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Original Article

Study of human sperm motility post cryopreservation

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ABSTRACT

Background: Cryopreservation of spermatozoa is a widely used technique to preserve the fertility of males. It can also benefit the armed forces personnel who are to be sent for long recruitments, while leaving their families behind. This study, apart from studying the effects of freezing and thawing, reveals the effect of the post thaw interval on the motility of the human spermatozoa and thus widens the insemination window period.

Methods: A detailed semen analysis was carried out as per the WHO guidelines for 25 samples. The samples were then washed, analysed and frozen in liquid nitrogen. The semen samples were subsequently thawed and similarly analysed after 20 min and 40 min of thawing. This was then followed by statistical analysis of the comparative motilities.

Results: Motility of sperms is found to decrease after cryopreservation. However, the study revealed that after thawing a significant increase in the motility of the sperms was noted with the progression of time ($p < 0.05$).

Conclusion: By simulating conditions similar to the in vivo conditions for the post thaw semen samples, we can safely wait, confirm the parameters like motility and count, and then inseminate the samples instead of blindly inseminating them immediately after thawing.

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Introduction

Cryopreservation is a process where cells, whole tissues or any other substances susceptible to damage caused by chemical reactivity or time are preserved by cooling to sub zero temperature. Semen cryopreservation is a process to preserve

sperm cells. Cryopreservation of tissues began with the freezing of Fowl sperms, which during the year 1957 was cryopreserved by a team of scientists in UK. The process was applied to humans during 1950 with pregnancies obtained after insemination of frozen sperm. Cryopreservation of spermatozoa is at present, the most valuable and used way to preserve reproductive function in men undergoing

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gonadotoxic treatment such as chemo- or radiotherapies.¹ In addition sperm cryopreservation is increasingly used even in cases of other disorders, such as auto immune diseases and myelodysplastic syndromes requiring treatments that may affect reproductive functions. Moreover, sperm cryopreservation is offered to patients with severe oligospermia or ejaculatory disorder for intracytoplasmic sperm injection (ICSI). Also, some non-malignant diseases such as diabetes and autoimmune disorders may lead to testicular damage, and cryopreservation is also advisable in these conditions.² It can be a boon in the armed forces scenario, for the couples who are unable to stay together due to their field or ship recruitments.

The first reported effects of low temperatures on spermatozoa were recorded by Lazaro Spallanzani in 1776, and the first to discuss the possible uses of sperm banks was the Italian neurologist, physiologist and anthropologist, Paolo Mantegazza. He wrote the following in 1866: "It might even be that a husband who has died on a battle-field can fecundate his own wife after he has been reduced to a corpse and produce legitimate children after his death".³

Cryopreservation is known to have detrimental effects on the sperm structure and function. Recovery of an optimal number of functionally intact spermatozoa from thawed samples has always been the main objective of semen cryopreservation technology.⁴ The principle involved is the prevention of intracellular ice crystal formation and optimal dehydration of the cells. For this a large variety of glycerol and non – glycerol based cryoprotectants have been used. Cryoprotectants act by decreasing the freezing point of a solution by increasing the amount of salts and solutes present in the liquid phase of the sample, thereby decreasing ice formation within the spermatozoa.⁵

Human uterus is considered as the best natural incubator for inseminated semen samples. Therefore most of the clinicians believe in inseminating semen samples immediately after thawing. This is because they fear that if the sample is kept in the in vitro conditions for a long time, the sperms might lose their functional capacity. The purpose of this prospective study was to carry out computer assisted semen analysis of the frozen sperm sample after thawing and predict their cryosurvival and evaluate the progressive motility recovery rate of the frozen spermatozoa 20 and 40 min post thawing.

Therefore by this study we intended to reconsider the ideal time for insemination after thawing of the semen sample.

Materials and methods

Subject selection: 25 patients who were enrolled at the ART centre for their own semen analysis were selected after a verbal informed consent. Semen samples were produced by

masturbation into 110 ml sterile containers (code 351006, BD Biosciences, USA) from these 25 patients after 3 days of abstinence. As per the inclusion criteria, samples with a volume ≥ 2 ml, total sperm count ≥ 15 million/ml, motility $\geq 50\%$ were included in the study and samples with round cells ≥ 1 million were excluded.

Assessment of spermatozoon count and motility: After leaving the sample for 20 min at 37 °C for liquefaction to occur, the motility of spermatozoa was evaluated using the Computer Assisted Semen Analysis (CASA), (ISAS, Proiser SL, Valencia, Spain). The motility of each spermatozoon was graded as "a," "b," "c," or "d" according to the WHO laboratory manual for semen parameters [WHO 1999 guidelines].⁶ (Table 1). As per this classification, the sperms with type 'a + b + c' motility were taken as the total number of motile sperms and the ones with type 'd' motility were taken as the total number of immotile sperms. Spermatozoa concentrations were evaluated using a Makler chamber (Sefi Medical Instruments, Haifa, Israel) (Fig. 1) as per the WHO 2010 guidelines. Samples with concentrations of 15×10^6 /ml or less (oligozoospermia)⁶ and round cells of $>1 \times 10^6$ /ml were excluded from the study. Minimal clumping and agglutination seen on visual analysis was ignored.

Semen preparation protocol: The sample was transferred to a 11 ml conical tube (Code CE 0543, Nunc, Denmark). It was mixed with 4 ml of gamete buffer media (Code K SIGB, William A Cook, Australia Pty. Ltd.) (Fig 2) and centrifuged for 10 min at 1500 rpm. After centrifugation a pellet containing the sperms was formed at the bottom of the tube. The supernatant was discarded, 2 ml of the gamete buffer media was again added without disturbing the pellet and the sample was left for swim up in the incubator for 20 min at an inclination. The motile sperms left the pellet and swim into the supernatant. The swim up containing the motile sperms constituted the post wash sample which was then analysed by CASA.

Sperm freezing and thawing protocol: 1 ml of sperm freezing media (Code HSISC-20 William A Cook, Australia Pty. Ltd.) was added drop by drop to the post wash sample (taking about 3–4 min to prevent osmotic shock to the sperms), with constant shaking to ensure thorough mixing of the two. The suspension formed was transferred to the 1.8 ml cryovials (Code CE 0543, Nunc, Denmark). The sample was subjected to sequential cooling first at room temperature for 10 min, then in the refrigerator (4 °C) for 10 min. Following this, the samples were frozen by static-phase vapour cooling and then plunged into liquid nitrogen (–196 °C) (Fig. 3).

After cryopreservation for at least 60 min, the sample was thawed at room temperature. During the procedure, universal safety precautions were taken and cryogloves were worn. The cryovial was opened keeping the mouth of the vial away from the face as sudden expansion of the air in the vial may lead to

Table 1 – Sperm motility – 4 groups (WHO 1999 guidelines).

WHO category	Code	Corresponding velocity
Rapid progressive	a	$>25 \mu\text{m/s}$ (≥ 1 monitor square, or 5 sperm head lengths)
Slow progressive	b	$5\text{--}24 \mu\text{m/s}$
Non progressive	c	$<5 \mu\text{m/s}$ (<1 sperm head length)
Immotile	d	–

Fig. 1 – Makler counting chamber for counting sperms.

expulsion of the cap of cryovial causing injuries to the eyes and face. As the sample is subjected to sudden change of temperature, small water droplets start to condense on the outer surface of the cryovials. This phenomenon is called sweating. We waited for some time and allowed the sweating to complete. After this, the entire sample was taken and added to an equal amount of HEPES containing buffer (Code K SIGB, William A Cook Australia Pty. Ltd.) at room temperature very gradually. The suspension was centrifuged for 5 min at 1500 rpm. Supernatant was discarded and the pellet was layered with half ml of gamete buffer media. The suspension was left for incubation at 37 °C for 20 min at an inclination. The supernatant was aspirated, and analysed by CASA. The suspension containing the supernatant was further kept at 37 °C and re-analysed after 20 min.

Semen parameters and spermatozoa motility were evaluated statistically according to the data obtained in the pre-freeze and the post thaw samples after 20 min and 40 min of thawing and wash procedure. Methods like unpaired T tests

Fig. 2 – Sperm cryopreservation buffer containing glycerol, Cook Australia Pty, Ltd.

Fig. 3 – Cryovials containing the semen samples, fixed on the aluminium cryocanes, being inserted into the liquid nitrogen cans.

and repeated measure ANOVA (analysis of variance) were used for statistical analysis.

Results

In our study we noted that mean motility of the raw sample was found to decrease by 45.2% after freezing and thawing procedure (Table 2).

When the semen samples were analysed after 20 and 40 min of thawing, it was observed, that the total number of immotile sperms (having type d motility) was found to decrease after 40 min post thaw interval and interestingly the total number of motile sperms (having type a + b + c motility) was found to increase (Table 3).

It was further observed that during in-vitro incubation the mean of rapidly progressive or code 'a' sperms (Table 1) was found to increase from 5.385 to 11.697 % post semen thawing, at 20 min and 40 min respectively. Total motility of the sperms is found to increase after 40 min of post thawing interval as compared to that after 20 min (Fig. 4).

Discussion

Human Spermatozoa endure a gamut of cooling and warming rates during semen banking. They are relatively less sensitive to the cellular damage sustained during rapid preliminary cooling, conceivably due to elevated membrane fluidity owing to the high-unsaturated fatty acids content of the lipid bilayer of the cell. The procedure may not have an adverse effect on human sperm function, particularly the motility if we modify the freezing thawing technology.

Sperm banking by cryopreservation is a time tested technology. Thawed semen samples can be used subsequently for artificial insemination or assisted reproductive techniques.

When compared with the other cell types, spermatozoa are found to be less sensitive to cryopreservation injury because

Table 2 – Descriptive analysis of motile sperms in raw, post wash and post thaw samples after 20 min and 40 min.

	Mean percentage of motile sperms (%)	Std. deviation	N	P value
Raw a + b + c	86.0286	16.53110	25	0.001
Post wash a + b + c	84.6536	23.37955	25	0.001
Post thaw (20 min) a + b + c	39.3013	23.90421	25	0.001
Post thaw (40 min) a + b + c	63.1180	24.72341	25	0.001

a + b + c: total no of motile sperms.
N: Sample size.

Table 3 – Table depicts comparison between motile and non motile sperms in the post thaw samples after 20 min and 40 min of thawing.

Type of sample	N	Mean percentage of sperms	Std. deviation	P value
	Motility of sperms (%)			
Post thaw (20 min)	a + b + c	39.3013	23.90421	0.003
	d	60.7307	23.89847	
Post thaw (40 min)	a + b + c	63.1180	24.72341	0.0001
	d	36.8820	24.75575	

a + b + c: Total no of motile sperms.
d: Total no of immotile sperms.
N: Sample size.

of high fluidity of the membranes and less water content (about 50%). Despite this, cryopreservation may result in deleterious changes to sperm structure and function.⁷ It has been reported that several damaging processes can occur during the freezing and thawing of human spermatozoa due to thermal shock with the formation of intracellular and extracellular ice crystals, cellular dehydration, and osmotic shock.⁸ The intracellular ice crystals thus formed, may breach the membranes and affect the functioning of the organelles. This can lead to impaired cell survival. On the other hand, a very slow cooling rate determines the efflux of water from the internal to the external environment, thereby increasing the concentration of solutes and the osmotic pressure inside the cell. This condition leads to cell volume changes due to

dehydration, and toxic damage due to high solute concentration.⁹ Cryoinjury is not only limited to the freezing process but it may also occur during the thawing process as the ice melts or recrystallizes.⁹

The decrease in motility of the spermatozoa has been attributed to damage to the mitochondrial membrane. The ATP generated by oxidative phosphorylation in the inner mitochondrial membrane is transferred to the microtubules, to drive motility.¹⁰ Therefore; an impairment of mitochondrial activity may explain the reduction in motility.¹¹

Although motility is not directly related to the fertilizing capacity, it is one of the most important factors affecting the sperm quality.¹² According to Keel and Webster, 1993, it has been documented that the motility of sperms is found to decrease by 50% after the freeze thaw cycle. However, a lot of variation can be seen in the susceptibility of individual donor sperms after cryopreservation.¹³

Having reviewed the pertinent literature most of the studies were found to focus on the effects on vitality, morphology and motility after cryopreservation. Also a few studies were found to compare the efficacy of the various techniques used for cryopreservation.

We compared the post cryopreservation motility of the spermatozoa in our study with the results of a few other studies. It was found that in our study, the total motile sperms after cryopreservation were 46.4% of the post wash sample. Another study showed that the freeze-thawing process caused a 66% reduction in rapid progressive motile spermatozoa, a 45% reduction in slow progressive motile spermatozoa and a 2% reduction in non-progressive motile spermatozoa.¹⁴ Yet another study showed that the progressively motile sperms were found to decrease by 41% and the motile sperms by 33% after freezing and thawing.¹¹

However, we could not find any study in the past that was found to study the effect of motility in the prolonged post

Fig. 4 – Comparison between total number of motile and immotile sperms in the raw, post wash and post thaw samples after 20 min and 40 min of thawing.

thaw interval. So we went a step ahead, waited for 40 min, while incubating the post wash swim up at 37 °C and then analysed it. It was found that the motility after 40 min was found to be 74.5% of the pre freeze value.

According to most of the protocols followed worldwide, thawing is done for 5 min, followed by incubation for 10–15 min at 37 °C before insemination.¹⁵ With our pioneering study we can conclude that instead of blindly inseminating the semen sample 20 min after thawing, we can safely incubate the sample till about 40 min post wash, analyse the motility and then inseminate. By doing this we can select the best possible samples (with confirmed motility), especially in case of donor inseminations.

However, our study is primarily laboratory based. More studies need to be undertaken in near future in the similar lines in terms of pregnancy in IUI cycles or fertilization rate in IVF/ICSI cycles before recommending the method for clinical use.

Conflicts of interest

All authors have none to declare.

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