Effects of Cryopreservation on the Ultrastructure of Human Testicular Sperm

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Objective  To investigate effects of cryopreservation on changes of the ultrastructure of human testicular sperm and evaluate the efficacy of cryopreserving testicular tissue as a source of sperm for assisted reproduction.

Methods  Testicular biopsy tissues were obtained from infertile patients (n=12) with obstructive azoospermia and cryopreserved. Testicular sperm motility was observed after in vitro culture procedure. The ultrastructure of testicular sperm (n=6) was examined by transmission electron microscope.

Results  After cryopreservation, 10 biopsy tissues frozen revealed motile sperm, and 2 samples showed non-motile sperm. Some testicular sperm in frozen-thawed group had normal morphology in fine structures. Sperm head in frozen-thawed tissue showed a proportion of nuclei with more electron-dense granules of chromatin. In a few frozen-thawed sperm heads, formation of vesicles and degeneration were observed. The frozen-thawed testicular sperm frequently showed swollen or/and ruptured of the plasma membrane and acrosome membranes.

Conclusion  Cryopreservation of testicular tissue is simple and efficacious for testicular sperm extraction. And the freezing-thawing procedure of testicular tissue causes damage to ultrastructural morphology of human testicular sperm.

Key words: testicular sperm; cryopreservation; ultrastructure; human

It has been shown that the combination of testicular sperm extraction and intracytoplasmic sperm injection (ICSI) is an efficient treatment for infertile men with obstructive
azoospermia\cite{1-3}. However, pregnancy rates with ICSI and testicular sperm still remain low, and more than one cycle of ICSI is often anticipated. Repetitive testicular sperm extraction procedures can not only cause the consequent risk of damage to the testes, but also may lead to adverse physiologic effects on the patient. Therefore, an alternative approach is cryopreservation of sperm obtained through testicular sperm extraction and use of cryopreserved testicular sperm for subsequent ICSI\cite{4, 5}. However, effects of cryopreservation on the ultrastructure of human testicular sperm have not been fully understood. In this study, we sought to observe changes of the ultrastructure of testicular sperm after thawing by transmission electron microscopy, and evaluate the efficacy of cryopreserving testicular tissue as a source of sperm for ICSI.

**Materials & Methods**

**Testicular tissue sample**

Testicular biopsy tissues were obtained from 12 infertile patients with obstructive azoospermia (age range: 27-35 years) as sources of sperm in the ICSI programme. Each tissue sample was cut into equal pieces in human tubal fluid (HTF) culture medium (Irvine Scientific, USA) supplemented with 2 mg/ml human serum albumin (HSA, Sigma) for ultrastructural analysis or culture.

**Cryopreservation of testicular tissue**

For cryopreservation the testicular tissue (n=12) was cut into small pieces in the culture medium. Glycerol-yolk-free cryoprotective medium was used\cite{6}. The cryoprotective medium was added slowly drop by drop to the medium containing the biopsy specimens. The biopsy pieces were frozen in 0.5 ml straws (IVM Co., France) which were held at 6°C waterbath for 30 min and in liquid nitrogen vapour being placed into the liquid nitrogen for 20 min. To thaw, the straws were taken out of liquid nitrogen and placed into a 37°C waterbath for 30 s, and the contents of the straw were transferred into the H-HTF medium in a tube. One biopsy piece was placed in fixative for ultrastructural analysis while the other was washed immediately for the removal of the cryoprotectant for further culture.

**Transmission electron microscopy**

From the same sample, the fresh and frozen-thawed tissue (n=6) was fixed and prepared for ultrastructural analysis using a routine method as previously described\cite{7}. Ultrastructural changes of testicular sperm were observed with transmission electron microscope (Philips Telnai-10).

**In vitro culture**

For observation of testicular sperm movement, the fresh (n=12) and frozen-thawed samples (n=12) underwent an in vitro culture procedure as previously described\cite{7, 8}. The testicular sperm-crushed tissue suspension was incubated for 3-4 h in 10-20 µl microdrops of
H-HTF medium under mineral oil in a Petri dish at 37℃ in 5% CO₂. Testicular sperm motility was observed under an inverted microscope. Besides, if there were only non-motile sperm in the testicular biopsies, the combined hypooosmotic swelling-eosin Y exclusion (HOS-EY) test was used to determine sperm viability[6].

**Statistical analysis**

The results were expressed as mean ± standard deviation. Difference between the frozen and the fresh group was evaluated by the Student’s t-test. A difference with P<0.05 was considered significant.

**Results**

Following testicular biopsy and in vitro culture, motile sperm were found in all 12 samples in their testicular suspension, and sperm motility in fresh group was 8.1 ± 3.4%. After cryopreservation, of the 12 biopsies frozen, 10 revealed motile sperm. Sperm motility in frozen-thawed group was 3.8 ± 2.9%, and was significant lower than that of the fresh group (P<0.01). In frozen-thawed group, however, the greater proportion of motile sperm showed slow progressive motility. And non-progressively motile sperm, including a very weak, shaking motility at level of the tail only, were frequently observed. In 2 samples with only immotile sperm after thawing, sperm viabilities were 19% and 11%, respectively.

Ultrastructural observations showed that some testicular sperm in frozen-thawed group had morphologically normal plasma membranes, acrosomal caps, and mitochondria. Sperm head in frozen-thawed tissue showed a proportion of nuclei with a degenerative morphology, presenting more electron-dense granules of chromatin. In a few sperm heads, formation of vesicles and degeneration were observed after the freezing-thawing procedure. Compared with those in the fresh tissue, the frozen-thawed tissue frequently showed rupture of the plasma membrane and acrosome membranes. And acrosomal matrix dispersed or leaked out. It was common to observe swollen plasma membrane and acrosome membranes in the frozen-thawed tissue (Figures 1 and 2).

**Discussion**

Recently, many studies have confirmed fertilization and pregnancies using cryopreserved testicular sperm[9-11]. However, cryopreservation of testicular sperm is difficult because of their very low numbers. For this reason, many attempts have been made to cryopreserve testicular tissue, such as freezing of seminiferous tubules, injecting of a small number of sperm into an evacuated zona pellucida, or an mixture of individual sperm and testicular tissue to form a frozen pill[12-15]. Relatively, the preparation and freezing protocol of testicular tissue can be easily performed. Furthermore, after thawing, the tissue can also be easily
identified in the medium, which benefits to retrieve testicular sperm. In the present study, our results showed that most frozen-thawed testicular samples could provide motile sperm. Besides, testicular sperm in tissue were more easily extractable after thawing. It indicated that cryopreserved testicular tissue can be used as an alternative method of sperm source for assisted reproductive program.

Figure 1  The nucleus presents more formation of vesicles. Plasma membrane and acrosome membranes are swollen and ruptured (original magnification, 27 000 ×).

Figure 2  The nucleus presents more electron-dense granules of chromatin. The acrosomal cap is swollen (original magnification, 17000 ×).
Several studies demonstrated that when cryopreserved testicular sperm are thawed, they are often non-motile\cite{10, 16-18}. In this study, we noticed 2 testicular specimens with no sperm movement, which might be related to serious cryoinjury\cite{19}. It suggested that cryopreservation had an adverse effect on the survival of testicular sperm. However, these 2 samples showed that viable sperm still existed by using the HOS-EY test. Nagy, et al. and Gianaroli, et al.\cite{18, 20} reported that the poor motility of testicular sperm recovered after thawing did not negatively affect the fertilization and embryo viability after ICSI. Testicular sperm with non-motile could maintain integrity and potential capacity to achieve fertilization. Therefore, ICSI attempt should be carried out if the frozen-thawed testicular sperm are viable though non-motile sperm are recovered in some testicular samples.

From our ultrastructural observations it shows that the freezing-thawing procedure of testicular biopsy samples changes and damages the fine structures of human testicular sperm. Similar to other studies that have assessed the ultrastructure of ejaculated sperm after cryopreservation, we observed that most obvious changes in frozen-thawed testicular sperm head were swollen or/and ruptured plasma membrane and acrosome membranes. These ultrastructural changes are related to the presence of cryoprotectant agents, especially due to the effects of glycerol. In addition, a degenerative chromatin of frozen-thawed testicular sperm could be observed, which was different from the ejaculated sperm. According to the physiology of sperm maturation\cite{21, 22}, nuclear maturation mainly occurs in the epididymis, which is responsible for the packaging of sperm DNA. Testicular sperm lacks the stage of epididymal maturation. So, it is possible that testicular sperm are more susceptible to cryodamage of DNA material than ejaculated sperm.

In conclusion, the freezing-thawing procedure causes similar damage to ultrastructural morphology of testicular sperm as that seen in ejaculated sperm. For the practical use, cryopreservation of testicular tissue is simple and efficacious, and the chance of losing testicular tissue during the entire process is low, which can benefit the testicular sperm extraction. Improving cryopreservation condition for reducing cryodamage to testicular sperm remains under investigation.

References

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