Follicular fluid the best medium of maturation, fertilization and development of immature oocytes

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Abstract

Introduction: In vitro maturation and development of immature oocytes, as an artificial reproduction technique, is useful especially in women who are affected by cancer and polycystic ovary syndrome. Despite using many types of in vitro media, an appropriate environment has not been reported yet. Present study was designed to assess the effect of heated human follicular fluid (hHFF), which is similar to in vivo environment for oocyte, on the maturation and fertilization potential of mouse immature oocytes.

Materials and methods: Healthy female mice, aged 4-6 weeks, were sacrificed via cervical dislocation and their ovaries were extracted under sterile conditions. After washing, the separated immature oocytes were divided into three groups: In the first group, 236 immature oocytes were placed in culture medium contained DMEM, HCG, FSC 25%, and rFSH. In the second group, 229 immature oocytes were put in culture medium contained 100% hHFF. In the third group, 255 immature oocytes were placed in culture medium contained DMEM, HCG, rFSH, and 25% hHFF. Immature oocytes were placed in an incubator for 24 hours. Then, the stages of oocyte maturation were assessed by invert microscope and mature oocytes in each group were transferred to sperm-contained drops. After 24 hr, rate of two-cell embryos was recorded using invert microscope. Data was analyzed by Chi square test.

Results: Maturation rate of oocytes in the second group (87.8%) was significantly higher than first (64.9%) and third (63.2%) groups ($p<0.0005$). The difference between first and third groups was not statistically significant ($P<0.2$). The formation rate of two-cell embryo in the second group (82.1%) was higher than first (50.2%) and third (54.3%) groups ($p<0.002$ and $P<0.01$, respectively).

Conclusion: It seems hHFF could improve in vitro maturation and fertility potential of immature oocytes and consequently the formation rate of two-cell embryos in mice, in comparison with DMEM even supplemented with 25% hHFF.

Keywords: Follicular fluid, maturation, fertilization, oocytes

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Introduction

Activation of the process of meiosis in oocyte and also its maturation has been a challenge of many investigators in the field of infertility for many years (1). For first time, Enzman and Pincus in 1935 introduced the theory of In Vitro Maturation (IVM), they activated myosis in oocytes of follicles of rabbit, without use of any hormone and took the Germinal Vesicles (GVs) to Germinal Vesicle Break down (GVBD) stage (2). After then, many tries had been done to modify this method, so that some researchers, maturing the oocytes, got embryos as well (3).

Today, induction of in vitro growth and development of oocyte is one of the common methods in assisted reproductive technologies (ART). This approach, of course, is very helpful for women who aren't able to mature their own oocytes as well as those with cancer, exposed to high doses of radiations (following oviducttomy) (4). During follicular stage in mammals, several follicles began to grow but only one or a few numbers of them can convert to a matured oocyte. Other remaining follicles, a few days before ovulation, undergo apoptosis and atheresis (5). In IVM method, oocytes removed from ovary in the stage of GV, followed by in vitro culturing; then, mature oocytes in metaphase II are fertilized by sperms. Since hormone therapy doesn't be used in the process of IVM, this inhibits abnormal over-activity of ovum, especially in patients with poly cystic ovarian syndrome and allows freezing and saving of immature ovums for further use. Any fault in development of cytoplasm of oocyte during the process of growth, can be considered as a difficulty to success in IVM (6).

Insufficiency of environments, in which oocyte starts to grow, is among the most important problems, investigators point to. According that, these environments such as oocyte culture environment, couldn't prepare the essential factors for its growth and development (7). Culture environment even may lead to large Off-Spring syndrome by their belonging stressful factors. So study of the effects of various cultural environments, as well as their effects on maturation and development of embryos is considered as one of the most important priorities of research topics (8).

Follicular fluid is one of the suitable natural cultural environments that is used in IVF labs. It is a yellow and low viscosity liquid that compromised of proteins, vitamins, steroid hormones, cholesterol, lipoproteins, hydrocarbons, and steroids (9). Entrance of this fluid into the ampoule of uterus is inevitable at the time of ovulation and oocyte transfer. It has been reported that follicular fluid could act as cultural environment for growth of embryo, granulosa and maybe somatic cells. By use of follicular fluid together with granulose, we can overcome to 2cell stop process; this is probably because of presence of substance(s), acting synergically with granulosa; on the other side, it also has been reported that cumulus cells and follicular fluid in the cultural environment, can lead to increase amount of acrosin (10). Also, toxins in the fluid can be removed due to process of heating. The aim of our study is to investigate effects of heated follicular fluid and a common cultural environment (DMEM) on Maturation, fertilization and development of oocytes.

Materials and methods

Mice by NMRI race selected for study. Ovaries of Normal female mice by 4 - 6 weeks age were drawn out in a sterile circumstance. After transfer into 500 micro liter drops of determined culture environments, we eliminated excessive layers of fat around. Immature oocytes containing germinal vesicles separated as well as granulosa cells around it by use of insulin syringe. Cutting off granulosa cells by use of pipette, immature nucleated

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oocytes with light cytoplasm and lucid and steady cortex and a suitable previtelline space were selected for 3 groups. Every 5 immature oocytes were put in the 25 micro liter of following each 3 culture environments for 24 hours:

Group1: DMEM (Invitrogen, UK) culture environment, containing 5mg/ml 25% FSC (sigma), 10 mg/ml rFSH sigma, 10mg/ml HCG (sigma)

Group2: Culture environment containing Human Heated Follicular Fluid 100%

Group3: DMEM (Invitrogen, UK) culture environment (sigma), containing hHFF 25%, 10mg/ml rFSH (sigma), 10mg HCG (sigma)

Preparation of follicular fluid: Follicular fluids have been prepared from women who underwent aspiration of follicles for IVF-ET. Range of age was 28-35 years and HCG used as follicle growth stimulator, following GnRH-a prescription. We investigated samples of follicular fluids resulted from follicles with one integrated and maturated oocyte. We also withdraw study of samples with more than one oocyte, immature oocyte as well as bleeding samples. Sterile dishes and tools are used in all stages of this work. After separation of oocyte, all samples centrifuged for 25 min at 2500g; then a definite volume of centrifuged follicular fluid was heated into ban Mary in 56c for 30 min and preserved in 4c until use. Heating method are common ways for inactivating enzymes and toxins in a ragin or normal fluids which are used in biochemistry and IVF laboratories for years.

Study of Maturation: Immature nucleated oocytes with light cytoplasm and lucid and steady cortex and a suitable previtelline space incubated for 24 h at 37c in an incubator containing (CO_2), then underwent study of maturity of oocytes by use of an invert microscope on the following day. They categorized in 3 groups. Those with broken nucleus as GV, those with broken nucleus without any polar body as GVBD and finally oocytes with first polar body as mII matured oocytes.

In Vitro Fertilization of Oocyte: The process of fertilization was done thorough 5 following stages:

1. Collecting all of the matured oocyte from all groups
2. Cutting off the epididymis of the sperms of NMARI race mice and transferring them into culture environments followed by incubation at 37c for 90min.
3. Incubation of healthy and active sperms in pre-balanced drops of cultural environment (1-2 x 106 sperms/ml(
4. Transfer of matured oocytes of each group into drops containing sperms.
5. Transfer of oocytes into IVF drops, 4-6 h later

At the end stage, we studied oocytes after 24h by invert microscope and registered the rates of 2 cell embryos formations.

Results

In this study total of 618 immature oocytes separated from ovaries and divided into 3 groups. The number of samples was 236 in DMEM(Invitrogen), 229 in hHFF 100% and 224 in hHFF 25%. Distribution of samples based on index of oocytes maturation and fertilization is shown in table 1. As mentioned in this table, the index of GV is 18.6% in DMEM (Invitrogen), 3.9% in hHFF 100% and 14.6% in hHFF 25% ($P<0.01$). Differences between groups 1&2 and also 2&3 was significant but between 1&3. In case of GVBD index, it wasn't seen any significant differences between 3 group. The rate of success among group in case of MII were 64.9%, 87.8% and 63.2% respectively ($P<0.0001$).

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Table 1. distribution of samples based on maturation and fertilization index of oocyte.

Maturation / Culture Environment	GV (Amount %)	GVBD (Amount %)	M II (Amount %)	Two Cell (Amount %)
FCS 25%, DMEM(Invitrogen) n=236	42 [a] (18.6)	39 (17.9)	134 [a] (64.9)	59 [c] (50.2)
hHFF 100% n=229	9 (3.9)	28 (12.6)	184 (87.8)	144 (84.6)
DMEM(Invitrogen), hHFF 25% n=224	34 [b] (14.6)	42 (17.8)	144 [b] (63.2)	84 [d] (53.6)
P value	P < 0.01	P < 0.4	P < 0.001	P < 0.001

GV: germinal vesicle, GVBD: germinal vesicle break-down, M II: metaphase II, hHFF: heated human follicular fluid.

[a]Comparison between cultural environments 1 and 2 (P < 0.0005)

[b]Comparison between cultural environments 2 and 3 (P < 0.0005)

[c]Comparison between cultural environments 1 and 2 (P < 0.02)

[d]Comparison between cultural environments 2 and 3 (P < 0.01)

The best type of maturation occurred in the hHFF 100% which had considerable differences with other two culture environments (P<0.001); However, difference between groups 1&3 wasn't significant (P<0.2). The greatest rate of fertilization was in hHFF 100% by 84.6% and then in hHFF 25% by 53.6% and finally the least one was DMEM (Invitrogen) by 50.2 % in case of 2 cell index (Figure 1). There were considerable differences between all 3 groups as well as each two groups.

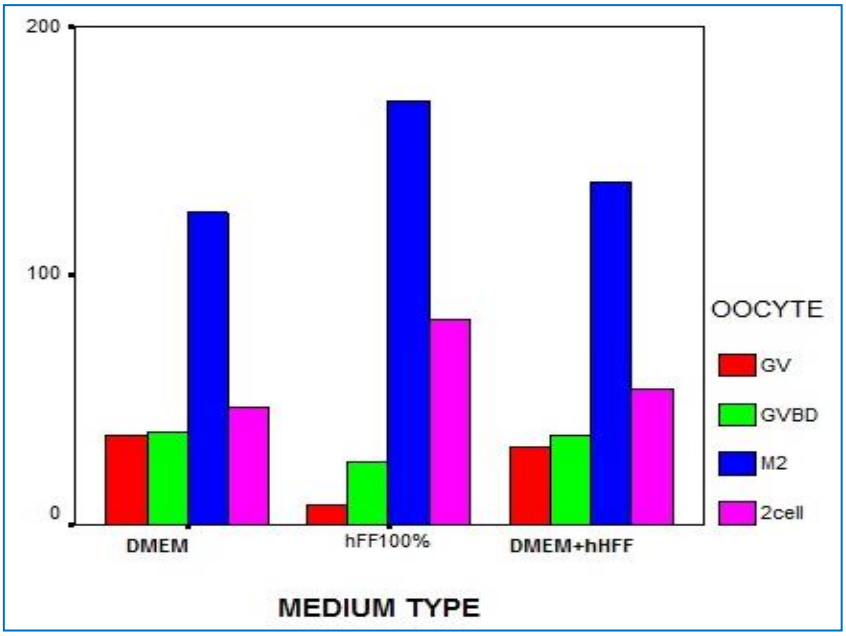

Figure 1. Histogram showed the percentage of GV, GVBD, MII and 2 Cell in three groups.

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Discussion

Study showed the positive effects of heated follicular fluid on maturation and fertilizing power of immature oocytes of mouse and this difference is significant even comparing to mixed media (11, 12, 13). Follicular fluid is one of the main secretions of female genital system and has the same composition as serum and plasma. This can guarantee the development of embryo after omitting the harmful factors (14,15). The required circumstances for growth and development of oocytes in culture environment are almost known, however, closer the circumstances to internal body situation, more favorable results. The least required circumstances are essential ions like sodium, K and CL which are found in follicular fluid (16,17). There are several reports on effects of heated follicular fluid on growth of 2cell embryos of rat. Until blastocyst stage, the main feature of these studies is about use of different volumes of follicular fluid but best results are gained in the 100% pure follicular fluid (18, 19). This demonstrates the sufficiency of follicular fluid for growth of embryos. Heated follicular fluid has many advantages on common media used for fertilizing of rat ovum, in preservation of unfertilized ovums and prevent of their degeneration (20). Comparing to media without human follicular fluid, use of this fluid as a complement may lead to better maturation and development of oocyte (21, 22). Use of follicular fluid in types of complement in higher proportions along with various hormones may also result in maturation and better development of immature oocytes (23).

There are many harmful factors in fresh follicular fluid, that affect development and growth processes. It seems that, toxicity of follicular fluid is in accordance with complement system. These factors are omitted during heating of follicular fluid by Heat Treatment Method (24).

Previous studies revealed that gonadotropin hormones are essential for maturation of immature oocytes and the process of maturation of oocyte will be disrupted in their absence in culture environment (25). Use of gonadotropin hormones with daily changes in culture environment, prepare a suitable complement for maturation of oocyte. Follicular fluid has all requisites for growth and development of immature oocytes(26,27). In this study, all experimental groups showed in vitro maturation of immature oocytes. It seems that this process occurs as a result of presence of hHFF and Gonadotropin hormones in culture environment, injection of both gonadotropins and presence of all essential factors. Many investigators believe that in this stage of development, follicular fluid has higher potential to increase the rate of maturation of immature oocytes than other culture environments. As results of our study, heated human follicular fluid has significant effects on maturation of follicles, so that, higher rates of matured oocytes as well as higher percentage of resulting embryos in experimental groups, which contained much more follicular fluid rather than other groups, approve it. In this study, rate of in vitro maturation as well as resulting embryos increased by 100% after heating of human follicular fluid, also because of this fact rate of 2cell embryo formation are more than other groups. In third group, 25% improve gained following heating and this differs from first group significantly. Comparing results of experimental groups with each other, yields more efficiency of heated human follicular fluid in the process.

Finally, based on enrichment of follicular fluid with essential ions for growth, factors which increase fertility and also fertilizing ability of sperm reinforce our belief about capability follicular fluid to be a suitable medium for growth and development of immature oocyte (28). We can conclude that, heated human follicular fluid may have positive influences on maturation of

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oocyte of rat, their fertilization and in vitro formation of two cells embryos.

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