Original Article

Impact of warming of vitrified semen at different temperatures on cryosurvival

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Abstract

Background: Vitrification is an ultra-rapid cryopreservation method in which cells are directly exposed to liquid nitrogen (LN_2). The warming protocol for vitrification is equally important as the freezing protocol because sperm damage can occur during the warming of sample as well. The warming rate is influenced by the warming medium (air, water) and temperature. A fast warming rate causes unbalanced glycerol efflux and water influx; however, a slow warming rate causes recrystallization of intracellular water microcrystals and leads to subcellular organelles damage. **Aim:** To determine the optimum devitrification temperature for vitrified human sperm by comparing prefreeze and postwarm motility parameters at different temperatures. **Setting and Design:** The prospective study was conducted on 100 patient semen samples. **Materials and Methods:** The semen sample was direct plunged into LN_2 and warmed at different temperatures, that is, at room temperature (RT), 37°C and 42°C for 5 minutes. Sperm parameters were evaluated by the computer-assisted semen analyzer system. Statistical analysis was carried out by applying one-way analysis of variance was carried out using SPSS-22. The level of significance was taken as $P \leq 0.05$. **Results:** The statistical significant difference was found in the case of recovery of all semen parameters, that is, motility ($P < 0.0001$), progressive motility ($P < 0.0001$), cryosurvival factor ($P < 0.0001$), curvilinear velocity ($P = 0.049$), straight-line velocity ($P = 0.033$), average path velocity ($P = 0.0001$), and except count ($P = 0.083$) between the RT, 37°C, and at 42°C. The cryosurvival factor of vitrified semen sample at RT, 37°C, and at 42°C was found to be 25.63 ± 13.885, 29.97 ± 13.212, and 41.99 ± 12.630, respectively. Hence, at 42°C, it was found to be maximum. **Conclusion:** The cryopreservation of the semen leads to a detrimental effect on spermatozoa. The basal semen parameters such as sperm motility, progressive motility, cryosurvival factor, and velocity parameters were affected by devitrification temperature. There was significantly high-sperm cryosurvival after warming of semen samples at 42°C when compared with warming at 37°C and RT. But velocity parameters were found to be better at 37°C.

Keywords: Cryosurvival factor, kinematics, sperm motility, sperm vitrification

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Key Message: The 42°C was found to be the optimum temperature for warming on the basis of cryosurvival factor.

INTRODUCTION

Vitrification is a semen cryopreservation technique that preserves male fertility. Optimum devitrification temperature of vitrified semen is important as it may result in intra- and extracellular ice crystallization that damages cell structure.[1] Also, Sanchez *et al.* have reported that for proper sperm function and for maintaining sperm membrane integrity, optimum devitrification temperature is vital.[2]

Mansilla *et al.* attempted to find out the optimum warming temperature and found that progressive motility was higher in those sperm samples, which were warmed at 42°C (65%) than those samples at 38°C (26%) and 40°C (57%) and also plasma membrane function was best at 42°C.[3] However, Vutyavanich *et al.* warmed the vitrified semen sample in 25°C to 28°C tap water and observed improved postwarming motility and cryosurvival than conventional freezing.[4] Another study showed warming of 21 vitrified samples of human sperm (30 µl pellets) in a water bath at 37°C for 5 minutes resulted in motile and fertile spermatozoa.[5] There is yet no optimum temperature which is known to give better postwarming results.

Therefore, this study was conducted to compare the postwarming semen parameters at different temperatures, that is, at room temperature (RT), 37°C, and 42°C to know about an optimum devitrification temperature. These three are the conventional temperatures used for devitrification; hence, our study aim was to find out the better one out of these. The temperature at which cryosurvival of spermatozoa was best recorded was the optimum devitrification temperature. The cryosurvival was defined as the viability of spermatozoa after 30 minutes postwarming incubation.

Study design

The prospective study was conducted on 100 patient semen samples. Semen sample of patients who came to our clinic for semen freezing for backup and the spare semen samples collected for semen examination or preparation for intrauterine insemination (IUI) were used for our study.

Inclusion criteria:

(1) Subjects having raw semen parameter values above lower limits of WHO-2010 criteria.
(2) Age range 25 to 50 years
(3) Signed patient consent for research.

Exclusion criteria:

Patients positive for hepatitis B surface antigen, hepatitis C virus, human immunodeficiency virus, Syphilis, and novel coronavirus disease 2019 were excluded. However, patients with pyogenic infection and diabetes were not excluded.

MATERIALS AND METHODS

The fresh semen parameters (count, motility, progressive motility, and kinematics) were analyzed using computer-assisted semen analyzer system after liquefaction of the raw sample.

Freezing and warming protocol

Vitrification was performed by initially warming the cryoprotectant (Quinn's Advantage™ Sperm Freezing Medium, Cooper Surgical Fertility Solutions, SAGE Media™, ART-8022) at 37°C for 15 to 20 minutes. About 1.0 ml of cryoprotectant was added to 1.0 ml of semen in a test tube, drop by drop. Mixed sample was kept at 37°C for 10 minutes. Mixed sample was transferred to three well-labeled cryovials. Finally, the cryovials were plunged into liquid nitrogen (LN_2).

After 5 to 10 minutes, the warming of vitrified samples was performed. Sample was placed at 3 different temperatures for warming for 5 minutes: (1) at RT, (2) 37°C water bath, and (3) 42°C water bath. Semen samples were incubated at 37°C for 30 minutes and then semen parameters (count, motility, progressive motility, and kinematics) of warmed samples were analyzed.

End points

The end points were:

(1) Percentage survival of motile sperm.

Cryosurvival factor: Postwarming motility/Prefreeze motility × 100

(1) Similarly, velocity parameters of prefreeze and postwarming were compared at different warming temperatures.

Statistical analysis

The results have been expressed as mean percentage ± standard deviation. Statistical analysis was performed using SPSS-22 (International Business

Machines (IBM), Statistical Package for the Social Sciences (SPSS) Statistics). Comparison of cryosurvival and kinematics was carried out using one-way analysis of variance. The level of significance was taken as $P \leq 0.05$.

RESULTS

The mean of sperm motility in raw semen sample, at RT, at 37°C, and at 42°C was 80.55 ± 13.631, 38.76 ± 14.279, 44.64 ± 12.465, and 56.69 ± 11.126, respectively. The sperm motility in postwarm samples was significantly lower at all temperatures compared to the raw sample.

Among various warming temperatures, sperm motility was the best at 42°C, and this difference was more significant when compared with RT and 37°C. Among various warming temperatures, cryosurvival factor was the best at 42°C, and this difference was more significant when compared with RT and 37°C.

The kinematic parameters, that is, curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP) showed a significant decrease in postwarming at different temperatures. The difference was significant at the RT, 37°C, and 42°C ($P < 0.049$, $P < 0.033$, and $P < 0.0001$, respectively). But kinematic parameters were the best at 37°C [Table 1 and Figure 1].

Table 1: Basal semen parameters, cryosurvival factor, and velocity parameter values after warming at room temperature, 37°C, and 42°C

		Mean	Std. deviation
Count (million/ml)	Raw sample	90.58	38.418
	At RT	48.71	27.539
	At 37°C	48.50	27.159
	At 42°C	56.47	31.755
Motility(%)	Raw sample	80.55	13.631
	At RT	38.76	14.279
	At 37°C	44.64	12.465
	At 42°C	56.69	11.126
Progressive motility(%)	Raw sample	75.97	15.338
	At RT	28.84	15.523
	At 37°C	34.81	13.382
	At 42°C	47.72	13.372
Cryosurvivalfactor(%)	Raw sample	100.00	0.000
	At RT	25.63	13.885
	At 37°C	29.97	13.212
	At 42°C	41.99	12.630
VCL(µm/sec)	Raw sample	101.78	16.134
	At RT	83.40	16.787
	At 37°C	88.37	18.376
	At 42°C	83.15	15.368
VSL(µm/s)	Raw sample	62.57	12.943
	At RT	59.04	14.897
	At 37°C	61.01	15.721
	At 42°C	55.53	14.074
VAP (µm/s)	Raw sample	62.89	9.759
	At RT	54.36	13.014
	At 37°C	59.73	14.136
	At 42°C	52.17	10.439

RT, room temperature; VAP, average path velocity; VCL, curvilinear velocity; VSL, straight-line velocity.

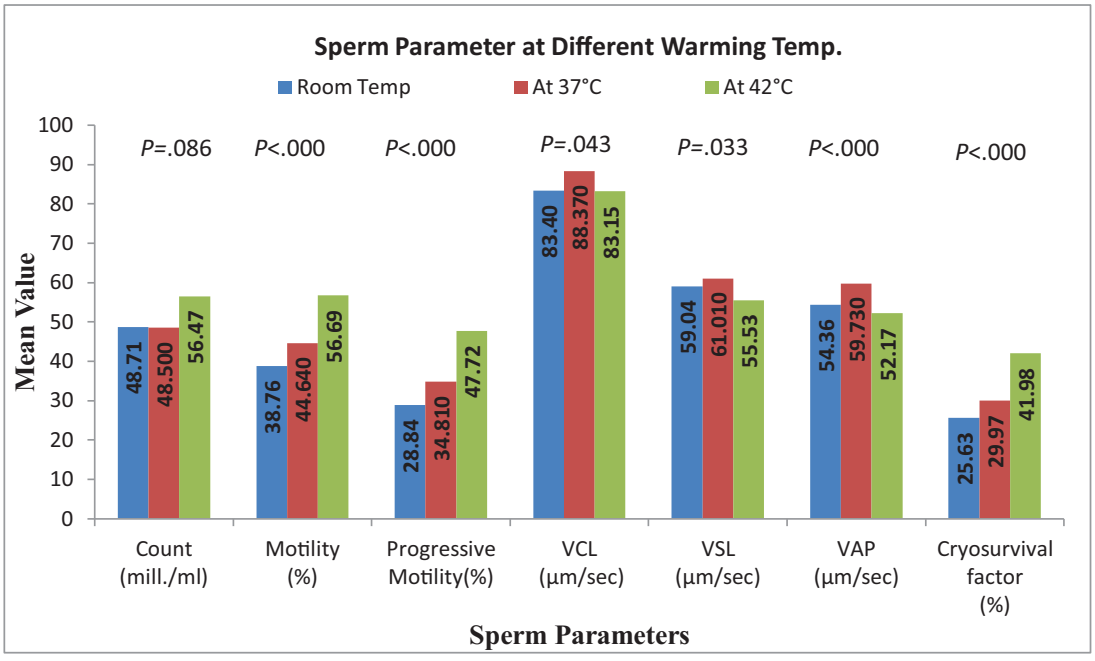

Figure 1: Comparison of count (million/ml), motility (%), progressive motility (%), curvilinear velocity (VCL) (µm/s), straight-line velocity (VSL) (µm/s), average path velocity (VAP) (µm/s), and cryosurvival factor (%) between different warming temperatures, that is, room temperature, 37°C, and 42°C.

DISCUSSION

There was significant decrease in all basal parameters in the semen samples warmed at different temperatures such as RT, at 42°C, and at 37°C compared to the raw semen sample. Stanic *et al.* reported that the various processes such as osmotic shock, cellular dehydration, and intracellular and extracellular ice crystal formation leading to thermal shock occur during freezing–warming procedure, affecting the various semen parameters.[6]

Sperm motility has been reported as a predictive factor of fertility.[7,8] It was found that there was a significant difference ($P < 0.0001$) of sperm motility when all three warming temperatures were compared to each other. The least sperm motility was found upon thawing the semen sample at RT, and the maximum sperm motility was found after thawing at 42°C. Similar to motility, the progressive motility was also significantly decreased $P < 0.0001$ in the semen samples that were cryopreserved. The maximum progressive motility was found in the semen samples that were thawed at 42°C.

The adenosine triphosphate (ATP) formed due to oxidative phosphorylation is transferred to the microtubules which is responsible for the sperm motility. The reason behind the decreased motility of sperm is due to the impairment of the mitochondrial activity of the spermatozoa. The mitochondrial bilayered membrane gets damaged due to which the oxidative phosphorylation and ATP generation is impaired leading to the decreased sperm motility.[9] However, oxidative phosphorylation in the mitochondria does not generate all of the ATP required for spermatozoa motility. Glycolysis is, in fact, another source of ATP that powers sperm movement.[10]

A faster recovery rate of sperm enzymatic antioxidant activity could be one explanation for the observed increase in motility recovery following warming at 42°C. There are two contradictory processes that indicate the rate of cell damage during warming: (1) the recovery rate of enzymatic antioxidant activity and (2) the magnitude of oxygen radical generation. Because the recovery of enzymatic antioxidant activity is faster at higher temperatures, sperm will be able to neutralize the increase in oxygen radical generation recorded after warming more effectively at 42°C than at RT and 37°C. Greater ATP production in the spermatozoa axoneme could be another cause for increased motility after warming at higher temperatures.[11] There are a few studies which showed that a high warming rate resulted in an increase in motility recovery.[12,13] Other research, however, reported no difference in motility recovery when different warming temperatures were used.[14,15]

There was a significant difference ($P < 0.0001$) in sperm cryosurvival factor, when all three warming temperatures were compared to each other. The maximum cryosurvival factor of sperm (%) was observed at 42°C. As the cryosurvival factor is directly dependent upon the post-thaw motility. As the motility was maximum at 42°C, the cryosurvival factor was found to be maximum at 42°C.

Hirano *et al.* reported that the velocity parameters can be the indicators of pregnancy outcome.[16] VCL, VSL, and VAP are the commonly used velocity values that describe the human sperm centroid movement.[17] Therefore, the motility parameters such as VCL, VSL, and VAP were compared between all warming temperatures. The significant difference in the VCL, VSL, and VAP, that is, $P = 0.049$, $P = 0.033$, and $P = 0.0001$, respectively, was found in the cryopreserved semen samples. Similar type of results have been reported in various studies.[18,19] In our study, the kinematic parameters resulted best at 37°C rather than at RT or 42°C.

CONCLUSION

The warming of semen samples at 42°C resulted in a significant increase in cryosurvival factor when compared with 37°C and RT. However, the velocity parameters were best at 37°C rather than 42°C and RT. The cryopreservation of the semen affected the basal sperm parameters such as sperm motility, progressive motility, cryosurvival, and kinematics. These were found to be decreased in the cryopreserved semen samples. It can also be concluded that upon thawing the semen sample at 42°C temperature, there was maximum sperm count, motility, and progressive motility in comparison to the semen samples thawed at RT and 37°C. The recovery of functioning human spermatozoa after cryopreservation may therefore be facilitated by warming it at 42°C for 5 minutes. Although results are significant, but to know if clinically this study will make any difference, the fertilization rates should be compared using a warmed sample at 37°C and 42°C.

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Conflicts of interest

There are no conflicts of interest.

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