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RESEARCH ARTICLE

Does Cryopreservation Affect the Morphology of Spermatozoa? – A Study in Rats

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ABSTRACT

The cryopreservation is an effective method used for germ cells for archiving valuable strains in biomedical research to the future of nature using successful cryopreservation tools. Despite the usefulness of it, cryopreservation may lead to deleterious changes. Henceforth the objective of study was to evaluate the changes that occur in rat sperm function and morphology during cryopreservation. The morphological changes of spermatozoa after cryopreservation and thaw were assessed by light microscopy. The epididymal rat spermatozoa were subjected to 15, 30, 45 and 60 days freezing separately, and then determined the motility, count, viability and morphological changes after thawing each sample. 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 showed decreased count and viability of spermatozoa ($P < 0.01$). Our research findings have suggest that cryopreservation makes rat spermatozoa susceptible to external and internal damage, in particular during cooling process. Thus, protection of sperm from these effects should give better results in reproduction and may help for biomedical research. Considering these results the question of entry of DNA/Chromosome into ovum is through sperm or by naked chromosomes during fertilization in compliment to the genes of ovum should be answered. This may provide clues to the future generations.

KEYWORDS

Count, Cryopreservation, Epididymis, Motility, Rat, Sperm

INTRODUCTION

Cryopreservation means a system of storage in a cold condition to maintain all biological systems intact and in almost live. It is of useful in storage of reproductive cells and tissues to provide benefits for animal husbandry programs, agriculture, human infertility treatments and biomedical research[1]. The process of cryopreservation of rat sperm is very challenging due to its sensitivity to various stress factors.

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Among all the animals rats are commonly used laboratory animals for biomedical and genomic research.[2] The Molecular and cellular biology techniques have generated thousands of new strains of laboratory animals and these are expected to accelerate in the future. The cryopreservation of germ cells such as sperm, oocytes and embryos are becoming very important both for reducing the maintenance cost and improving distribution of strains.[3] Cryopreservation of sperm provides a simpler and more economical alternative to cryopreservation of embryos, and reduces the cost and space needed for keeping a large number of rat strains having a single mutation.[4]

Sperm preservation protocols vary among species due to their inherent characteristics. There are marked species differences in spermatozoa size and morphology. In addition, there is a subtle difference in membrane phospholipid composition and metabolism of spermatozoa. [5] Rat sperm cryopreservation is an effective method of archiving valuable strains for biomedical research and handling of it is an easy process. The semen of rat is known to have extreme sensitivity towards suboptimal conditions such as centrifugation, pipetting, chilling, osmotic stress,[6] freezing and thawing possibly due to long tail, head shape and membrane composition.[7] Thus, acceptable and repeatable rat sperm cryopreservation protocol has not been achieved and post-thawed sperm quality is yet unsatisfactory for intrauterine insemination or in vitro fertilization (IVF) in rats for genetic diversity.[8] Despite species variation, there are common stages in sperm freezing protocol. All protocols involve sperm collection and extension, addition of cryoprotective agents (CPA), storage and thawing.[9] In all these stages, spermatozoa are exposed to a number of potentially damaging stresses such as the change in temperature, osmotic and toxic stresses presented by exposure to high molar concentrations of CPA and the formation and dissolution of ice crystals in the extracellular space.[10] Extenders, CPA, optimal cooling and thawing rates play important role for successful cryopreservation of sperm. However success of cryopreservation depends on sperm endurance to this insults.[11]

Cryopreservation leads to deleterious effects on sperm of all organisms.[12] Freeze thawing process causes a decrease in the percentage of motility, lifespan of the spermatozoa and oxidative stress which finally leading to a reduction in the fertilizing ability.[13,14] In general, damage to sperm during cryopreservation have been attributed to several factors including cold shock, freezing injury, oxidative stress, alterations in membrane compositions, chemical toxicity of CPA, and osmotic stress.[15] Procedure for rat sperm cryopreservation and subsequent IVF are still under development.[16] Although successful IVF and production of live offspring have been demonstrated with the use of fresh rat spermatozoa,[17] successful IVF of cryo preserved rat spermatozoa have not been well established. The developmental competence of frozen–thawed rat spermatozoa and the pregnancy rate are very limited and current success rates for rat sperm cryopreservation are still inadequate for intrauterine insemination, or IVF preservation of most rat strains. The first live-born rat derived from frozen– thawed rat spermatozoa was reported by using intrauterine insemination (IUI).[6]

It has been reported that the cause of changes in morphology, damage to DNA and sperm tail is due to cryopreservation.[18] The proportion of the fully functional sperm that retain cellular activities are low in frozen and thawed sperm.[19] Thus, a detailed study of the motile sperm structure along with different steps of cryopreservation might help to improve the protocols currently used for bovine semen. The success of cryopreservation depends largely on the specific susceptibility of sperm cells to low temperature. Cooling and freezing–thawing, two major steps in cryopreservation of spermatozoa, have major effects on cell structure and function.[20] Which may result in damage to the plasma and acrosome membrane,[20] reduction of motility and fertilizing ability of spermatozoa and induction of premature capacitation and nuclear decondensation.[21] Moreover, part of the reduction in sperm motility and fertility associated with cryopreservation may be due to oxidative damage from excessive or inappropriate formation of ROS.[22]

Although it is well known that rat spermatozoa are more difficult to cryopreserve than the spermatozoa of other species,[6] however it is necessary to establish more reliable sperm cryopreservation method for efficient management of a vast number of rat models. In order to better understanding of the cellular changes that occur during cryopreservation and physical stress it may be necessary for developing strategies of storage to overcome low post-thaw viability and fertility of rat

spermatozoa. This study would design to obtain information which finding out cellular, morphological changes, motility, viability and count differences in rat sperm function during cryopreservation. In our study epididymal rat spermatozoa were subjected to freezing–thawing processes and then determination of motility, count and viability were compared control with 15, 30, 45 and 60 days after preservation.

MATERIALS AND METHOD

Experimental Animals

The male albino rats weighing about 250gm and 12-15 weeks old were purchased from Sri Venkateswara Enterprises, Bangalore, India. The animals were housed in polyacrylic cages with not more than six animals per cage and maintained under standard laboratory conditions. The animals were fed with standard pellet diet with fresh water *ad libitum*. All the animals were allowed to acclimatize to laboratory condition for a week before commencement of an experiment. All procedures described were reviewed and approved by the University Animals Ethical Committee.

Cryopreservation of Sperm

Male 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 (DPBS). Later using scissors it was cut into three slits. The epididymis was then placed in the selected cryopreservation medium (1.0:0.7), i.e. Sperm Freeze™, a 15% glycerol based cryoprotectant in HEPES 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. This sample was subjected to static vapour phase cooling for 15min before being plunged into liquid nitrogen. Samples were subsequently thawed at 37°C for 10min. Once totally thawed, cryovials were centrifuged at 200Xg for 6min to remove any traces of sperm freeze cryoprotectant. Then the samples were subjected to morphological 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* (1992).[23] with minor modifications. For staining, Trypan blue was used at a concentration of 0.27%. Spermatozoa were diluted 1:10 with 0.9% NaCl, one drop (5µl) of diluted semen and one drop (5µl) of Trypan blue were mixed on a slide and two smears were prepared by using a single semen droplet. Slides were air-dried in vertical position then put into a fixative consisting of 86ml 1N HCl and 14ml of 37% formaldehyde solution with the addition of 0.2gm Neutral Red in a slide staining jar for 2min and then rinsed with distilled water. Slides were put in to jars containing the Giemsa solution and placed in a water bath at 37°C for 2h. For improving the Giemsa staining quality water bath used, reported to be more effective at 25–40°C, and for reducing the length of the procedure, avoiding the Giemsa staining overnight. The Giemsa staining solution was freshly made by adding 7.5% (v/v) of Giemsa stock solution to distilled water. The slides after air dry cover slipped and observed using microscope.

Eosin-nigrosin staining was used to assess sperm viability according to WHO protocol.[24] Briefly, eosin and nigrosin was prepared in distilled water. One volume of sperm suspension was mixed with two volumes of 1% eosin. After 30 sec, an equal volume of nigrosin was added to this mixture. Thin smears were then prepared and observed under a light microscope at 1000X magnification. Viable sperm remained colourless while nonviable sperm was stained blue.

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[24]. Assessment of sperm count and motility were performed according to Freund and Carol.[25] Briefly, both *cauda epididymis* from each rat were placed in 2ml of normal saline pre-warmed to 37°C. Small cuts were made in the two on *cauda epididymis* from where the spermatozoa were obtained and suspended in to the saline solution. Two hundred microlitres of the suspension was diluted with 800µl of saline. A small amount of the diluted suspension was transferred to both chambers of a Neubauer haemocytometer using a pasteur pipette by touching the edge of the cover slip and allowing each chamber to be filled by capillary action.

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

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 Samples Analysis

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.

Post-Thawed Semen Analysis

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 motility of frozen-thawed rat epdidymal sperm for 15, 30, 45 and 60 days cooling rates are given in Tables 1. Sperm motility after dilution ranged between 67.66% and 31.03%, 29.50%, 28.16%, 26.16% for the frozen thawed

Table: 1- 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

M ± SD = mean ± standard deviation

samples. The cryopreservation has decreased the motility levels of compared to the control group. After 15 days cryopreservation, sperm motility loss was under 54.13%, after 30, 45, 60 days of cryopreservation sperm motility was reduced to 56.39%, 58.38% and 61.33%, respectively. Freezing and thawing processes has resulted in loss of sperm motility compared to control. Spermatozoa were affected by cryopreservation in motility characteristics, while frozen–thawed spermatozoa showed more than 50% loss in total motility. The viability of sperm is frozen semen were almost equal with their respective motility rates.

The Effects of Cryopreservation on Rat Epididymal Sperm

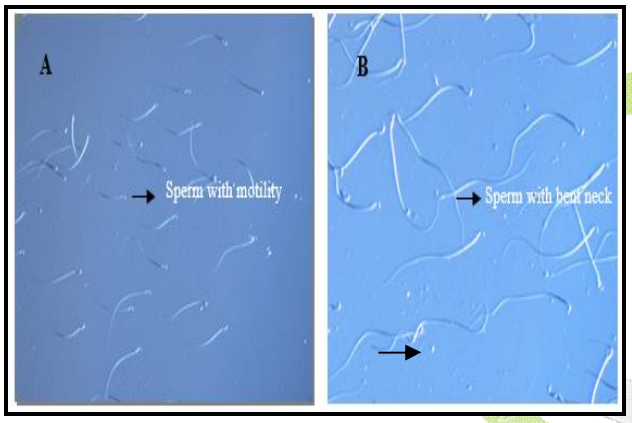

Figure 1: Light microscopy of rat spermatozoa stained with Trypan blue and showing for fresh and cryopreserved sperm. A. Fresh sperm, B. 15 days Frozen Thawed sperm

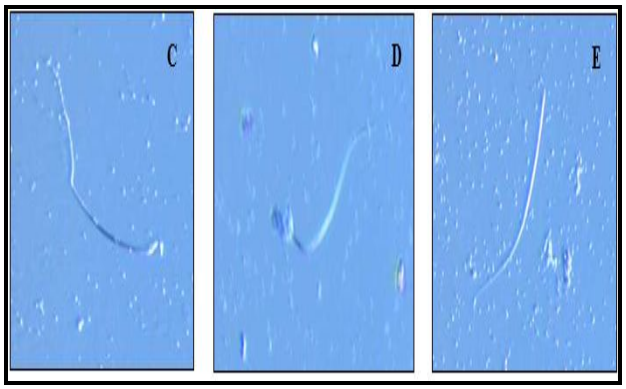

Figure 2: Different categories of sperm stained with Trypan blue/ Giemsa: C. Sperm with bent tail, D. Sperm with Intact acrosome damage, E. Sperm without head

Frozen Thawed Rat Epididymal Sperm

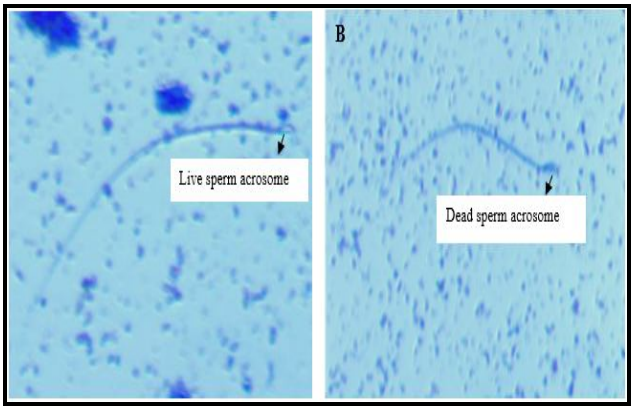

Figure 3: Patterns of rat sperm stained with the eosine-nigrosin technique. (Light microscopy; Magnification 1000 x). A: Represents the live sperm. B. Represents the dead sperm

Based on the observations found in Table 1 on the structural elements motility and viability characteristics during cryopreservation the changes in morphology of spermatozoa were assessed by using light microscopy. The results have demonstrated the frozen sperm in microscopic analysis has showed morphological variations such as (1) Sperm with bent neck (Figure B), (2) Sperm with bent tail (Figure C), (3) Sperm with acrosome damage (Figure D), (4) Sperm without head (Figure E) and sometimes dead sperms were found on eosine nigrosine acrosome specific dye staining (Figure F)

DISCUSSION

The cold storage technology is used to preserve food grains, meat foods, vegetables and almost all biomolecules including cells. In cold storage cryopreservation is specific method used for maintenance of genetic material of cells to use in the future and protect the endangered species to show for its existence in the earlier periods. The technology of cryopreservation for rat sperm preservation has been developed for selection of genetically superiors and transport of genetic lines across countries. The detrimental effect of cryopreservation on spermatozoa has been reported, e.g. cold-shock, osmotic stress and oxidative stress from reactive oxygen species (ROS), which impair normal sperm function and fertilizing potential.

Cryopreserved sperm or oocyte is routinely used in IVF to produce next generation/modified generation. However the detailed examination has suggested that the intensity of complete function of the sperm in a freeze and thawed sperm was considerably reduced.[12] A typical mammalian spermatozoon consists of a head partly covered by an acrosome, a neck and a flagellar like tail. The head of the mammalian spermatozoon is ovate and dorsoventrally flattened. The neck typically consists of the connecting piece and the centriole.[26] The mammalian sperm tail contains an axonemal complex of microtubules,[27] in the midpiece of the mammalian spermatozoon, the axoneme and outer dense fibers are enclosed by a long sheath of mitochondria. The mitochondria itself are elongated and arranged around the core of the sperm tail in a helical fashion. Sperm motility, viability, acrosomal integrity enables more accurate description of spermatozoa's fertilization capacity.[28] For this reason, all sperm parameters should be taken into consideration to evaluate sperm fertility capability.

Motility is a strong predictor of the ability of given semen to achieve fertilization in *in vitro.*[29] The reduction in the motility is an irreversible looping of the flagellum occurred in rat sperm tail.[30] Freeze-thawed spermatozoa could be motile but incapable of fertilization due to acrosomal damage.[31] In our study, the increase in the tail abnormalities in rat epididymal sperm freezing and thawing was observed due to the exposure of sperm to the cold shock. 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 this step of the cryopreservation protocol was done carefully at a low cooling rate. Yet, it seems that even with slow cooling, this change in temperature induce a certain degree of cellular damage, transitions of lipids and altered permeability of membranes.[10] The increase in the spermatozoa damage observed in this study might be a symptom of severe effects of cryopreservation. In this context, it would be interesting to see if rat spermatozoa also would show an increase of morphological damage to decrease in motility during cryopreservation were observed (Table 1).

Consequently, the freeze–thaw procedures caused poor viability, count and impaired functional characteristics due to sub lethal damage of sperm.[32] The motility reflects the detrimental effects from cryopreservation on the metabolism of spermatozoa, as well as on the status of sperm membrane domains.[33] The cryopreservation usually causes cryodamage of spermatozoa for decreasing post thaw cell viability more rapidly compared to fresh cells. [34]

In addition to the known effects of cryopreservation on spermatozoa our results showed three hypothetical effects may explain further alterations of rat spermatozoa due to freeze–thaw procedure: (a) tail bendings (Figure.2), (b) disturbed acrosome membrane function (Figure.2), which is detectable by staining and (c) sperm count reduction due to the damage of head (removed heads, Figure.2). These three supposed effects which extent is responsible for the altered morphology of spermatozoa after cryostorage has elucidated to decline 50% of motility. We present here, for the first time, an objective analysis of the sperm morphology changes that take place during cryopreservation in rats. They are able to indicate alterations of cellular functions and may promote our understanding of effects of cryopreservation on sperm biology. The addition of dead sperm resulted in a decline in percent viable sperm as measured by staining and sperm motility measures that was consistent with the derived expected decline of frozen thawed sperm (Figure. 3).

CONCLUSION

In conclusion, the current results clearly demonstrate that there are numerous clear changes that occur in spermatozoa during cryopreservation. Freezing procedure significantly decreased the motility, count and viability of rat sperm, but there was enormous difference between 15, 30, 45 and 60 days

cryopreserved samples and control. In addition, we found that sperm morphological changes, which were highly affected from freezing leads to reduction in motility, count and viability. Future work will also need to extend all these results to assessment of fertilizing ability of frozen thawed rat sperm. 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.

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