Goat oocytes quality after vitrification using difference of glycerol concentration and exposure time

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Abstract. The aim of the study was to analyze the effect of glycerol intracellular cryoprotectant concentration and the length of exposure to the morphology of oocytes after cryopreservation using the vitrification method. The research material was Mt-II goat oocytes which had been carried out in IVM media in 24 hours. The study design employed a random design of factorial patterns of 6 x 3, the first factor was glycerol concentration 0,15, 20, 25,30, and 35% and the second factor was the exposure time of 1,3,5 minutes respectively. Data analyses using analysis of variance. Oocyte morphology is classified as normal if it shows a round shape with intact plasma membrane and round cytoplasm with homogeneous compact granulation, while the morphology is abnormal if it shows a non-spherical shape, zone of the pellucida fracture, the cytoplasm is not spherical and degeneration. The analysis of variance and Duncan Multiple Range Test were employed to analyze the data. The results showed glycerol concentration and exposure time had an impact (P <0.05) on oocyte morphology. The highest proportion of normal oocyte morphology resulted from vitrification using 25% glycerol concentration and 3-minutes exposure time.

1. Introduction
Significant progress has been made through the cryopreservation of spermatozoa and embryos in a number of species. However, oocytes are different among themselves in terms of sensitivity towards low temperature environment, hence oocytes cryopreservation results have not been satisfactory. In fact, oocytes are more sensitive to cryopreservation than embryos. They are very sensitive to cold temperature and have low permeability to cryoprotectants [1]. Compared to embryos, oocytes are distinguishable in regard to their plasma membrane, the presence of cortical granules, and the formation of spindles in the metaphase II (MII) meiosis stage [2].

Long-term storage of oocytes will allow female genetic materials to be stored and later be fertilized with desired or planned male germplasms. Furthermore, oocyte cryopreservation is useful to facilitate a number of reproductive technologies. For instance, oocyte cryopreservation shall enable researchers to salvage genetic materials from infertile, pre-pubertal, pregnant or even dead cattle, which in turn will be useful for the establishment of an oocyte bank.

Albeit in vitro fertilization (IVF) technology is able to utilize both fresh and frozen oocytes, fresh oocytes have a limited survival ability at room temperature. Hence, it is necessary to develop an oocyte cryopreservation method to maintain normal morphology and cell viability.

There are 2 types of oocyte cryopreservation methods, which are conventional/slow freezing and vitrification. Initial studies of oocyte cryopreservation were conducted by using conventional method, but currently the vitrification method has been applied more frequently due to its relatively easy
requirements. The vitrification method can be easily applied by using a liquid nitrogen container and does not require special tools to reduce temperatures gradually and minimize the formation of ice crystals [3]. It has also served new viewpoints in regard to oocytes and embryos cryopreservation, both for somatic nuclear transfer and in vitro fertilization procedures. However, vitrification of oocytes has been tricky because of their sensitivity to cold temperature and complex structure.

During the cryopreservation process, an intracellular and extracellular cryoprotectant is needed. In addition to protecting cells, intracellular cryoprotectants can also cause damage to cells due to their toxicity. The combination of intracellular cryoprotectants with low molecular weight such as glycerol, ethylene glycol (EG) and Dimethyl sulfoxide (DMSO) will establish hydrogen bonds with intracellular water molecules, which reduce freezing temperature and curb crystallization, whereas extracellular cryoprotectants such as fructose, trehalose and sucrose, will pull water from cells through osmosis, produce intracellular dehydration [5]. The degree of protection of cryoprotectants against crystallization throughout the course of freezing depends on the category and concentration of cryoprotectants used and exposure time. Since cryoprotectants are cytotoxic, the selection of concentration as well as exposure time are critical to minimize the toxic effect [6].

The purpose of the study was to examine the quality of oocytes after vitrification using glycerol intracellular cryoprotectants in different concentrations and time of exposure.

2. Materials and methods

2.1. Preparation of oocytes and in vitro maturation procedure
Ovaries were obtained from abattoir and put in Penicillin 100 IU/L + NaCl 0.9% + Streptomycin 100 IU/L at 30 °C less than 3 hours after slaughtered. Immature oocytes were collected by aspiration through 18-G needle from 3 to 6 mm diameter of follicles. Oocytes with compacted ooplasm and cumulus cells were placed into the maturation medium TCM 199 (Gibco Cat no 212000760) boosted with 10% fetal bovine serum (Gibco) + Carnitine 1.2 mg/L + antibiotics (100 μg/ml Streptomycin + 100 IU/ml of Penicillin G, Meiji, Japan). In vitro maturation process according to Wahjuningsih et al, 2014 [7].

2.2. Freezing and thawing
Mt-II oocytes were exposed to different vitrification solutions (15, 20, 25, 30, and 35%) + 0.5 M of sucrose with time of exposure of 1.3.5 minutes. Fresh Mt-II oocytes were used as control. Two hundred oocytes were used for in-vitro maturation without vitrification that served as control. The oocytes were then placed into a 0.25 cc transparent French straw, each containing 10 oocytes. After exposure in nitrogen vapor for 10 seconds, the ministraw containing oocytes was put in a liquid nitrogen container and stored for 4 weeks for further analysis. Thawing process according to Wahjuningsih et al, 2019 [8].

2.3. Evaluation of oocyte morphology
The morphological evaluation of the cryopreservation of Mt-II oocytes was carried out by using an inverted microscope with 400 X of magnification. The oocytes’ morphology is deemed as normal if they are round with intact plasma membrane and round cytoplasm with homogeneous compact granulation. While the morphology is abnormal if it shows a non-spherical shape, the zone of the fracture pellucida, the cytoplasm is not spherical and degeneration occurs [9]. The percentage of normal morphology was derived from the number of normal oocytes divided by the total number of vitrified oocytes.

2.4. Data analysis
This research employed a random design of factorial patterns of 6 x 3, with the first factor was glycerol concentration 0, 15, 20, 25,30, and 35% and the second factor was the exposure time of 1, 3, and 5 minutes respectively. Data analyses was carried out by using variance analysis.
3. Result and discussion

The rate of normal morphology differs significantly between Glycerol concentration of cryoprotectants and exposure time but was significantly higher in non-vitrified control group. Table 1 shows the difference between morphologically normal oocytes under different concentration of cryoprotectants and time exposure.

The results of this experiment suggest that the concentration of Glycerol 25% and 3-minute time of exposure is the optimum concentration and time of exposure for vitrification of goat oocytes. Glycerol concentration of 25% as intracellular cryoprotectants and 0.5 M trehalose as extracellular cryoprotectants can inhibit oocyte damage caused by vitrification. Glycerol concentration of 25% and time of exposure of 3 minutes may intercept the intracellular ice crystal’s formation as well as prevent cell from being damaged. Water molecules in the cell have left the cell and intracellular cryoprotectants enter the cell until intracellular ice crystal formation does not occur. Exposure time is a very important parameter when selecting cryoprotectants. Optimal exposure time for successful vitrification must prevent toxic injury and intracellular ice formation [10].

Table 1. Morphologically normal oocytes recovered after 4 weeks of cryopreservation at different Glycerol concentrations and exposure time

| Glycerol concentration (%) | Exposure time (minute) | Normal morphology of oocyte (%) |
|----------------------------|------------------------|--------------------------------|
| Fresh oocyte (control)     |                        | 89.14 b                        |
| 15                         | 1                      | 24.86 a                        |
|                            | 3                      | 24.29 a                        |
|                            | 5                      | 33.29 b                        |
| 20                         | 1                      | 25.86 a                        |
|                            | 3                      | 36.71 c                        |
|                            | 5                      | 37.25 c                        |
| 25                         | 1                      | 45.14 c                        |
|                            | 3                      | 65.29 e                        |
|                            | 5                      | 58.86 f                        |
| 30                         | 1                      | 40.00 d                        |
|                            | 3                      | 41.19 d                        |
|                            | 5                      | 41.29 d                        |
| 35                         | 1                      | 25.19 a                        |
|                            | 3                      | 24.57 a                        |
|                            | 5                      | 23.86 a                        |

Different superscripts indicate significant (P<0.05)
Many oocytes are damaged at a glycerol concentration less than 25% and more than 25%. Improperly concentrations of intracellular cryoprotectant and time of exposure can not to protect the oocytes during the vitrification process, so that the oocytes are damaged when exposed to cold temperatures. In suboptimal concentrations, the lost hydrophobic environment is not completely replaced by glycerol, that too low cryoprotectant concentration cannot prevent the formation of intracellular ice crystals due to very rapid temperature drops and cell damage [10]. At low concentrations cryoprotectants are unable to protect the oocytes during the vitrification process so that the oocytes are damaged when exposed to cold temperatures. At suboptimal concentrations, the lost hydrophobic environment is not completely replaced by Glycerol. Cryoprotectant concentrations that are not optimum cause many oocytes are damaged. This is because cryoprotectant concentrations that are too high can be toxic and damage the cytoplasm of cells [4, 10].

Ice crystal formation factors and toxicity are the main factors in cryopreservation by vitrification methods. The balance between achieving the maximum cooling rate to prevent ice crystals’ formation and minimizing the concentration of cryoprotectants that are toxic to cells is essential to be considered. The strategy used to avoid the toxicity of vitrified solutions is to shorten the exposure time, but if the exposure time is too short, cryoprotectant absorption is not enough, so intracellular ice is still formed ice crystals. Therefore, optimal exposure time is needed as a consideration to maintain toxicity and prevent it from being prevented and ice crystal formation. Hence, a successful vitrification calls for the optimization of cryoprotectant concentration and exposure time [10, 11].

Cell damage can happen from increased osmolarity of cryopreservation media so that cryoprotectants become toxic, physical damage due to the emergence of extracellular ice crystals, or osmotic swelling, zona fracture, changes in intracellular organelles and cytoskeleton, disturbances in distribution of cortical granules, shrinking of cytoplasm, change in shape of oocyte and partially or fully removal of cumulus cells layer from oocyte, increased polyspermy, and stiffening due to premature zones released by cortical granule which leads to a decrease in fertilization rate [12,13,14]. The extent of damage throughout the course of cryopreservation mainly depends on the cell’s size, membrane’s permeability, oocyte’s quality [15]. Furthermore, cryoinjuries are yet to be fully researched, thus it is difficult to attempt any improvement to the quality of oocyte cryopreservation [16].

4. Conclusion
Glycerol concentration and duration of exposure affect the quality of Mt-II goat oocytes after cryopreservation using vitrification methods. Glycerol concentration of 25% and duration of exposure of 3 minutes produced the highest normal morphological percentage.
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