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Cryopreservation of Limited Sperm Using A Combination of Sucrose and Taurine, Loaded on Two Different Devices, and Thawed at Two Different Temperatures

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Abstract

Background: Cryopreservation of sperm is essential for patients with low sperm counts and couples undergoing infertility treatment. The aim of this study was to compare the effects of Taurine (T) and Sucrose (S) in individual sperm cryopreservation utilizing cryotop and petri dish and thawing at 37 and 42°C.

Materials and Methods: In this experimental study, 17 normospermic semen samples were processed using the "Swim-up" procedure and progressively motile sperm were then isolated from these samples using an inverted microscope. Sperm were added to droplets of "sucrose medium" with 25 mM Taurine antioxidant (S+T) and the commercial cryoprotectant "Sperm Freeze" (CPA), loaded on a petri dish and cryotop. After rapid freezing of the samples, they were thawed at two different temperatures (37°C and 42°C), and the sperm classical parameters, viability, and DNA fragmentation were assessed.

Results: Statistical analysis displayed a significant increase in total and progressive motility in individual sperm freezing on cryotop with CPA and thawing at 42°C ($P<0.05$). Other parameters did not show any differences between the CPA and S+T groups and two thawing temperatures in either of the cryopreservation methods.

Conclusion: Although, both cryoprotectants (CPA and S+T) may preserve individual sperm effectively using cryotop, the CPA and thawing at 42°C showed a better effect on the motility percentage of the small number of sperm.

Keywords: Antioxidant, Cryotop, Sperm Cryopreservation, Taurine

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Introduction

Following the introduction of the sperm cryopreservation method in the 1960s, sperm cryopreservation approach has become an essential part of assisted reproductive technology (ART). These days, cryopreservation of sperms is essential in the treatment of infertile couples and in maintaining fertility in male cancer patients if sufficient sperm concentration is available (1). However, the traditional methods are associated with some significant challenges in scenarios where the sperm count is limited, such as in cases of azoospermia who undergo testicular sperm extraction (TESE) and severe oligospermia. The fact that the sperm sticks to the carrier vessel and demands centrifugation and thorough washing procedures, which may lower the sperm count when utilizing conventional sperm preservation techniques, makes the situation worse (2). Researchers and clinicians have considered carriers with small surface areas and high recovery ability, such as cryopiece (3), cell sleeper (4), cryoleaf (5), and culture dish (6), as potential solutions to the current problems. The cryotop is a carrier with a simple structure and easy handling that was used first time by Endo et al. (7) for the sperm freezing procedure. The cryotop carrier is the most up-to-date method for lowest volume vitrification. The vitrification technique by cryotop is intuitive to use. Anyone with basic knowledge of embryology may do it correctly after only a short amount of training. The procedure is straightforward and trustworthy, yielding the same outcomes with minimum variance among operators. After vitrification of human embryos and oocytes around the world, cryotop vitrification has led to the highest number of live births, and it is currently also successfully utilized in a number of areas of animal biotechnology (8).

Similar to traditional approaches, the cryopreservation of

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gametes poses significant challenges related to oxidative stress and osmotic damage caused by cryoprotectants (CPAs). In the case of sperm, which are a limited resource, the impact of such damage is particularly consequential. Various studies on the sperm cryopreservation have observed the utilization of antioxidants and the omission of cryoprotectants as two viable strategies (9-11). An essential natural antioxidant in the body, Taurine (T) is a sulfur-containing non-proteinogenic -amino acid that exhibits inhibitory effects on the reactive oxygen species (ROS) production and membrane stabilization via a redox balance maintenance linked to the control of different transcription factors (12). In recent years, there has been an increased consideration of the potential benefits of the T concerning male infertility and the mitigation of oxidative damage in the preservation of sperms (13). In this regard, previously we reported that the utilization of microdroplet and CPA-free method for the vitrification of a limited quantity sperm, coupled with the incorporation of T, has demonstrated the capacity to effectively safeguard the sperm parameters against cryo-damage, such as a reduction in motility, morphological changes, and the loss of acrosome integrity (14).

Moreover, the thawing rate is also an important factor in maintaining quality and motility of a sperm during the cryopreservation procedure. Traditionally, in most species, frozen sperm is thawed at 37°C (15). Mansilla et al. (16) showed that increasing the thawing temperature enhances the progressive motility of sperms following a vitrification of 5×10^6 sperms in a straw. They reported a significant difference in sperm motility at 38, 40, and 42°C after thawing. The motility of thawed sperm at 42 °C increased significantly in comparison with the two other temperatures.

As far as we know, there are no reports on the T utilization in the vitrification media for the limited sperm count. Nevertheless, in our previous study, the vitrification of sperm using microdroplets and T supplementation demonstrated the preservation of sperm parameters (14). Therefore, in the present study, we examined the effects of using a mixture of Sucrose (S) and T using two separate devices and two different thawing temperatures in the case of limited sperm cryopreservation.

Material and Methods

This experimental study was performed after the approval of the Royan Research Institute Ethics Committee, Tehran, Iran (IR.ACECR.ROYAN.REC.1395.112).

Semen sample collection

Seventeen semen samples according to previous studies (17) were collected after 3-4 days of sexual abstinence in a sterile container from men who had been referred to the Royan Institute (Tehran, Iran), with normal parameters according to World Health Organization (WHO) 2010 (18). All the experiments and stages used in this investigation are depicted in Figure 1. Following the semen liquefaction, macroscopic and microscopic examinations were performed. Using a Computer Assisted Sperm Analyzing system (CASA, SCATM motility module; Microptic, Version 4.2, Barcelona, Spain) with 10x magnification, the motility of sperms was determined. The Papanicolaou staining was used for assessment of sperm morphology at least 200 spermatozoa were checked under a light microscope 100x and immersion oil (18).

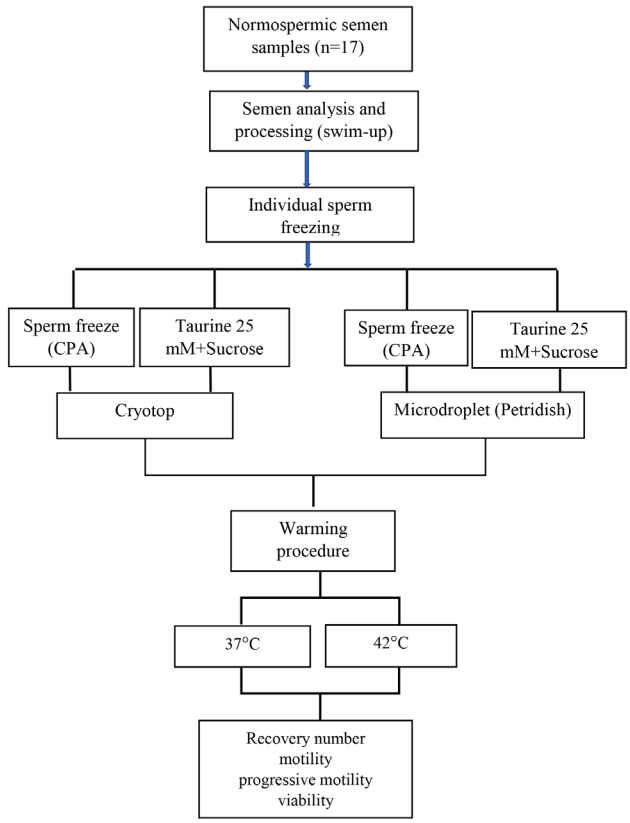

Fig.1: Flow chart of study design. CPA; Cryoprotectant.

Motile sperm isolation by swim-up method

The 1 ml of each semen sample was centrifuged at 3000 rpm for 5 minutes. The sediment was then removed and 1 ml of human tubal fluid (HTF) with 1% human serum albumin (HSA) (70024-90-7, Sigma-Aldrich, USA) that was made in the laboratory (Sodium Chloride 97.8 mM, Potassium Chloride 4.69 mM, Magnesium Sulfate 0.20 mM, Potassium Phosphate, Monobasic 0.37 mM, Calcium Chloride 2.04 mM, Sodium Bicarbonate 25.0 mM, Glucose 2.7 mM, Sodium Pyruvate 0.33 mM, Sodium Lactate 21.4 mM, Gentamicin 10 µg/mL, Phenol Red 5 mg/L) was added to the remaining and centrifuged for 3 minutes at 3000 rpm for the second time. Removing supernatant, 1.2 ml of HTF/HSA was added slowly to the pellet and then incubated for 30-45 minutes in a 5% CO_2 incubator at 37°C. After collection 600 µl of the medium above the pellet, concentration and motility of sperm were re-evaluated using the CASA software. Also, sperm morphology was examined using the Papanicolaou staining method. The swim-up sample was diluted at a 1:20 ratio with a HTF/HSA medium to

prepare for freezing procedure. Then, a limited number of sperm (3 to 10) were separated from the diluted medium by Pasteur pipettes (19). All experimental tests were performed individually on each semen sample, and the sample was divided into four experimental groups (CPA_{37}, CPA_{42}, $S+T_{37}$, $S+T_{42}$).

Cryopreservation and thawing of sperm by microdroplet method

After diluting the sample, a metal strainer was covered with a metal foil and placed in a foam box containing liquid nitrogen. We used two tools: a cryotop and a petri dish and two treatment groups were considered for freezing. The first group was frozen with the commercial cryoprotectant Sperm freeze media (CPA group), and the second group was frozen with a composition S (94474, Sigma, USA) and T antioxidant (107-35-7, Sigma, USA) (S+T). The S+T group was treated with a mixture of 90 µl HTF media+1% HSA, 90 µl of 0.2 M S and 20 µl of 50 mM T antioxidant (final concentration 25 mM) (14). The CPA group contained an equal ratio of the Sperm freeze (FertiPro Glycerol, HEPES, 4 g/Liter Human Serum Albumin, NV, Beernem, Belgium) and HTF.

After supplying the freezing medium, one µl of the frozen medium droplets was placed on the petri dish. A limited number of sperms (3-10) was transferred into each droplet. The droplets were covered with a 2.5 ml of mineral oil. The droplets inside the petri dish were examined by an inverted microscope (Nikon, USA) with 20x magnification to confirm the presence of transferred sperms. The petri dish was closed by a lid and covered with a plastic wrap and then was placed on a metal sheet in a foam box for 3 minutes to freeze its droplets mineral oil. The Petri dish was immersed in a liquid nitrogen and finally stored in a nitrogen tank for one week (Fig.S1, See Supplementary Online Information at ww.ijfs.ir). After removing the petri dish from liquid nitrogen, samples were placed quickly in the incubator at two different temperatures 37°C (for 10 minutes) and 42°C (for 5 minutes). The motility of sperms was evaluated immediately by an inverted microscope with 20x magnification. The sperms were transferred to 0.5 µl of wash drop (HTF with 1% HSA), and then the sperm parameters were re-evaluated (20, 21).

Rapid freezing by cryotop method and thawing samples

After diluting the swim-up samples, a metal strainer coated with a sheet of metal was placed one centimeter from the nitrogen surface in a foam box containing liquid nitrogen. Later, one microliter of the frozen medium droplets was placed on the cryotop strip. A limited number of sperm (3-10) were pipetted into the prepared freeze medium droplets on a cryotop strip. The cryotop was placed on the metal sheet in the foam box for 10 seconds to freeze the drop on the cryotop strip. The covered cryotop strip with a plastic cap was immersed in liquid nitrogen and finally stored in a nitrogen tank (Fig.S2, See Supplementary Online Information at ww.ijfs.ir).

For thawing, the washing medium (HTF with 1% HSA) was placed in an incubator for 20 minutes at the intended thawing temperature (37 and 42°C). Because of the bottom surface thickness, the petri dish was heated up two degrees higher than the intended thawing temperature, which this procedure gave our intended temperatures. Then, 1 ml droplets were placed on the petri dish and covered with the mineral oil and placed on a heating plate at the intended temperature for 30 minutes.

The cryotop strips were removed from the nitrogen and immediately transferred to the mineral oil-coated washing droplets. The droplets were examined under an inverted microscope at 20x magnification to measure the recovery and motility of the frozen samples.

Assessment of sperm viability

After thawing the frozen samples on cryotops and petri dishes in two different temperatures and investigating sperm retrieval and motility, the cells were transferred into 0.5 µl droplets of hypoosmotic solution (150 ± 5 mmHg) to equivalent to Ham's medium and sterile distilled water. These samples were incubated for 5 minutes at 37°C, and then their survival was examined according to WHO (2010) criteria by the Invert microscope (18).

Measurement of DNA fragmentation

To evaluate the sperm DNA fragmentation, the semen samples from three normospermic men were collected and after washing and dilution, a limited number of sperm were selected and frozen with two different cryopreservation medium as described above. The sperm transfer site was marked on the slide using a marker and at least 10 sperm were transferred to the slide. After drying the droplets (20 minutes), the dried droplets were covered using Carnoy solution as fixation and kept in a cool place for 2 - 24 hours. The slides were taken to a dark room, and acridine orange dye (65-61-2, Sigma, USA) was placed on the fixed droplets for 5 minutes, and then washed once with distilled water or phosphate buffered saline (PBS, MP Biomedicals, USA). After that, the marked sites on slides were examined immediately with a fluorescent microscope (AX70, Olympus Japan) with 100x magnification (22).

Statistical analysis

The present study used descriptive and inferential statistics to analyze the data. Descriptive statistics were used to prepare tables, draw graphs, and calculate statistical indicators. After confirming the normal distribution of values by using the Kolmogorov-Smirnov test, inferential statistics, including one-way ANOVA using Post-hocand Tukey range's test, were used to compare the four groups (CPA_{42}, CPA_{37}, $S+T_{37}$, $S+T_{42}$) in each method. Data were analyzed by the SPSS software version 16 (IBM company, USA), and the values of $P<0.05$ were considered significant.

Results

The study groups in the microdroplet on a petri dish and the cryotop method

The freezing groups included $(S+T)_{37}$ and $(S+T)_{42}$ freezing with S+T antioxidant, which was thawed at 37 and 42°C, and $(CPA)_{37}$ and $(CPA)_{42}$ included cryopreservation with CPA and thawed at 37 and 42°C, respectively. The results of statistical analysis after thawing at two different temperatures of 37 and 42°C have been reported in Tables 1 and 2.

Table 1: The mean of classical parameters studied after freeze/thawing by the microdroplet method

Studied groups	Parameters examined (%)	Mean ± SEM	Maximum	Minimum
$(S+T)_{37}$	Recovery number	100	100	100
	Total motility	11.9 ± 8.30	100	0
	Progressive motility	0	0	0
	Viability	22.60 ± 7.68	100	0
$(S+T)_{42}$	Recovery number	100	100	100
	Total motility	6.35 ± 4.97	60	0
	Progressive motility	1.67 ± 1.67	20	0
	Viability	34.74 ± 5.90	60	0
$(CPA)_{37}$	Recovery number	100	100	100
	Total motility	7.98 ± 3.09	25	0
	Progressive motility	0	0	0
	Viability	34.18 ± 4.97	50	11.11
$(CPA)_{42}$	Recovery number	100	100	100
	Total motility	16.77 ± 9.06	80	0
	Progressive motility	3.23 ± 2.32	20	0
	Viability	40.11 ± 9.09	81.82	0

SEM; Standard error of the mean, S+T; Sucrose+Taurine, and CPA; Cryoprotectant.

Sperm parameters of microdroplet on petri dish method

The recovery rate of frozen sperms in the microdroplet on the petri dish method was 100% and no difference was observed between the frozen groups. We did not observe significantly different of motility among our groups (Fig.2). Also, both cryopreservation groups thawing at 37°C, did not show any progressive motility, and this rate was zero. In contrast, there was not a significant increase in the progressive motility of $(CPA)_{42}$ group when compared to the group $(S+T)_{42}$ (Fig.2). Also, it was observed that there was no significant difference in the sperm viability rate among our groups (Fig.S3, See Supplementary Online Information at ww.ijfs.ir).

Table 2: The mean of classical parameters studied after freeze/thawing by cryotop

Studied groups	Parameters examined (%)	Mean ± SEM	Maximum	Minimum
$(S+T)_{37}$	Recovery number	99.60 ± 0.4	100	94.44
	Total motility	41.25 ± 6.79	100	0
	Progressive motility	27.69 ± 5.4	60	0
	Viability	62.49 ± 5.60	100	28.57
$(S+T)_{42}$	Recovery number	97.52 ± 1.45	100	83.33
	Total motility	44.55 ± 7.50	75	0
	Progressive motility	25.83 ± 6.6	64.29	0
	Viability	63.66 ± 6.64	100	20
$(CPA)_{37}$	Recovery number	98.05 ± 1.33	100	85.71
	Total motility	60.28 ± 6.58	100	25
	Progressive motility	38.38 ± 7.89	100	0
	Viability	68.42 ± 15.98	100	50
$(CPA)_{42}$	Recovery number	96.52 ± 3.02	100	66.67
	Total motility	75.50± 9.33	100	0
	Progressive motility	52.92 ± 8.13	100	0
	Viability	76.46 ± 9.41	100	0

SEM; Standard error of the mean, S+T; Sucrose+Taurine, and CPA; Cryoprotectant.

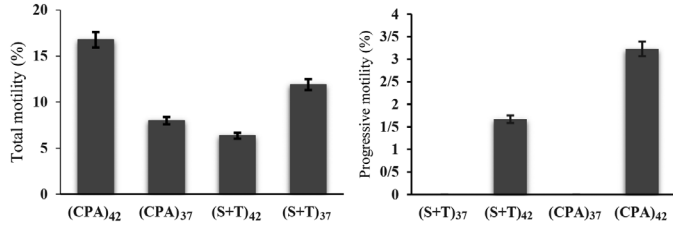

Fig.2: Comparison of total and progressive sperm motility in terms of mean percentage in frozen samples with two different cryoprotectants and by microdroplet method on the petri dish and thawing at two different temperatures (37°C and 42°C). Data were presented as mean values ± standard error of the mean using One Way-ANOVA (Post-hoc test: Tukey's range test). The significance level was considered as $P<0.05$. S+T; Sucrose+Taurine and CPA; Cryoprotectant.

Sperm parameters of cryotop method

Table 2 provides information on the classical sperm parameters in the presence of commercial cryoprotectant and S with the antioxidant T in the cryotop method.

We observed a more than 97% rate of recovery in all groups of the cryotop method, including, $(S+T)_{37}$ group: 106/107 cells (99.07%), $(S+T)_{42}$ group: 114/117 cells (97.44%), $(CPA)_{37}$ group: 104/106 cells (98.11%) and $(CPA)_{42}$ group :105/107 cells (98.13%). However, this rate of cell loss was not significantly different between groups. The average percentage of sperm recovery was not

significantly different among groups: $(S+T)_{37}$: 99.60%, $(S+T)_{42}$: 97.52%, $(CPA)_{37}$: 98.05%, and $(CPA)_{42}$: 96.52%.

Comparing the total motility of thawed sperms, although the average motility percentage in the $(S+T)_{42}$ group (44.55%) was higher than the $(S+T)_{37}$ group (41.25%), this increase was not statistically significant. Also, the mean percentage of motility in the $(CPA)_{42}$ group (75.50%) did not show a considerable increase in comparison with the $(CPA)_{37}$ group (60.28%). In the $(CPA)_{42}$ group, motile sperms (75.50%) were significantly higher than in the $(S+T)_{37}$ and $(S+T)_{42}$ groups (P =0.005, Fig.2). Also, the mean percentage of progressive sperm motility was not statistically significant among the groups, $(S+T)_{37}$ and $(CPA)_{37}$, $_{42}$ (Fig.3). The mean rate of progressive sperm motility in the $(CPA)_{37}$ group (38.38%) was not significantly higher than $(S+T)_{37}$ group (27.69%). While in the $(CPA)_{42}$ group progressive motility (52.92%) showed a significant increase in comparison with $(S+T)_{37}$ and $(S+T)_{42}$ groups (25.83 and 27.69%, respectively) (P=0.001, Fig.3). The rate of sperm viability in the $(S+T)_{42}$ group was more than the $(S+T)_{37}$ group, this difference was not statistically significant. Moreover, we observed an insignificant increase in the motility rate of the $(CPA)_{42}$ group in comparison with the $(CPA)_{37}$ group. The live sperm rate in the $(CPA)_{37}$ and $(CPA)_{42}$ groups, respectively, was not significantly higher than the $(S+T)_{37}$ and $(S+T)_{42}$ groups (Fig.S4, See Supplementary Online Information at ww.ijfs.ir).

Regarding DNA damage, statistical analysis revealed that both groups of sperm that were frozen with and without commercial cryoprotectant noticed an increase in DNA damage. The DNA fragmentation rate showed a non-significant decrease in the $(CPA)_{42}$ and $(S+T)_{42}$ groups in comparison with the $(S+T)_{37}$ and $(CPA)_{37}$ groups. There was also no significant difference in the percentage of DNA fragmentation in each temperature group.

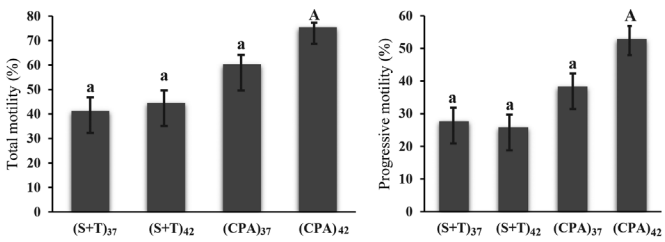

Fig.3: Comparison of the total and progressive motility of frozen sperms with two different cryoprotectants and cryotop methods and thawing at two different temperatures (37°C and 42°C) as a mean percentage in the semen sample. Data were presented as mean values ± standard error of the mean using One Way-ANOVA (Post-hoc test: Tukey's range test). Capital and small letters show significant differences among the evaluated groups (P<0.05). S+T; Sucrose+Taurine and CPA; Cryoprotectant.

Discussion

Sperm cryopreservation is an essential method for treating male infertility, especially in patients with severe oligospermia, obstructive and non-obstructive azoospermia. As is well known, intracytoplasmic sperm injection (ICSI) is the most impressive treatment for the serious cause of male infertility and sperm cryopreservation plays a significant role in maintaining male fertility throughout ART and ICSI strategy (2). However, the occurrence of injury during the freezing process significantly diminishes the probability of finding best motile sperm with higher morphology. The use of inanimate sperm during ICSI may result in complications about both fertilization and pregnancy (23). In this study, we compared the effect of a complex of S and T with the commercial CPA on the classical parameters during the rapid freezing of limited sperm by two different methods (microdroplet on the petri dish and cryotop).

Here, we found that freezing on a petri dish, brings a 100% sperm recovery rate in all our groups, while recovering sperms were easily visible after thawing that it seems because of their cryopreservation method. This outcome was in line with the Bouamama et al. study, that reported a complete recovery rate (100%) after thawing in a culture dish with motility around 50%. Also, they observed less than 2 sperms were recovered at thawing phase when used the straw classical technique to freeze 20 sperms (24).

The reduction rate of sperm parameters, including motility and viability is a common consequence of a freezing process, regardless of methods and materials. Microdroplet-based cryoprotectant freezing techniques are secure and advantageous in terms of both cost and safety. In these methods, sperm recovery rate, motility, and fertilization rate are highly significantly different from the results before sperm preparation. Methods currently used to freeze a sperm without adding any cryopreservation medium and usually use S (25). The Cryotop acts as a freezing vessel for the embryos and eggs with a 99% survival rate after thawing, and a good chance for small quantities sperms using. The cryotop technique used in this study was based on the Endo et al. (7) protocol. But we added T as an antioxidant, to try to increase the motility percentage in the free-cryoprotectant group (S+T). Therefore, we evaluated the effects of two freezing mediums . However, the motility rate was lower in the S in comparison with Sperm Freeze Medium. In the present study, a successful cryopreservation of a limited number of sperm, with an effective quick recovery rate was achieved. Moreover, it was determined that the cryotop approach was more appropriate for viral infections during nitrogen storage due to the convenience and speed of freezing-thawing and less infection than the petri dish method, as well as the lack of problems such as enough space and storage in a nitrogen tank (2).

Also, it was shown that the thawing at the 42°C maintains the survival and motility rates of all sperm better than 37°C. Similar results were obtained by Mansilla et al. (16) who reported a better sperm motility rate in the 42°C than the 37°C and 40°C, when thawed vitrified sperm (5×10^6/ml). The HOS test showed the

viability of motile sperm at 38, 40, and 42°C is 26.48 ± 8.4%, 56.6 ± 16.3%, and 65.4 ± 15%, respectively. The Plasma membrane integrity was supported better than at 42°C compared to other thawing temperatures (26). The results of another study (27) suggest that higher temperatures have beneficial effects on the metabolism restoring and membrane stability of sperms. It seems due to the thawing rate; the latter researchers observed that 60°C temperature for 5 sec resulted in a better sperm quality rate in comparison with the 37°C for 30 seconds conditions. The sperms motilities, total and progressive, were consistently and significantly better in the rapid thawing condition (60°C for 5 seconds). According to Malo et al., for thawing temperatures of 60°C, the overall motility rate at 0 hour and 1 hour was higher than 37°C (27). Also, El-Ahwany et al. (28) reported an increase in motility recovery rate in 42°C thawing condition in comparison with the 37°C temperature.

Studies drew attention to the possibility of induced sperm DNA fragmentations through the freezing/thawing procedure. It has shown that a cryopreservation procedure increases the ROS production level, impairs DNA repair enzymes, induces apoptotic changes in a sperm, and produces high frequencies of single- and double-stranded DNA fractures. Conversely, other studies have shown that cryopreservation does not affect the stability of a sperm DNA (23). In the present study, we did not encounter a significant increase in the DNA damage index in the groups subjected to thawing at 42°C, it is reasonable that a brief duration of thawing is less likely to induce DNA damages. Therefore, it can be concluded that 42°C thawing condition is suitable temperature than 37°C thawing. It is suggested that when sperm are incubated at lower temperatures, they become dormant, allowing them to retain their energy. As per the current research, it has been observed that upon entering the female reproductive system, sperm undergo a state of inactivity due to exposure to elevated temperatures. This hypothesis potentially elucidates the reduction in sperm motility observed at 37°C in contrast to lower temperatures (29, 30).

The creation of adequate space for preserving the culture dish and preventing nitrogen infiltration into this device was the main restriction, even though this study demonstrated the feasibility of using both a culture dish and a cryotop for the cryopreservation of limited sperm. Another difficulty we encountered was keeping the medium culture temperature constant while the sperm were freezing that be considered in next studies.

Conclusion

The combination of S and T showed a good ability to protect a frozen small number of sperms in both cryotop and petri dish procedures. The cryotop approach also features a safer thawing procedure and is quicker and easier to use than the microdroplet on a petri plate method. Limited sperm characteristics such total motility, progressive motility, and viability are better maintained at 42°C than at 37°C. Furthermore, there was no change in DNA damage between 37°C and 42°C when using the cryotop technique.

Acknowledgments

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Authors' Contributions

M.T., L.R.G.; Investigation, Data analysis, and Manuscript writing. A.D.; Methodology, Validation, Manuscript reviewing and Editing of manuscript. M.R.V.; Conception, Study design, and Project administration. All authors read and approved the final manuscript.

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