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Original Article

The Effect of follicular fluid on in Vitro Maturation of Mouse Immature Oocytes

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In vitro maturation is a highly effective method for achieving mature. Oocytes to treat infertility based on in vitro methods (IVF-ICSI). This technique is effective for treating women who suffer from polycystic ovary syndrome (PCO) and cancer. 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 25% human follicular fluid.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% CO_2 at 37 ºC for 24 hours. Although the resumption of meiosis in the experimental group was more than the control group (44 and 33%, respectively), the difference was not statistically significant (PV>0.05). Moreover, among immature oocytes, the development of GV oocytes to mature oocyte was more than MI oocytes. 50%, 38% of GV oocytes and 28%, 38% of MI oocytes, respectively in the experimental and control groups reached to mature oocyte. Results of the present study demonstrated that a higher percentage of immature oocytes in the experimental group matured in to MII oocytes. It seems that adding follicular fluid to the culture media has positive effects on oocyte maturation. Moreover, GV oocytes are more capable to reach in MII stage compared with MI oocytes in vitro.

Keywords: In vitro maturation, follicular fluid, Germinal Vesicle

Infertility is observed in about 10 to 15% of couples who decide to conceive (1). Infertility is treated through various methods and techniques called Assisted Reproductive Techniques (ARTs) (2). In vitro maturation (IVM), in vitro fertilization (IVF) and embryo transfer are the major assisted reproductive techniques (3). IVF is the most common assisted reproductive technique for infertility treatment (4). Based on this technique, gonadotropin is daily injected to provide growing antral follicles. The medicines used in IVF lead to ovarian stimulation and subsequently, simultaneous production and growth of several follicles. Some women are sensitive to these medicines including women with polycystic ovary syndrome (PCO), who are at risk for ovarian hyper stimulation syndrome (ohss) (5). Thus for these patients, we need to use a technique to collect the oocytes and mature them in vitro. The best and the most reliable method is retrieval of immature oocytes through IVM (6). So far, women with polycystic ovary syndrome constitute most patients who experience in vitro maturation of oocytes (7). IVM is an advanced in vitro technique in which the immature oocytes are

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obtained from ovarian antral follicles and unstimulated ovaries or ovaries with minimal stimulation before in vivo growth. The immature oocytes are cultured for 24-48 from Germinal Vesicle (GV) stage until the second cell division in Meiosis is complete and the polar body is formed (7).

Since late 1985, a great deal of researches on the development of in vitro maturation (IVM) shifted from GV stage to telophase I (T1). Currently, IVM is used as a modern assisted reproduction technique to increase fertility and is accompanied by several advantages (8, 9). Due to technological reasons such as long period of culture and difficulty of separating follicles, the IVM technique develops slowly (10). One of the major problems of IVM development is inadequate development of oocyte cytoplasm during different stages of growth. Most researchers attribute this problem to inappropriate culture media in which the oocyte develops (11). These media could not provide the necessary and appropriate conditions for the growth of the oocyte. Even some culture media may create a disorder called “Large offspring syndrome”, which is caused by stressors in the culture media (12). Therefore, one of the research priorities of IVM is investigating various media culture and their effect in the maturation and development of embryo. We don’t yet know exactly why co-culture supports in vitro maturation. However, there is a possibility that the co-cultured cells secrete growth factors or other compounds that improve oocyte (13). One of the appropriate natural culture media for IVM is the follicular fluid, mostly used in IVF labs. Follicular fluid is the main female genital tract secretion with a combination similar to serum and plasma (14). Peptides, proteins and phospholipid fatty acids of follicular fluid are appropriate media for the development of oocyte, which can contribute to fertilization and fertility in ART (15, 16). It has been reported that follicular fluid can act as culture media for embryo growth, granulosa cells and maybe somatic cells and we can prevent cessation of development in the 2-cell stage embryo using follicular fluid along with granulosa cells; maybe there are materials in follicular fluid that act as synergist with granulosa cells (14). Reports indicate that follicular fluid may increase in vitro maturation of horse oocyte, yet has no effect on IVF (17). Moreover, compared with other supplements added to the culture medium, bovine follicular fluid significantly increases the mitochondrial activity of oocytes and this effect is directly related to intracellular ATP content. In addition, this fluid considerably increases fertilization in the oocytes selected for IVF (18). On the other hand, it has been reported that the presence of cumulus cells and follicular fluid in culture media accelerates and increases acrosin(14). Therefore, considering the role of human follicular fluid in maturation and development of oocyte, this study aims to analyze the effect of human follicular fluid on oocyte maturation as a supplement added to the culture medium compared with conventional culture media.

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

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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 (100 immature oocytes in each group) 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 25% human follicular fluid (14). 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.

Preparation of follicular fluid

Follicular fluid were prepared from women undergoing ovarian puncture for fertility treatment using IVF or ICSI techniques in Fatemeh Zahra Infertility Center in Babol. Stimulating the growth of follicles in these patients was done using HCG after administration of GnRH-a. Follicular fluid samples containing follicles with 1 mature and healthy oocyte were analyzed. Immediately after isolating oocyte, all samples were centrifuged at 2000 rpm for 15-20 minutes. Then, the follicular fluid supernatant, was heated at 56 ˚C for 30 minutes (to deactivate the enzymes and toxins found in follicular fluid) in water bath and was kept in the refrigerator (at 4 ˚C) until the time of use (one week maximum) (14).

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. These media were cultured in humid weather with 5% CO2 at 37 ˚C. After 24 hours, the maturation stage was analyzed by inverted microscope and changes in nuclear morphology were considered by the release of the first polar body, which is a criteria for nuclear maturation of immature oocytes. Oocytes without deformation of the nucleus were identified as immature oocytes (GV), oocytes with broken nucleus were identified as oocytes of GVBD stage at the beginning of meiotic division and oocytes with polar body were identified as matured oocytes or metaphase II (Figure 1).

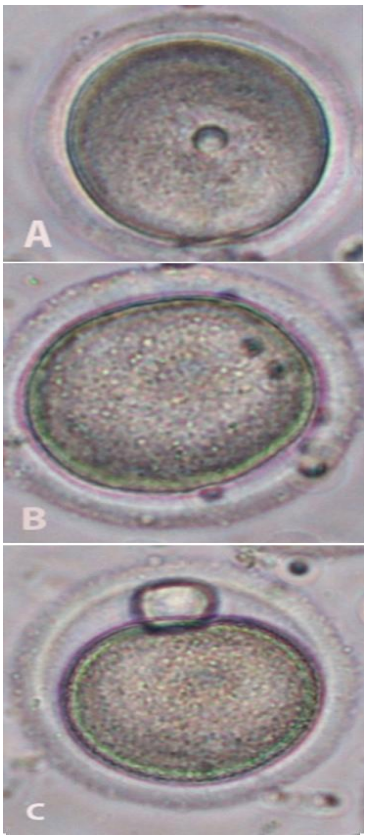

Figure 1. Mouse oocyte maturation stages: A: Germinal Vesicle ،B: Metaphase I orGerminal Vesicle Break Down, C: Metaphase II.

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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 oocytes, the immature oocytes were divided into two groups of GV, MI and the two groups were then compared while the following results were achieved:

In the group of GV immature oocytes, the number of immature oocytes that matured and developed to MII oocytes were respectively 50 and 38% in the experimental and the control groups. Although a higher percentage of GV oocytes matured in the experimental groups, the difference was not statistically significant (PV>0.05). The resumption of meiosis or the maturation of GV oocytes to MI stage were 28 and 24% in the experimental group and the control group, respectively, while the difference was not statistically significant (PV>0.05). Although this difference was not statistically significant, more oocytes reached MI stage in the experimental group. Moreover, the proportion of oocytes that stated in GV stage was 8 and 14% in the experimental and the control group, respectively. This result shows that the percentage of oocytes that stayed in GV stage was more in control group, but the difference was not statistically significant (PV>0.05). The degeneration of immature GV oocytes was 14 and 24% in the experimental and the control group, respectively. This result shows that the percentage of the degenerated oocytes was more in control group, but the difference was not statistically significant (PV>0.05) (Figure 2).

In the group of MI immature oocytes, the percentage of MI immature oocytes developed to

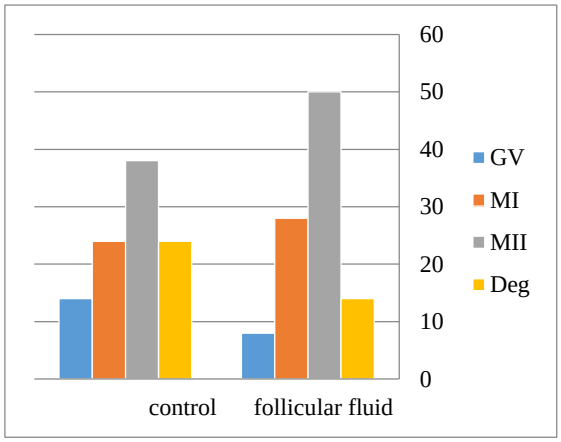

Figure 2. Comparing the maturation of immature oocytes in the group of immature GV oocytes based on percentage.

MII oocytes were 38 and 28% in the experimental and control groups, respectively. Although higher percentage of MI oocytes matured in the experimental group, the difference was not statistically significant (PV>0.05). Moreover, the proportion of oocytes that stayed in MI stage was 36 and 42% in the experimental and control groups, respectively, indicating that the percentage of oocytes that stayed in MI stage was more in control group, but the difference is not statistically significant (PV>0.05). The degeneration of immature MI oocytes was 26 and 30% in the experimental and the control group, respectively. This result shows that the percentage of the degenerated oocytes was more in control group, but the difference was not statistically significant (PV>0.05) (Figure 3).

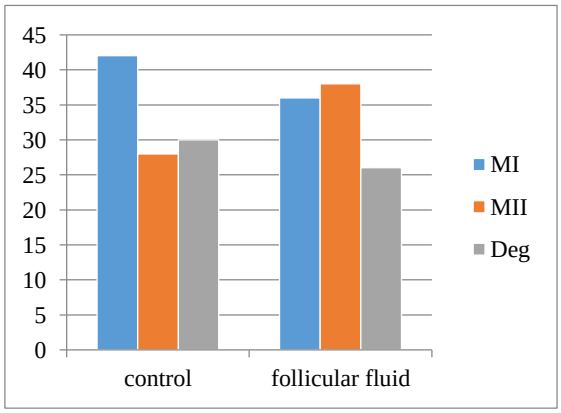

Figure 2. Comparing the maturation of immature oocytes in the group of immature MI oocytes based on percentage.

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Discussion

Result of this study demonstrated that higher percentage of immature oocytes in the experimental group developed to MII oocytes. Therefore, heated human follicular fluid can increase the maturation of rat oocytes in vitro.

The follicular fluid contains all raw materials, such as essential ions and the energy source for cell growth (13). The follicular fluid provides the conditions for growth and maturation of oocyte during follicle growth (18). The follicular fluid is created around follicle more than blood plasma, which exudates through follicular basement membrane and accumulates in follicle cavity. This fluid also contains specific components such as steroids, which are secreted by granulosa cells. Therefore, the follicular fluid contains growth factors, hormones and various nutrients for the growth and maturation of oocytes (19). The ability of oocytes increases through hormonal regulation, which leads to final oocyte maturation (20). Therefore, it is one of the major controlling factors of growth and development of follicles, hormones, estrogen and progesterone (21, 22). In addition to steroid hormones and gonadotropins, metabolic factors such as IGF-1 and insulin hormones also participate in follicular development (23). These hormones along with gonadotropins can stimulate growth and development of ovarian oocytes (24). Moreover, IGF-1 and insulin increase the production of estrogen and progesterone (25). IGF-1 concentrations of follicular fluid increase during growth and development of follicles and the growth hormone increases by gonadotropins (26). During the process of follicular development, nutrients and growth factors lead to oocyte maturation due to the secretion of granulosa cells into the follicular fluid (19) and in this respect, it is not much different from a synthetic medium. However, the advantages of granulosa cell culture over synthetic medium include having steroid hormones, growth factors, protein I and II such as insulin-like growth factor inducing the synthesis of steroids, tumor necrosis factor-α, vascular endothelial growth factor, cytokines such as IL-1, IL-6 and prostaglandins (27). Most of these factors have mitogenic effects on somatic cell. Some studies have shown that the follicular fluid can apply its mitogenic effects and stimulate cell proliferation (28).

There has been reports regarding the effect of follicular fluid on the growth of two-cell mice embryos until blastocyst stage. The noteworthy point in these researches is the use of various percentages of follicular fluid and achieving the best development conditions in 100% follicular fluid. This indicates the appropriate and adequate conditions of follicular fluid for embryonic growth (29).

Davoodi et al. investigated the effect of humanfollicular fluid and synthetic serum substitute on embryonic growth and development and cell cleavage using $Hamsf_{10}$ basic culture medium, synthetic serum supplements and follicular fluid. The follicular fluid had more effects on cell cleavage compared with synthetic serum, but the effects on the embryonic development were similar (30).

In his research, Zhang used umbilical cord blood serum and follicular fluid in TCM 199 culture medium and investigated their effects on oocyte maturation and development. He found that these supplements have direct effects on oocyte maturation and development in IVM culture medium and increases pregnancy rate (31).

BudiyantoAgung et al. conducted a study on matured pig oocytes infollicular fluid. Their basic culture medium was follicular fluid plus FSH. They used two different concentrations (2 ml and 3.5 ml) for the medium. Fertilization, pregnancy, 2PN creation, cleavage and blastocyst formation increased in the medium with higher concentration of follicular fluid. This indicates that the concentration of follicular fluid has significant effects on oocyte maturation (32). In the same year, BudiyantoAgung et al. investigated the effect of follicular fluid on IVF and development of pig oocytes and found that follicular fluid is an

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appropriate culture medium for oocyte maturation,sperm penetration, fertilization, pregnancy and cleavage (32). The presence of oxidative stress and the adverse effects of reactive oxygen species (ROS) in vivo or in vitro may reduce the quality of germ cells (33). Although its effect on women's pregnancy is not precisely clear, studies show that the presence of ROS in the culture medium reduces the quality of embryo and increases fragmentation (34). and plays a key role in decreasing the chance of pregnancy (35). We need antioxidants with protective effects to prevent vulnerability and cell damage. The antioxidants can prevent oxidative decomposition in different parts of body tissues. Vitamin E, C and beta-Carotene are among the natural antioxidants with protective effects. Vitamin C is an anti-oxidizing substance. Vitamins, minerals and antioxidant substances (Vitamin A, B, C, E, zinc, selenium and copper) play a key role in the production of sperm and oocytes (36). T.Nakazawa et al. investigated various effects of amino acids in human plasma and follicular fluid on single-cell mouse embryo in vitro and found that the amino acids in follicular fluid has greater effects on growth and culture of single-cell mouse oocytes and embryos compared with other culture media (37). M.E.Dell et al. investigated the composition of the IVM culture medium containing follicular fluid and demonstrated that it has positive effects on fertilization and development of horse oocytes after IVF or ICSI (17). A.Romero Arredondo et al. investigated that effect of follicular fluid on IVF and early embryonic development during cow IVM and concluded that some unknown factors in follicular fluid may have significant effects on increasing the pregnancy rate (38). Moreover, the follicular fluid decreases oocyte degeneration and prepares an appropriate medium for cow oocytes (39).

Conclusion

Results of the present study demonstrated that a higher percentage of immature oocytes in the experimental group matured in to MII oocytes.Overall, adding follicular fluid to the culture media during oocyte maturation affects oocyte schematization and improves the formation of metaphase II oocytes and thus improves nuclear maturation.Therefore, GV oocytes are more capable to develop and transform into MII oocytes in laboratory conditions

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Conflict of interest

The authors declared no conflict of interest.

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