Controlled cooling versus rapid freezing of teratozoospermic semen samples: Impact on sperm chromatin integrity

ABSTRACT

AIM: The present study evaluates the impact of controlled slow cooling and rapid freezing techniques on the sperm chromatin integrity in teratozoospermic and normozoospermic samples. SETTING: The study was done in a university infertility clinic, which is a tertiary healthcare center serving the general population. DESIGN: It was a prospective study designed in vitro. MATERIALS AND METHODS: Semen samples from normozoospermic (N=16) and teratozoospermic (N=13) infertile men were cryopreserved using controlled cooling and rapid freezing techniques. The sperm chromatin integrity was analyzed in fresh and frozen-thawed samples. STATISTICAL ANALYSIS USED: Data were reported as mean and standard error (mean ± SEM) of mean. The difference between the techniques was determined by a paired t-test. RESULTS: The freeze-thaw induced chromatin denaturation was significantly (P<0.01) elevated in the post-thaw samples of normozoospermic and teratozoospermic groups. Compared to rapid freezing, there was no difference in the number of red sperms (with DNA damage) by the controlled slow cooling method in both normozoospermic and teratozoospermic groups. Freeze-thaw induced sperm chromatin denaturation in teratozoospermic samples did not vary between controlled slow cooling and rapid freezing techniques. CONCLUSIONS: Since the controlled slow cooling technique involves the use of expensive instrument and is a time consuming protocol, rapid freezing can be a good alternative technique for teratozoospermic and normozoospermic samples when sperm DNA damage is a concern. KEY WORDS: Chromatin denaturation, controlled slow cooling, rapid freezing, teratozoospermia

INTRODUCTION

Sperm experience physiological and chemical stress during the freeze-thaw process which reduces the sperm viability and functional capabilities. The quality of a frozen-thawed sperm depends on several factors such as the composition of the cryoprotectant, cooling rate, and quality of the semen sample stored. In addition to the well documented detrimental effect of cryopreservation on sperm functional characteristics, cryopreservation is also known to affect the sperm chromatin integrity. Sperm morphology is known to be an important factor in predicting the fertilization potential of a sperm and treatment outcome in assisted reproductive technology (ART) program. Although it has been shown that bovine sperm with morphologically abnormal head have defective chromatin structure, the association between sperm head deformities and an abnormal chromatin structure in sperm is still debated. Intact human sperm DNA is an essential prerequisite for successful fertilization and embryo development whereas abnormal DNA can lead to derangements in the reproductive process. Recently, we have shown that sperm with head abnormality are more susceptible to undergo chromatin denaturation when cryopreserved by conventional rapid freezing method. Since the controlled cooling method is found superior to other methods in retaining sperm functional capabilities in post-thaw samples, this study was undertaken to find out the
benefit of controlled cooling in preventing sperm DNA denaturation in teratozoospermic and normozoospermic samples.

MATERIALS AND METHODS

Sample collection and evaluation
This prospective study was conducted on 29 infertile men aged 22–31 years attending Division of Reproductive Medicine for semen analysis. The study was approved by the institutional ethics committee and a written consent was taken from the subjects before their participation in the study. Subjects who had a sexual abstinence of 3–5 days were asked to collect their semen sample by masturbation in a sterile container. The sperm concentration, motility and morphology were assessed according to WHO criteria.[12,13]

Briefly, the sperm concentration was calculated using Makler’s counting chamber (Sefi Medical Instruments, Israel) by observing 10 μl of the semen sample under a microscope (Olympus, India). To assess the sperm motility, a drop of the ejaculate was placed on a microscopic slide and a cover slip was placed carefully avoiding any air bubbles. The motility was graded in 200 spermatozoa by observing them under microscope using ×40 objective. For viability and morphology assessment, a drop of the ejaculate was mixed with 2 drops of 1% eosin and 3 drops of 10% nigrosine. A thin smear was prepared on a clean glass slide by the feathering technique. The slides were air dried. The viability and morphology were scored by observing the slide under the light microscope using an oil immersion objective (Olympus, India).

Assessment of sperm DNA damage
Smears were prepared from the semen samples on a clean grease-free glass slide and air dried. The slides were fixed in Carnoy’s fixative (1:3 glacial acetic acid and absolute methanol) for 2 h and then stained with 0.1% acridine orange (AO) in a citrate buffer (pH 2.5) as described earlier.[9] After 1 min of staining, the excess stain was removed by repeated washing in a citrate buffer and observed under the fluorescent microscope (Imager A1, Zeiss, Germany). Orange-red fluorescence is an indication of the presence of single-stranded DNA and sperms with intact DNA fluoresce green. A minimum of 200 spermatozoa were evaluated from each slide and the percentage of sperms with denatured chromatin was calculated.

Semen cryopreservation
The semen samples were divided into three aliquots to allow the direct comparison of the efficiency of slow cooling and rapid freezing methods for the same ejaculate. The samples were carefully mixed with a 10% glycerol-based cryoprotective medium (glycerol-egg-yolk-citrate medium) at room temperature and cryopreserved.

Controlled slow cooling
The equilibrated samples were transferred to cryovials (Nunc, Denmark) and subjected to cooling as described earlier[14] using an automated programmable freezer (Kryoplaner, USA). Briefly, the semen samples were cooled from an ambient temperature to −4°C at a rate of −5°C/min, from −4°C to −30°C at a rate of −10°C/min, and from −30°C to −140°C at a rate of −20°C/min. Finally, the cryovials were plunged into the liquid nitrogen (−196°C).

Rapid freezing
The cryovials were subjected to static cooling at 4°C for 10 min and then vapor-phase cooling for 1 min before being plunged into liquid nitrogen (−196°C).

Thawing
After 2 weeks, the cryovials were removed from the liquid nitrogen and thawing was accomplished at 37°C. When the samples were completely thawed, an equal volume of the EBSS medium was added to each vial and the samples were centrifuged at 1800 rpm for 8 min to remove the cryoprotective medium. The sperm chromatin integrity was assessed using the acridine orange bindability assay.

Statistical analysis
The data represent mean and standard error (mean ± SEM) of mean. The statistical significance level was calculated by the paired t-test using SPSS (version 16.0). Differences between the means were considered to be significant when P<0.01 was achieved.

RESULTS

Out of the 29 semen samples analyzed in this study, 16 were normozoospermic and 13 were from teratozoospermic subjects (<30% morphologically normal sperm). The average number of morphologically normal sperms in normozoospermic samples was 34 ± 1.4%, whereas the teratozoospermic sample had 22.5 ± 1.2% morphologically normal sperms [Table 1].

The average number of red sperms (with DNA damage) were 15.8 ± 1.2% and 16.6 ± 1.2% in normozoospermic and teratozoospermic samples respectively. The freeze-thaw induced DNA damage was significantly (P<0.05) elevated in the post-thaw samples of normozoospermic and teratozoospermic groups and the enhancement was approximately 2.3 folds in both controlled cooling and rapid freezing groups [Figure 1]. There was no difference between percentages of denatured sperms subjected to the
controlled slow cooling method in both normozoospermic and teratozoospermic groups.

**DISCUSSION**

The survival rate of sperms after cryopreservation is highly variable and it has been shown that sperm with abnormal head morphology are highly susceptible to cryodamage than sperm having normal morphology.\(^9\) Hence, the use of an appropriate freezing method is essential to minimize the freeze-thaw induced DNA modification in the morphologically abnormal spermatozoa. In the present investigation, the efficacy of two freezing techniques, i.e., controlled cooling and rapid freezing protocols, on freeze-thaw induced sperm denaturation was tested. We found that both techniques are equally effective in maintaining the sperm DNA integrity in teratozoospermic samples. It has been reported that the controlled cooling of semen from men with testicular tumor or Hodgkin’s disease preserved spermatozoa significantly better than the standard rapid freezing method in terms of functional characteristics.\(^9\) Hence we wanted to test whether controlled cooling is beneficial in maintaining the chromatin integrity of teratozoospermic samples as defects in the sperm chromatin structure have been shown to cause increased DNA instability and sensitivity to denaturation stress.\(^{15,16}\)

The results from the present study suggested no difference in the sperm DNA damage level in post-thaw samples subjected to controlled cooling and rapid freezing protocols. Sperms have an unusual cryobiological behavior due to their low or negligible amount of cytoplasm and hence the penetrating cryoprotectant may have a minimum role to play in dehydrating the cell and preventing the ice crystal formation. This could be the major reason behind the similar effect of controlled slow cooling and conventional rapid freezing techniques on DNA damage observed in the present investigation. The results of present study agree with the earlier observation in which sperm survivals after controlled cooling and ultra-rapid freezing or vitrification was compared.\(^{17}\) Cryopreservation is known to induce chromatin denaturation. Our earlier study\(^9\) has shown that the teratozoospermic semen has a higher susceptibility to chromatin denaturation and DNA fragmentation than normozoospermic semen samples. However, in this study there was no difference in DNA damage between normozoospermic and teratozoospermic samples. One possible explanation for this observation is the difference in the subject’s inclusion criteria where only head abnormalities were considered in our earlier study and the teratozoospermic group in this study included all kinds of morphological abnormalities. Poor chromatin packaging and possible DNA damage may contribute to the failure of sperm decondensation and results in the failure of fertilization and embryo development\(^{18}\) which makes it very crucial to prevent the freeze-thaw-induced alteration in the sperm chromatin structure.

In conclusion, cryopreservation techniques do not have any influence on the DNA damage of teratozoospermic samples. Since the controlled cooling technique involves the use of expensive instrument like a biological freezer and is a time-consuming protocol, rapid freezing can be a good alternative for teratozoospermic samples when sperm DNA damage is a concern.

**Table 1:** Efficiency of controlled slow cooling and rapid freezing protocols on post-thaw parameters of normozoospermic and teratozoospermic semen samples

| Sample     | Motility (%) | Viability (%) | Morphology (%) | Sperm with denatured chromatin(%) |
|------------|--------------|---------------|----------------|-----------------------------------|
| Fresh      |              |               |                |                                   |
| Normozoospermic | 76.1±1.9  | 80.5±1.5      | 34.0±1.4       | 15.8±1.2                          |
| Teratozoospermic | 73.3±3.2  | 74.0±1.6      | 22.5±1.2       | 16.6±1.2                          |
| Slow cooling  |              |               |                |                                   |
| Normozoospermic | 41.8±1.2  | 52.7±1.4      | 27.2±1.1       | 37.2±3.3                          |
| Teratozoospermic | 42.2±1.4  | 53.3±1.5      | 18.1±1.4       | 37.8±3.1                          |
| Rapid freezing |              |               |                |                                   |
| Normozoospermic | 41.2±1.7  | 52.6±1.6      | 26.8±1.3       | 40.2±3.1                          |
| Teratozoospermic | 40.0±1.2  | 51.8±1.2      | 17.2±1.4       | 41.2±3.6                          |

**Figure 1:** Sperm denaturation level in fresh and post-thaw spermatozoa from normozoospermic and teratozoospermic samples cryopreserved by controlled slow cooling and rapid freezing methods. (a) \(P<0.01\) vs. fresh normozoospermic group, (b) \(P<0.01\) vs. fresh teratozoospermic group
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