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Cryoprotectant combination ethylene glycol and propanediol on mice blastocyst viability post vitrification

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Abstract. The best method to store embryo is vitrification using cryoprotectant, but so far, the viability of the embryo after warming process is decreasing. This objective of this research was to know the effect combination ethylene glycol and propanediol as intracellular cryoprotectant on mice blastocyst viability. The experimental animals were female mice strain Balb/C which was super ovulated using *Pregnant Mare Serum Gonadotropin* (PMSG) and *Human Chorionic Gonadotropin* (hCG). The oocyte collected from the ampulla of tuba fallopian. In Vitro Fertilization was done by placing the sperm and the oocyte in the same medium then cultured until became blastocyst. The blastocyst was classified into: T1: ethylene glycol 30%, T2: propanediol 30%, T3: ethylene glycol 20% and propanediol 10%, and T3: ethylene glycol 10% and propanediol 20%. Each group was vitrified for a week then warmed immediately. Examination of the viability of blastocyst was done using an inverted microscope after warming process. The statistical analysis showed that there were significant differences between single cryoprotectant and combination cryoprotectant group $p < 0.05$. The best cryoprotectant combination was ethylene glycol 10% and propanediol 20%.

1. Introduction

Vitrification is the most suitable technique to cryopreserve living cell. The cell is being hydrated and froze into $-196\text{ }^{\circ}\text{C}$. In vitrification, cells or tissues are placed into a medium with high concentration of cryoprotective agents, known as cryoprotectants [1]. Vitrification is the best method to store frozen embryo because it is protecting the embryo from environmental disasters and infectious diseases. The advantage of vitrification that makes researcher like to use is the absence of crystallization that caused embryo become death. Intracellular crystallization mechanically can make the organelle of cell damage and finally the embryo is deceased [4]. The use of vitrification generally is using single intracellular cryoprotectant, there is some intracellular cryoprotectant that commonly used in vitrification, and two of them are ethylene glycol (EG) and propanediol (PROH).

Ethylene glycol (EG) has a small molecular weight that makes it has high permeability, and it also has low toxicity [6]. EG is usually used as oocyte cryopreservation but it is also used to cryopreserve embryo. Ethylene glycol has the ability to penetrate the cell membrane rapidly than any other cryoprotectant, this is because the molecular weight is smaller (62.07) compared to the other cryoprotectants. In the other hand, the excessive shrinkage observed suggest the risk of an intense osmotic stress by exposing the embryo to the ethylene glycol concentration [3].



Propanediol (PROH) is cryoprotectant that can be used as an alternative in cryopreservation because of their effectiveness in protecting the cell. Research result show relatively low in morphological abnormality. Furthermore, PROH is cryoprotectant with small molecular weight so that they will easily penetrate cell membrane [10]. The level of success in vitrification is the use of cryoprotectant in high concentration but the toxic effect is relatively low [7]. PROH has great ability to maintain morphology of the cell during vitrification it is more stable [10].

The cryoprotectant used for embryo vitrification commonly is single cryoprotectant, but after warming process commonly the viability of embryo is decreasing, and this becomes one of the disadvantages of vitrification using single cryoprotectant. The use of cryoprotectant is the most important thing in vitrification, but unfortunately, there is no cryoprotectant that is safe for living tissue. All cryoprotectant has a toxic effect that can cause osmotic damage depending on its concentration and duration of exposure. During cryopreservation with vitrification method, there is physicochemical changing due to physiologic stress. Morphological changing like fracture zone, lysis and degeneration of the membrane, and intracellular damage that cannot be seen with a usual microscope. The toxic effect of cryoprotectant can be minimized by giving the right concentration during vitrification process [11].

Cryopreservation of embryos was mainly performed on pronucleus-stage or cleavage-stage embryos in the past. However, recently, a great amount of research on blastocyst stage freezing has been performed by increasing the incidence of blastocyst formation through improvement of the culture medium and culture conditions. It has been known that blastocyst-stage freezing has a higher viability than early cleavage-stage embryo freezing and that frozen-thawed blastocyst-stage embryo transfer could improve implantation and pregnancy rates because it is physiologically suited to the environment of the uterus [8].

Both EG and PROH have its own advantages and disadvantages, EG because of its high permeability has the higher potency to cause osmotic stress meanwhile PROH with its bigger molecular weight make it more stable. Research done by [9] showed EG is safer to use than any cryoprotectant because of its lower toxic effect.

This research was aimed to find the effectiveness of vitrification using a combination of intracellular cryoprotectant especially EG and PROH in the same stage of the embryo, especially blastocyst.

2. Materials and method

This research was using experimental laboratories. This research was using mice embryo in blastocyst stage as the sample. There were 4 treatment groups in this research, Treatment Group 1 (T1) : PBS + EG 30%, Treatment Group 2 (T2): PBS + PROH 30%, Treatment Group 3 (T3): PBS + EG 20% + PROH 10% and Treatment Group 4 (T4): PBS + EG 10% + PROH 20%.

2.1 Research Ethical Clearance

This research received ethical clearance number: 717-KE released by Animal Care and Use Committee, Universitas Airlangga, Faculty of Veterinary Medicine.

2.2 Research Materials and Equipment

Materials used in this research were female and male mice Balb/C strain, vasectomized mice (all the mice obtained from PUSVETMA Surabaya), Phosphate Buffer Saline (PBS), sterile aquadest, physiologist NaCl 0,9%, propanediol 30%, 20%, and 10% (Sigma®, St. Louis, USA), and ethylene glycol 30%, 20% and 10% (Sigma®, St. Louis, USA), liquid nitrogen (N2), Minimum Essential Medium (MEM) (Sigma®, St. Louis, USA), oil mineral (Sigma®, St. Louis, USA), Pregnant Mare Serum Gonadotropin (PMSG) (Folligon®, Intervet, Boxmeer, Holland), human Chorionic Gonadotropin (hCG) (Chorion®, Intervet, Boxmeer, Holland), dilution of sucrose also 0.25 M, 0.5 M and 1 M. Equipment that used in this research were CO2 5% incubator (Thermo Fisher Scientific), container for liquid of N2, laminar air flow, scissor, infusion set, nippers, inverted microscope (Meiji Techno America), scalpel, beaker glass, syringe disposable (1 cc, 3 cc, 10 cc), Pasteur pipet, mini

straw 0.25 ml (Sigma®, St. Louis, USA), petri dish disposable (Thermo Fisher Scientific), Millipore split (Thermo Fisher Scientific), and Bunsen burner.

2.3 Research Method

2.3.1 Superovulation

Superovulation was done by injecting Pregnant Mare Serum Gonadotropin (PMSG) 5 IU with dosage 0.1 cc into female mice intraperitoneally and after forty-eight hours then the mice were injected with human Chorionic Gonadotropin (hCG) 5 IU intraperitoneally. After HCG injection, each female mouse were mono-mated by placing in a cage with one vasectomized mouse. Eighteen hours later after mono mating, vaginal plug examination was performed. If there was vaginal plug means copulation has occurred.

2.3.2 Oocyte and sperm collection

Oocytes of female mice with the vaginal plug was collected from ampulla of tuba fallopian by using dislocation of *os. Cervicalis*. The tuba fallopian was flushed by using PBS solution and poured into the petri dish. The oocyte obtained from the ampulla of a fallopian tube of the mice. Under the inverted microscope, the tuba fallopian was flushed by ripping off ampulla. The oocyte was washed in MEM medium and prepared for in-vitro fertilization.

Sperm collected from epididymis cauda of fertile male mice, after dislocating the cervical from male mice. The cauda of epididymis was taken with avoiding the blood, tissue fluid and fat as much as possible. The cauda of epididymis washed in the MEM medium and put in the disposable dish. Sterile scissor was used to chop the cauda of epididymis so that the sperm will spill out from the duct, and after that MEM medium was added into chopped epididymis cauda. The sperm was collected and washed in the washing medium. The sperm then capacitated in MEM medium.

2.3.3 In Vitro Fertilization and embryo culture

In vitro fertilization was started right after oocyte and sperm preparation and in vitro fertilization medium preparation. The fertilization started by putting the oocyte and the sperm in the same fertilization drop medium, then was incubated in the CO₂ incubator, after seven hours, the medium was examined and oocyte that had been being fertilized was washed to remove the granuloza cells. The oocyte that had been being fertilized was moved into the medium culture and then incubated. In the day two and four the culture medium was changed to give enough nutrition for supporting embryo development until blastocyst stage. Mice embryo was reached blastocyst stage in the day five.

2.3.4 Vitrification

The blastocyst was exposed in the cryoprotectant in the different concentration filled with PBS + EG 30% and PBS + PROH 30%, and the other two petri dishes was filled with PBS + EG 20% + PROH 10% and PBS + EG 10% + PROH 20%, the exposure duration was two minutes. The blastocyst was kept in the tip of hemi-straw and the equilibrated by placing the hemi-straw in the evaporation of N₂ then dipping the embryo in the N₂. The hemi-straw then was packed in the bigger straw and fixated the tip of the hemi-straw. Finally, the bigger straw was kept in the straw's disc and placed in the liquid N₂ container.

2.3.5 Cryopreservation

Cryopreservation was done in three stages by exposure embryo three times into sucrose solution with different concentration (0.25 M for 1 minute, 0.5 M, and 1 M for 2 minutes).

2.3.6 Data Analysis

The data then analyzed using Kruskal Wallis in SPSS (Statistical Programs for Social Scientific) and continued with Mann Whitney U test.

3. Result and discussion

This research was examining the viability of embryo. The analytic result from the viability of blastocyst after warming process was shown in table 1 below.

Table 1. Kruskal Wallis Test result on embryo viability

Group	Mean \pm SD
T1 ^a	(60.00 \pm 13.693 ^b)
T2 ^b	(80.00 \pm 11.180 ^{ab})
T3 ^c	(88.00 \pm 10.954 ^{ab})
T4 ^d	(100.00 \pm .000 ^b)

*The different superscript showed the significant difference.

^a Treatment Group 1 (T1): Ethylene glycol 30%

^b Treatment Group 2 (T2): Propanediol 30%

^c Treatment Group 3 (T3): Ethylene glycol 20% + propanediol 10%

^d Treatment Group 4 (T4): Ethylene glycol 10% + propanediol 20%

Blastocyst that had been being vitrified with a combination of cryoprotectant had higher viability and the highest viability was blastocyst which vitrified with a combination of ethylene glycol 10% and propane diol 20%. The most significant difference was between blastocyst which vitrified with ethylene glycol 30% and blastocyst which vitrified with a combination of ethylene glycol 10% and propane diol 20% (Table 1).

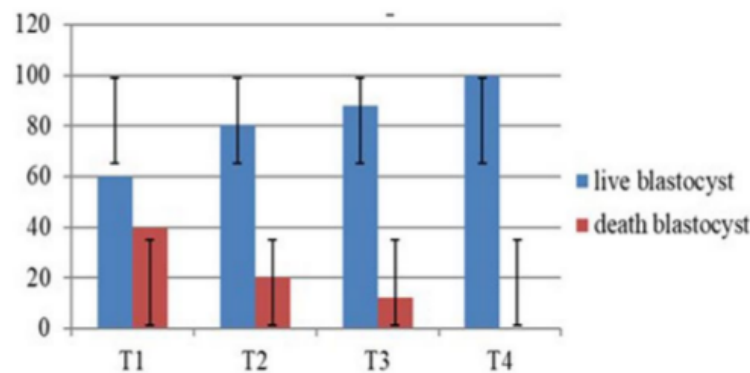


Figure 1. The graph from the percentage of viability blastocyst

Live expanded blastocyst after warming process could be identified by its morphology. The membrane plasma was clear, there was no granular in the embryo, the cytoplasm was clear, there was no degeneration and zona pellucida and cytoplasm defined clearly (Figure 2b).

Death blastocyst after warming process also could be identified by its morphology. The membrane plasma was irregular, there were granular in the embryo, the cytoplasm was full of granular, there was degeneration and zona pellucida and cytoplasm cannot be defined clearly. The size of the blastocyst was also shrunken (Figure 2c).

Vitrification is very simple method yet improves cryopreservation because it eliminates mechanical injury from ice formation, eliminates the need to find optimal temperature and warming rates, enables freezing to be rapid enough to prevent injury [5].

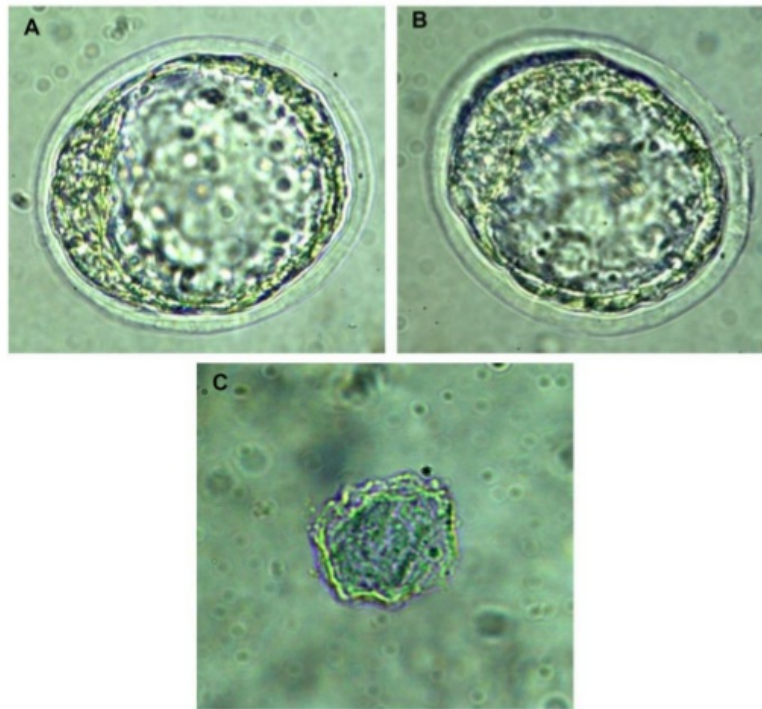


Figure 2. Mice blastocyst. A) showed expanded blastocyst before vitrification method, B) showed expanded blastocyst after warming process, C) showed death blastocyst

The best stage to cryopreserve embryo is in the blastocyst stage, the presence of Na^+/K^+ ATPases and aquaporins ensures channeling of ions and water, allowing the formation of a fluid-filled cavity, denominated blastocyst cavity. In this stage the cryoprotectant slowly was defused in aquaporins protein on the plasma membrane of mice blastocyst. The diffusion of cryoprotectant made perivitelline space became looser. After cryoprotectant penetrated into the cell membrane, there was the formation of zona pellucida hardening on blastocyst [2].

From this research showed that vitrification using ethylene glycol 30% as treatment group one (T1) had the lowest viability of blastocyst after warming process which was 60%. Ethylene glycol has the ability to penetrate to the cell membrane rapidly than any other cryoprotectant, this is because the molecular weight is smaller (62.07) compared to the other cryoprotectants. This condition enables its rapid influx during cryoprotectant exposure and its rapid efflux. During warming process water movements across the membrane of the cells made the blastocyst regain the initial size after warming [3]. The small molecule of ethylene glycol made them crossed the membrane rapidly then it is plausible to assume that the rapid efflux during warming is related to an osmotic damage resulting from an excessive swelling when they are suddenly placed at 37 °C and the cell membrane became unstable.

Propane diol is intracellular cryoprotectant that has small molecular weight (76.09), so that propane diol can easily penetrate the cell membrane. Propane diol molecular weight was made the cell membrane more stable when the cryoprotectant was penetrated, so the osmotic stress from the influx of cryoprotectant could be reduced. During the warming process which was the efflux of cryoprotectant, the osmotic damage also could be reduced because of its stable plasma membrane [11].

In this research showed the viability of blastocyst used propane diol 30% as treatment group two (T2) is higher than treatment group one, the viability of blastocyst was 80% and significantly different ($p < 0.05$) with treatment group one. It was similar to research done by Szurek & Eroglu (2011), that propane diol is the best cryoprotectant than the other cryoprotectant.

From the data showed that using ethylene glycol 20% and propane diol 10% as treatment group three (T3), the viability of blastocyst was 84.6% which was significantly different ($p < 0.05$) with treatment group one but it was not significantly different ($p > 0.05$) with treatment group two. The data showed that this combination could reduce the toxic effect from propane diol with combination ethylene glycol 20%, and the used of propane diol with concentration 10% could help ethylene glycol molecule more stable during penetration in the cell membranes but still there were blastocyst that became death caused by the osmotic stress from ethylene glycol 20% still affected the blastocyst during vitrification process.

While using ethylene glycol 10% and propane diol 20% as treatment group four (T4), showed the viability of blastocyst was 100%. The result was significantly different ($p < 0.05$) from treatment group one and treatment group two, with this result it was known that using propane diol with concentration 20% could make the membrane plasma more stable during cryoprotectant penetration and warming process, also ethylene glycol with concentration 10% could reduce the toxic effect from propane diol.

4. Conclusion

In conclusion, the combination of ethylene glycol and propane diol as an intracellular cryoprotectant in vitrification can maintain the viability of mice blastocyst. The best concentration combination based on this research was 10% ethylene glycol and 20% propane diol.

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