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Original article

Impact of time between repeated sperm freezing cycles on sperm quality

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ABSTRACT

Refreezing of sperm samples would provide the possibility of performing more cycles of fertility treatments. Although the effect of repeated cycles of freezing on sperm quality was studied, the effect of the length of the time interval between each freeze-thaw cycle has not been reported. Hence, we assessed the effect of incubation time on the sperm quality of thawed sperm after repeated freezing.

One-hundred samples of potential sperm donations with normal sperm quality were evaluated. The fresh semen samples were analyzed and cryopreserved in liquid nitrogen until use. After thawing, the samples were divided randomly to two groups and reanalyzed for motility, vitality, and DNA fragmentation. They were incubated at room temperature and reanalyzed after either 90 min (group A) or 180 min (group B) of incubation, and once again after a repeated cycle of freezing and thawing.

Our results showed that the sperm parameters of fresh samples of both groups were similar. After one freeze-thaw cycle, both groups still had comparable values. At the end of their respective incubation time periods, however, there was a significant difference in the mean values of the assessed parameters between the two groups ($p < 0.01$). An additional freeze-thaw cycle further exacerbated those differences, with group B undergoing an even more substantial decline ($p < 0.001$).

Our data suggest that thawed human spermatozoa sustain a significant decline in sperm parameters in association with longer incubation time, which is further exacerbated by an additional freeze-thaw cycle.

1. Introduction

Cryopreservation of human spermatozoa has been widely used in assisted reproductive technology (ART) since the 1960s [1]. In addition to sperm donation, cryopreservation of ejaculated spermatozoa is an option of fertility preservation for patients diagnosed with severe oligoteratozoospermia or malignant diseases that may cause sterility following chemotherapy, radiation, or surgical treatment. However, the capacity of spermatozoa to survive the freeze-thaw processes varies between patients [2–4]. It is known that sperm cryopreservation may result in membrane injury with consequent loss of sperm motility and viability [5–7]. The cryopreservation process has also been shown to induce apoptosis in sperm as measured by the amount of DNA fragmentation [8–11]. Repeated freezing and thawing of sperm has more drastic effects with each freeze-thaw cycle, which is exemplified by the severe decline in motility and vitality [12–14]. Furthermore, Thomson et al. reported a significant increase in DNA fragmentation following each freeze-thaw cycle [15]. This fragmentation was shown to negatively affect success rates in ART and to possibly be implicated in the increased rate of miscarriages associated with ART [16–23].

Despite the adverse effects of cryopreservation, there are several conditions that would serve to justify the re-freezing of human spermatozoa. For example, donor sperm may be refrozen in order to maximize the number of cycles that a sperm sample can be used. Similarly, oncology patients who have been rendered infertile often request to have their sperm refrozen due to its limited supply [4].

Previous studies that assessed the effect of repeat freeze-thaw cycles on human spermatozoa have been limited by their small sample size and the absence of any documentation on the time intervals between each freeze-thaw cycle [12,14]. The latter is of particular importance, since sperm motility is known to decrease with each hour and ART procedures can take anywhere from 0.5–8 hours [24,25]. The aim of

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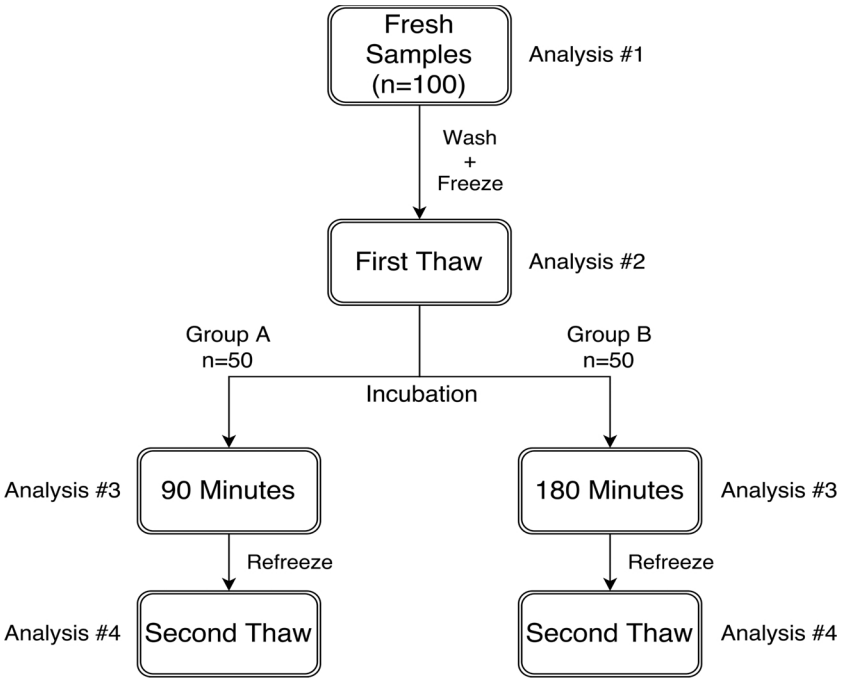

Fig. 1. Flow Diagram.
The samples were analyzed at four separate time points. The baseline analysis was performed on fresh semen. After the first freeze-thaw cycle, the samples were assessed for the second time and then randomized to either group A or group B, which underwent 90 or 180 min of incubation at room temperature, respectively. At the end of their respective incubation periods, the samples were evaluated for the third time, cryopreserved, thawed, and analyzed for the fourth and last time.

this study, therefore, was to assess the effect of an additional freeze-thaw cycle followed by set incubation times (90 min and 180 min) on thawed human sperm quality.

2. Materials and methods

2.1. Specimens

The study population included potential sperm bank donors. They were comprised of young males, most of them students, and all of Caucasian origin. Men with oligospermia, teratospermia, asthenozoospermia or any combination of the three diagnoses were excluded. The study was approved by the local institutional review board committee in accordance with the Helsinki Declaration of 1975. All participants signed a consent form.

2.2. Study design

Sperm samples were analyzed at four separate time points (Fig. 1). Following the *First Thaw* time point, the samples were randomly assigned to either group A (incubation at room temperature for 90 min), or group B (incubation at room temperature for 180 min). Simple randomization was performed in a 1:1 fashion.

To calculate the sample size, we hypothesized that we would need at least 35 samples in each group in order to detect a 7 % difference between the means in group A and group B with a power of 80 %. We chose to analyze 50 samples to account for intra-observer bias. Type I error was defined as 0.05.

2.3. Semen analysis

The laboratory of the Institute for the Study of Fertility successfully participates in various quality control exercises (UK NEQAS, External Quality Assessment Schemes) for sperm concentration, motility and morphology.

2.3.1. Fresh samples

Semen samples were collected by masturbation into a sterile plastic container after 2–3 days of abstinence. After liquefaction, the fresh semen samples were analyzed following the WHO manual guidelines for the examination and processing of human sperm [26]. Two-hundred spermatozoa from each aliquot were analyzed for sperm concentration, total motility (progressive and non-progressive), and vitality. Each aliquot was analyzed twice by the same observer. Vitality was assessed through eosin-nigrosin staining and examined under x200 magnification. Sperm morphology was evaluated by Papanicolaou staining under x1000 magnification and scored as being “normal” or “abnormal” according to the strict criteria of Kruger et al. [27].

2.3.2. Thawed samples

Frozen sperm samples were thawed on a hotplate at 37 ˚C for five minutes and then transferred to 1.5 ml tubes which were mixed thoroughly to ensure a homogeneous mixture. The samples were then incubated at room temperature according to their randomly assigned group. Sperm parameters were analyzed as above. DNA fragmentation was evaluated by TUNEL assay.

2.4. Assessment of DNA fragmentation by TUNEL assay

The In-Situ Cell Death Detection Kit (Roche, Basel, Switzerland) was used to assess the extent of DNA fragmented cells. Semen was smeared on a microscope slide and fixated in a phosphate-buffered solution of 4 % formaldehyde. After TUNEL labeling, the nuclei were counterstained with DAPI (Sigma-Aldrich, St. Louis, Missouri). The cells were observed under fluorescent microscopy (Olympus Provis AX70, Tokyo, Japan), and 100 stained sperm cells were counted as a percentage of TUNEL-positive cells. Positive and negative controls were included in each TUNEL assay in order to ensure experiment quality. The positive control was prepared by incubation with DNase I for 10 min followed by TUNEL labeling. The negative control was prepared by omitting the enzyme from the TUNEL labeling solution.

2.5. Freezing process

2.5.1. Fresh samples

Semen samples were mixed with mHTF medium (Irvine Scientific, Santa Ana, CA) in a 1:2 ratio and centrifuged for 10 min at 300 *g*. The washed sperm pellet was resuspended in 0.3 ml mHTF and carefully diluted by adding an equal volume of test yolk buffer freezing medium (Irvine Scientific). The mixture was equilibrated for 20 min at room

Table 1
Comparison of sperm parameters at different time points between groups A and B.

Sperm Parameters (%)	Group A (n = 50)	Δ (%)	Group B (n = 50)	Δ (%)	p value[†]	p value[‡]
Baseline						
Motility	63.9 ± 6.0	–	61.5 ± 7.5	–	0.78	–
Vitality	83.2 ± 5.5	–	82.7 ± 6.2	–	0.695	–
First Thaw						
Motility	26.2 ± 12.9	−59.5 ± 21.3	24.97 ± 11.4	−59.8 ± 17.6	0.634	0.933
Vitality	46.7 ± 12.3	−44.0 ± 14.9	48.0 ± 11.9	−41.9 ± 14.3	0.578	0.474
DNA Fragmentation	20.8 ± 10.4	–	25.3 ± 10.9	–	0.122	–
Incubation						
Motility	17.22 ± 9.7	−31.2 ± 32.0	12.1 ± 7.8	−50.8 ± 20.5	0.007	< 0.001
Vitality	37.8 ± 10.5	−18.4 ± 11.8	31.9 ± 9.69	−33.5 ± 13.1	0.005	< 0.001
DNA Fragmentation	29.5 ± 13.3	+54.2 ± 41.4	45.3 ± 12.8	+98.9 ± 73.5	< 0.001	0.008
Second Thaw						
Motility	8.9 ± 5.3	−47.0 ± 18.6	6.0 ± 3.9	−46.9 ± 28.4	0.006	0.979
Vitality	27.1 ± 8.5	−28.0 ± 13.2	20.7 ± 7.4	−34.9 ± 13.5	< 0.001	0.011
DNA Fragmentation	37.7 ± 14.0	+32.2 ± 23.9	56.4 ± 13.0	+28.5 ± 26.3	< 0.001	0.608

Values are represented by mean ± standard deviation. Δ Values were calculated as a change in percentage from the previous time point. p values were calculated using Fisher's independent t-test. Significance level was set at $p < 0.05$.

[†] p value represents the statistical significance of the differences between average sperm parameters of the groups.

[‡] p value signifies the statistical significance of the change between the deltas of both groups.

temperature, then sealed in 0.5 ml straws (minitube, Tiefenbach, Germany) and cooled in a semi-programmable freezer (Nicool LM-10; Air Liquid, Paris, France). The straws were cooled from room temperature to -5 °C at a rate of 1.7 °C/min, and then to −100 °C at a rate of 5 °C/min. The straws were then transferred directly to liquid nitrogen (−196 °C) for storage.

2.5.2. Thawed samples

At the end of the incubation process, the samples were re-analyzed and sealed in 0.5 ml straws and re-frozen as above.

2.6. Statistical analysis

All statistical analyses used the Statistical Package for Social Sciences (SPSS 25.01 for Windows, Chicago, IL). Means and standard deviation of motility, vitality, and DNA fragmentation percentages for all time points (baseline, first thaw, incubation, and second thaw) are presented in Table 1. Differences in the means at each time point were established using the independent *t* test. A repeated measures model was applied to evaluate the changes over time within and between group A and group B. Significance was defined as $p < 0.05$.

3. Results

The effect of repeated freezing and thawing on sperm quality was assessed on a total of 100 samples from potential sperm bank donors. Their average age was 25.7 ± 3.5 years and their body mass index was 22.04 ± 1.14 kg/m^2. Data on other potentially pertinent factors, such as tobacco and alcohol consumption, were not available.

3.1. Comparison between group A and group B

Semen analysis at baseline revealed a similar average motility and vitality in both groups A and B, and comparisons between the groups did not reach a level of significance (Table 1). Differences between the average sperm concentrations of the two groups were also non-significant (74.9 ± 30.2 million/mL vs. 74.8 ± 32.8 million/mL, $p = 0.99$). Following the first round of freezing and thawing, there was a similar decrease in the average motility and vitality values in both groups. In addition, they both had similar DNA fragmentation percentages (Table 1).

Following incubation times of 90 min for group A and 180 min for group B, the motility and vitality of the sperm samples in group A decreased from the *First Thaw* time point by an average of 31.2 ± 32.0 % and 18.4 ± 11.8 %, respectively, while the percentage of cells showing DNA fragmentation increased by an average of 54.2 ± 41.4 % (Table 1, Fig. 2). Group B was severely affected by the longer incubation time, with a steep decrease in motility and vitality by 50.8 ± 20.5 % and 33.5 ± 13.1 %, respectively, and an increase in DNA fragmentation percentage by 98.9 ± 73.5 % (Table 1, Fig. 2). All changes in sperm parameters from the *First Thaw* time point to *Incubation* were all significant in both groups (Table 1).

An additional freeze-thaw cycle further exacerbated the decrease in sperm quality. Group A and group B showed a similar rate of decline in motility (47.0 ± 18.6 % vs. 46.9 ± 28.4 %, $p > 0.05$) and increase in DNA fragmentation percentage (32.2 ± 23.9 % vs. 28.5 ± 26.3 %, $p > 0.05$, Table 1). However, the decrease in vitality was much greater in group B (28.0 ± 13.2 vs. 34.9 ± 13.5, $p = 0.011$, Table 1).

3.2. Repeated measures analysis

We opted to perform a repeated-measures statistical analysis in order to assess whether there was a significant change over the various time points. The analyses generally indicated that there was a significant decrease in sperm quality. Specifically, sperm motility underwent a significant decrease over time in each group independently ($p < 0.001$), as well as when they were directly compared ($p = 0.042$). Sperm vitality also decreased significantly over these time points ($p < 0.001$), however, the difference between the two groups was of borderline significance ($p = 0.064$). The DNA fragmentation percentage increased dramatically and was significant over time in each group independently ($p < 0.001$), as well as between the two groups ($p < 0.001$).

4. Discussion

Cryopreservation of human sperm is considered a routine procedure in ART. Refreezing of human semen allows the retrieval of viable spermatozoa and provides additional conception opportunities for patients with limited supply. Our results demonstrated that the length of time between thawing and refreezing sperm is crucial and has a negative association with sperm characteristics, while an additional freeze-thaw cycle further reduces its quality.

After the initial freeze-thaw cycle, there was a reduction in sperm motility and vitality in both groups (Fig. 2A and B). Henry et al. hypothesized that the decreased motility following a freeze-thaw cycle may occur as a result of damage from the cryopreservation process that was likely due to osmotic shrinkage, a rise in solute concentration, and

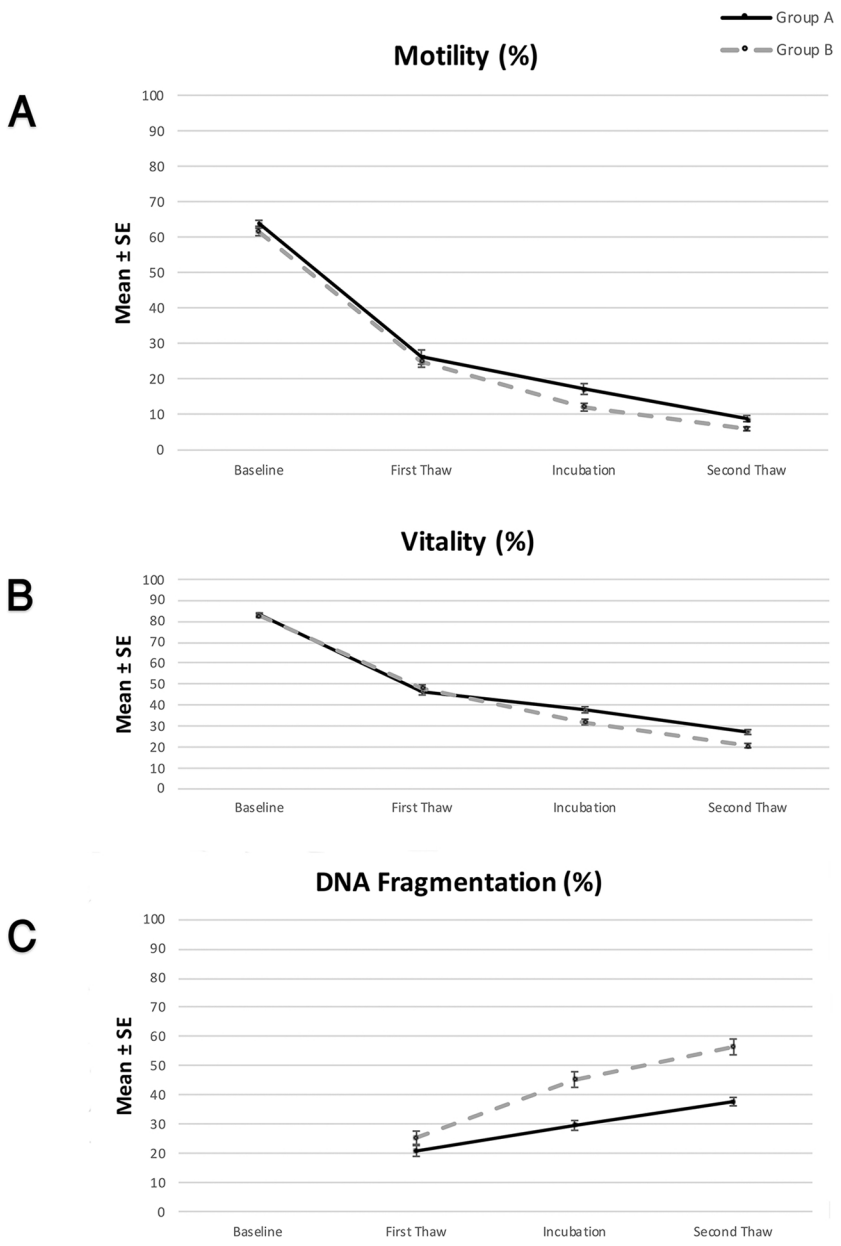

Fig. 2. Time Point Measurements of Sperm Parameters.
Samples were analyzed at four separate time points: Baseline, First Thaw, Incubation, and Second Thaw. The dots and error bars represent the mean ± standard error.

a decrease in the unfrozen fraction, all of which can lead to the formation of intracellular ice and damage to the sperm cell [28].

The incubation of samples also resulted in a decline in sperm motility and vitality in both groups. This can be attributed to the fact that sperm motility requires high levels of ATP, which is associated with the production of reactive oxygen species (ROS) that have detrimental effects on spermatozoa over time [29]. This would explain the prominent decline in motility and vitality of group B, which had a longer incubation time (Table 1, Fig. 2A and B). An additional freeze-thaw cycle further decreased the motility and vitality for both groups by a similar percentage ($p > 0.05$), suggesting that the cryopreservation process itself decreases the sperm quality.

After the first freeze-thaw cycle, the percentages of cells showing DNA fragmentation were comparable for groups A and B, with values of 20.8 ± 10.4 % and 25.3 ± 10.9 %, respectively, similar to those reported by Thomson et al. [15] (Table 1, Fig. 2C). Several studies have suggested that the increase in sperm DNA damage after freezing and thawing may be due to oxidative stress generated by lipid oxidation, by the lack of seminal antioxidants, and/or by the process of cryopreservation [15,30,31]. Incubation of the samples increased the DNA fragmentation percentage in both groups, however, it was significantly more severe in group B ($p < 0.001$). This is probably due to the continued oxidation-mediated degradation of DNA and the generation of ROS with time [32,33]. An additional freeze-thaw cycle further increased the percentage of fragmented DNA in both groups by a similar ratio ($p = 0.608$), suggesting that the cryopreservation process increases the DNA fragmentation rate independent of the length of the time interval between each freeze-thaw cycle.

In summary, our study demonstrated that there is a decline in sperm motility and vitality and an increase in the DNA fragmentation percentage following each freeze-thaw cycle. Longer incubation times were associated with significantly poorer sperm motility, vitality, and a higher percentage of DNA fragmentation. An additional freeze-thaw cycle further exacerbates the declining quality of sperm, irrespective of

the interval between cycles. Based on these findings, we recommend that spermatozoa should be thawed very close to the time of utilization. Any subsequent cryopreservation should be carried out as soon as possible to prevent untoward consequences and to maximize the success of sperm retrieval.

Further research into cryopreservation techniques which limit the formation of ROS for decreasing the extent of associated spermatozoa damage and apoptosis is warranted. Future studies may also consider the effect of incubation at various temperatures.

5. Limitations

Limitations of our study included the use of sperm samples of young males with normal values of sperm parameters. In addition, since DNA fragmentation is not routinely performed on fresh samples of potential sperm bank donors, these data were not available. The decision to use two different groups that included samples with similar baseline characteristics instead of testing the same sample at 90 min and 180 min was due to the potential bias on sperm parameters, that may derived from differences in volume between each sample tested as has been reported elsewhere [34]. Moreover, our simple randomization method may lead to selection bias.

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Authors' contribution

Benjamin Zaghi - performed the research, analyzed the data and authored the paper.

Shimi Barda - made substantial contributions to conception and design, acquisition of data, analysis and interpretation of data, was involved in drafting the manuscript and gave final approval of the version to be published.

Sandra Edith Kleiman - given final approval of the version to be published.

Ron Hauser - made substantial contributions to conception and design and gave final approval of the version to be published.

Declaration of Competing Interest

The authors declare that they have no conflict of interests. All authors read and approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.repbio.2019.12.003.

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