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DNA DAMAGE IN SPERM DUE TO CRYOPRESERVATION – A STUDY IN RATS

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ABSTRACT

Background: Evaluating cryoinjury of rat spermatozoa is crucial to improving the probability of fertilization. **Methodology:** The epididymal rat spermatozoa were subjected to 15, 30, 45 and 60 days freezing separately, and then determined the motility, count, viability, morphological changes and DNA intactness after thawing each sample. **Results:** The results of thawed samples showed that the cryopreservation has significant effect on decrease in sperm motility ($P < 0.01$) to more than 50% and increase in percentage of dead or membrane damaged sperm formation. Therefore the frozen and thawed samples had decreased count and viability of spermatozoa ($P < 0.01$). Genetic damage was determined by comet assay and was compared in fresh and frozen-thawed samples, there were significantly More DNA strand breaks after cryopreservation. **Conclusion:** Our research findings have suggest that cryopreservation makes rat spermatozoa susceptible to external and internal damage, in particular during cooling process.

KEYWORDS: Comet assay, Count, Epididymis, Motility.

LIST OF ABBREVIATIONS: DNA: Deoxy ribonucleic acid, µl- Microlitre.

INTRODUCTION

The reduction of fertilizing ability of semen after freezing and thawing has been associated with a reduced rate of sperm motility, normal sperm morphology and damage of the sperm membranes. [1] In this sense, Zilli *et al.* [2] showed that sperm cryopreservation promotes DNA fragmentation, it has been reported that fertilization with DNA damaged sperm could

increase the rate of abnormal karyotype in the offspring and increase the percentage of abortions, as well as the risk of cancer and abnormal weight in newborn mice. [3] Rats are commonly used laboratory animals for biomedical and genomic research[4]. Molecular and cellular biology techniques have allowed production of thousands of new strains of laboratory animals and this process is expected to accelerate in the future. Hence, the evaluation of this parameter and reduction of DNA injury shall be a priority in the design of new freezing thawing procedures and extenders, in order to increase fertilization rates, and also to ensure good embryo development, the health of the offspring, and the maintenance of the genotypes to be preserved. [5]

The mechanisms by which DNA damage is produced are not completely understood but several studies point out the possibility that oxidative and mechanical events could be responsible for DNA fragmentation and base modification. [6, 7] Recent works developed on sperm revealed that DNA fragmentation significantly increases after cryopreservation, and suggested that oxidation of specific bases could occur during freezing-thawing. [8] reported that the effects of cooling rates and role of freezing extenders on cryosurvival of rat sperm. In our study epididymal rat spermatozoa were subjected to freezing thawing processes and then determination of morphological changes and DNA damage were compared fresh with 15, 30, 45 and 60 days after preservation.

MATERIAL AND METHODS

Cryopreservation of sperm

The male albino rats weighing about 250gm and 12-15 weeks old were purchased from Sri Venkateswara Enterprises, Bangalore, India. Rats were euthanized via ethanol inhalation followed with induction of bilateral pneumothorax. Immediately following confirmation of sedation, the distal end of the scrotal sac was removed and dissection of the tunics allowed for exteriorization of the testis and *cauda epididymidis*. The *cauda epididymidis* was removed from the testicle and adherent fat, and was rinsed in warm phosphate buffered saline. The epididymis was then placed in the selected cryopreservation medium (1.0:0.7), i.e. Sperm Freeze™, a 15% glycerol based cryoprotectant in buffer (Ferti Pro N.V., 8730 Beernem, Belgium), and was incubated for 10min to allow the sperm to swim out of the tissues. The liquid medium was collected and tested for sperm count. Samples were subsequently thawed at 37°C for 10min. and were subjected DNA analysis.

Staining of semen samples

Samples of all dilutions of semen were analyzed by using Trypan blue/Giemsa staining at collection and after frozen as reported by Kovacs *et al.* [9] with minor modifications. The stained slides were observed using microscope for morphology of sperm.

Epididymal spermatozoa motility, viability and count

The fresh spermatozoa immediately after collection from cauda epididymis were used as control. A sperm viability test was done by the method described by World Health Organization. [10] Assessment of sperm count and motility were performed according to the method of Freund and Carol. [11]

Comet assay

The Sperm DNA intactness was investigated using the single cell gel electrophoresis (COMET) assay[12] at alkaline conditions. Each precleaned slide was pre-coated with a layer of 1% normal melting point agarose in PBS (Ca^{2+} and Mg^{2+} free) and then dried at room temperature. Approximately, 100,000 sperm cells (10µl) were mixed with 0.75% low melting point agarose (80µl) at 37^0C, and this suspension was dropped onto the first agarose layer. Slides were allowed to solidify for 20min at 4^0C and the slides were immersed in freshly prepared cold lysis buffer. The slides were then incubated at 37^0C in lysis buffer for 2h. The slides were removed from the lysis buffer, drained and placed in a horizontal electrophoresis unit filled with fresh alkaline electrophoresis buffer at 4^0C for 20min incubation to allow the DNA to unwind. Electrophoresis was performed at room temperature, at 25V for 30min. Following electrophoresis, slides were stained with 80µl 1x Ethidium Bromide, for 5min and then dipped in chilled distilled water to remove excess stain and later kept them for 20min in cold absolute 100% ethanol for dehydration. The slides were stored in a dry area and images were observed on a Fluorescent microscope (BX53) at 400x magnification.

Statistical Analysis

Results obtained using microscope analysis was expressed as the Mean ± Standard Deviation (SD). All statistical analysis was performed using the statistical software SPSS 11.0 (SPSS Ltd., Surrey, UK). The P value of less than 0.01 ($P \leq 0.01$) was considered as statistically significant.

RESULTS

Table1. Effect of cryopreservation on rat sperm motility, count and viability.

Parameters	Motility (%)	Count (No. of sperm/rat×10^6)	Viability (%)
Fresh sperm (control)	67.66±0.51	54.05±0.35	68.0±0.63
15 days frozen	31.03±0.75	28.35±0.79	34.83±0.75
30 days frozen	29.50±1.04	27.75±0.41	32.33±0.51
45 days frozen	28.16±0.75	25.10±0.36	30.83±0.75
60 days frozen	26.16±0.75	24.15±0.56	29.0±0.89

The effect of freezing-thawing processes on motility, count and viability characteristics of epididymal rat spermatozoa before and after cryopreservation is shown in Table 1. Fresh sperm was analyzed for morphology, motility, viability and count. A significant difference in semen qualities was found in frozen thawed samples when compared to control (Table 1). In fresh semen 54 million sperms per mm, with 68% motility and viability were observed. After being stored for 15, 30, 45 and 60 days, the samples were thawed following the specified procedures. Significant differences among fresh and frozen- thawed semen motility, count and viability were found (Table 1). The viability of cells after storage and cooling was found to be 50% less compared to fresh samples.

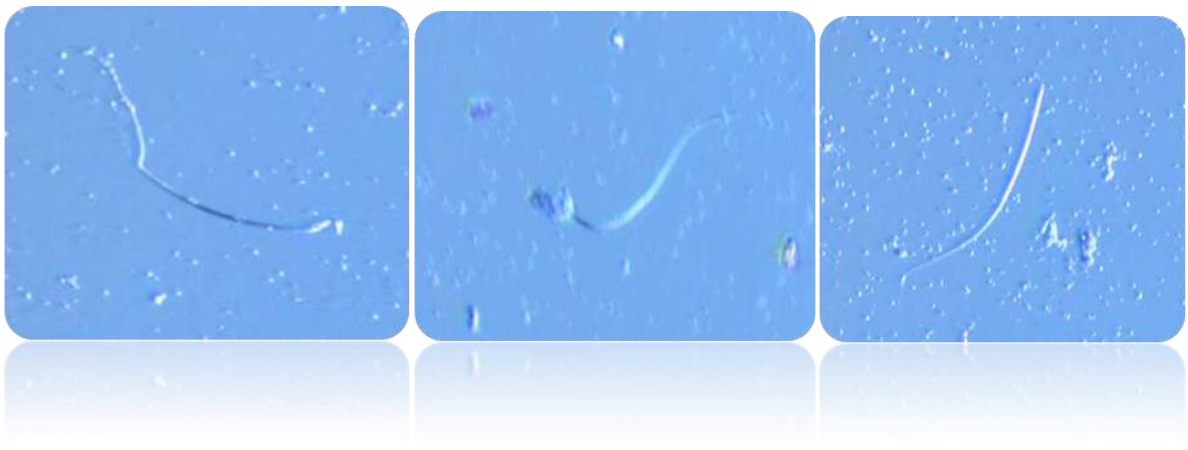

Figure 1: Different categories of sperm stained with Trypan blue/ Giemsa: A. Sperm with Bent tail, B. Sperm with Intact acrosome damage, C. Sperm without head.

The results have demonstrated the frozen sperm in microscopic analysis has showed morphological variations such as (1) sperm with bent neck (Figure 1A), (2) sperm with bent tail (Figure 1B), and (3) sperm with acrosome damage (Figure 1C).

Effect of cryopreservation on DNA damage

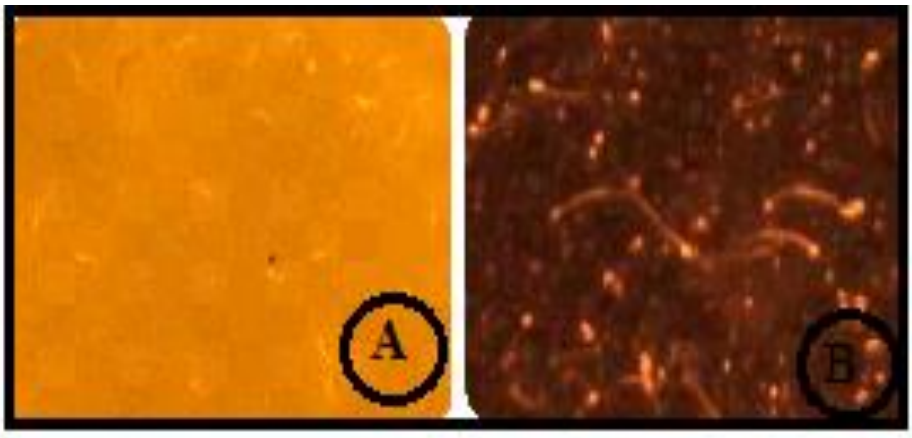

Figure 2: Comets observed under Fluorescent Microscope A) Bright feild, (B) Flourescent.

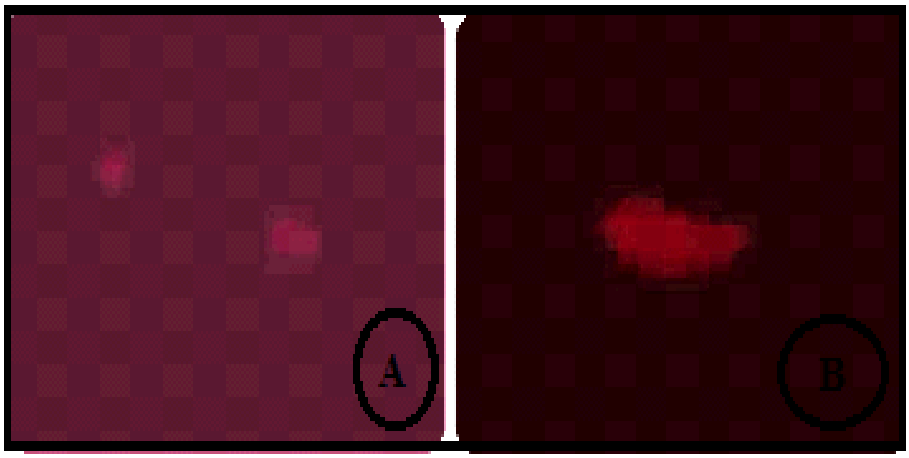

Figure 3: Comets observed under Fluorescent Microscope A) Normal and Comet, (B) Damaged DNA with comet.

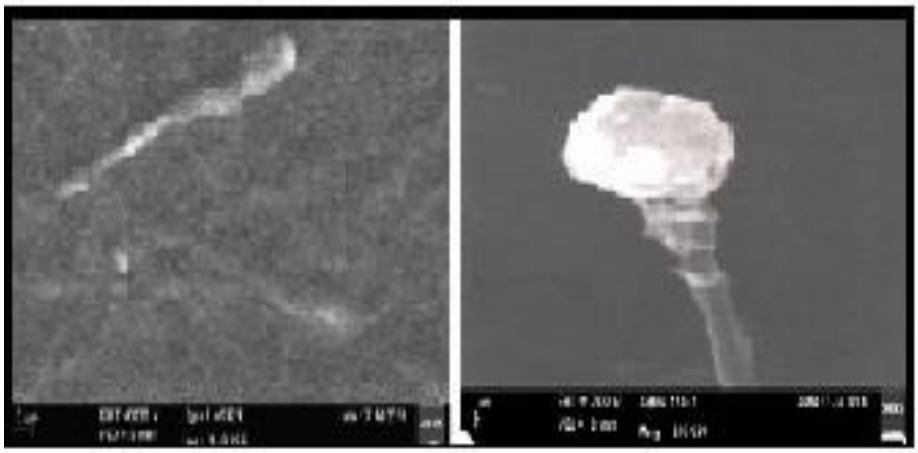

Figure 4: Scanning Electron Micrograph (SEM) of cryopreserved rat sperm with damaged acrosome (Fertil Steril., 2007).

Sperm DNA fragmentation could be a consequence of the freeze-thaw process. [13] The sperm DNA fragmentation was detected by Comet assay which unfurls the damaged DNA like the tail of a comet (Figure 2&3) in acrosome of sperm head. The analysis of scanning electron microscope further showed that the acrosome damage due to cryopreservation (Figure 4). Compared with the fresh, cryopreservation induced DNA damage as indicated by the presence of the DNA tail (Fig. 3 B). A greater DNA tail area and longer DNA tail length (distance from DNA head to the end of DNA tail) reflect more extensive DNA damage.

DISCUSSION

Motility is a strong predictor of the ability of given semen to achieve fertilization *in vivo* or *in vitro.* The reduction in the motility is an irreversible looping of the flagellum normally occurs in rat sperm tail. [14] Cryopreserved spermatozoa could be motile but incapable of fertilization due to acrosomal damage. [15] In our study, the increase in the tail abnormalities in rat epididymal sperm due to freezing and thawing was observed. The fact is that mass motility is more affected by freezing process than individual sperm motility. Because a rapid cooling of semen between room temperature to sub-zero temperatures is known to induce cold shock injury in rat spermatozoa hence this step of the cryopreservation in protocol may require skill and care.

The process of cryopreservation in our laboratory has resulted in an increase in oxidative damage and DNA fragmentation. This suggests that DNA damage, due to oxidative stress was induced by cryopreservation in the sperm. The irreversible deterioration would enlarge the sperm during storage (Figure 3B) and result in the reduction of sperm viability. The comet assay is a sensitive and simple method to detect DNA damage. [16] Induction of sperm chromatin breakage may be attributed to the cryopreservation. The occurrence of sperm chromatin breakage may be considered within the acceptable range. Reports in human male fertility suggest that DNA fragmentation below 30% does not affect the fertilizing potential of a semen specimen. [17] The present investigation showed that the mean values of fragmented sperm nuclear DNA in control group of animals significantly differed from the other groups. This may be explained by the existing association between sperm DNA integrity and semen quality parameters. In this study, we found that sperm morphological changes, which were highly affected from freezing leads to reduction in motility, count and viability. Our results indicate that understanding both the stresses of cryopreservation would

offer useful information to effectively preserve and distribute valuable rat models and to reduce viable sperm loss after thawing of spermatozoa.

CONCLUSIONS

In conclusion, we have shown that sperm DNA fragmentation is associated with cryopreservation. There is a need for long-term studies to determine the possible impact of subtle genomic changes resulting from cryopreservation. Therefore the susceptibility of morphologically abnormal sperm to DNA damage and chromatin modification during the freeze-thaw process is significantly higher than that of sperm with normal morphology.

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