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Research Article

The Effect of Vitrification of Oocytes Cumulus Complex Apoptosis of Mice (*Mus musculus*) to Apoptosis, Rate of Fertilization and Embryo Quality

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ABSTRACT

The purpose of this study was to determine the quality of cumulus oocyte complex (COC) after vitrification process toward apotosis of COC, fertilization rate and embryo quality. Frozen occurs during the cold shock that can lead to changes in the molecular level COC. These changes will affect the quality of frozen thawed oocytes after COC. The study used two groups, There are COC not frozen and frozen COC. Parameter observed were apotosis of blastomere cells, fertilization rate and embryo quality. Apoptosis of COC were observed with tunnel apoptec staining, fertilization rates were measured based on number of zygotes and embryo quality were observed through number of blastocyst. The data of apotosis of blastomere cells were analyzed by Kruscal Wallis. The result showed that the apoptosis number, fertilization rate, and morula number between are significantly ($p<0.05$) between groups. The administration of frozen COC increase number of apoptotic blastomer cells, decreased fertilization rate up to 51.1% and embryo quality up to 69.2 %. In conclusion, Frozen on COC increased apotosis of COC, decreased both of fertilization rate and embryo quality. The embryo Frozen of vitrification is required in the ART technology necessary to add an antioxidant to improve the fertilization rate and embryo quality.

Keywords: COC, apoptosis, fertilization rate, embryo quality.

INTRODUCTION

The aid of reproductive technology (ART) is nowadays developing to support the success of test tube baby program. One of the supporting methods is Frozen tissues, embryos, or oocytes. Frozen is conducted by storing tissues, excessive embryos, or oocytes so that the embryos can be utilized if they are needed someday. However, storing embryos or oocytes are not allowed in some countries. In vitro fertilization (IVF) usually produce many embryos, but not all embryos are transferred to the patient, and the excess embryos are stored in a frozen state. The number of embryos transferred to the patient depends on patient's age, and maximally 3 embryos can be transferred to the patient. The remaining embryos that are not transferred to the patient can be saved through the Frozen process. However, until recently it was reported that the quality of frozen embryos is still very low. Many researchers reported poor quality of the embryo, followed by the low percentage of successful pregnancies, because of the occurrence of a failure of implantation.

In recent years, it has developed a method of Frozen either COC or ovaries of patients suffering from cancer[1,2,3]. Many methods have been developed to freeze the COC, which is critical of this method is the ability to maintain the level of maturity of oocytes after in vitro maturation[4]. This time the results of Frozen oocytes have a low level of maturation and will be followed by difficulties in reaching the metaphase II stage[5].

Low oocyte maturity level after thawing is supposedly caused by cell death or blastomer cell apoptosis. Blastomere cell death caused by the temperature, the change from Frozen conditions to room temperature. Oocyte quality highly influences fertilization rate and embryo quality produced. A number of blastomeres cells undergoing apoptosis will result in lower fertilization rate, and directly reduce the quality of the blastocyst in vitro. Therefore, research is needed to determine the effect on the number of oocytes vitrified oocytes result of apoptosis, the rate of fertilization and embryo quality.

MATERIALS AND METHODS

The study used female mice (*Mus musculus*), age of 8 weeks. The used animal in this research was approved by Committee Ethic of Animal, Airlangga University (500 KEP).

Superovulasion and Egg Collection

Female mice were injected by hormones of Pregnant Mare Serum Gonodotropin (PMSG Foligon) with a dosage of 5 IU. 48 h later they were also injected by hormones of Human Chorionic Gonadotropin (Chorulon) and directly were mated with male mice which were

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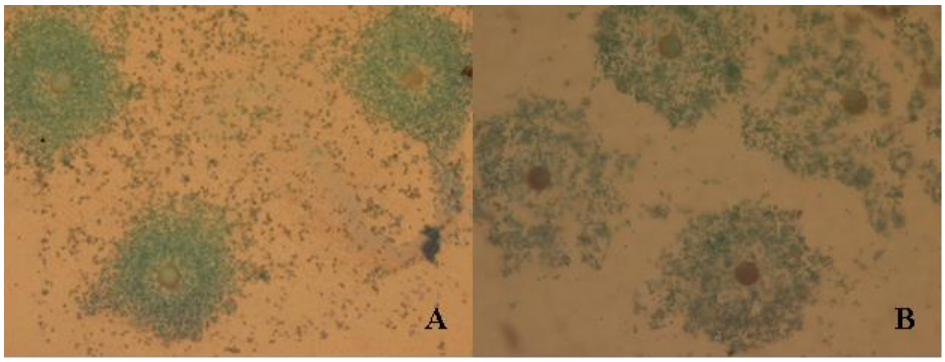

Figure 1: Expression of COC apoptotic in Frozen and no Frozen group (Nikon microscope H600L, *camera DS Fi2 300 megapixel, magnification of 100x)*

The brown color indicates a positive expression of apoptosis on COC
A: No Frozen COC
B: Frozen COC

Table 1: Number apoptosis cumulus oocyte complex.

Group	Mean Rank	Asymp. Sig
Control (Not Frozen)	8.50	0.00
Treatment (Frozen)	24.50	

castrated monomattingly. Vagina plug examination was carried out after 17 h after mated, followed by collecting eggs. The eggs were washed and frozen using vitrification method.

Vitrification and Oocyte Thawing

This vitrification used the method with slight modification[6]. Cumulus oocyte complex was put into vitrification medium 1 of PBS for 2 min, then moved into vitrification medium 2 of EG 30 % for 2 min, next moved into vitrification medium 3 of sucrose 1 M for 30 seconds, after that oocytes were put at the tip of hemi straw. Then hemi straw was immersed in liquid N_2 was put at the tip of hemi staw into big straw. Putting hemi straw into big straw must be done in liquid N_2 so that COC at the tip of hemi straw is not gone. Next, big staw was fixed at the tip of hemi straw and put it into cassette staw and cassette staw was put into goblet container of liquid N_2.

Warming cumulus oocyte complex was carried out by previous methods[6]. Cumulus oocyte complex was discharged from straw, next was dropped on vitrification medium 4 containing PBS medium + sucrose 1 M for 2 min, then moved into vitrification medium 5 containing PBS medium + sucrose 0.5 M for 2 min, put into PBS medium for 2 min. Embryo was cultured in CO_2 incubator for in vitro fertilization.

In vitro Fertilization

Cumulus oocyte complex resulted from thawing was washed 3 times in PBS and MEM media respectively. Cumulus oocyte complex washed was moved into fertilization medium. Spermatozoa was taken from cauda epididymis of male mice, then soaked in fertilization medium containing eggs with a dosage of 200.000. Cumulus oocyte complex mixed with spermatozoa was incubated in CO_2 5 % with the temperature of 37^{o}C for

Table 2: Percentage of fertilization rate of treatment group.

Group	Fertilization rate (%)
Control (no Frozen)	99.6
Treatment (Frozen)	51.03

7 h and zygotes was observed, next it was moved into a new culture medium to prevent it from being contaminated by granulose cell fall off.

In vitro Embryo Culture

Fertilized embryo was moved into culture medium and incubated in incubator of CO_2 5% at the temperature of 37^{o}C. Embryo culture was changed every two days until embryo reached blastocyst stage.

Examination of Apoptosis

Microslides were deparaffinized in xylol and alcohol series. Put PBS on the slide for 10 min, absorbed the liquid with a tissue. Then it were covered with 50 mL proteinase K for 15-30 min, washed twice with diionized water (dH_2O) each for 2 min. Put drops on the samples with sequencing solution for 5 min, washed the slides by using PBS for 1 min. Put drops of TdT labeling buffer to the samples for 5 min, covered the sample with 50mL of labeling reaction mix for 2 reactions: TdTdNTP Mix(Cat #4828-30-04) 2mL, TdTEnzyme(Cat #4810-30-05) 2 mL, 50 x Cation Stock 2 mL, 1 X TdT labeling Buffer 100mL, incubated for 1 h at 37°C in a humid place. Washed slides with1xTdT stop buffer for 5 min, washed slides every 5 min with dH_2O. Put drops on sample with 50 mL streptavidin-HRP solution and incubated for 10 min at 37 °C in a humid place to prevent evaporation. Washed slides with PBS twice each for 2 min. Dropped with DAB solution for 2-7 min, washed slides with dH_2O each 2 min. Put drops of 1% methylgreen on the slides for 30 seconds to 5 min, soaked and washed slides10 times with dH_2O. Then microslides were dried overnight and covered with entellan.

RESULT AND DISCUSSION

The results of the analysis oocyte cumulus complex apoptosis showed significant ($p<0.05$) between groups.

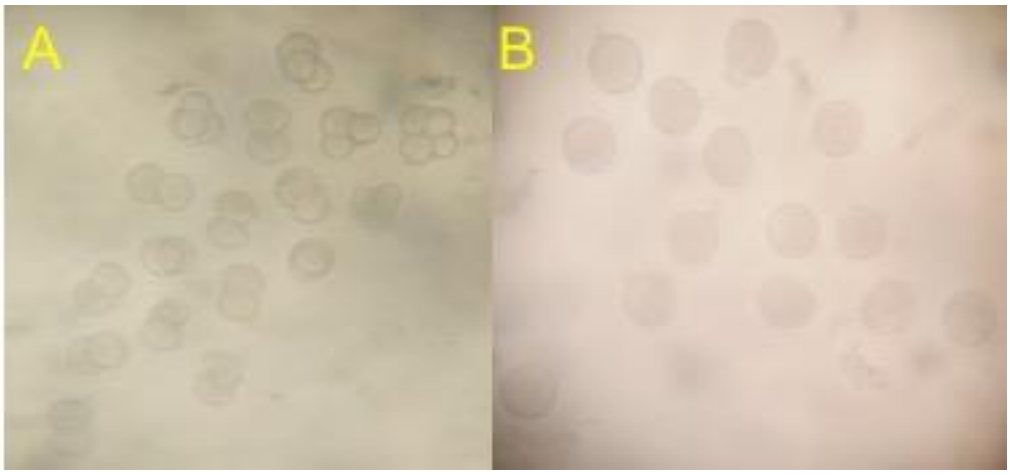

Figure 2: Embryos of in Vitro fertilization.

(A) No Frozen COC
(B) Frozen COC

Table 3: Percentage of Developping Embryo Frozen.

Groups	Developping Embryos (%)					
	Zygotes (%)	2 cells	4 cells	8 cells	Morula	blastocyst
No Frozen	99,6	99.3	98,9	99,6	99,6	99,3
Frozen	51,03	74,5	79,3	70,5	62,9	69,2

The number of apoptotic cumulus oocyte complexes (COC) from Frozen group was higher than the group without Frozen COC observed with immunocytochemistry (Table 1, Figure 1).

The COC vitrification process more difficult than embryos, vitrification on intact cumulus cell hinder cryoprotectants enters the cell, so that the COC is not well protected by cryoprotectants extra-cellular or intra-cellular. Cold shock will cause cell damage. The previous report stated on vitrification of COC cyoprotectant difficult to penetrate the cell membrane, thus forming intra-cellular ice crystals in the oocyte, which will cause damage organell intra-cellular, followed by damage to the spindle or mitochondria[7,8].

The results showed that the number of frozen COC higher than the group with no frozen COC. The results are consistent with previous research which states that the COC is vitrified cause spindle damage and mitochondria, and further causes the COC apoptosis

Macroscopically, after vitrification process in group of no frozen COC confirmed there are no cell damage, and cumulus cells, oocytes and cytoplasm look clear. However, in Frozen COC group induced oxidative stress, apoptosis, low fertilization rate. Frozen COC group normally exposured by cryoprotectant. Intra-cellular damage takes place when cryoprotectant is not able to protect COC from cold shock. The previous research reported that on in vitro vitrification condition, spindel of microtubuli can depolarized and followed by fragmentation of DNA[9,10,11]. The no Frozen group determined that fertilized COC result developed into embryo on second stage cells, or even reached fourth stage cells, but frozen COC group still in the form of zygotes and have low fertilization rates.

The development of the embryo reaches the blastocyst stage at COC group that is not frozen reaches as high as 99.3%, while the frozen COC reached 69.2%. Frozen OCC affect the ability of embryos pass through a block of cells, in rats this happens in embryonic development stages of one cell into two cells.In this study generated the number of embryos progressed to stage 2 cells reached 74.5% and 99.3% in no frozen and frozen group, respectively. The COC quality conditions determine the quality of the embryos that developed to the blastocyst stage. Stress caused by differences in temperature will affect the quality of COC. Quality of blastomeres in the group no lower than frozen COC.

CONCLUSION

The frozen process increase apotosis of COC, decrease both of fertilization rate and embryo quality. The embryo Frozen of vitrification is required in the ART technology necessary to add an antioxidant to improve the fertilization rate and embryo quality

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