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Effect of Cryopreservation on Sperms Function Parameters and *In vitro* Fertilization Rate in Mice

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Summary

The study was carried out to investigate the effect of cryopreservation in liquid nitrogen (-196ºC) on sperm quality (concentration, motility, morphology) and *in vitro* fertilization rate (IVF), 24 hours post insemination in mice. The sperms were obtained from caudal epididymis of 20 mature male mice and prepared by Direct Activation Technique after cryopreservation followed by insemination the oocyte of 20 female mice using *in vitro* fertilization procedure. Results showed that there were significant ($P < 0.05$) decrease in sperms concentration, motility and morphology post cryopreservation. In addition, the results demonstrated that cryopreservation significantly decreased ($P < 0.05$) fertilization rate in treated mice (36%) compared to control mice (56%). It is conclusion that frozen sperm can be used without compromising pregnancy chances.

Keywords: Cryopreservation, Sperm, *In vitro* Fertilization, Male mice.

Introduction

The procedure that makes it possible to stabilize the cells at cryogenic temperatures is called cryopreservation, also known as an applied aspect of cryobiology or the study of life at low temperatures (1). Many advances in the cryopreservation technology have led to the development of methods that allow for low-temperature maintenance of a variety of cell types including male and female gametes, small multicellular organisms, and even more complex organisms such as embryos.

Cryopreservation of human spermatozoa introduced in the 1960's has overcome many space and time limitations and now forms integral part of assisted reproduction technologies (ARTs)(2). This technique becomes particularly important in cases of preservation of male fertility before radiotherapy or chemotherapy (3) which may lead to testicular failure or ejaculatory dysfunction. In fact, semen cryostorage seems to be the only proven method that may offer these couples a chance of having children in the future: cancer therapy could in fact lead to damage, resulting in subfertility or sterility due to gonad removal or permanent damage to germ cells caused by adjuvant therapy. In particular, the risk associated to therapy depends on several factors: the age of the patient at the time of treatment, the dose, site, and type of treatment (4). In addition, some nonmalignant diseases, such as diabetes and autoimmune disorders, may lead to testicular damage. Cryopreservation is advisable also in these conditions (5). In countries in which heterologous fertilization is allowed by law and in donor insemination programmes cryopreservation is necessary to have enough time to screen donors for infectious agents, such as the human immunodeficiency (HIV) and hepatitis B viruses, before the cryopreserved semen is used for clinical purposes (6). In azoospermic patients, who have undergone testicular sperm extraction or percutaneous epididymal sperm aspiration, sperm cryostorage is also used to avoid repeated biopsies or aspirations (7). Furthermore, cryopreservation is routinely performed in patients who having to start an assisted reproduction treatment decide to preemptively freeze the semen sample to avoid inconveniences due to fail ejaculation often associated with “semen collection stress,” certain emotional states, or other commitments at the time of oocyte retrieval (8). Finally, male gamete freezing is largely recommended to preserve fertility in those subjects who for one reason or another are exposed to potentially toxic agents, which may interfere with gametogenesis (6). The aim of this study is to

evaluate the effect of cryopreservation in liquid nitrogen on sperm quality.

Materials and Methods

The Preparation of cryoprotective medium by formulations 100 ml of commonly used cryoprotective medium was added to the sperm in 1:1 dilution as described in (9). After preparation, the medium is divided into aliquots of 5 ml in sterile tubes and stored frozen at -20ºC until needed. Thawing was carried out at 37 ºC.

Sperms were collected from twenty mature male mice (Balb/C St Can BR Strain) 8-12 weeks old were sacrificed by cervical dislocation, and the 2 caudal epididymides were removed aseptically and placed into 1ml of PBS in a 35-mm culture dish. The epididymides were minced 5 to 6 times using forceps and scissors, sperms were allowed to disperse by gently shaking the dish by hand for 3 to 5 min at room temperature (10). *In vitro* Sperms Activation Technique was performed by divided the spermatozoa suspensions into two parts, first: incubated at 37 ºC in 5%CO_2 at least for 30 minutes, using Direct Activation Technique, the caudal epididymis sperms were allowed to swim-up through 30 minutes. This technique for sperm activation was characterized by direct effect of culture medium on sperm parameters, and then the sperms were counted (11).

The microscopic field was scanned systematically and the motility of each spermatozoa encountered were graded, according to (10): A- Rapid and linear progressive motility, B- Rapid nonlinear or linear non rapid progressive motility, C-Non progressive motility and D- Immotile.

The values were expressed as percentage, and then the sperms were used to inseminate the control group. The second part of spermatozoa suspensions were diluted 1:1 (v/v) with the cryoprotectant medium, glycerol-egg yolk-citrate buffer (GEC), which was added dropwise, (over 10 min) to minimize hyperosmotic stress. The mixture was placed in a thermostatic water bath at 37 ºC for 10 min for equilibration. The sperm-medium mixture was transferred in a plastic bubbler dish provided with a comb (IMV, cat.no.GC 000 and GC 001). The diluted spermatozoa suspension was drawn into straws using a peristaltic pump. Straws, sealed at one end by a cotton plug, then provided in 20 colors and two different volumes of 250 and 500 µl. After aspiration each straw was placed on the comb to create an air bubble. This air space is important to avoid explosion of the straw after thawing. After removing the straws from the comb they were sealed by dipping in polyvinyl alcohol powder, (available in eight colors IVM, cat. no. A501-A508). The powder – sealed ends of the straws were immersed in water, which polymerizes the powder. Excess powder and water were wiped off using paper tissue. Finally, the air bubble was moved to the center of straw. Straws were marked individually with the date and the numbers of the mice. Sperms were cooled and frozen by the static vapor method; the straws were placed horizontally as a single layer in a copper mesh tray, which was placed in an aluminum mesh basket at level of 35 cm above the liquid-nitrogen surface and left for 15 min. The basket is then moved down to a level of 15 cm (6 in) above the liquid surface and left for another 15 min (12) and, the straws are immersed in liquid nitrogen (-196℃). Straws were thawed at 37℃ for 10 min, before analysis, the sperms were immediately washed, by mixed with equal volume of IVF medium and centrifuged at 2500 (rPM) for 10 minutes (13), then using Direct Activation Technique was described previously (11).

According to estrus cycle detection procedure, superovulation was induced by IP injection of 7.5 IU of PMSG, and then followed by IP injection of 7.5 IU of hCG after 42-48 hours (8). Oocytes were collected from 20 females mice (8-12 weeks old) sacrificed by cervical dislocation at approximately 15-18 hrs post-hCG injection, the oviducts were isolated. For flushing the oocytes, Nagy *et al.* (10) method was used the ampulla was tearing to release the cumulus masses. Then, all the cumulus masses were transferred to a single fertilization dish by using a mouth-controlled pipette under aseptic conditions. Pipettes were used to pick up the oocytes, then they were transferred to a central well-culture dish containing 1 ml of culture medium (PH=7.4-7.6) and kept at 37°C with 5% CO_2 and 95%

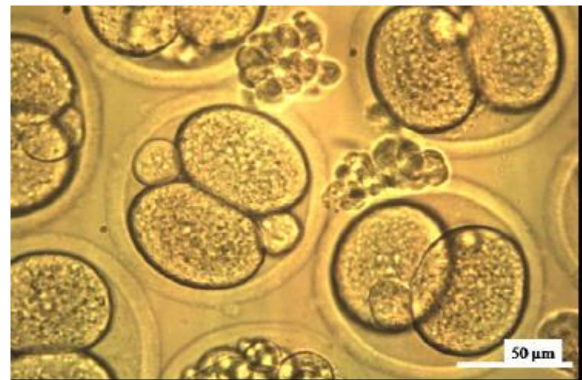
humidity (10). Insemination by IVF procedure was performed by loaded IVF dishes with 1 ml IVF medium, and covered with paraffin oil. Insemination of the oocytes was done by adding 1-2 x10^5 of the incubated sperm to the IVF well containing 4 oocytes. Fertilization of mice ova takes about 6-12 hours. Thus, 12 hours after fertilization the egg start to divide into two cells, and subsequently into four and so on. Fertilization rates were assessed by recording the number of zygote, two cells stage and three-four cells stage embryosafter 24 hours after insemination, and were gained by dividing the number of preimplantation embryos on the number of collected oocytes.

Results and Discussion

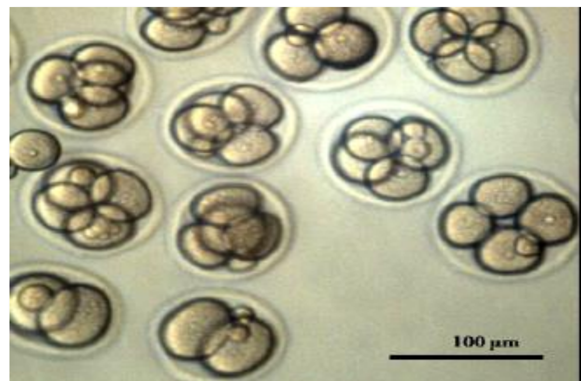
The results of *in vitro* activation techniques on sperm functions parameters (sperm concentration, sperm motility, grade of activity and sperm normal morphology) between fresh sperm (control group) and frozen-thawed group (treated group) were shown in (Table, 1).

The mean sperm concentration (x10^6 sperm /ml) following activation of frozen-thawed sperm (treated group) was significantly ($P< 0.05$) decrease when compared with fresh sperms (control group). Active sperm motility (grade A and grade B) was significantly ($P< 0.05$) decreased. The percentage of morphologically normal sperms showed a significant ($P< 0.05$) difference between control and treated group. There were 150 collected oocytes obtained from 20 female mice, 75 oocytes were inseminated by frozen-thawed sperm (treated group), and cultured in IVF medium in the CO_2 incubator after insemination. The *in vitro* fertilization (IVF) rate was 36%(27 embryos out of 75 oocytes).The other 75 oocytes were inseminated by fresh sperms (control group), the *in vitro* fertilization rate was 56% (42 embryos out of 75 oocytes).There was a significance ($P<0.05$) decrease in the number of *in vitro* fertilization rate between control and treated groups, as shown in (Table, 2).

In treated group, the total numbers of embryos were 27 of them, 15 (55.6%) at two cell stage embryos (Fig.1) and 12(44.4%) at 3-4 cell stage embryos (Fig. 2). While in control group the total numbers of embryos were 42, there were 18(42.8%) at two cell stage embryos, and 24 (57.5%) at 3-4 cell stage embryos. There was significant ($P<0.05$) increase at the number of two cells stage embryos in treated group as compared to the control group. While there was a significant ($P<0.05$) decrease at the number of 3-4 cells stage embryos in treated group compared to control group as shown in (Table, 2).



Figure, 1: Two cell stage embryos



Figure, 2: 3-4 cell stage embryos

Table, 1: Sperms function parameters of treated and control mice following *in vitro* direct activation (Mean± SE).

Parameters	*In vitro* sperm activation (Mean± SE)		P-Value
	Control group	Treated group	
Sperm concentration (10^6/ml)	36.33±4.323	22.46±1.405	S
Sperm motility grade A (%)	16.73±0.918	11.40±2.315	S
Sperm motility grade B (%)	29.73±2.472	21.33±2.622	S
Progressive motility (A+B)%	45.07±3.065	32.73±4.362	S
Morphologically normal sperms (%)	33.93±3.084	25.43±4.410	S

Table, 2: *In vitro* fertilization rate in treated and control mice, 24 hours post insemination.

In vitro Fertilization Rate		Total No. of embryos after 24 hrs	P-Value	Total No. of two cell stage embryos after 24 hrs	P-Value	Total No. of 3-4 cell stage embryos after 24 hrs	P-Value
Treated Group	27/75 (36%)	27	S	15 (55.6%)	S	12 (44.4%)	S
Control Group	42/75 (56%)	42		18 (42.8%)		24 (57.2%)	

There were significant (P<0.05) decrease in certain sperms functions parameters (Table, 1) and fertilization rate, 24 hours post insemination (Table, 2), and the differences in the means were 20% between the treated and control groups. Hence it is a large percentage of difference in the fields of experimental embryology and Assisted Reproductive Technologies (ARTs), and there were a lot of factors those might interfere with this observation; one of those is sperm concentration. The result found that, there is a significant (P<0.05) decrease in the total active sperm concentration after direct activation of frozen thawed sperm. Sperm concentration had the largest reliability coefficient for conception, followed by motility and morphology, and that sperm concentration has an influence on fertilization if patients were treated with IVF (13). Meanwhile, all the ovulated oocytes in this work were inseminated with the same sperm concentration (1-2 x10^5 sperm to the IVF well containing 4 oocytes). Therefore, the differences in FR did not due to the increments in the sperm concentration but it might be belonged to the sperm functions affected by cryopreservation and to the increase in the insemination volume to each fertilization well (insemination volume was increased to overcome with a decrease in sperm quality). That may elevate the decapacitation factors and contaminants from the seminal plasma. These may be in the form of cellular debris (gelatinous pieces, epithelial cells), bacteria, mycoplasmas, Chlamydia, Trichomonas, and various blood components like leukocytes and erythrocytes (14). Although motility is not completely related to fertilizing capacity, it is generally a considered the primary parameter to evaluate freeze-thawing efficiency, it is not the only parameter that is correlated with postthaw functionality in general and, more specifically, with the fertilizing ability of frozen sperm. Percentage recovery of motility (PR) is defined as the final postthaw percentage motility × 100/initial fresh percentage motility (15). A wide individual variability in PR after freezing and thawing is reported, ranging from 10% to 95% (3). In most articles a mean PR of 40%-60% immediately after thawing was observed when using an appropriate cryoprotective medium (16). According to O'Connell *et al.* (17), two-thirds of the postthaw decrease in motility is due to cryoaggression and one-third to hyperosmotic stress induced by glycerol addition (17). The sperm plasma and mitochondrial membranes have susceptibility to cryopreservation (17); mitochondria are placed along the midpiece between the plasma membrane and the nine fibrous columns, to form a coating that provides energy necessary for sperm motility (4). The greatest amount of

energy is provided by molecules of ATP synthesized either by glycolysis in the cytoplasm (16) or through oxidative phosphorylation in the mitochondria (18). Therefore, an impairment of mitochondrial activity may explain the reduction in motility (19). In addition, cryopreservation has been shown to diminish the antioxidant activity of the spermatozoa making them more susceptible to reactive oxygen species (ROS) damage (20). High concentration of ROS and fall of antioxidant enzymes lead to cell apoptosis (21). The release of apoptosis-inducing factors from the mitochondria leads to DNA fragmentation (22). Several studies examined the role of *in vitro* antioxidant supplementation in protecting the sperm DNA from oxidative damage. For example, when added to the seminal fluid during cryopreservation, genistein (23), resveratrol (24), and ascorbic acid (25) seem to reduce DNA damage; on the contrary, vitamin E (15), ascorbate, and catalase (25) seem to improve motility and reduce ROS levels, though they do not improve spermatozoal viability and do not reduce DNA damage (22). Cryopreservation of sperm significantly reduced the percentage of sperm with normal morphology. Light-microscopic analysis was clearly shown an increase in bent and coiled tails. It has been described severe impairment of sperm in terms of ultrastructural morphology. Several investigators have confirmed this damage at the level of the membranes and acrosomes after freezing (26). It has been reported postthaw recoveries of sperm with intact membranes and with intact acrosomes of only 27.7% and 40.8%, respectively, while the average motility recovery for the same group was 69.2%. These results indicate that morphological damage is even more pronounced than the impairment of motility. Sperm, however, which showed mechanically intact acrosomes after thawing, maintained their acrosomal function (14). Compared with other cell types, spermatozoa seem to be less sensitive to cryopreservation damage because of the high fluidity of the membrane and the low water content (about 50%). Despite this, cryopreservation may lead to deleterious changes of sperm structure and function (13). It was largely reported that several damaging processes could occur during freezing-thawing of human spermatozoa, such as thermal shock with formation of intracellular and extracellular ice crystals, cellular dehydration, and osmotic shock (25). The primary cause of cellular damage during cryopreservation is the formation of intracellular or extracellular ice crystals. During the freezing process, the cooling rate plays an important role in determining the extent of cryoinjury to the spermatozoa (27). Ice crystals formed breach the membranes and affect the organelle function. This condition leads to impair cell survival. Cryoinjury is not limited to the freezing process but may also occur during the thawing process as the ice melts or recrystallizes (27). The phenomenon of recrystallization of both intracellular and extracellular ice, in frozen samples, occurs as smaller ice crystals with a rate of recrystallization that increases with increasing temperature (28). It has largely been reported that chilling injury can modify the structure and integrity of plasma membranes (29 and 30) mainly composed by phospholipids and cholesterol (31). Even though high concentrations of cholesterol and polyunsaturated fatty acids give more fluidity to the membrane at lower temperature (32), during cryopreservation the cooling process causes phase transition of membrane lipids and impairs membrane protein function. In particular, the outer layer of the spermatozoal plasma membrane consists of a glycocalyx, a carbohydrate-rich zone that mainly contains oligosaccharide chains that bind to the integral protein of the plasma membrane (glycoproteins) or lipids (glycolipids) (33).

Sperm thawing at room temperature or at 37℃ preserves motility, vitality (12) and fertilizing ability (17 and 34) better than slower thawing in ice baths or faster thawing in warm-water baths. While the addition of glycerol was indispensable for cryosurvival of sperm, sperm cells are subjected to hyperosmotic stress when exposed to the cryprotectant due to its high osmolarity (6). To minimize osmotic injury and prevent severe dehydration the cryoprotectant solution were added dropwise (27). Sperm show a very high permeability to glycerol, which is temperature- dependent. An osmotic equilibrium was obtained within a few

minutes at room temperature or at 37℃, while equilibration was delayed at lower temperatures. On the other hand, prolonged exposure of sperm to glycerol can cause chemical toxicity to the sperm cells. The chemical toxicity of glycerol requires its removal after thawing, before the sperm can be used with safety in IVF program (35).

When comparing fertilizing ability of fresh and frozen- thawed sperm, the literature did not provide well-controlled studies. Reliable results were obtained only when alternating oocytes of one patient collected in an IVF cycle were inseminated either with fresh or with frozen- thawed sperm from the same donor or husband (18). The few properly controlled studies on the fertilizing ability of fresh, as compared with frozen-thawed, sperm have been carried out in vitro by means of the zona-free hamster oocyte penetration test. While one group described an individual variability in the postthaw maintenance of fertilizing capacity, with a mean fertilization rate decreasing from 75% with fresh to 51% with frozen sperm (7), the conclusions are contradictory in that some groups found no differences between fresh and frozen sperm as regards fertilization rate (14) while others observed impaired fertilizing capacity with frozen- thawed sperm (19). It is, however, remarkable that the investigators unanimously found similar pregnancy rates after replacement of embryos derived from insemination with either fresh or frozen sperm (26). This had led to the hypothesis that freezing and thawing act more aggressively on a subpopulation of weaker, probably infertile sperm (12) and it is conclusion that frozen sperm can be used without compromising pregnancy chances.

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تأثير التجميد على وظائف النطف والإخصاب خارج الجسم في الفئران

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الخلاصة

أجريت الدراسة لبيان تأثيرتجميد نطف الفئران في سائل النتروجين بدرجة حرارة (-196º)مئوية على وظائف النطف (تركيز النطف ونشاطها والمظهر الخارجي) وعلى معدل الاخصاب خارج الجسم بعد مرور 24 ساعة بعد التلقيح في الفئران. تم الحصول على النطف من ذيل البربخ لذكور 20 فأرة وإجريت عملية تنشيط النطف بعد تجميدها في سائل النتروجين ثم إجراء الاخصاب خارج الجسم لبويضات 20 انثى. أظهرت النتائج وجود تأثيراً تثبيطياً معنوياً (P <0.05) في تركيز النطف ونشاطها والمظهر الخارجي ومعدل الإخصاب خارج الجسم (36%) بعد مرور24 ساعة من التجميد عند مقارنتها مع مجموعة السيطرة (56%). لذلك يمكن الاستنتاج ان التجميد بسائل النتروجين لا يعطي فرص فعالة لحدوث الحمل.

الكلمات المفتاحية: تجميد النطف،النطف، الاخصاب الخارجي، ذكور الفئران.