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Effect of cryoprotectants on sperm vitrification

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ABSTRACT: One of the problems of using high concentrations of cryoprotectants for sperm vitrification is the cytotoxic effect that affects sperm recovery after warming. Therefore, in this study, we determined the recovery rate after vitrification with and without cryoprotectants. Ejaculates with progressive motility and viability above 50% were used as samples. The samples were divided into two groups: (1) samples were mixed with a basic solution (2) samples were mixed with a vitrification solution. Sperm were vitrified by direct plunging into LN2. Sperm motility and viability were observed to evaluate the quality of sperm before and after vitrification. Overall, the sperm samples vitrified with cryoprotectants had a significantly higher proportion of sperm motility (56%) and viability (58.15%) compared with those vitrified without cryoprotectants (35% and 48%, respectively, $p < 0.05$). However, vitrification of human sperm without cryoprotectants could be recommended for routine assisted reproductive technology.

Keywords: sperm vitrification, cryoprotectants, sperm motility, sperm viability

1 INTRODUCTION

Vitrification is known to establish a glass-like solid state during the cooling process. It also has an economic advantage compared with the slow freezing method such as a lack of ice crystal formation due to an increase in the speed of temperature conduction, which provides a significant increase in cooling rates. This rapid cooling process circumvents the ice crystalline formation phase by converting solutions or water into a glass-like amorphous solid (Dinnyes et al., 2007). Vitrification conditions can be achieved by using high concentrations of cryoprotectants. The high concentration of cryoprotectants and extremely rapid rates of cooling are responsible for the formation of the solid state, thereby preventing the formation of intracellular ice crystals. One of the disadvantages of using high concentrations of cryoprotectants for sperm vitrification is the cytotoxic effect (Özkavukcu and Erdemli, 2002), which affects sperm recovery rates after the vitrification process. Therefore, it is particularly detrimental to patients who have low counts of sperm.

The use of cryoprotectants in the vitrification of human sperm requires further study because sperm is gamete cells having an intracellular matrix with high viscosity that may function as an internal cryoprotectant. Moreover, sperm has a compact structure and a small number of cytosols. This structure determines whether the sperm requires a small amount of intracellular cryoprotectants or not. The aim of this study was to observe the recovery rate of human sperm that were vitrified using different methods: (1) with cryoprotectants and (2) without cryoprotectants. Furthermore, this study determined whether the use of toxic intracellular cryoprotectants for sperm vitrification can be

avoided and whether sperm vitrification without cryoprotectants can be suitable for use in assisted reproductive technology.

2 MATERIALS AND METHODS

2.1 *Sample*

Ejaculates were obtained from 20 men as donors by masturbation after 2–7 days of sexual abstinence. The ejaculates were selected after liquefaction for 30 minutes, and those having a concentration of 15 million or more sperm/ml and showing at least 50% progressive sperm motility and viability were used as samples. Semen analysis was performed according to the published guidelines by the World Health Organization (2010). Each ejaculate was divided into three equal parts. Part 1 (P1) was the control group. Part 2 (P2) and part 3 (P3) were diluted with Earle's Balanced Salt Solution (EBSS) medium, and then centrifuged at 600 g for 10 min to remove seminal plasma. After centrifugation, sperm pellets were diluted with 500 µl EBSS medium. The suspended sperm was again diluted (1:1) with a vitrification medium containing (P2) EBSS (without cryoprotectants) and (P3) EBSS + 0.25 M sucrose + 1% Human Albumin Serum (HAS).

2.2 *Vitrification and warming*

After dilution, the P2 and P3 groups were allowed to equilibrate at room temperature for 10 minutes before vitrification, and then sperm motility and viability were assessed. Afterwards, each specimen was loaded into a 0.25 ml plastic straw using a syringe and then sealed. The straws were vaporized in liquid nitrogen for 5 seconds, and then plunged into liquid nitrogen directly and stored until 24 hours. The straw was taken out from liquid nitrogen, warmed at 37°C water for 5 seconds, and then the tip of the straw was cut, and the sample was put into a microtube. Sperm motility and viability were evaluated.

2.3 *Evaluation of sperm motility and viability*

Sperm motility was evaluated immediately after liquefaction, dilution with the vitrification medium, and after warming the samples. Here, we used two different categories of sperm motility, i.e., 'a' category for motile sperm and 'b' category for immotile sperm. Sperm viability was observed under a microscope using eosin–nigrosin staining.

2.4 *Statistical analysis*

Statistical analysis was performed using Minitube version 14. Data were analyzed using ANOVA and Tukey's test with a significance level of $p < 0.05$.

Table 1. Sperm motility during the vitrification process.

Stage of vitrification	Sperm motility (%)		
	P1	P2	P3
Equilibration	$100.0 \pm 0.0\%^{a}$	$88.50 \pm 6.30\%^{a}$	$94.20 \pm 3.40\%^{a}$
Warming	$100.0 \pm 0.0\%^{b}$	$56.90 \pm 4.38\%^{c}$	$35.00 \pm 10.30\%^{d}$

*Values for the same volume with different letters are significantly different ($P < 0.05$).
**P1: control, P2: EBSS (without cryoprotectants), P3: EBSS + 0.25 M sucrose + 1% HAS (with cryoprotectants).

Table 2. Sperm viability during the vitrification process.

Stage of vitrification	Sperm viability (%)		
	P1	P2	P3
Equilibration	$100.0 \pm 0.0\%^{a}$	$92.1 \pm 5.50\%^{a}$	$95.90 \pm 2.00\%^{a}$
Warming	$100.0 \pm 0.0\%^{a}$	$58.15 \pm 4.70\%^{b}$	$48.06 \pm 3.20\%^{c}$

*Values for the same volume with different letters are significantly different ($P < 0.05$).
**P1: control, P2: EBSS (without cryoprotectants), P3: EBSS + 0.25 M sucrose + 1% HAS (with cryoprotectants).

3 RESULTS

The results of sperm motility and viability during the vitrification process are summarized in Table 1 and Table 2. Sperm quality after vitrification was decreased in both methods compared with the control group. Overall, the sperm samples vitrified with cryoprotectants had a significantly higher proportion of sperm motility (56%) and sperm viability (58.15%) compared with those vitrified without cryoprotectants (35% and 48%, respectively, $p < 0.05$).

4 DISCUSSION

There are two types of cryoprotectant: intracellular cryoprotectant that will cross the cell membrane to buffer the intracellular salt, and extracellular cryoprotectant that has an important role in the cell dehydration process. Human sperm contains a large amount of protein, sugar, and other components that make the intracellular matrix highly viscous. As a result, it can be speculated that we can achieve intracellular vitrification for human sperm (Best, 2015, Isachenko et al., 2004). The high protein content in the cells

leads to high viscosity, so it is highly sufficient to achieve intracellular vitrification. Thus, high concentrations of cryoprotectants are necessary for extracellular vitrification than intracellular vitrification.

In our study, vitrification of sperm using 0.25 M sucrose and 1% HAS as cryoprotectants showed higher recovery rates of sperm than that without cryoprotectants because sucrose and HAS act as extracellular cryoprotectants that stabilize sperm membrane during the vitrification process. Extracellular cryoprotectants have a size that is too large to diffuse into cells, but they help water vitrification and devitrification, inhibiting the extracellular space. Sucrose is a type of sugar that cannot diffuse into the plasma membrane. Therefore, sucrose will produce an osmotic pressure that induces the dehydration process and reduces the formation of intracellular ice crystals. Sucrose also interacts with phospholipids in the plasma membrane, so that sperm membranes will reorganize the survival rate during freezing. High viscosity will increase the glass transition temperature in the cytosol of sperm.

Based on Table 1 and Table 2, human sperm vitrification without cryoprotectants can still be applied, because human sperm contains a large number of proteins, sugars, and other components that make the intracellular matrix highly viscous and to have compartments that enable the function as natural cryoprotectants (Isachenko et al., 2004b). These results are supported by (Isachenko et al., 2004a), indicating that frozen sperm motility without the use of cryoprotectants in the vitrification method showed a decrease of about 40% when compared with conditions before freezing. A previous study has reported a novel vitrification method for a single spermatozoon using the Cryotop method. The vitrification and warming techniques were simple, and the recovery rate of sperm after warming was relatively high (Endo et al., 2011). The size of the sperm also plays a role in the success of vitrification without cryoprotectants. The sperm is smaller than the embryo, so it is less likely that intracellular ice nucleation is followed by the growth of crystals during the freezing stage, or recrystallization during the warming stage (Isachenko et al., 2003).

5 CONCLUSION

Vitrification of human sperm can be achieved without intracellular cryoprotectants. Moreover, vitrification by directly plunging into liquid nitrogen without cryoprotectants was effective and could be recommended for routine assisted reproductive technology.

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