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Research Article

In Vitro Oocyte Maturation Based on Presence or Absence of Granulosa Cells in the Methods of ART in Mehr Institute

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Abstract

Introduction: Usually, 10 percent of couples in reproductive age are faced with the problem of infertility and need *in-vitro* fertilization treatment. Choosing a suitable egg and sperm in IVF before fertilization is one of the most important steps. Typically, the egg and sperm for fertilization are selected based on appearance. Adult sperms and eggs can ultimately lead embryo to a successful pregnancy. The presence of cumulus cells surrounding the oocyte cytoplasm injection and time are important factors that are discussed in this project.

Methods: Patients with a mean age of 32.7 participated in this project, and after the puncture obtained, eggs were divided into two groups. 94 couples as the "Control group" with the conventional method, and 95 couples as the "Test group" with the proposed method were prepared and punctured. Obtained embryos classified, and embryos transfer into the uterus of the patient was done on the third or fifth day.

Results: 1070 oocytes in control group were containing 721 M2 oocytes (67%), 206 M1 oocytes (19%) and 143 GV oocytes (13%). 927 oocytes (91%) out of 1070 were injected rapidly, and 530 of them (57/1%) became embryos. 1107 oocytes in test group consist of 789 M2 oocytes (71%), 170 M1oocytes (15%) and 145 GV oocytes (13%) which was assaying into groups with 1, 2 or 3 hours postpone in preparing to inject. Finally, 618 (63/7%) out of 970 oocytes (87%) became embryos which is about 6/5% better than conventional method.

Analysis: If oocytes after parrying red blood cells and plasma are incubated for 15 to 30 minutes, obtaining good quality embryos is resulted. The chance of M1 oocytes becoming M2 oocytes at the first hour is high, and generally, obtained embryos of test group (63/7%) in compare with control group embryos (57/1%) have better quality. Also, the quantity of embryos increased about 6/5 percent.

Keywords: *In Vitro Oocyte Maturation; Granulosa Cells; ART; Mehr Institute*

Introduction

In the process of *in vitro* fertilization (IVF), when oocyte and sperm are come close together, oocytes are surrounded by cumulus and corona cells. Studies show that the best result of IVF comes when oocytes are rested about 2 to 6 hours after gathering and then imposed

to sperm [1,2]. On the other hand, the scientists believe that the oocyte incubation before IVF leads to completion of nuclear and cytoplasmic maturation.

There is an oocyte (female sexual cell) in which ovarian follicular are surrounded by ovarian texture. Follicular cells consist of Oocyte, granulose cells and inner and outer layers' cells. Recent studies show that the oocyte has the vital role in regulating oogenesis, ovulation rate and fertility. The oocyte this by releasing growth factors. These elements act close to follicular cells and regulate the majority of granulose and cumulus cell's activities.

Recently, the scientists have more concentrated on molecules which released from oocyte. These molecules form the basis of relation axis. The lack of growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) caused infertility. There is more attraction to study about these factors as the new members of super family transforming growth factor beta (TGFβ). In addition the need for primary flocculation, these molecules are the central regulator for granulose and oocyte distinguishes [3].

The release factors from oocyte (GDF9, BMP15, BMP6…) play the vital role in key reproduction events like regulating the action of follicle cells, phenotype keeping of cumulus cells, regulating the distribution of cumulus cells. These factors also have the basic role in regulating the development of vital processes such as the action of the granulose cells in follicular growth steps, the action of cumulus cells through oocyte maturation and the events lead to ovulation and afterwards the surrounded cells are responsible to release the growth factors paracrine, these factors have a very important role in nuclear and cytoplasmic maturation [4].

The cytoplasmic maturation consists of mRNA transcription [5], protein translation [6], the instructive changes [7] and post translation in proteins [7] which all are effective in cell cycle.

In the method ICSI, introduced in 1992, in preparation phase, at first the cumulus and corona cells were removed from the round of the oocyte and then sperm was injected to oocyte. There are two convenience methods for this removal, mechanical and enzymatic methods [8]. In this method, soon after the oocyte maturation was observed, the sperm will be injected just into the oocytes in which nuclear maturation was completed and the first polar body can be observed on them. The main question is whether the oocytes matured enough, in cytoplasmic view, for activation and development into embryo implantation, or not? The cytoplasmic maturation cannot be detected by microscopy. The oocytes that remained in metaphase I in 1 to 2 hours after oocyte pick up and matured after 4 hours in *in vitro* situation bare of cumulus and corona cells, shows less maturation rate. Furthermore, the quality of embryos will not be affected. The main concern is the time between oocyte collection and sperm injection. This process should not take a long time. The embryo quality is decrease significantly when the ICSI is done after 11 hours [9].

There are several different opinions about the incubation of oocytes with keeping cumulus and corona complexes surrounded them before ICSI.

Javier and colleagues [10] have studied the effect of incubation time of oocytes in two different groups, before IVF and ICSI on the fertilization rate. The first group spent 1 - 3 hours' pre-incubation and the second spent 4 - 6 hours. The results showed that the pregnancy and implantation rate were significantly increased in second group.

In the other study, the ICSI results compared within 4 different groups with different incubation time. These groups are: First: less than 3 hours, Second: 3 to 6 hours, Third: 6 to 9 hours, and the fourth: 9 to 12 hours. The results showed that the increase in pre-incubation period leads to the improvement of the fertilization rate and embryos quality [11].

Imani and colleagues conclude that using mono layer of granulose cells is very useful for media development and increase in embryo maturation [12].

All these results and many other evidences [13-17] show that the pre-incubation of oocytes with keeping the granulose and corona mono layers, have a significant effect on the maturation rate and increase in pregnancy rate.

Materials and Methods

Materials

All the materials used in this project are clinical grade. Total global, (Human Serum Albumin) HAS, All-Grad 100%, Hyaluronidase enzyme purchased of Life Global. Ham's F-10 powder, sodium bicarbonate, Calcium L-lactate Hydrate, Penicillin G Sodium Salter purchased from Sigma Aldrich.

Methods

Media preparation

In order to preparing the Ham's medium the stock solution should be prepared first.

Stock solution

1 package of Ham's powder contains about 9.88 gr powder that was dissolved in 250 ml deionized water. Then, 0.075 gr penicillin was added to the solution. 0.075 gr Streptomycin was consequently added to the solution. The solution was extremely shacked for a few minutes to achieve the homogeneous solution. The solution, followed by filtering by a 22 μm volumetric filter, was assisted by applying vacuum.

Preparation of Ham's media

0.248 gr of Calcium Lactate was dissolved in 840 ml deionized water followed by adding 240 ml Stock's solution. Then 2.104 gr Sodium Bicarbonate was added to the solution. The solution was extremely shacked for a few minutes to achieve the homogeneous solution. The Osmolality of the media was checked; this should be in the range of 283 - 285. Then final approved solution finally was filtered by 22-micron volumetric filter assisted by applying vacuum.

Method

Based on the treatment protocol, the microinjection candidates got hormone that entered them to induction process. After performing the regular tests, hormone test and patient visit were performed by the experts. The following steps were passed:

1. The sonographer test was performed in the second or third day of menstrual period in order to evaluate the ovary and uterus condition.
2. After sonography, Gynecologist prescribe the drugs that would stimulation the ovulation.
3. 5 - 6 cycle sonography was performed, depended on the reaction of the ovary, during the drug usage step.
4. The HCG drug was injected when the follicles reached to suitable size and patient would be ready to pick the eggs up.
5. The *in vitro* fertilization just 36 hr after HCG injection, followed by taking the sperm from man.

The IVF candidates were selected in the average age of 32.7 years old. 94 patients were considered as the control group and 95 patients followed the proposed method of the study.

Steps

Sperm preparation

The sperm sample collected in sterile container and transferred to incubator at 35°C in order to fluidize. The container contained fluidized sperm removed from the incubator after 15 - 20 minutes. 1 ml pure All Grad solution poured into a conical bottom falcon tube with

the capacity of 15 ml. Then 1 ml All Grad 50% (prepared by dissolving 0.5 ml of pure all Grad and 0.5 ml Ham's + HSA) was slowly added into the solution. Final solution was transferred into the CO_2 containing incubator at 37°C for 1 hour. The fluidized sperm sample was slowly added into the upper phase of the solution into the falcon tube. Then the falcon tube was placed into the centrifuge for 15 minutes at 5000 rpm. The sediments at the bottom of the falcon tube, that contains alive sperms, was collected by Pasteur pipette. These sperms were transferred into a 5 ml falcon tube containing Ham's + HSA (that previously gas treated). The falcon tube was placed into the centrifuge for the second time. The centrifuge adjusted to 500 rpm for 4 minutes. Finally, the upper phase into the falcon tube was collected by Pasteur pipette that is rich from sperm ready to transfer and fertilization.

Oocyte preparation

The oocytes were picked up by sonographer observation through vaginal in Mehr institute.

As the first step, the granulose cells around oocytes were removed by using the Hyaluronidase enzyme at several pipetage steps. Then, the oocytes were injected into the certain drops made by Total Global medium. The couples with the almost same characteristics were immediately separated into 2 different groups just after oocyte collection. The first group was dissected with regular method, and the second group, after puncture and removing the blood and the particles with risk of oxidants, separated into three main groups; dissected with the delay 60, 120 and 180 minutes. These oocytes were dissected by three different pipettes, Number 1, 2 and 3, which are different in diameter, 1 refers to largest diameter and 3 refers to smallest. The only follicles entered in this procedure that curtained about those are in metaphase 1 level. The obtained follicles placed into the drops contains c for 10 - 15 seconds then picked up by pipette number 1 in order to remove some granulose cells from round of oocyte, but they were now insisting to remove all the granulose cells. Some granulose cells remained around the oocytes which would be removed through picking up pipette number 3 that have diameter a little bigger than oocyte diameter. By this method, almost all the granulose cells would be removed. The collected oocytes were separated into three main groups: A: don' dissected, B: Partially dissected; and C: totally dissected. In group A; the red globules and additional materials were removed but the cumulus cell was totally remained around the oocytes. This group divided into three subgroups; A1: the cumulus cells were removed after 1 hour; A2: the cumulus cells were removed after 2 hours; and A3: the cumulus cells were removed after 3 hours. In group B; some cumulus cells (without any insist in totally removal) were removed from around the oocytes metaphase 2, metaphase 1 and GV so that some cumulus cells were remained around the oocytes. This group divided into 3 subgroups; B1: the cumulus cells were removed after 1 hour; B2: the cumulus cells were removed after 2 hours; and B3: the cumulus cells were removed after 3 hours. In group C, cumulus cells were removed by Hyaluronidase enzyme just after oocyte collection.

Finally, the all oocytes in different groups were evaluated and compared in following items:

1. The amount and quality of cell maturation
2. The fertilization rate and zygote quality
3. The percentage of embryo formation and the quality of result embryos
4. The fertilization rate and the chance of twinning.

Results and Discussion

The patients were separated into two different groups. The first were passed the regular procedure, this named group control, and the second group passed the proposed procedure that called test group.

At first, the whole candidates were investigated for age and characteristics. Two groups were examined by statistical methods to predict the standard deviation of the age in two different groups. Figure 1 can show the result.

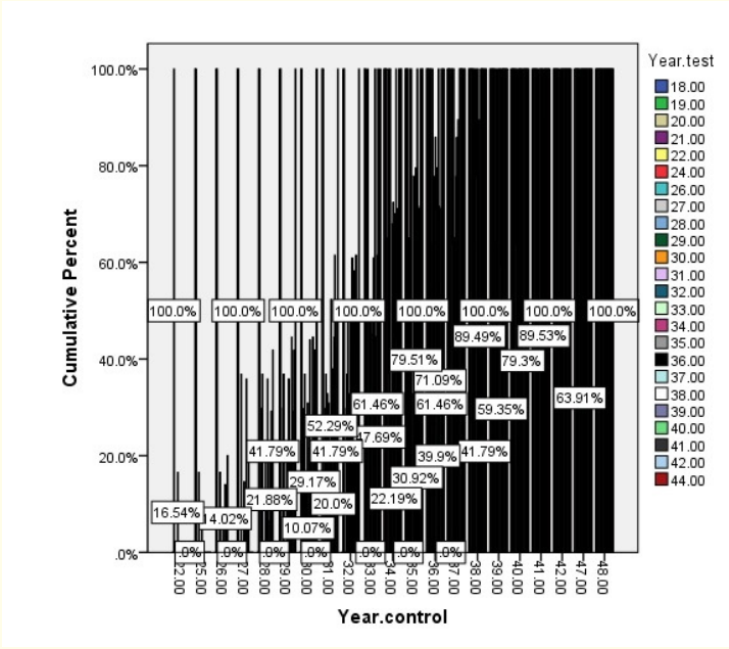

Figure 1: *Two different groups of patients were candidate for IVF, divided into test and control groups. The figures show the ages of them*

The standard deviation between two groups was 0.008 that is very smaller than the confidence limit, 0.05. It shows that the two groups test and control are the same in the manner of age and characteristics that allows us to compare them.

Table 1 can show the situation of the group control.

GV	MI	MII	Total Oocytes	LO	RO	Group
143	206	721	1070	554	516	Control

Table 1: *The control group overall situation.*

According to table 1: the total number of oocytes was 1070 distributed in GV, MI and MII. Only 927 of these oocytes were being able to inject. Finally, 530 embryos were formed from these 927 injected oocytes.

95 candidates were selected from 189 patients to pass the proposed procedure. The total 1107 oocytes were collected that distributed in different phases as showed in table 2.

GV	MI	MII	Total Oocytes	LO	RO	Group
145	170	789	1107	571	536	Test

Table 2: *The overall situation of test group.*

The whole number of the oocytes was not being able to inject. Table 3 shows the number of injected oocytes and formed embryos for both two groups.

Group	Total oocytes	Injected oocytes	Embryos
Control	1070	927	530
Test	1107	970	618

Table 3: *Number of Injected oocytes and formed embryos for both two groups.*

The group test were/was divided into three mentioned groups and followed the aforementioned procedure to study the effect of time and the presence of the cumulus cells around the oocytes.

The effect of incubation time

Table 4 shows the number of matured oocytes in different hours.

3hr	2hr	1hr	GV	MI	MII	Oocytes	Group
3	12	25	145	173	789	1107	Test

Table 4: *The number of matured oocytes in different hours.*

Only oocytes in the phase of MII and MI are able to inject. On the other hand, the other oocytes (GV) should be matured into the upper maturation phases. In the control group, the oocytes were injected immediately after gathering, so the GV oocytes were ignored, but in the test group the GV oocytes had chance to mature. The only 8 oocytes of the GV oocytes were matured and able to be injected. This is less than 1% but is important; 1 oocyte injectable, 1 chance to born.

The oocytes have separated into 3 different groups in order to find the best time for resting before injection. The result shows that the best results have reached after 1 hour. 25 oocytes matured into MII just after the first hour, (25/40 = 63%). This result can prove that the best resting time is 1 hr. It was proved before by Patrat [18]. It can be concluded that: the immature oocytes (MI and GV) should be rested for 1 hr; after that just the immature oocytes should be kept in incubator for one hour more.

The effect of dissection on oocyte maturation

The best result in oocyte maturation is for partially dissected oocytes. 53% of the matured oocytes are the oocytes that are partially dissected. After that the 33% is related to fully dissect and the rest refer to non-dissected ones. The main reason is that the partially dissection of oocytes leads to remove the blood and unnecessary cells around the oocytes, that leads to better maturation rate than non-dissected oocytes. Also, the fully dissection on oocytes remove all the necessary and unnecessary cells around the oocytes, that limits the feeding of the oocytes during maturation period.

Conclusion

The best result for the maturation of oocytes before injection are approached by partially dissection before injection and the rest time of about 1 hour. The presence of necessary granulose cell around the oocytes can feed the oocyte and reduce the stress, and then the resting for 1 hour in this state can help the oocyte to be matured in the best way.

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