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IMPROVING EWE OOCYTE VIABILITY AFTER VITRIFICATION WARMING USING COMBINATION OF DIFFERENT CRYOPROTECTANT AND CARRIER SYSTEM

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ABSTRACT

The aim of this study was to determine the best combination of cryoprotectant (Ethylene glycol, EG), dimethyl sulfoxide (DMSO) and propanediol (PrOH) and carrier system (hemistraw and cryotop) in improving ewe oocytes viability during cryopreservation. Oocytes with multi layers of compact cumulus cells were colleted from abbatoir and matured in TCM 199 medium supplemented with 10% fetal calf serum for 24-26 h at 38.5° C under 5% CO_2 in the air. Matured oocyte was divided into six parts and vitrified in three different vitrification solutions; (i) 17% EG+17% DMSO with hemistraw as carrier system, (ii) 34% EG with hemistraw as carrier system, (iii) 17% EG+17% PrOH in hemistraw (iv), 17% EG+17% DMSO with cryotop as carrier system (v), 34% EG with cryotop as carrier system (vi), and 17% EG+17% PrOH in cryotop. Oocytes were cryopreserved for one week before revived and evaluated for viability. The result showed that oocytes vitrified in media containing EG and DMSO in cryotop had the highest viability (88.16%) compared to media containing EG only or EG and PrOH (70.95% and 68.76%, respectively) ($P<0.05$). Moreover, oocytes viability that vitrified using cryotop and hemistraw as carrier system were not significantly different. The present results indicated that vitrification using combination of EG and DMSO as permeable cryoprotectant and cryotop as carrier system was the best system to maintain oocyte viability after vitrification-warming.

Key words: carrier system, cryoprotectant, oocyte viability, oocytes vitrification

ABSTRAK

Tujuan dari penelitian ini adalah menentukan kombinasi cryoprotectant (ethylene glycol (EG), dimethyl sulfoxide (DMSO) dan propanediol (PrOH) dan sistem carrier (Hemistraw dan cryotop) untuk kriopreservasi dengan menggunakan oosit domba. Oosit yang mempunyai beberapa lapisan sel cumulus diperoleh dari ovarium yang dikoleksi dari Rumah Potong Hewan dimaturasi secara in vitro dalam media TCM 199 yang disuplementasi dengan 10% fetal bovine serum selama 24-26 jam di dalam inkubator dengan suhu 38,5 ° C dan 5% CO2. Oosit yang telah matang dibagi menjadi enam bagian, selanjutnya divitrifikasi dalam tiga larutan berbeda (i) 17% EG+17% DMSO dengan hemistraw sebagai sistem carrier (ii) 34% EG dengan hemistraw sebagai sistem carrier(iii) 17% EG+17% PrOH di hemistraw (iv) 17% EG+17% DMSO dengan cryotop sebagai sistem carrier(v) 34% EG dengan cryotop sebagai sistem carrier(vi) 17% EG+17% PrOH dalam cryotop. Oocytes disimpan selama satu minggu, kemudian dicairkan kembali dan dievaluasi untuk melihat viabilitasnya. Hasil menunjukkan bahwa viabilitas oosit yang divitrifikasi pada media yang mengandung EG dan DMSO dalam cryotop memiliki viabilitas tertinggi (88,16%) dibandingkan dengan media yang hanya mengandung EG atau atau EG dan PrOH (masing-masing 70,95% dan 68,76%) (P<0,05). Selain itu, viabilitas oosit yang menggunakan cryotop dan hemistraw sebagai sistem system carrier tidak berbeda secara signifikan. Hasil ini menunjukkan bahwa vitrifikasi menggunakan kombinasi EG+DMSO sebagai krioprotektan dan cryotop sebagai sistem carrier adalah sistem terbaik untuk mempertahankan viabilitas oosit pasca vitrifikasi.

Kata kunci: sistem carrier, krioprotektan, viabilitas oosit, vitrifikasi oosit

INTRODUCTION

Vitrification is a technique to maintain fertility from a patient who undergoing gonadal-toxic chemotherapy or radiotherapy and ovariohysterectomy by freezing and storing their oocytes in Assisted Reproduction Technologies (ART) (Cobo *et al*., 2013). Oocyte vitrification is also applied for women who wish to delay having offspring by freezing their oocytes during reproductive age (Daniluk and Koert, 2017) and to be used in the future. In the field of veterinary medicine and animal husbandry, vitrification has been used to guarantee the availability of gamete cells for in vitro embryos production, safeguard of wildlife or endangered species (Comizzoli, 2016), and cryobanking of farm animals genetic (Mara *et al*., 2013).

Vitrification is known to establish a glass-like solid state during the cooling process. The successful rate of oocyte vitrification is still limited (Vajta, 2000; Paulini *et al*., 2014). Vitrification needs a high concentration of cryoprotectant and rapid cooling rate to prevent the formation both of intracellular and extracellular ice crystal. One way to reach the high cooling rate is by using a carrier system that able to minimize the cyryopotectant volume. Some types of carriers that have been used for vitrification of oocytes are cryoloop, cryotop, fibreplug, and electron microscope

grid (Bartolac *et al*., 2015). The difference in the volume of cryoprotectant in vitrification will result the different degree of viability of the oocyte after vitrification-warming cycle.

The disadvantage of using the high concentration of cryoprotectant for oocyte vitrification is the cytotoxic effect that damages the structure of oocyte organelles. Various methods have been developed to enhance the efficacy of oocyte vitrification, for example by evaluating the type and concentration of cryoprotectants (Aye *et al*., 2010; Lee *et al*., 2010), combining one or more intracellular and extracellular cryoprotectants to reduce the cytotoxic effect, and reducing the freezing point when the cells are exposed to the vitrification solution (Vatja and Kuwayama, 2006).

Three major permeating cryoprotectants that used in commercial vitrification media are dimethyl sulfoxide (DMSO), 1,2 propanediol (PrOH), and ethylene glycol (EG). All of cryoprotectants have a potency to degrade or alter over preservation time and most of these degradation products are also toxic. EG is cryoprotectant that less toxic and low molecular weight. EG is mostly used as cryoprotectants for cryopreservation of embryos and also applied for cryopreservation of oocytes (Gordon, 2004). The low molecular weight of EG (62.07 g/mol) gives a favorable effect of higher permeability.

The aim of this study was to evaluate the effects of EG in combination with other cryoprotectants as vitrification solution and two different carrier systems towards oocyte viability after vitrification-warming cycle. Sheep oocytes were used as models for oocytes vitrification, and the result can be applied for oocytes cryopreservation in a human fertility clinic or embryo transfer program in veterinary medicine.

MATERIALS AND METHODS

Oocytes Collection

Ovaries were collected from a local abattoir and brought to the laboratory in physiological saline (0.9% NaCl) at 25-30° C within 4 hours. Oocytes were collected by slicing method in PBS media supplemented with 5% fetal bovine serum (FBS). The cumulus-oocyte-complexes (COCs) with at least 3-4 layers of cumulus cells and having homogeneous cytoplasm were washed twice in DPBS supplemented with gentamycin. The COCs were suspended in DPBS with 10% FBS and antibiotic-gentamycin. Oocyte selection and manipulation were performed at room temperature.

In Vitro Maturation

Five to ten COCs from all grades were introduced to 50 µL maturation media and covered by mineral oil (Sigma, USA) in a sterile petri dish. The COCs were cultured for 24 hours in the incubator (5% CO_2; 90% humidity; 38° C). Each of COCs was examined under a stereomicroscope and evaluated for maturation based on extrusion of the first polar body (Widyastuti *et al*., 2017a).

Vitrification of Oocytes

Oocytes with first polar body extrusion were divided into six groups that used cryotop and homemade hemistraw as a carrier system. Each of group was transferred into three treatments; (i) vitrified with 17% EG+17% DMSO, (ii) vitrified with 34% EG, (iii) vitrified with 17% EG+17% PrOH. All vitrification mediums were supplemented with 0.65 M sucrose and 20% FBS in PBS. Oocytes were equilibrated in the solution containing a half concentration of permeable cryoprotectant of vitrification solution before introduced to vitrification solution. After 15 minutes, oocytes were transferred into vitrification solution for 30 seconds then about 6µl of vitrification solution containing oocytes was loaded directly into cryotop or hemistraw, exposed to liquid nitrogen and stored in liquid nitrogen-filled cryo tank (Widyastuti *et al*., 2017b).

Warming and Evaluation of Vitrified Oocytes

The vitrified micro drops were exposed to dilution medium containing 1.0 M sucrose in TCM-199 supplemented with 20% FBS. Samples were incubated for 3 hours at 38.5° C under 5% CO_2[16]. The post warming viability of oocytes was determined by morphologically examinationd under an inverted microscope (200x). The oocytes were considered viable if they have spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, or cytoplasm degeneration.

Data Analysis

Data were analyzed using analysis of variance (ANOVA). The differences between control and experimental group were evaluated using Tukey's HSD test as Post-Hoc Multiple Comparison Tests. The significantly different level was considered significant at $P<0.05$.

RESULTS AND DISCUSSION

The general steps of oocyte vitrification process are equilibration, vitrification, and warming. Oocytes are exposed to an intermediate concentration of permeable cryoprotectant to avoid osmotic shock and lessened the damaging effect during the equilibration before introduced to the vitrification solution and stored in the liquid nitrogen. The permeating cryoprotectant forms a hydrogen bond with phospholipid cell layer to replace intracellular fluid. A precise concentration of permeable cryoprotectant is very important, because a high concentration is toxic for cells, while a low concentration induces the forming of the intracellular and extracellular ice crystal. The cytotoxic and damaging effect of the permeable cryoprotectant can be reduced by choosing permeable cryoprotectant with low toxicity and or combining one or more permeable cryoprotectant. In our study, we used a high concentration of cryoprotectants and an extremely rapid rate of cooling to form solid state and prevent formation of intracellular ice crystals. The oocyte morphology after vitrification warming cycle is illustrated in Figure 1.

Three combinations of cryoprotectant were used as vitrification solution. All treatments were processed through the corresponding equilibration and vitrification solutions. The viability of oocytes that preserved in cryotop and hemistraw as carrier system are presented in Figure 2. The oocytes viability was not significantly different between oocytes vitrified using cryotop and hemistraw as a carrier system. Both carrier systems used in this study were similarly effective for oocytes vitrification. The previous study showed that the sheep oocytes viability was not significantly different in both carrier systems (Winangun *et al*., 2017). Both types of carrier are open system that allows oocytes and media directly contact with liquid nitrogen (Begin *et al*., 2013). The cryoprotectant volume used on both carriers was very small (<3 µL), so it allowed the sample to reach the freezing point quickly and minimized cell damage due to cold temperature (chilling injury) during vitrification. The most critical factor during freezing that induces a chilling injury is an intermediate zone of temperature -15 to -60° C (Gao and Critser, 2000). Under these conditions, cells will begin to damage indicated by morphological changes such as mitochondrial, lipid and cytoplasmic destruction and spur abnormalities of cell chromosomes (Boiso *et al*., 2002; AlbarracÍn *et al*., 2005; Gautam *et al*., 2008).

The type and concentration of cryoprotectant were also a key factor for an oocyte vitrification successful rate. This experiment showed that EG alone as permeable cryoprotectant gave the lowest viability of oocyte after warming compared with the other groups. The highest viability of oocyte after warming was gained in oocytes vitrified with a combination of EG and DMSO ($P<0.05$).

EG as permeable cryoprotectant will diffuse through the cell membrane and maintain the balance of intracellular and extracellular fluid. It can increase the degree of dehydration, by keeping the volume of intracellular water to balance (Gilmore *et al*., 2000). This agent has a small size and thus penetrates the membrane rapidly (Shaw and Jones, 2003). EG as single permeable cryoprotectant may induce shock osmotic and cytotoxic effect to lead biochemical damage. As the results of biochemical damage are the inactivation or denaturation of specific enzymes, damage from transmembrane ion pumps, and disturbance to cellular structures and functions caused by the direct interaction between cryoprotectants with proteins and biological membranes (Lin and Tsai, 2012). It indicated that using EG only as a permeable cryoprotectant was not adequate for vitrification solution and needs another permeable cryoprotectant for oocyte vitrification.

Our results showed that combination of permeable cryoprotectants was advantageous for oocyte vitrification. It reduces the toxic and damaging effect to the cell, increase the viscosity of vitrification solution, and prevent the formation of an intracellular and extracellular ice crystal. The combination permeable cryoprotectant will act with several possible mechanisms during vitrification process including decreasing the freezing point of the vitrification solution, preventing oocytes damage from high electrolyte concentration and preventing the expose of oocytes to a high concentration of intra and extracellular electrolytes by linking to the electrolytes (Pegg, 2007).

Based on this study, it seem that the combination of EG and DMSO gave the better result than a combination of EG and PrOH. DMSO has two a polar groups and a highly polar domain that make this molecule soluble in organic and inorganic solution. DMSO is able to penetrate and leave cells without significant damage and it was reported that vitrification solutions with DMSO protect embryo membrane integrity better than solutions without DMSO (Kartberg *et al*., 2008).

Similar to this present finding, previous study also found that the best combination of cryoprotectants were

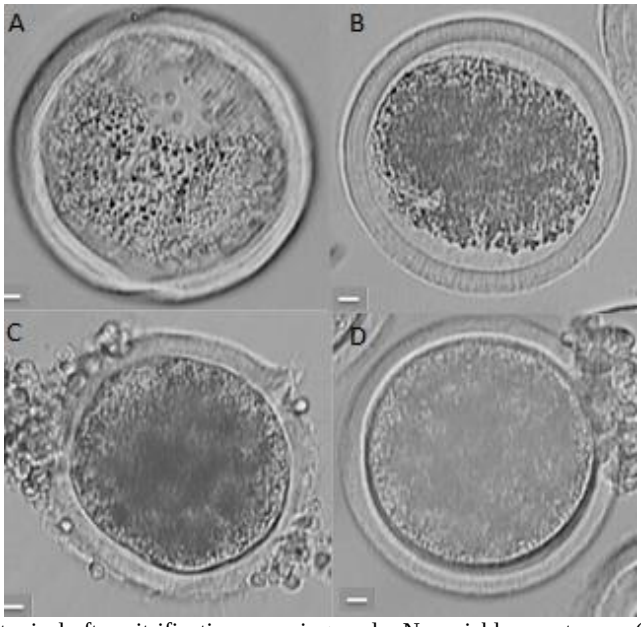

Figure 1. Oocyte morphology typical after vitrification-warming cycle. Non-viable oocyte was (A) lysis cytoplasm, (B) shrunk or un-recover cytoplasm, and (C) degenerated cytoplasm with dark cytoplasm. (D) Viable oocyte was showed by normal round shape, clear, and smooth cytoplasm. Bar scale= 10 µm

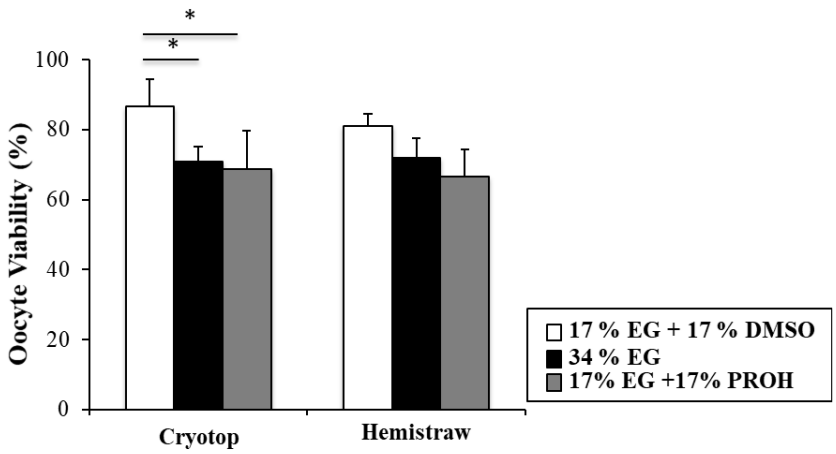

Figure 2. Oocyte viability after vitrification-warming cycle using three different combinations of cryoprotectant in cryotop and hemistraw as a carrier system (The highest oocytes viability were oocytes vitrified in 17% EG+17% DMSO, but the differences were not statistically significant, n= 50/group; *= P<0.05)

EG and DMSO for vitrification of the immature buffalo oocytes using straw or open pulled straw methods (Mahmoud *et al*., 2010). The combination of EG and PROH showed the lower oocyte viability after vitrification and warming compared with a combination of EG and DMSO. It is probably due to PrOH natural breakdown product, formaldehyde, which is considered toxic to living cells. Mahdevan *et al*. (1998) reported that formaldehyde is an unavoidable bi-product during a production of PrOH, with each batch containing formaldehyde in variable amounts. Karran and Legge (1996) reported that the level of formaldehyde increased in PrOH under storage indicating a continual breakdown of the product.

CONCLUSION

Combination of EG and DMSO as permeable cryoprotectant and cryotop as carrier system was the best system to maintain owe oocyte viability after vitrification-warming.

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