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Effect of Dosage on Combination Ethylen Glycol and Dimethyl Sulfoxide as Cryoprotectant using Vitrification on Viability of Mice (*Mus musculus*) Blastoscyst Post Warming

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ABSTRACT

Cryopreservation is a method of reproductive technology that is useful for embryo and preservation creatures that are almost extinct. Cryoprotectants are often used for vitrification are PROH, DMSO and EG because they have good and efficient results. The study objective was to know does the viability of mice blastocyst increase with the combination of intracellular cryoprotectant ethylene glycol (EG) and dimethyl sulfoxide (DMSO) in vitrification. This study used mice oocytes then carried out in vitro fertilization and cultured until reaching the blastocyst stage. The mice were superovulated using 5 IU PMSG and 5 IU hCG after 48 hours, then monomatting using infertile males. Egg cells are taken by tearing the fertilization bag and washing using MEM. Embryo is cultured so that it reaches the blastocyst phase and is then divided into 4 groups: vitrified using 30% EG, 30% DMSO, 20% combination EG + 10% DMSO and 10% P4 combination EG and 20% DMSO. The four groups were inserted into the hemi straw which has been modified with 0.25 berurn and then put back into the hemi straw with a size of 0.5. Hemi Straw which already contained embryos was inserted into a liquid N_2 container with a temperature of -196. Warming was done after the embryo was vitrified for a week. After being removed from the container, heating is done at room temperature. The embryo hemi straw was inserted into the sucrose level alternately, i.e. 0.25 M, 0.5 M and 1M respectively for 2 minutes. Warming process to remove cryoprotectants that are in the embryo. Examination of the viability of blastocyst was done using an inverted microscope. The results of this study that there were significant differences between groups using combination cryoprotectant EG 20% and DMSO 10% ($p<0.05$) with 84% viability. The combination of EG and DMSO could increase the viability of mice blastocyst. The ethylene glycol 20% and dimethyl sulfoxide 10% was the best concentration combination based on this research.

Keywords : *Food production, Vitrification, Ethylene glycol, Dimethyl sulfoxide, Blastocyst.*

Introduction

Mice are one of the experimental animals that are widely used for experiments because the gene structure is similar to humans and is easy to obtain. Cryopreservation is a method of reproductive tech-

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nology that is useful for embryo and preservation creatures that are almost extinct (Marcao, 2010). Cryopreservation has good results in various studies and one of the most widely used is vitrification. Vitrification is a fast freezing embryo at -196^{O}C without forming ice crystals (FAO, 2012). The advantage of vitrification is that it does not form ice crystals which can be deadly to cells (Gao and Critser, 2000). In vitrification method of cell or tissue incubated usingcryoprotectantthat has high concentration (Fay and Wowk, 2015). There are many studies using cryoprotectant combinations, but still showing low results, decreased embryo viability often occurs due to the warming process, the temperature changes that cause damage to cells (Pamungkas, 2010) but also because cryoprotectants cannot reduce the osmotic shock during vitrification (Pamungkas, 2010).

Cryoprotectants are often used for vitrification are PROH, DMSO and EG because they have good and efficient results (nateghi, 2017 HP). Dimethyl Sulfoxide has a molecular weight 78.13 g/mol, EG has 70 g/mol and PROH has 76.06 g/mol. Cryoprotectant size and density have different effects on various species (19 categories). Cryoprotectants are toxic, therefore their use must be done properly, according to Kartberg, many studies using a combination of EG and DMSO showing good results. Research publication show that vitrification with different cryoprotectant solutions is possible for human and mammal embryo, such as DMSO combination with ethylene glycol (EG), or EG alone (Rama *et al*., 2005; Takahashi *et al*, 2005). The combination of DMSO and EG has been used successfully by Desai *et al*. (2007). The ultrarapid freezing and vitrification most commonly employed for mouse cryopreservation. The ultrarapid method provides dehydration similar to the obtained with the conventional curve, even without cooling, allowing embryos to be plunged directly into membrane plasma, In previous studies (Mezzalira, 1991) freezing of mice morulae showing good developmental rates (83.6 to 92.9% of blastocysts) were obtained with the ultra-rapid, using 30% glycerol. Another reserch showing good result using morulae stage with viability 80% (Cseh *et al*., 1997). This research is to know the viability of mice blastosis were vitrified using a combination of cryoprotectant EG and DMSO.

Materials and Methods

This study use mouse oocytes then carried out in vitro fertilization and cultured until reach the blastocyst stage. Female mice were superovulated using 5 IU PMSG and 5 IU hCG after 48 hours, then monomatting using infertile males. The next day a vaginal plug is checked to ensure marriage. Female mice were then sacrificed with cervical os dislocation. Egg cells are taken by tearing the fertilization bag and washed using MEM. Embryo is cultured using MEM under mineral oil and stored in a 5% CO_2 incubator. Embryo is cultured so that it reaches the blastocyst phase and is then divided into 4 groups. The first group was vitrified using 30% EG, 30% DMSO, 20% combination EG + 10% DMSO and 10% combination EG and 20% DMSO. The four groups were inserted into the hemi straw which has been modified with 0.25 berurn and then put back into the hemi straw with a size of 0.5. Hemi Straw which already contained embryos was inserted into liquid N_2 container with a temperature of -196.

Warming is done after the embryo is vitrified for a week. After being removed from the container, heating is done at room temperature. The embryo hemi straw was inserted into the sucrose level alternately, i.e. 0.25 M, 0.5 M and 1M respectively for 2 minutes. Warming process to remove cryoprotectants that are in the embryo. Blastosis that has gone through a warming process is observed under an inverted microscope.

Results and Discussion

The results obtained after observing blastosis under an inverted microscope all groups survived with

Table 1. The mean and standard deviation of the amount of blastocyst between treatment groups.

Treatment	Percentage of Viability
Vitrified using 30% EG	60.00^{ab} ± 13.693
Vitrified using 30% DMSO	67.00^{ab} ± 9.747
Vitrified 20% combination EG + 10% DMSO and 10%	84.00 ^{a} ± 4.00
Vitrified combination EG and 20% DMSO	42.00 ^{b} ± 2.00

different viability. In groups with the highest viability, group of Vitrified 20% combination EG + 10% DMSO and 10% (84%) and significantly different from group of Vitrified using 30% EG (60%) p <0.05. the lowest result was in group of Vitrified combination EG and 20% DMSO with only 42% viability and significantly different from group of Vitrified using 30% DMSO (67%) p <0.05. from the results obtained group of Vitrified 20% combination EG + 10% DMSO and 10% has the best results and group of Vitrified combination EG and 20% DMSO has the worst results.

Vitrification has different successes in each study, in the process of successful vitrification by optimizing each stage of the vitrificai process, namely at the stage of oocyte collection, sperm retrieval, in vitro fertilization, clotting and warming processes. Vitrification uses cryoprotectant as a supporting material that has high toxicity, so the use and time of cryoprotectant exposure are important. Blastocyte viability can be determined by microscopic morphological examination that is not found in the pelucidic degeneration zone, the blastosol is large and the shape of the blastocyst is compact. In accordance with the study of Lieberman who used 20% EG and 20% DMSO and produced a viability of 84.5%. Lieberman's results are not much different from the group of Vitrified 20% combination EG + 10% DMSO and 10%. Recent publication shows most successful vitrification method with combination cryoprotectant using EG and DMSO each 15% concentration (Matsui *et al.*, 1995; Selman *et al.*, 2006; Desai *et al.*, 2007).

Ethylene glycol is a cryoprotectant which has a low molecular weight and high penetration power, as well as a low level of toxicity (nateghi). Dimethyl sulfoxide has a molecular weight that is not much different from Eg, besides that DMSO is relatively polar and has a compact and stable structure so that to enter the cell does not cause significant damage (Kartberg, 2008). The cryoprotectant combination of EG and DMSO eliminates the deficiencies of each cryoprotectant, according to Kartberg (2008) who said the use of cryoprotectant combinants EG and DMSO causes low toxicity, because EG reduces toxicity from DMSO as well as DMSO makes penetration of this combination more stable due to more DMSO structures compact compared to EG.

According to Mukaida *et al.*, (2003) the process of vitrification and warming occurs prior to adjusting the culture medium, the effect of cryoprotectant toxin, the occurrence of physical damage due to

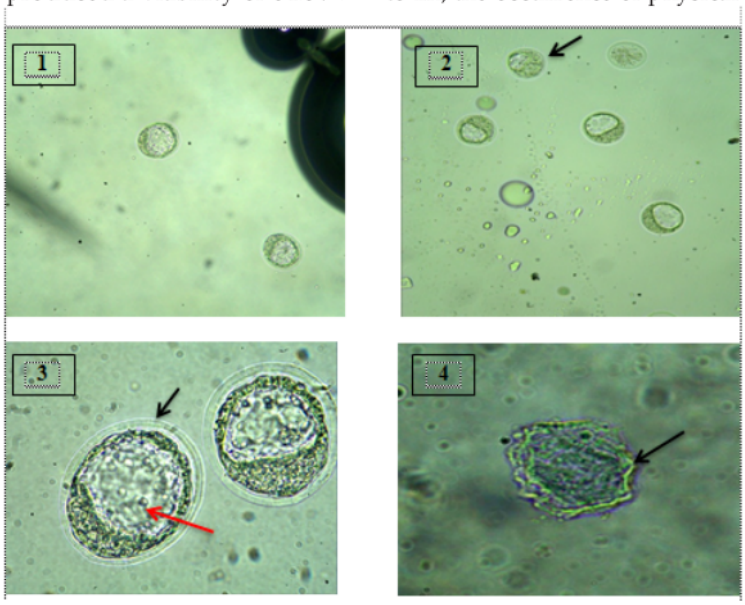

Fig. 1. Blastocyst cells. 1: blastocyst cells before vitrification (magnification 100x); 2: blastocyst cells after vitrification (100x magnification) there are dead blastocysts (signs); 3: blastocyst cells after vitrification (400x magnification), clearly visible pelucidic zones (black arrows), large blastosols (red arrows); 4: blastocyst cells die (400x magnification), the pelucid zone looks degenerated (black arrow) and irregular shape 2: blastocysts expand

crystalline ice formation during clotting, osmotic stress during cryoprotectant release of cells during warming, and lack of embryo quality good. Therefore cell death in vitrification or warming process is determined by many influencing factors. In the group of vitrified combination EG and 20% DMSO, the results of blastocyst cell viability after waming were 33%, this was very significant (P <0.05) with group of Vitrified 20% combination EG + 10% DMSO and 10%. The P4 group was a combination of 10% cryoprotectant EG and 20% DMSO. The combination of group of Vitrified combination EG and 20% DMSO showed a decrease in post-warming blastocyte cell viability, this happened because the DMSO concentration was 20% so that cryoprotectant had a high toxicity effect, 10% EG had a less stable structure when penetrating, so in the group of EG 10 combination % and 20% DMSO can reduce post-warming viability.

The results obtained in this study with 30% EG cryoprotectants had good results, 60% of these results were very significantly different (P <0.05) with group of vitrified using 30% DMSO with 66% viability. Cryoprotectant use in the (Vitrified using 30% EG) and (Vitrified using 30% DMSO) groups gave viability presentations above 50%, this proves that the use of a single cryoprotectant is able to provide good results, blastocyst cell death can be caused due to the nature of each cryoprotectant.

Conclusion

The conclusion of this research is combination cryoprotectant DMSO and EG can increase the viability of mice blastocyst. Because the bad characteristic each cryoprotectant, the other concentration of combination EG and DMSO decrease the viability of mice blastocyst and the most suitable and show good result is combination cryoprotectant EG 20% + DMSO 10%.

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Authors' contributions

Research concept and design:Zakiah Saumi, Collection and/or assembly of data: Epy Muhammad Luqman, Data analysis and interpretation:Viski Fitri Hendrawan, Writing the article: Zakiah Saumi, Widjiati; Critical revision of the article: Epy Muhammad Luqman; Final approval of the article: Zakiah Saumi, Epy Muhammad Luqman.

Competing interests

The authors declare that there is no conflict of interest.

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