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## Low Concentration of Ethylene Glycol Improve The Recovery Rate of Human Spermatozoa After Vitrification

(ETILEN GLIKOL KONSENTRASI RENDAH MENINGKATKAN RECOVERY RATE SPERMATOZOA MANUSIA PASCAVITRIFIKASI)

Rini Widyastuti<sup>1</sup>, Sony Heru Sumarsono<sup>2</sup>,  
Arief Boediono<sup>3</sup>, Siti Darodjah Rasad<sup>1</sup>

<sup>1</sup>Laboratorium of Animal Reproduction and Artificial Insemination,  
Animal Husbandry Faculty, Padjadjaran University,

<sup>2</sup>Research Groups of Physiology, Developmental Biology and Biomedical,  
School of Life Science and Technology, Bandung Institute of Technology;

<sup>3</sup>Lab of Embryology, Department of Anatomy, Physiology and Pharmacology,  
Faculty of Veterinary Medicine, University of Bogor Agriculture

Jl. Raya Bandung-Sumedang Km 21 Jatinangor, Sumedang, Indonesia 45363  
Phone: (022) 7798241; Fax: (022) 7798212; E-mail: r.widyastuti@unpad.ac.id

### ABSTRACT

The use of cryoprotectants for the cryopreservation of human spermatozoa, oocytes, zygote, early cleavage stage of embryos and blastocyst is an integral part of almost every human In Vitro Fertilization program. Moreover, the cryopreservation of these types of cells by direct plunging into liquid nitrogen (-196°C) usually requires a high concentration of cryoprotectant with a consequent of cytotoxic effect. The aim of this study was to observe the effect of ethylene glycol concentration on the spermatozoa recovery rate following the vitrification process. Earle's balanced salt solution + 0.25 M sukrosa + 1 % human albumine serum as basic solution supplemented with some different concentrations of ethylene glycol (ie: 36.25%; 18.25%; 9.12%; 4.56%; 1.14% and 0.57%) was used to evaluate the motility and viability of spermatozoa following vitrification. Human's spermatozoa from ejaculates with progressive motility and viability above 50% were used as samples. Samples were mixed with vitrification solution and then loaded into 0.25 mL straws, equilibrated for 10 minutes at room temperature before plunged into liquid nitrogen. Spermatozoa thawing was done 24 hours after the vitrification. The result showed that the highest decreases of spermatozoa motility and viability (100%, 96.70%) was found in the samples that were added with vitrification medium contained 36.25% of ethylene glycol concentration. On the other hand, the lowest decrease of the spermatozoa motility and viability (14.11%, 43.81 %) found in the samples without ethylene glycol supplementation. It can be concluded that the highest spermatozoa recovery rate was obtained from the vitrification using a low concentration of ethylene glycol.

Keywords: cryoprotectant, vitrification, ethylene glycol, spermatozoa motility, spermatozoa viability

### ABSTRAK

Penggunaan krioprotektan sebagai zat pelindung pada kriopreservasi spermatozoa, oosit, zigot, tahap awal pembelahan embrio, dan blastosis merupakan bagian integral pada Fertilisasi *in vitro*. Kriopreservasi dengan memaparkan secara langsung ke dalam nitrogen cair (-196°C) memerlukan krioprotektan dengan konsentrasi yang tinggi. Kondisi demikian menimbulkan efek negatif berupa toksisitas pada sel yang dibekukan. Penelitian ini bertujuan untuk mengetahui pengaruh konsentrasi etilen glikol pada *recovery rate* spermatozoa setelah proses vitrifikasi. *Earle's balanced salt solution* + 0,25 M sukrosa + 1% *human albumine serum* sebagai media dasar, ditambahkan dengan etilen glikol dengan konsentrasi yang berbeda (yaitu: 36,25%; 18,25%; 9,12%; 4,56%; 1,14% dan 0,57%) digunakan untuk mengevaluasi motilitas dan viabilitas spermatozoa selama proses vitrifikasi. Sampel yang digunakan adalah spermatozoa manusia yang berasal dari ejakulat dengan motilitas progresif dan viabilitas lebih dari 50%. Sampel dicampur dengan media vitrifikasi dan kemudian dimasukkan ke dalam *straw* 0,25 mL, equilibrasi dilakukan selama 10 menit pada suhu kamar sebelum dipaparkan ke dalam nitrogen cair.

*Thawing* dilakukan 24 jam setelah vitrifikasi. Hasil penelitian menunjukkan bahwa penurunan tertinggi terhadap motilitas dan viabilitas spermatozoa (100%, 96,70%) ditemukan pada sampel yang ditambahkan dengan media vitrifikasi yang mengandung 36,25% etilen glikol. Di sisi lain, penurunan terendah terhadap motilitas spermatozoa dan viabilitas (14,11%, 43,81%) ditemukan dalam sampel tanpa suplementasi etilen glikol. Dapat disimpulkan bahwa *recovery rate* tertinggi diperoleh dari vitrifikasi yang menggunakan etilen glikol dengan konsentrasi paling rendah.

Kata-kata kunci : krioprotektan, vitrifikasi, etilen glikol, motilitas spermatozoa, viabilitas spermatozoa

## INTRODUCTION

Vitrification as a cryopreservation method has many advantages, such as no ice crystal formation through a significant increase in the cooling rates and the use of a high concentration of cryoprotectant. Compared to a slow freezing method, a vitrification has an economic advantage and its cost benefit, because it does not need freezing instruments and requires only a few second on the process. Vitrification has been applied successfully in the preservation of mouse embryos (Rall and Fahy, 1985). It should be possible to apply the same technique to spermatozoa cryopreservation. The key success in the use of cryoprotectant for vitrification is the type and the concentration of cryoprotectant which affect the recovery rate of spermatozoa after the vitrification process. Generally, a good vitrification solution is containing a single cryoprotectant with a high concentration of about 30-50% (Ali and Shelton, 1993). However, the use of a high concentration of cryoprotectant could induce the toxic effects to the spermatozoa.

Some spermatozoa vitrification methods have been developed to find the best way to decrease the toxic effects of cryoprotectant, such as addition of serum and sucrose into the base medium (Isachenko *et al.*, 2008), glycerol (Saki *et al.*, 2009; Peirouvi *et al.*, 2007), ethylene glycol, trehalose and dextran serum substitute (Schuster *et al.*, 2003), carbohydrate (glucose, sucrose and trehalose) for ultra-rapid cryopreservation by exposing the sample into the liquid nitrogen directly (Schulz *et al.*, 2006), or without using a cryoprotectant (Isachenko *et al.*, 2004). Reducing the toxicity effects of cryoprotectant could be done by: (i) using a type of cryoprotectant that has low toxicity level (Boediono, 2003), (ii) combining both impermeable extracellular cryoprotectant and permeable intracellular cryoprotectant (Vatja and Kuwayama, 2006). An impermeable cryoprotectant is a chemical which has a large molecule size and is unable to diffuse into the

cell, such as glucose, sucrose, raffinose and some protein. Permeable cryoprotectant as dimethyl sulphoxide (DMSO), propylene glycol (PG) and ethylene glycol (EG) has small molecular size thus, they easily diffuses into the cell (Bautista *et al.*, 1998). Among those, EG has the lowest toxicity effect (Bent, 2008). Ethylene glycol is used as a permeable cryoprotectant for freezing spermatozoa of gorillas, monkeys, and Rhesus monkey (Li *et al.*, 2005). Ethylene glycol diffuses through the membrane of the spermatozoa with a higher speed than other permeable cryoprotectants (Gilmore *et al.*, 1997). It was expected that the use of EG, sucrose and human albumin serum (HAS) in this study could minimize the toxic effects of cryoprotectant and increased the recovery rate of spermatozoa after vitrification. Therefore, the aim of this study was to evaluate the effect of different concentrations of EG on spermatozoa recovery rate following vitrification process.

## RESEARCH METHODS

### Sample

Ejaculates were obtained from 10 donor men by masturbation after at least 2-7 days of sexual abstinence. The ejaculates were selected after liquefaction for 30 minutes. From those that had a concentration of 20 million or more spermatozoa/mL and showed at least 50% progressive spermatozoa motility and viability were used as samples. Semen analysis was performed according to the published guidelines of the World Health Organization (2010).

Each ejaculate was divided into eight equal parts. Each part was diluted with earle's balanced salt solution (EBSS) medium then centrifuged 600 g for 10 minutes to remove seminal plasma. After centrifugation, spermatozoa pellet was diluted with 500  $\mu$ L EBSS medium. The diluted suspensions of spermatozoa were again diluted (1:1) with vitrification medium/EBSS + 0.25 M sukrosa + 1% HAS as a basic solution

supplemented with EG at various concentrations (36.25%; 18.25%; 9.12%; 4.56%; 1.14%, 0.57%; or 0%).

### Measurement of Osmolarity

Osmolarity measurements conducted to determine the influence of seminal plasma and cryoprotectants on spermatozoa motility and viability. Osmolarity of ejaculates, (i). Ejaculates + basic medium, (ii). Ejaculates + basic medium +36.25% EG (iii) were measured directly by using osmometer.

### Vitrification and Thawing

After dilution, each specimen was allowed to be equilibrated at room temperature for 10 minutes before vitrification, then spermatozoa motility and viability was assessed. Following the assessment, each specimen was loaded into a 0.25 mL plastic straw using a syringe and then sealed. The straws were vaporised in liquid nitrogen vapor for five seconds, then plunged into liquid nitrogen directly and stored until 24 hours. The straw were taken out from the liquid nitrogen, thawed at 37°C water for five seconds and then the tip of the straw was cut and the sample was put into a microtube. Spermatozoa motility and viability was evaluated.

### Evaluation

Samples were assessed for percentage of progressive forward motility and viability by counting 200 spermatozoa. Spermatozoa motility and spermatozoa viability evaluation performed three occasions, immediately after liquefaction, after diluted with vitrification medium and after thawing. The Makler's chamber was used for motility evaluation under the light microscope using 400 times magnification. Two different categories of spermatozoa motility was used, ie: 'a' category for motile spermatozoa and 'b' category for immotile spermatozoa.

The spermatozoa viability was evaluated using eosin-nigrosin staining. Spermatozoa were mixed with eosin-nigrosin (1:1), put on the object glass and smear, and then dried. Spermatozoa viability was observed under a light microscope with a magnification of 400 times. Viable spermatozoa was characterized by pale color while the nonviable spermatozoa gave a red color on the head area. Data were analyzed by descriptive analysis.

## RESULTS AND DISCUSSION

The osmolarity of samples decreased around 123 mOsm/L compare to fresh ejaculates. On the other hand, when the ejaculates supplemented with basic solution + 36.25% EG, the osmolarity increased around 74 mOsm/L compared to the fresh ejaculates. It means, when ejaculates were supplemented with the basic solution, they become hypo-osmotic in contrast; they become hyperosmotic when supplemented with the basic solution + 36.25% EG. The osmolarities of ejaculates and samples were shown in Table 1.

After additional of vitrification medium and the samples were equilibrated at the room temperature, the motility and viability of spermatozoa were assessed. The data showed that the highest decrease of the spermatozoa motility (100%) was found in the samples 36.25% of EG was added. However, the lowest decrease of the spermatozoa motility (14.11±6.63%) was found when samples were added with the basic solution without EG. Between both concentrations, the decreases of the spermatozoa motility have gradually occurred parallel with the decreases of EG concentrations. Twenty-fourth hours after vitrification, the samples were then warmed into the water with a temperature of 37°C for five seconds, and the decreases of the motility and viability of the spermatozoa were assessed. We found that the decrease of the motility of the spermatozoa was the highest (100%) in the samples that added with the basic solution supplemented with EG at three different concentrations (i.e.: 36.25%; 18.25%; and 9.12%), and the lowest decrease of the spermatozoa motility (44.03±6.63%) was found in the samples without EG supplementation (the basic solution only) (Figure 1).

Table 1. Osmolarity of human ejaculates and vitrification media

Samples	Mean ± SD (mOsm/L)
Ejaculates	511.80±51.71
Ejaculates+basic solution+0 % EG	388.89±28.72
Ejaculates+basic solution+36.25% EG	585.80±95.89

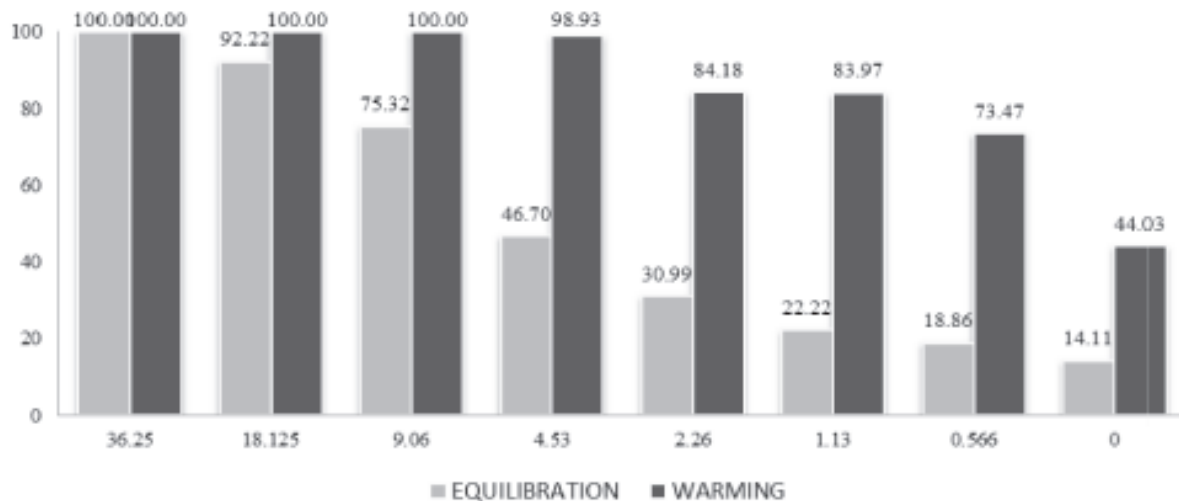


Figure 1. Decreases of the motility of spermatozoa before (equilibration) and after vitrification (thawing) using basic solution supplemented with various concentrations of ethylene glycol/EG.

Based on the results in Figure I, an impairment decrease of the spermatozoa motility at the equilibration stage was caused by increasing the osmolarity of the medium used. It can be seen that at the time of spermatozoa exposed to the medium containing EG with lower concentrations, the percentage of progressive spermatozoa motility was getting higher. The increase of osmolarity caused a hypertonic condition, hence it increased the friction of flagella and inhibited microtubule filament's sliding the spermatozoa (Santymire *et al.*, 2006).

The decrease of the spermatozoa motility after thawing was caused by the stress from the freeze-thawing process. That stress might be caused by the cold shock which results from reactive oxygen species (ROS) and damages the mitochondria. Reactive oxygen species influenced the spermatozoa function by decreasing the spermatozoa kinetic. The damage of the mitochondria caused spermatozoa less energy for motility because mitochondria as the main source of oxidative energy production through adenosine three phosphates (ATP) with the electron transport chain (ETC). Formation of ATP through oxidative phosphorylation in the inner mitochondrial membrane was transferred to the microtubules and initiate the movement of spermatozoa. Adenosine three phosphate that was synthesized in the mitochondria would initiate spermatozoa motility by activating dynein ATPase under the mitochondria (Breitbart and Ness-Arden, 1995). The

spermatozoa motility occurred because of the existence of asymmetry and the rhythmic movement of the three dimensions of the flagella. The movement resulted from the complex arrangements by flagella, except at the distal (end piece), only contained a pair of microtubules that were in the middle (Luconi *et al.*, 2006).

Similar phenomena occurred in the viability of spermatozoa. During the equilibration stage, the highest decrease of the spermatozoa viability ( $58.36 \pm 28.11\%$ ) was found in the samples added with the basic solution supplemented with the highest concentration of EG (36.25%), and the lowest decrease of the spermatozoa viability ( $6.44 \pm 5.03\%$ ) was found in the samples added with the basic solution without EG. The viability of spermatozoa was gradually decreased in the samples added with the basic solution supplemented with various concentrations of EG. After vitrification process, the highest decrease of the viability of the spermatozoa ( $96.70 \pm 0.77\%$ ) was found in the samples that were added with the basic solution supplemented with the highest concentration of EG (36.25%), and the lowest decrease of the viability of the spermatozoa ( $43.81 \pm 22.56\%$ ) was found in the samples that were added with the basic solution without EG. Between these concentrations, the decreases of the spermatozoa viability gradually declined in parallel with EG concentrations (Figure 2).

The hypertonic also decreased the percentage of the spermatozoa viability at equilibration. During equilibration process, dehydration and

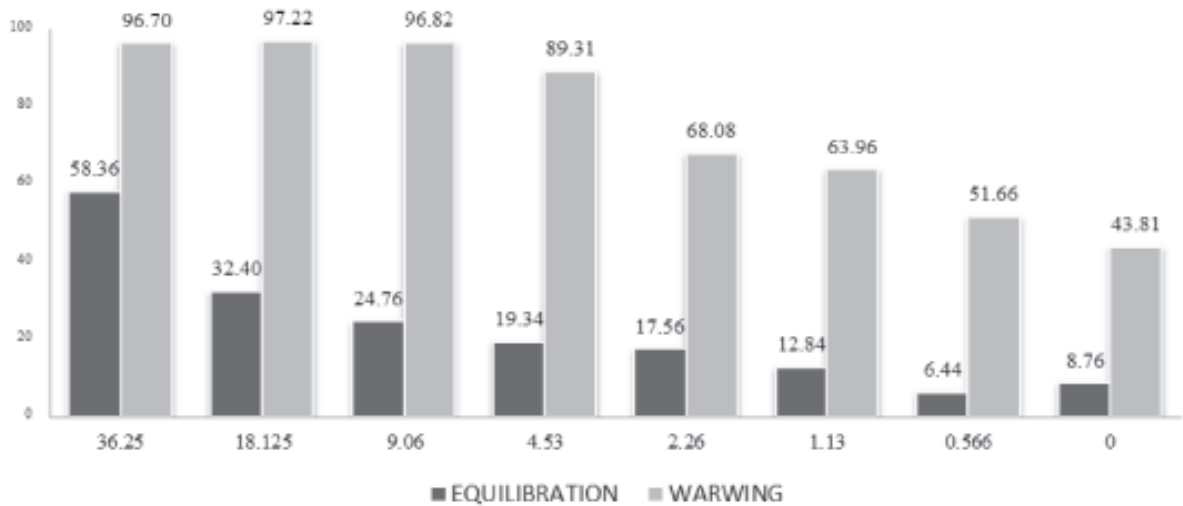


Figure 2. Decreases of the viability of spermatozoa before (equilibration) and after vitrification (thawing) using the basic solution supplemented with various concentrations of ethylene glycol/EG.

rehydration happened, the liquid of spermatozoa was pulled out and replaced by the intracellular cryoprotectant that used. Therefore, it could impair the membrane of spermatozoa and decreased the spermatozoa viability. At the thawing phase, the damage on the plasma membrane was caused by the stress from the freeze-thawing process. Membrane stress has resulted from the combined effects of dehydration and rehydration of spermatozoa with membrane lipid phase transition (Hammerstedt *et al.*, 1990). The damage on the plasma membrane increased the permeability of the cell so that the chemicals freely passed through the plasma membrane and got into the cell. However, the damage on the plasma membrane in non-viable spermatozoa caused the function of sodium pump system did not work properly, thus sodium ions bound with eosin-nigrosin dye get into the cell and remained in and colored spermatozoa, especially on the head area. In addition, the percentage decrease in the spermatozoa viability after thawing was often associated with the increase production of ROS that would induce membrane lipid peroxidation and reduced antioxidant capacity in these cells, such as dismutase and glutathione superoxide to defense free radicals (Watson, 2000).

Based on the data of this study, the decrease on motility and viability of spermatozoa vitrified without EG was lower than those vitrified with EG-contained vitrification medium. The previous study found that the spermatozoa motility and

viability gradually decreased in parallel with the increase of the concentration of EG as a permeable cryoprotectant on Test-Tris-Egg yolk based dry diluent (TTE). Ping *et al.* (2012) reported that about 10% EG showed the lower spermatozoa motility than 3% of that on tree wild shrew epididymis spermatozoa cryopreservation. This result showed that higher EG concentrations caused more damages in the spermatozoa. This was probably related closely to the toxic effects of EG. At the high concentrations, EG was absorbed into the spermatozoa in a large amount, so it could not be tolerated and caused spermatozoa death.

Another result showed that spermatozoa could be vitrified without EG as intracellular permeable cryoprotectant rather it worked in the presence of extracellular cryoprotectant that was in the basic solution. In this study, the decrease of progressive spermatozoa motility from samples vitrified with the basic solution (buffer solution which contain sucrose and HAS) was higher when compared to the results reported by Isachenko *et al.* (2008). The difference in these results might be caused by the difference of optimization techniques that were used during vitrification process. Isachenko *et al.* (2008) optimized samples using a swim-up method prior to vitrification, which could separate motile spermatozoa from immotile, while in this study separation of motile and immotile spermatozoa was not performed. The previous study showed that the spermatozoa of mice was successful to



be frozen using skim milk in high protein added sugar and raffinose without using intracellular cryoprotectant (Kusakabe *et al.*, 2001).

Spermatozoa has a very compact structure and contains a large number of proteins, sugars and other components that make an intracellular matrix to have a high viscosity; however, it also has compartments that allow functions as a natural cryoprotectant (Isachenko *et al.*, 2004). The high protein content will lead solution in the cells to have high viscosity so that it can help to the intracellular vitrification. As a result, the spermatozoa requires higher concentrations of cryoprotectant for the vitrification of extracellular than that in intracellular vitrification. Sucrose is a sugar group that is not able to diffuse through the plasma membrane, thereby sucrose will produce osmotic pressure that induces dehydration process and reduces the formation of intracellular ice crystals. Sucrose also interacts with phospholipids in the plasma membrane hence the spermatozoa membrane adjust to survive during freezing.

### CONCLUSION

The highest spermatozoa recovery rate was obtained from the vitrification system using a low concentration of ethylene glycol.

### SUGGESTION

The high decreases spermatozoa motility and viability during vitrification process showed the sample preparation did not optimal, it is necessary to optimize sample before vitrification with swim up method. For the further research, is need to evaluate the DNA integrity and acrosomal status of spermatozoa after vitrification process.

### ACKNOWLEDGEMENT

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