovarian patients. Fertil Steril. 1994;62(2):353-62.

6. Chanson A, Nocera D, Senn A, De Grandi P, Germond M. Animal Experiece: Development of a well-defined medium for the in vitro maturation of immature bovine cumulus- oocyte complexes. J Assist Reprod Genet. 2001;18(2):97-105.

7. Wani N. In vitro maturation and in vitro fertilization of sheep oocytes. Small Rumin Res. 2002;44(2):89-95.

8. Chian R-C, Lim J-H, Tan S-L. State of the art in in-vitro oocyte maturation. Curr Opin Obstet Gynecol. 2004;16(3):211-9.

9. Hardy K, Wright CS, Franks S, Winston RM. In vitro maturation of oocytes. Br Med Bull. 2000;56(3):588-602.

10. Heidari M, Malekshah A, Azami N, Mirhoseini S. In vitro maturation of mouse preantral follicles in two culture media: a comparative study. Ann Biol Res. 2012;3(10):4937-41.

11. Nasr-Esfahani MH, Moulavi F, Hosseini SM, Hajian M, Shahverdi A. The effect of vero cell co-culture on in vitro maturation of bovine immature oocytes and further embryo development. Cell J (Yakhteh). 2008;9(4):240-7.

12. Lin Y-H, Hwang J-L, Seow K-M, Huang L-W, Chen H-J, Tzeng C-R. Effects of growth factors and granulosa cell co-culture on in-vitro maturation of oocytes. Reprod Biomed Online. 2009;19(2):165-70.

13. Duque P, Diez C, Royo L, Lorenzo P, Carneiro G, Hidalgo C, et al. Enhancement of developmental capacity of meiotically inhibited bovine oocytes by retinoic acid. Hum Reprod. 2002;17(10):2706-14.

14. Morriss-Kay G, Wardt S. Retinoids and mammalian development. Int Rev Cytol. 1999;188:73-131.

15. Berisha B, Sinowatz F, Schams D. Expression and localization of fibroblast growth factor (FGF) family members

during the final growth of bovine ovarian follicles. Mol Reprod Dev. 2004;67(2):162-71.

16. Mohan M, Thirumalapura NR, Malayer J. Bovine cumulus-granulosa cells contain biologically active retinoid receptors that can respond to retinoic acid. Reprod Biol Endocrinol. 2003;1(1):104.

17. Mohan M, Malayer J, Geisert R, Morgan G. Expression of retinol-binding protein messenger RNA and retinoic acid receptors in preattachment bovine embryos. Mol Reprod Dev. 2001;60(3):289-96.

18. Eimani H, Tahaei L, Parivar K, Rezazadeh M, Kazemi S, Shahverdi A. The effect of granulosa cells co-culture and retinoic acid on maturation and development of immature mouse oocytes in vitro. J Iran Anat Sci. 2007;5(19):137-46.

19. Whaley S, Hedgpeth V, Farin C, Martus N, Jayes F, Britt J. Influence of vitamin A injection before mating on oocyte development, follicular hormones, and ovulation in gilts fed high-energy diets. J Anim Sci. 2000;78(6):1598-607.

20. Lelièvre-Pégorier M, Vilar J, Ferrier M-L, Moreau E, Freund N, Gilbert T, et al. Mild vitamin A deficiency leads to inborn nephron deficit in the rat. Kidney Int. 1998;54(5):1455-62.

21. Vahedi V, Zeinoaldini S, Kohram H, Farahavar A. Retinoic acid effects on nuclear maturation of bovine oocytes in vitro. Afr J Biotechnol. 2009;8(16).

22. Nasiri E, Mahmoudi R, Bahadori MH, Amiri I. The effect of retinoic acid on in vitro maturation and fertilization rate of mouse germinal vesicle stage oocytes. Cell J (Yakhteh). 2011;13(1).

23. Reinblatt SL, Son W-Y, Shalom-Paz E, Holzer H. Controversies in IVM. J Assist Reprod Genet. 2011;28(6):525.

24. Domej W, Oettl K, Renner W. Oxidative stress and free radicals in COPD–implications and relevance for treatment. Int J Chron Obstruct Pulmon Dis. 2014;9:1207.

25. Golkar-Narenji A, Eimani H, Samadi F, Hasani S, Shahverdi AH, Eftekhari-Yazdi P, et al. Effect of Papaver rhoeas extract on in vitro maturation and developmental competence of immature mouse oocytes. Reprod Med Biol. 2010;9(4):211-5.

26. Pfeifer N, Baston-Büst D, Hirchenhain J, Friebe-Hoffmann U, Rein D, Krüssel J, et al. Selection of the in vitro culture media influences mRNA expression of Hedgehog genes, Il-6, and important genes regarding reactive oxygen species in single murine preimplantation embryos. Sci World J. 2012;2012.

27. Cetica P, Pintos L, Dalvit G, Beconi M. Antioxidant enzyme activity and oxidative stress in bovine oocyte in vitro maturation. IUBMB Life. 2001;51(1):57-64.

28. Livingston T, Eberhardt D, Godkin J, editors. The effects of retinol on bovine embryo development in vitro. Biol Reprod; 2002: SOC Study Reproduction 1603 Monroe ST, Madison, WI 53711-2021 USA.

29. Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update. 2001;7(2):175-89.

30. Ahlemeyer B, Bauerbach E, Plath M, Steuber M, Heers C, Tegtmeier F, et al. Retinoic acid reduces apoptosis and oxidative stress by preservation of SOD protein level. Free Radic Biol Med. 2001;30(10):1067-77.

31. Tahaei LS, Eimani H, Yazdi PE, Ebrahimi B, Fathi R. Effects of retinoic acid on maturation of immature mouse oocytes in the presence and absence of a granulosa cell co-culture system. J Assist Reprod Genet. 2011;28(6):553-8.

32. Byskov AG, Andersen CY, Hossaini A, Guoliang X. Cumulus cells of oocyte-cumulus complexes secrete a meiosis-activating substance when stimulated with FSH. Mol Reprod Dev. 1997;46(3):296-305.

33. Carolan C, Lonergan P, Monget P, Mermillod P. Role of insulin family of growth factors in bovine oocyte maturation (IVM) and embryo development (IVC) in vitro. J Reprod Fertil. 1995;15:71.

34. Lima P, Oliveira M, Santos M, Reichenbach H-D, Weppert M, Paula-Lopes F, et al. Effect of retinoids and growth factor on in vitro bovine embryos produced under chemically defined conditions. Anim Reprod Sci. 2006;95(3):184-92.