

## Effects of Trehalose and Sucrose on Human Sperm Motility, Vitality and Morphology after Cryopreservation

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### Abstract:

**Objective:** To compare human sperm motility, vitality and morphology after cryopreservation among sucrose, and different trehalose concentrations.

**Material and Methods:** A total number of 124 normozoospermic semen samples were collected. Each semen sample was divided into 4 portions, and cryopreserved in a human sperm-preserving medium along with cryoprotectants, including; 50 milimolar (mM) trehalose, 100 mM trehalose, 200 mM trehalose and 50 mM sucrose, respectively. All semen samples were frozen by using a vapor phase method. Post-thawed sperm motility, vitality and morphology were assessed. R program was used for data analysis. A p-value of <0.05 was considered statistically significant.

**Results:** Post-thawed semen evaluation indicated that 50 mM trehalose was better than 50 mM sucrose in all sperm parameters, which included progressive motility (p-value=0.037, total motility (p-value<0.001), vitality (p-value<0.001) and morphology (p-value<0.001). The sperm parameters were not significantly different among 100 mM trehalose, 200 mM trehalose and 50 mM sucrose.

**Conclusion:** The use of 50 mM trehalose, as non-permeating cryoprotectant, showed superior post-thaw sperm parameters over sucrose, and other trehalose concentrations.

**Keywords:** cryopreservation, human sperm, sucrose, trehalose

## Introduction

Sperm cryopreservation is an integral part in preserving male fertility. Although several cryopreservation methods have been developed, over several decades, they still involve significant loss of spermatozoa motility and viability. Human sperm cryopreservation is notoriously unsuccessful, and the 40.0–60.0% survival rates observed today are little in difference from those of 40 years ago. In contrast oocyte and embryo cryosurvival can be as high as 90.0%.<sup>1</sup>

There are two main factors of sperm death, after sperm has been frozen–thawed, including; intracellular ice crystal formation and osmotic shock.<sup>2</sup> Intracellular water is the main component of any cell. Without cryoprotectants, when cells are frozen, intracellular water may become ice crystals, which can cause irreversible damage. Therefore, cryoprotectants should be used to prevent ice crystal formation. They can also help to maintain osmotic change, during the frozen–thawed process.

The selection of types of cryoprotectants, either permeating or non–permeating, is particularly important for the cryopreservation process. To date, sucrose, a non–permeating cryoprotectant, is most commonly used in sperm cryopreservation<sup>3</sup>; however, studies demonstrated the benefits of using trehalose, as a non–permeating cryoprotectant, are limited.

The beneficial effects of using trehalose, on the post–thawed viability of mammalian sperm cells, has been reported in many studies. The studies in buffalo, boar, mouse, and goat showed that: trehalose improved sperm motility, compared with using cryoprotectant without trehalose.<sup>4–8</sup>

Although there is a number of animal studies supporting that trehalose has a positive effect on sperm parameters, the evidence in human sperm is very limited. Therefore, to optimize cryopreservation protocol in human sperm, the objective of this study was to compare human

sperm motility, vitality and morphology after cryopreservation, among sucrose and with different trehalose concentrations.

## Material and Methods

### Study population

Semen samples were obtained from healthy male donors (age range 20–45 years old), who came to the infertility clinic at Songklanagarind Hospital. One hundred and twenty–four samples with normal semen parameters were recruited into the study. The approval of the Prince of Songkla University Research Ethical Committee was obtained, prior to the study, and all subjects were informed with respect to this study.

### Semen samples

All donors had maintained sexual abstinence from 3 to 5 days. After signing an informed consent, fresh, ejaculated semen samples were obtained by masturbation into sterile containers. After ejaculation, samples were allowed to liquefy for 30–60 minutes, at 37 °C. Sperm count and motility were scored using Makler<sup>®</sup> counting chamber (Sefi medical instruments, Haifa, Israel) under a microscope according to World Health Organization criteria 2010.<sup>9</sup> Sperm vitality was determined using an eosin–nigrosin staining technique, while sperm morphology was assessed by using Kruger strict criteria.<sup>10</sup> All semen analysis was performed by a well–trained obstetrics and gynecology resident.

### Reagents and media

Human sperm preserving medium (HSPM)<sup>11</sup> (1:1) was prepared in our laboratory as follows: 100 millimolar (mM) NaCl, 5.365 mM KCL, 2.72 mM CaCl<sub>2</sub>, 0.492 mM MgCl<sub>2</sub>, 12.856 mM sodium lactate, 0.321 mM NaH<sub>2</sub>PO<sub>4</sub>, 30.949 mM NaHCO<sub>3</sub>, 20 mM Hepes–free acid, 133.21 mM glycine, and 5.506 mM glycerol plus 4.9g/L human

albumin, 15.0% volume/volume glycerol, streptomycin 0.1 mM and penicillin G 0.2 mM. The cryoprotectants including 50 mM trehalose, 100 mM trehalose and 200 mM trehalose were added in experimental groups, respectively; and 50 mM sucrose was added in the control group.

### Freezing and thawing protocol

Each semen sample was divided into 4 equal portions, and each portion was diluted 1:1, with a freezing medium consisting of varying concentrations of trehalose, to obtain a final concentration of trehalose of 50 mM, 100 mM and 200 mM and 50 mM of sucrose (control group). Sperm had been mixed and incubated, with the freezing medium for 10 minutes. After 10 minutes of incubation, each sample was loaded by sperm straw, and then held 15 cm above the surface of LN<sub>2</sub>, on a metal rack in the vapor phase for 15 minutes. The straws were finally plunged into LN<sub>2</sub> at -196 °C for storage. All semen samples were frozen for about a week. To thaw, each straw was quickly immersed into a 37 °C water bath. Straw content was

expelled into a 5 ml tube. Post-thawed sperm parameters were examined.

### Statistical analysis

The variables were expressed as mean±S.D. The mean difference was calculated by subtracting means of post-thawed sperm motility, vitality and morphology by means of pre-freeze parameters for fresh specimens. Mean differences of all parameters were tested for data normality as well as the sperm parameters between three different trehalose concentrations, comparing to sucrose as control group were compared by the Wilcoxon signed-ranks test, in paired samples. R program was used for data analysis. A p-value of less than 0.05 was considered statistically significant.

## Results

The standard semen quality parameters, namely; progressive motility, total motility, vitality and morphology of spermatozoa in fresh semen, and after the frozen-thawed process of the 124 samples, are shown in Table 1.

**Table 1** Sperm parameters before and after, the frozen-thawed process

Parameters (n=124)	Pre-freeze (mean±S.D.)	Post-thaw (mean±S.D.)				P-value*
		Sucrose 50 mM	50 mM	Trehalose 100 mM	200 mM	
Volume (mL)	4.0±1.1					
Conc (10 <sup>6</sup> /mL)	56.9±26.6	24.3±16.1	25.1±16.1	24.5±16.1	24.2±16.1	
Total mot (%)	73.2±9.6	9.9±10.9§	16.8±12.8	11.3±12.8§	7.6± 9.5§	<0.001
PR (%)	50.6±11.8	3.2±4.8§	5.9±6.6§	3.7±6.5§	2.2±3.7§	<0.001
Vitality (%)	76.1±9.0	18.2±11.0	27.2±12.1	19.7±11.4	15.4±10.1	<0.001
Morp (%)	17.8±3.9	10.2±3.4	12.7±3.8	10.6±3.7	9.2±3.3	<0.001

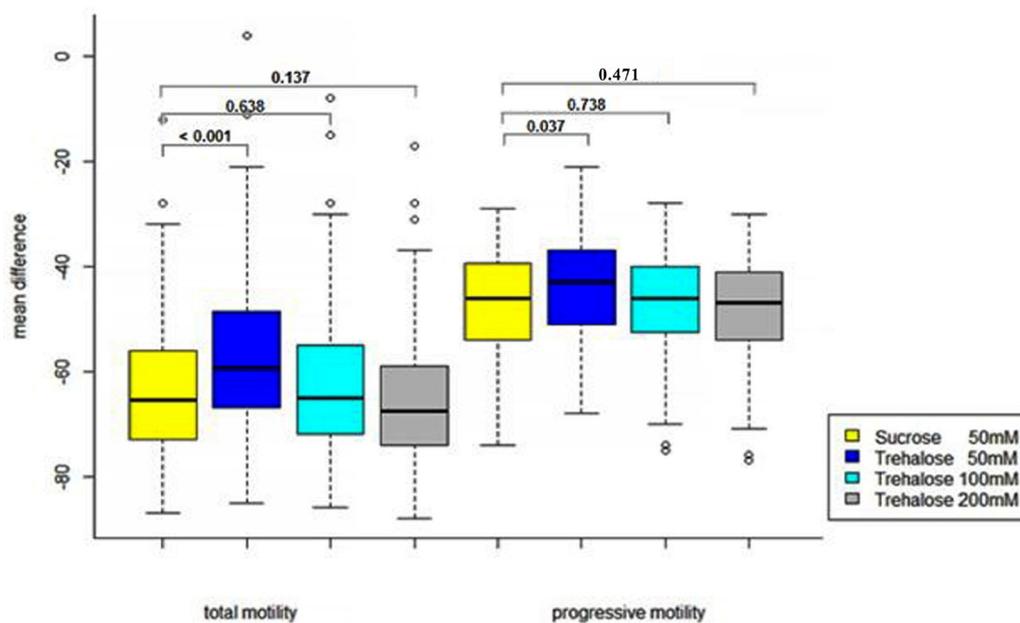
Conc=concentration, Total mot=total motility, PR=progressive motility, Morp=sperm morphology, S.D.=standard deviation, mM=millimolar, mL=milliliter, § Data were not normally distributed, \*Significance of differences between fresh semen samples, and post-thawed semen samples of each group

Cryopreservation resulted in a significant reduction in sperm motility parameters. Progressive motility had a mean value of 50.6%, before freezing and a mean value of 5.9%, 3.7%, 2.2% and 3.2% after cryopreserved, with 50 mM trehalose, 100 mM trehalose, 200 mM trehalose and 50 mM sucrose, respectively ( $p$ -value<0.001). Reflecting the total number of motile spermatozoa, progressive motility plus non-progressive motility, was also considered. This value decreased significantly after the frozen-thawed process ( $p$ -value<0.001). Comparing with different cryoprotectants, the best post-thaw sperm parameters were obtained within the 50 mM trehalose group.

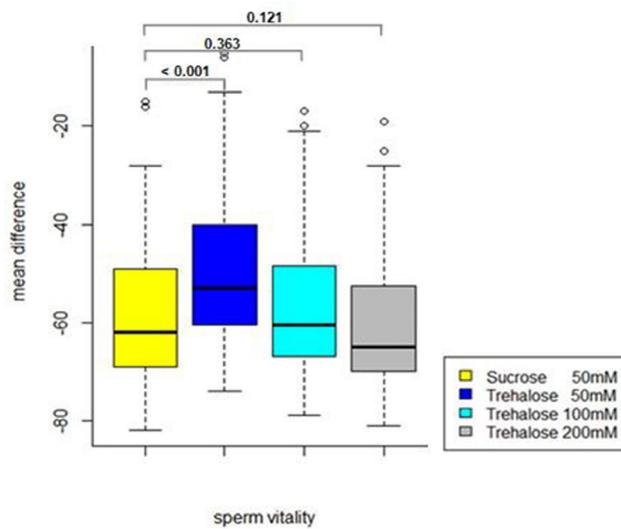
Figure 1 shows the percentages of difference, between mean of both total and progressive sperm motility, from 124 cryopreserved semen samples comparing between 50 mM sucrose, and three different trehalose concentrations. The sperm motility was significantly higher after 50 mM trehalose, than it was after 50 mM sucrose for both total motility and progressive motility ( $p$ -value<

0.001 and  $p$ -value=0.037), respectively. To compare 50 mM sucrose, there were no significant differences regarding the statistically compared post-thaw sperm motility frozen in 100 mM, and 200 mM trehalose.

The percentages of difference between; mean of post thawed sperm vitality frozen in 50 mM sucrose and three different trehalose concentrations are presented in Figure 2. Sperm vitality, which was determined using the eosin-nigrosin staining method, revealed a mean value of 76.1% before freezing. This value diminished to 27.2%, 19.7%, 15.4% and 18.2%, after cryopreserved with 50 mM trehalose, 100 mM trehalose and 200 mM trehalose and 50 mM sucrose, respectively. After cryopreserved in 50 mM trehalose, the percentages of differences between mean of post-thawed sperm vitality were significantly better than 50 mM sucrose ( $p$ -value<0.001), however, post-thawed sperm vitality were not different in both 100 mM trehalose, and 200 mM trehalose, when compared with 50 mM sucrose.

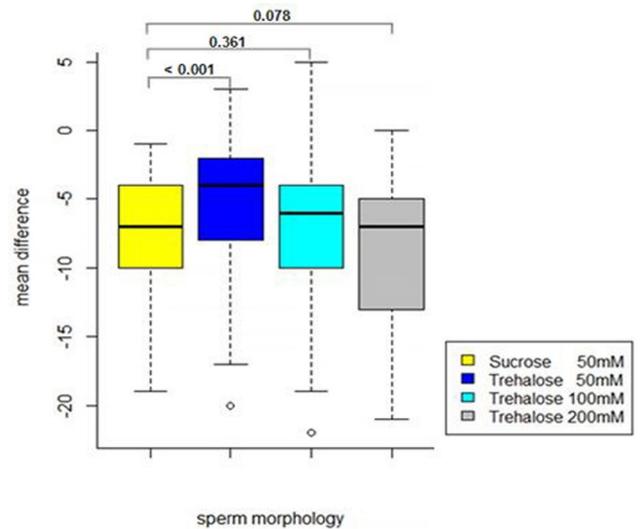


**Figure 1** The percentage of difference between; mean of post thawed total sperm motility and progressive motility frozen in 50 millimolar sucrose, and three different trehalose concentrations.



**Figure 2** The percentage of difference between; mean of post thawed sperm vitality frozen in 50 millimolar sucrose, and three different trehalose concentrations.

Assessment of sperm morphology, using light microscopy, revealed a statistically significant decrease in the percentage of normal spermatozoa, after thawing, in that there was a mean value of 17.8% before freezing, and a mean value of 12.7%, 10.6%, 9.1% and 10.2% after cryopreserved, with 50 mM trehalose, 100 mM trehalose, 200 mM trehalose and 50 mM sucrose ( $p$ -value<0.001). Figure 3 shows the percentages of differences between, mean of post-thawed morphology. The sperm cryopreserved in 50 mM trehalose were significantly better than that of 50 mM sucrose ( $p$ -value<0.001) in post-thawed morphology, but no significant differences in both 100 mM, and 200 mM trehalose comparing with 50 mM sucrose were found.



**Figure 3** The percentage of difference between: mean of post thawed sperm morphology frozen in 50 millimolar sucrose, and three different trehalose concentrations.

## Discussion

Our study showed that only 50 mM of trehalose has better post-thawed sperm parameters than sucrose, and other trehalose concentrations. Although animal studies showed the benefits of trehalose over sucrose in sperm freezing, only a few studies have investigated the effects of trehalose on human sperm cryopreservation.<sup>3,12</sup>

Cryopreservation adversely affected the cryo-survival of human spermatozoa. Unsurprisingly, in our study, progressive motility, total motility, vitality and morphology of spermatozoa; after the frozen-thawed process, was significantly reduced when compared with the fresh semen values. These deleterious effects on spermatozoa were similar to results obtained in a previous study.<sup>13</sup>

Only supplementation of 50 mM trehalose led to a significant increase in the post-thawed human sperm parameters, including sperm motility, vitality and morphology, compared with other higher trehalose concentrations in our study. This demonstrated that the osmolarity in the cryopreservation medium is of importance to sperm parameters. Higher concentrations of trehalose, over 50 mM, may cause the osmolarity to become too high, and can produce substantial osmotic damage, and again this is consistent with another, previous study<sup>3,12</sup>; whereas the osmolarity has a significant effect on human sperm motility. Gao et al.<sup>14</sup> suggested that appropriate osmolarity of the cryopreservation medium is between 250 and 350 milliosmoles per liter. However, our study did not determine osmolarity of the medium.

To compare between trehalose and sucrose, the study also showed the benefits of using trehalose, over sucrose, at the same concentrations. These beneficial effects have also been reported in animal studies conducted on buffalo, boar, goat, and mouse.<sup>4-6,15</sup> Although sucrose, and trehalose are also a non-reducing disaccharide, they are significantly different in their structures. The bonding of trehalose has a clam shell conformation, which has more stability than those of the more open conformation of sucrose.<sup>15,16</sup> The key as to why trehalose showed more of a benefit, over sucrose, in the cryopreservation process may be the  $\alpha,\alpha$ - (1 $\rightarrow$ 1) glycosidic linkage of trehalose.<sup>16</sup> In vivo studies demonstrated that: the  $\alpha,\alpha$ -trehalose binds with the cell's lipid membrane and forms solid, supported lipid bilayers.<sup>15-18</sup> This helps to maintain cell integrity as well as decreasing cell damage during the cryopreservation process.<sup>8,16,18</sup> In contrast, Liu et al.<sup>3</sup> showed that trehalose and sucrose were both effective, while our study found that trehalose was significantly better than sucrose in terms of recovering post-thaw's sperm motility. This discrepancy result may be that our study

added glycerol in the freezing medium, which reported a synergic effect of trehalose on cell integrity.<sup>8</sup>

One strength of our study is that the large number of human semen samples used, which were then compared between trehalose and sucrose as a non-permeating cryoprotectant. However, the study was limited by the fact that sperm parameters (motility, vitality and morphology), that were used in this study, may not accurately predict the invitro fertilization outcome (IVF).<sup>19</sup> A systematic review<sup>20</sup> in the evaluation of sperm function has been recommended using the hemizona assay, for the prediction of IVF outcomes.

## Conclusion

In conclusion, the usage of trehalose, instead of sucrose, as a non-permeating cryoprotectant improved post-thawed human sperm parameters, with a concentration of 50 mM trehalose demonstrating the best sperm parameters. However, further investigations may be needed to determine the optimal concentration of trehalose, or types of cryoprotectant to achieve the best quality of sperm after cryopreservation. In addition, studies to evaluate the effective of trehalose, for patients presenting with oligospermia, would be more useful.

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## Conflict of interest

The authors have no conflict of interest to declare.

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