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ANDROLOGY

Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa

Sinan Ozkavukcu · Esra Erdemli · Ayca Isik · Derya Oztuna · Sercin Karahuseyinoglu

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Abstract

Purpose Cryopreservation of sperm is a widely used technique to maintain and protect the fertility in various occasions such as infertility and malignancy treatments. This study aims to reveal the effects of freezing and thawing on human spermatozoa.

Materials and methods To evaluate the effects of freeze–thawing, semen samples were evaluated by light microscopy by means of morphology, motility and viability, by scanning and transmission electron microscopy for detailed ultrastructural changes.

Results After cryopreservation, a significant decrease in spermatozoa viability was observed ($p<0.01$). Group a, b and c motility according to World Health Organization criteria decreased considerably ($p<0.05$, $p<0.01$, $p<0.05$, respectively), whereas there was a substantial increase in group d motility. A strong correlation between rise in number of immotile spermatozoa and decrease in viability was also noted ($r=-0.848$, $p<0.01$). Post-thaw light microscopic studies revealed a considerable decrease in rate of normal spermatozoa ($p<0.05$). A considerable decline in the rate of normal sperm was also observed by TEM ($p<0.05$). Statistically, acrosomal changes and subacrosomal swelling were found to be significantly increased (both $p<0.05$), where the latter appears to be a novel finding in literature.

Conclusion Cryopreservation has deleterious effects on spermatozoa, especially on plasmalemma, acrosomes and tails. Electron microscopy is the ultimate modality to investigate spermatogenic cells.

Capsule This manuscript enlightens the ultrastructural cryo-injury mechanisms of human spermatozoa with supplementary novel acrosomal pathologies, revealing correlations between different sperm parameters following cryopreservation.

S. Ozkavukcu (✉)
Department of Histology and Embryology, Department of Obstetrics and Gynecology, Assisted Reproduction Center, Ankara University School of Medicine, Ankara Universitesi Tip Fakultesi, Ankara, Turkey
e-mail: sinozk@gmail.com

E. Erdemli · S. Karahuseyinoglu
Department of Histology and Embryology, Ankara University School of Medicine, Ankara Universitesi Tip Fakultesi, Ankara, Turkey

A. Isik
Zekai Tahir Burak Maternity Hospital, IVF Centre, Ankara, Turkey

D. Oztuna
Department of Biostatistics, Ankara University School of Medicine, Ankara, Turkey

Keywords Spermatozoa · Cryopreservation · Transmission electron microscopy · Scanning electron microscopy · Viability

Introduction

Over the past decades, infertility treatments are gaining momentum because of advances in assisted reproduction and cell manipulation techniques. Men with azoospermia or oligozoospermia now have the opportunity to children if only a single spermatozoon is harvested.

Cryopreservation of spermatozoa and testicular tissue preserves male fertility for years, regardless of infertility etiology [1, 2]. Thus, research concerning the effects of cryopreservation on these cells has been delayed, since a single viable spermatozoon is usually sufficient for clinical treatments. Cryo-injury models in previous studies are

contradictory [3, 4] and many of the findings are now obsolete, as newly developed chemicals and cryoprotective agents (CPA) are being produced each day [5]. Therefore, cryostorage of spermatozoa or testicular tissue is becoming more important because of novel clinical needs and current clinical practise: assisted reproduction, preservation of fertility following chemotherapy, radiotherapy or various surgical procedures, and confirmation of seronegativity for sexually transmitted diseases in semen banking [6–13].

As spermatozoa are extremely small compared to other cells, examination of organelles such as cell membranes, acrosomes, mitochondria and tail skeletons require higher magnification and/or special staining methods [14, 15]. In this purpose, we used advanced cell imaging techniques including electron microscopy to reveal possible detrimental effects on sperm from normal samples in order to achieve a basic knowledge of sperm cryo-injury. Motility assessment and light microscopic morphology studies were also performed.

Materials and methods

Subject selection

Semen samples were produced by masturbation into sterile containers from 15 healthy volunteers with proved fertility. The mean age was 24.5 years; percentage of non-smokers was 70%. Samples were obtained after an abstinence period of 3–4 days. The approval of the Ankara University Research Ethical Committee was obtained prior to the study, and all subjects were informed with respect to this study. Samples were allowed to liquefy at 37°C for 45 min. Semen of one subject that failed to liquefy in that time period was excluded. Liquefied semen samples were divided into two aliquots. One of the aliquots was immediately mixed with CPA and frozen for further investigation, while the other aliquot was processed for motility, vitality and morphology tests.

Assessment of spermatozoon morphology

Light microscopy (LM), scanning (SEM) and transmission electron microscopy (TEM) were used to determine morphological changes. For light microscopy, a small drop of liquefied semen was smeared on a slide. The slides were air-dried and fixed in ethanol before the modified Papanicolaou staining method was performed. Stained preparations were examined by the same observer according to the Kruger strict criteria using bright field illumination and 1,000× magnification under an oil immersion objective [15]. Two hundred spermatozoa in each aliquot were evaluated. Defects were subdivided according to head, midpiece or tail abnormalities.

For TEM, liquefied semen samples were centrifuged at 400×*g* for 15 min and the pellet was fixed in 2.5% glutaraldehyde and 2% paraformaldehyde overnight at +4°C. Samples were post-fixed in osmium tetroxide before block staining with uranyl acetate. Araldite was used for embedding the samples, and ultra-thin cut sections of 50–70 nm (Leica Ultracut R Vienna, Austria) were contrasted with uranyl acetate and lead citrate. Preparations were observed using an 80 kV LEO 906E TEM (Oberkochen, Germany).

In order to perform SEM observations, samples were washed with a sperm washing medium (SpermWash, Nidacon, Sweden) then fixed in Trump's fixative. Cells in suspension were dropped on to poly-L-lysin coated glass-slides, dehydrated, dried in a critical point drier (Emitech K850, Kent, UK), covered by 10 nm of Au/Pd (Emitech K550X, Kent, UK) and examined under a LEO 438VP (Cambridge, England) microscope. One hundred cells were evaluated for each subject according to Atlas of Human Sperm Morphology [15].

Assessment of spermatozoon motility and concentration

Motility of spermatozoa was evaluated using the Makler chamber. The motility of each spermatozoon was graded "a," "b," "c," or "d" according to the WHO laboratory manual [16]. Spermatozoa concentrations are also evaluated using a Makler chamber after sample collection. Two samples with concentrations of 20×10^6/ml or less (oligozoospermia) were excluded from the study.

Assessment of spermatozoon vitality

Spermatozoon vitality was determined using one-step eosin–nigrosin staining technique [17]. Spermatozoa that were white or unstained were classified as live, while those that showed pink or red coloration in the head region were considered dead. At least 200 spermatozoa were assessed for each preparation.

Sperm freezing and thawing

After semen analysis, liquefied samples were split into two equal aliquots. While one was processed through observations as the control, the second aliquot was prepared for cryopreservation. CPA, (CryoProtec™, Nidacon, Sweden) was added in droplets to semen samples to reach a 1:1 dilution. Then the mixture was transferred to a sterile cryo-vial (Cryo.s Cellstar®, Greiner bio-one, Austria) at a volume of 1.0 ml maximum. Vials were subjected to a static vapour phase cooling 15 cm above the surface of liquid nitrogen (approximately −180°C) for 25 min and then were plunged into liquid nitrogen (−196°C).

Thawing was performed in a 37°C water bath after exposing the cryo-vials to room temperature for about one minute. Once totally thawed, CPA was removed by adding a sperm wash medium and centrifuging at 300×*g* for

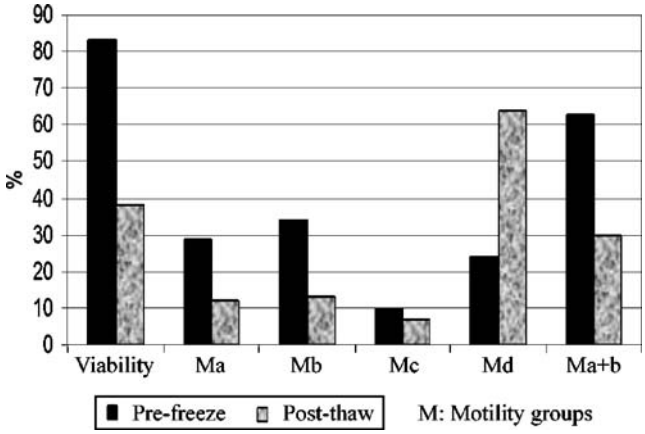

Fig. 1 Changes in viability and motility after cryopreservation

10 min. Thawed and washed samples were assessed for motility, morphology, vitality and ultrastructural changes.

Statistical analysis

The non-parametric Wilcoxon matched pairs test was used to assess differences between fresh and freeze–thawed sperm within each parameter. The strength of the relationship between the increase in group "d" motility and the rates of non-viable spermatozoa was expressed using Spearman rank correlation coefficients.

Results

Sperm parameters and spermatozoon morphology were evaluated statistically according to the data obtained pre-freeze and post-thaw.

Vitality

Spermatozoon vitality, which was determined using the eosin–nigrosin staining method, revealed a mean value of 83% before freezing (min 76%, max 94%). This value diminished to 38% (min 21%, max 55%) after thawing. The difference was statistically significant at $p<0.01$ (Figs. 1, 2).

Motility

Cryopreservation resulted in a significant reduction in spermatozoon motility parameters (Fig. 1). Rapid progressive spermatozoa (group "a") had a mean value of 29% before freezing and a mean value of 12% after thawing ($p<0.05$). The slow progressive group (group "b") decreased from 34% to 13% after cryopreservation ($p<0.01$). Reflecting the total number of motile spermatozoa, group "a" plus group "b" motility was also considered. This value declined from 63% to 30% ($p<0.01$). The non-progressive motile group (group "c") also decreased significantly after thawing ($p<0.05$). After freezing and thawing, the rate of immotile spermatozoa increased significantly from 24% to 64% ($p<0.01$). All the changes mentioned above were found to be statistically significant.

Decrease in vitality and increase in immotile spermatozoa rates were compared using Spearman's correlation test. There was strong correlation between these parameters ($r=-0.848$, $p<0.01$; Fig. 3).

Morphology

Assessment using LM revealed a statistically significant decrease in the percentage of normal spermatozoa after thawing ($p<0.05$). There was no significant change in the morphologies of head, midpiece and acrosome in general; nevertheless morphologic abnormalities of the tail increased significantly ($p<0.01$; Fig. 4). When the organelle structures of the spermatozoa were considered in detail, the rate of tapered heads was significantly decreased and the rate of loose heads (detachment of head and the tail) was increased after thawing ($p<0.05$; Fig. 5a,b). The parameter among tail abnormalities which showed significant change was tail coiling, which increased after thawing ($p<0.05$; Fig. 5c).

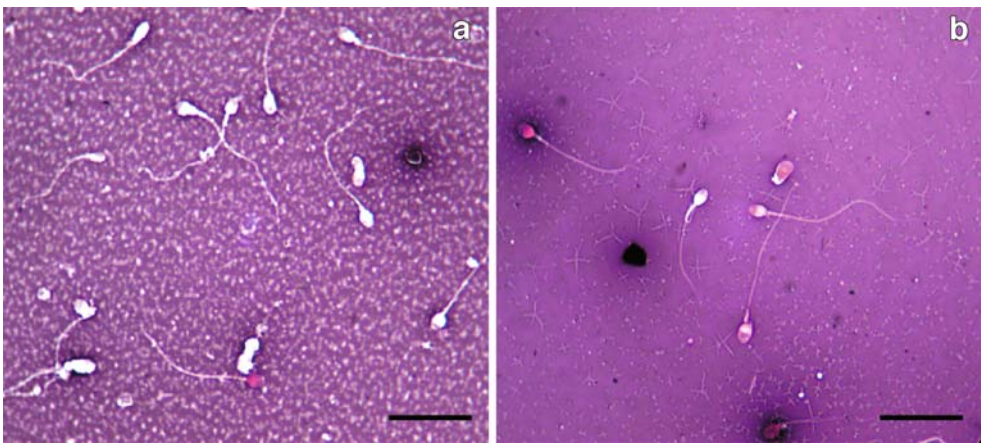

Fig. 2 Appearance on viability assessment slides of the same subject using eosin–nigrosin staining. Mean percentage of viability decreased after thawing (**b**) significantly when compared to pre-freeze samples (**a**). *Scale bars* indicate 20 μm

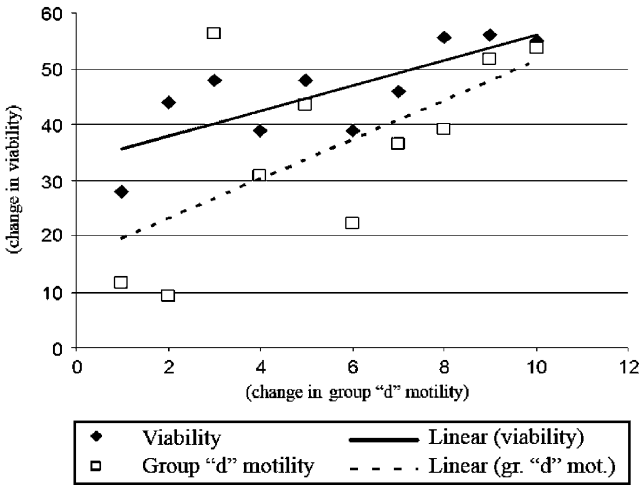

Fig. 3 Correlation between viability and group "d" motility

Post-thaw acrosomes were also evaluated using LM and the rate of spermatozoa with small acrosome decreased significantly ($p<0.05$; Fig. 6).

Pre-freeze and post-thaw spermatozoa were evaluated by means of TEM in three regions: head, neck, and tail. Pre-freeze evaluation of native spermatozoa revealed mostly normal cells with the typical shape of head, intact cell membranes, acrosomes, and homogenous nuclei. Beneath the plasmalemma, inner and outer acrosomal membranes were intact and electron dense homogenous contents of acrosomes were noted. Inner membranes of the acrosome were in close contact and tight with the nuclear membranes of spermatozoa (Fig. 7a). Different ultrastructural abnormalities were also noted, such as decomposition of the plasmalemma, outer acrosomal membrane, and acrosomal content, as well as early acrosomal reaction, chromatin condensation abnormalities, and diadem defects. Rounded, early spermatogenic cells were also detected.

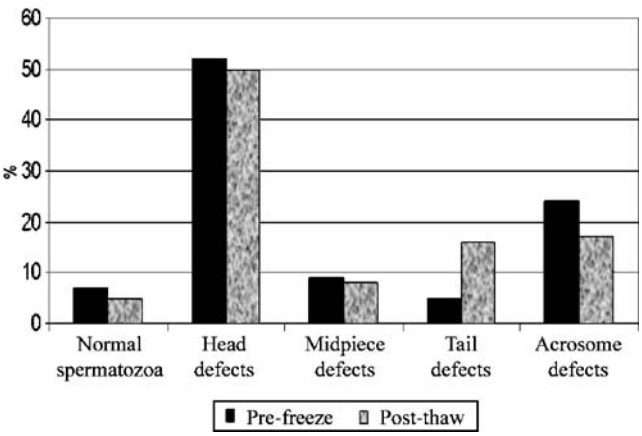

Fig. 4 Light microscopic morphology assessment

After thawing, the rate of spermatozoa that were considered normal by means of TEM evaluations declined significantly ($p<0.05$). Among defined abnormalities, the defects of acrosomal change and subacrosomal swelling increased significantly ($p<0.05$; Fig. 8). In general, changes in integrity of the membranes and morphology of the head regions were especially conspicuous. In many cells, wrinkling (undulation) on the plasmalemma was noted. Acrosomal change defect, which is characterized by unaffected equatorial acrosomal content but an altered apical acrosomal region, was also determined. Apical head alterations in acrosomal change defect are lack of continuity, loss of acrosomal content and appearance of vesiculations (Fig. 7c,d). Some spermatozoa were observed to

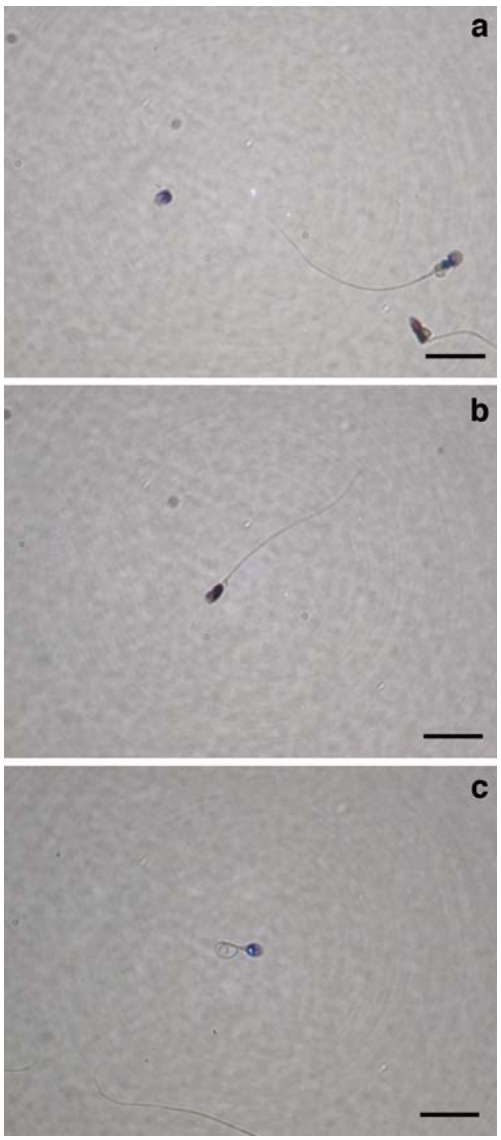

Fig. 5 Light microscopic, morphology assessment using Papanicolaou stain. **a** Mean percentage of loose heads increased significantly after cryopreservation. **b** Tapered headed spermatozoon. **c** Coiled tailed spermatozoon. *Scale bars* indicate 40 μm

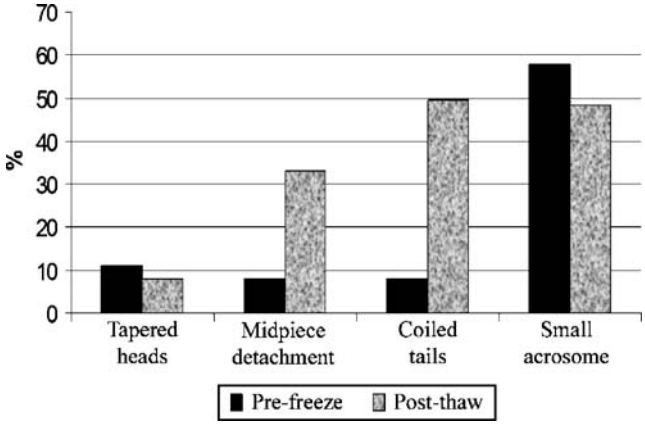

Fig. 6 Detailed assessment of light microscopic morphology

have diffuse or local condensations and granularities in their acrosomal content (Fig. 7d). Another statistically significant observation was subacrosomal swelling, which is characterised by detachment of the inner acrosomal membrane from the nuclear envelope and filamentous widening of the subacrosomal space (Fig. 7b). Evaluation of nuclear content revealed some condensation defects and chromatin irregularities. However, these changes in nuclear material were not statistically significant. Similarly, morphological changes in neck and tail regions were not statistically significant.

SEM was found to be more sensitive to expose surface alterations that were all statistically significant ($p<0.05$) as loose heads, acrosomal alterations and broken, bent, coiled tails (Fig. 9). Round immature spermatogenic cells were also a common feature of SEM examinations while both

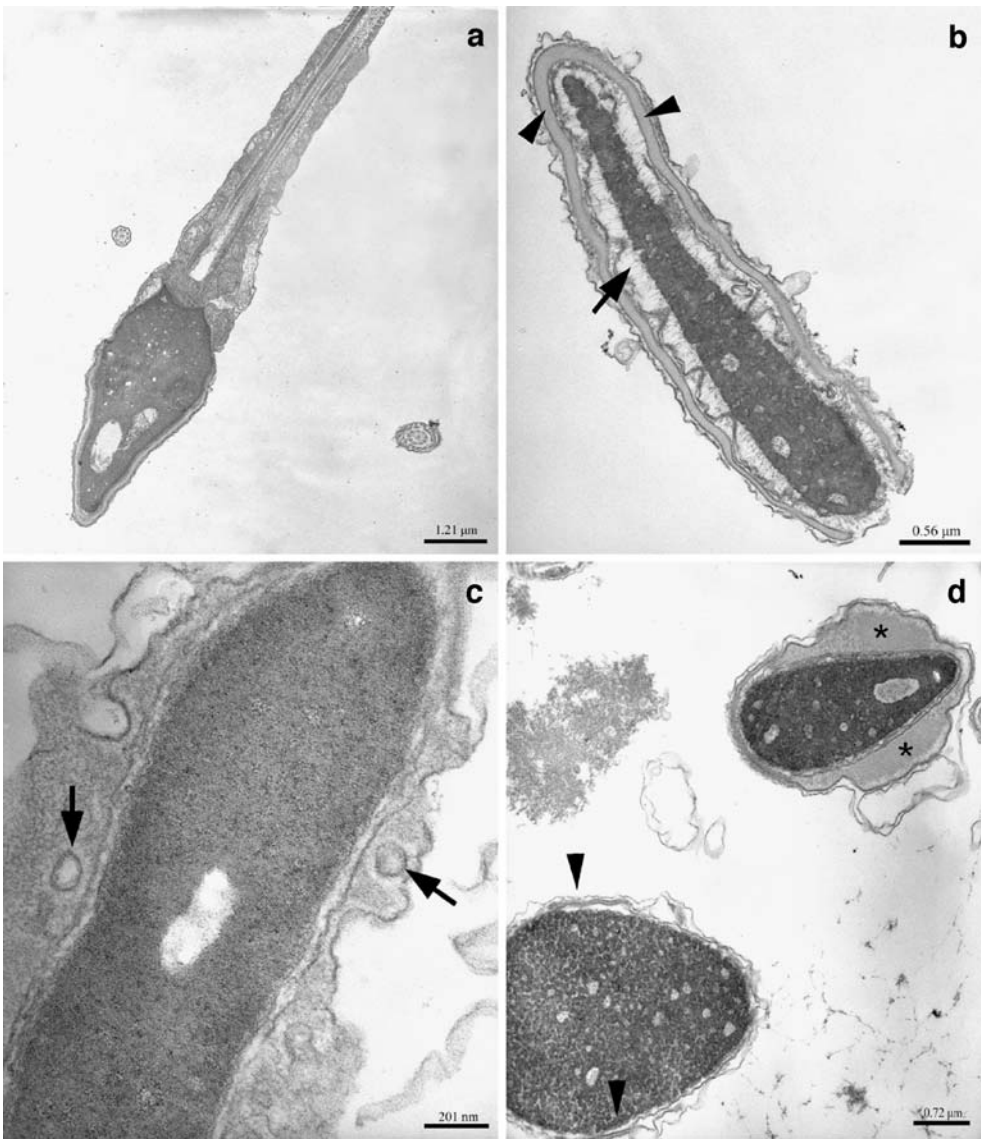

Fig. 7 Transmission electron microscopic evaluation. **a** Normal spermatozoon with intact head, acrosomes and midpiece structure. **b** Subacrosomal swelling. Detachment of the inner acrosomal membrane from the nuclear envelope and filamentous widening of the subacrosomal space can be seen (*arrow*). Note the intact acrosomal content (*arrow heads*). **c** Destruction of cell membranes and vesiculations in an acrosome (*arrows*). **d** Acrosomal change defect (*lower*): Unaffected equatorial acrosomal content (*arrow heads*), and altered apical acrosomal region. Acrosomal swelling (*upper*): Diffuse and nodular accumulation inside the acrosome (*asterisk*)

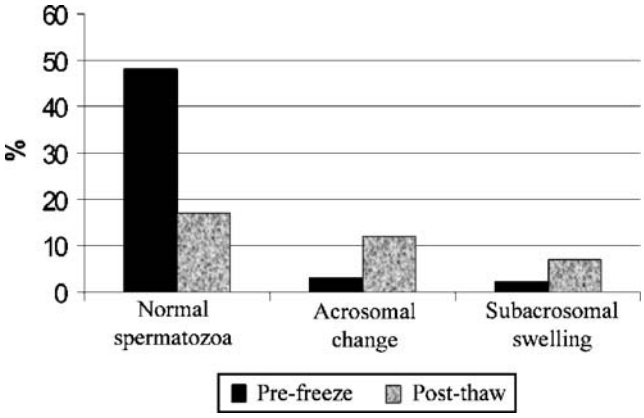

Fig. 8 Electron microscopic morphology assessment

TEM and SEM observations revealed cytoplasmic droplets most of which were larger than one-third of the head size.

Discussion

There are several features of a spermatozoon necessary for fertilizing an egg which must be conserved after cryopreservation. The most important of these features can be summarised as DNA content, acrosomal integrity, motility, and viability. In this study, these vital parameters of spermatozoa in a fertile and healthy population were evaluated by means of several histological criteria, both before freezing and after thawing in order to assess the effects of cryopreservation. Our study is unique in that it combines the studies of ultrastructural changes (both for TEM and SEM) with sperm parameters, and also reveals a basic knowledge about the durability of sperm cells of normal population after cryopreservation. Sperm parameters and the vulnerability of spermatozoa may vary among populations which are healthy, infertile, or suffer from chronic diseases such as malignity [18–20]. Our detailed morphological research provides valuable data for further studies of cryopreservation and is essential for the literature to understand the cryo-injury mechanisms of sperm.

As has been previously reported, we found that the viability of spermatozoa decreased significantly after freezing and thawing [21]. The most probable reason for this regression seems to be the physical and chemical environments to which a spermatozoon is exposed. Crystal ice formation outside the cell is the main factor which physically affects cell morphology. Ice formation around the cells concentrates the surrounding matrix rapidly, leaving the cells in fluids containing high solute content [22]. Also, cell water–CPA exchange during the early stages of the procedure causes cell swellings and shrinkages which may be intolerable for the majority of organelles [5]. Moreover, toxic effects of glycerol, which is widely used for sperm cryopreservation, have been reported in the literature [23, 24]. Rapid changes in osmolarity often occur during freezing–thawing which cause deformations on the membranous structures [25]. Tail defects after cryopreservation have been previously reported, and plasma membrane destruction in this region has been suggested as the probable reason for these defects. We examined an increase in coiled tails, which usually occur after osmotic changes. Deleterious effects of low temperatures have been reported on membrane lipid structure and trans-membrane water canal proteins. Media such as CPAs, addition–removal of them and also changes occurring in water content during freezing of the compounds may lead to coiling of the tails [26].

Although there are several studies suggesting that seminal plasma has a protective role during the freezing processes, there are also papers reporting advantages of swim-up or other separation methods [27–29]. Other studies claim that there is no significant difference between washed and native sperm for freezing [30]. In our study we assumed that one of the major deleterious effects of freezing/thawing is formation of reactive oxygen species. Radical oxygen species are blamed for deleterious effects such as declines in spermatozoan motility and peroxidation of the plasma lipid membrane [5]. Thus, we used native semen for freezing, allowing any antioxidant features of the seminal plasma to exert their protective effects [30–33].

In our study a significant post-thaw decrease in motility was detected. According to Critser et al, glycerol has particular effects on motility, although freezing and thawing seem to have more deleterious impact [34]. We have found a significant increase in the rate of immotile spermatozoa after thawing. As we detected a strong correlation between the increase in immotile spermatozoa and the decrease in viability after thawing, it is likely that the main reason for the decrease in motility is the loss of vitality. This finding partially excludes an organelle defect in causing immotility.

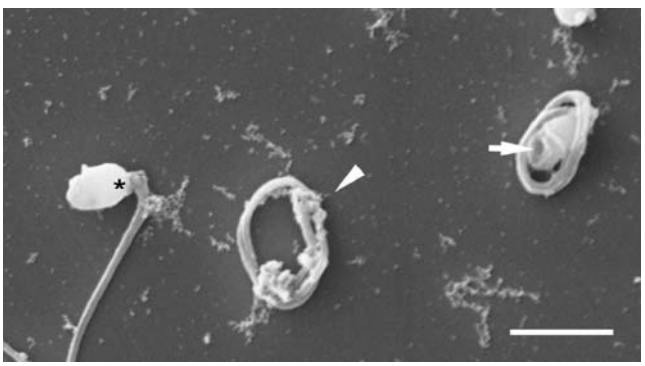

Fig. 9 Scanning electron microscopic evaluation. Bent mid-pieced sperm (*asterisk*), loose head and tails (*arrow head*) as well as acrosomal defects (*arrow*) were evident after cryopreservation. *Scale bar* indicates 5 μm

Mitochondrial defects after cryopreservation have been mentioned by O'Connell et al. and a correlation has been found with loss of motility [35]. Our TEM studies showed that the ultrastructure of the mitochondria and plasma membranes are altered together in diminished spermatozoa, and that destruction of mitochondria generally occurs as a consequence of widespread cellular destruction. Only rarely were cells with mitochondrial defects in the absence of other cellular destruction seen using TEM.

Changes in DNA structure have been studied by many groups after cryopreservation, and contradictory results have been reported. Some groups reported destruction in DNA while others claim such changes occur only or more in men with diminished fertility [21, 36]. Moreover, Hammadeh et al. revealed that slow freezing, rather than nitrogen vapour technique, is more advantageous in all patient groups, especially by means of chromatin protection [37]. Although no specific nuclear markers were used in this study, TEM observations allowed detailed nuclear examinations, which identified defects such as chromatin decondensation and physical morphological destruction. We found no statistically significant changes relating to chromatin and nuclear content morphology. These findings support the data published by Donnelly et al. [19]. Usage of nuclear markers identifying defects in DNA strands and advanced genetic assays may help to settle these controversies [38].

It is clear that the morphologic structure of the spermatozoon is extremely important for the processes of fertilization and embryo development. Thus, it is critical to select the most viable spermatozoa, and exclude the damaged cells following cryopreservation. Kam et al. stressed the usage of zeta method in order to improve the selection of quality sperm after cryopreservation; however the technique is currently experimental [39]. In this study TEM, SEM and light microscopic examinations have shown that the rate of sperm cells having normal morphology decreased significantly. This finding is mentioned by the majority of the papers in the literature dealing with this topic [21]. Our study demonstrated that rates of tapered headed spermatozoa decreased significantly, which is probably caused by uncontrolled liquid influx inside the cell during the thawing procedure and tapered headed sperm cells swell during thawing (Fig. 5b). Loose head and tail fractions of spermatozoa were seen separately (Fig. 5a). Light and scanning electron microscopic assessments showed a significant increase in loose heads after thawing. This detachment defect is probably due to ice crystals that form during the freezing of extra cellular fluids. SEM is found to be more sensitive to the defects in neck and tail regions compared to TEM observations.

We observed that, among all of the organelles, acrosomes were affected most severely. It is likely that acrosomal structure, with its delicate and fragile membranes, is most susceptible to physical and chemical effects and to ionic changes. Moreover, the acrosome is a vital organelle which facilitates the passage of the spermatozoa through the zona pellucida of the oocyte prior to fertilization. As mentioned in other studies, detailed study of acrosomal structure is impossible with LM, despite use of specific acrosome stains [40]. Although the preparation methods for TEM are difficult and require experienced staff, this method is the most precise for evaluating acrosomal structure. SEM also has a high magnification factor as TEM but is limited to evaluations of cell surface. Acrosomal abnormalities are evident as cracks or peelings by SEM. Low temperatures increase cytoplasmic Ca^{+2} levels, capacitation-like reactions, ionic leakage, and distinct exocytosis of acrosomal content [41]. In this study we found no evidence concerning acceleration of the natural progress of the acrosomal reaction after freezing and thawing. Instead, increases in pathological features were detected. Acrosomal change was most significantly increasing pathology after freezing and thawing. Acrosomal loss, which is a marker of the acrosomal reaction, was not a significant finding, indicating that defects observed after thawing result from pathological mechanisms rather than induced physiological processes. It is well known that the equatorial region is rich in vimentin protein accumulation and, as a result, membrane structures are closely and strongly associated with cell skeleton elements [42]. These facts partially explain the survival of the equatorial region despite defective acrosomal changes. Another statistically significant finding in our TEM evaluations is the increase in the widening of the subacrosomal region. This pathology, which probably originates from a post-acrosomal sheath defect, has never been reported before.

Conclusions

Today, in most reproductive medicine centers, the classical vapour freezing method is being used; which is also tested in our study. Vitrification and dry storage of spermatozoa without using CPAs would be preferable methods in cryopreservation. Thus, osmotic and chemical toxicity of CPAs would be avoided if viability after thawing can be maintained [43–47]. It has been reported that, while freezing sperm on liquid nitrogen vapour is sufficient for healthy donors, it is more desirable to use slow, controlled freezing devices for those who are sub- or infertile [48, 49]. In our opinion, different and novel cryopreservation methods (slow freezing, vitrification, vapour freezing, etc.) for different donor groups (normal population, infertile or oligospermic men and malignancy patients) with different CPAs should be considered in basic studies, which include ultrastructural

evaluations [50–52]. As light microscopy and ICSI practice can only help to distinguish between normal or abnormal spermatozoa to a certain point, it is impossible to detect the effects of cryopreservation without using ultrastructural evaluations. That may be one of the possible reasons of further growth failures in ICSI-fertilized embryos especially after gamete thawing. TEM and SEM have critical roles to reveal the ultimate morphology, injury mechanisms and pathways, regarding spermatozoa. Devices which allow high magnification and resolution need to be produced and used for assisted reproduction treatments in order to detect abnormalities on gametes.

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