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Vitrification of Small Volume of Normal Human Sperms: Use of Open Pulled Straw Carrier

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The objective of this study was to evaluate whether cryopreservation of small volume of sample (sperm+cryoprotectant) was feasible using open pulled straw and also compared the outcomes of open pulled straw and conventional straw as carrier for normal human sperm cryopreservation. Semen samples were obtained from 10 men undergoing evaluation for infertility after 3-4 days of abstinence. Washed normal sperm samples were divided into three aliquots as follows: (1) Fresh; (2) cryopreserved in open pulled straw and (3) crypreserved in conventional straw. In order to do cryopreservation of sperm in open pulled straw first washed normal sperm samples were mixed with equal volume of test yolk buffer and 12% v/v glycerol, later on 3-4 μL from the prepared mixture was loaded in each straw by using syringe. The loaded straws were plunged into liquid nitrogen and after 3 months recovered and thawed. Each straw was emptied of their fluid content in drop of 10 μL medium covered with mineral oil. Motility of vitrified-thawing sperm was assessed by using inverted microscope. The results show as percent progress motility±SD and the $p<0.05$ were suggested as significant. The percent progress motility±SD of fresh sperm was evaluated as follow in study groups: For fresh group was evaluated as 59.2±7.6; the value of 37.5±8.2 in cryopreserved sperm in open pulled straws group; and value of 26.3±6.4 for conventional straw group, respectively. Statistical analysis shows that the difference between cryopreserved sperm in open pulled straws and conventional straw groups is significant ($p = 0.001$). Because of the significant difference between cryopreserved sperm in open pulled straws and conventional straw groups, we concluded that vitrification of human sperm is feasible using open pulled straw. The results of this study shows that open pulled straw could be a good carrier for cryopreservation of small volume of normal human sperm.

Key words: Vitrification, open pulled straw, cryopreservation, sperm

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INTRODUCTION

Cryopreservation of human sperm is used to preserve the individual's fertility before several situations: surgical procedures that can harm fertility, vasectomy, chemotherapy and radio therapy treatments for specific cancers, which generally cause germinal aplasia (Agarwal *et al*., 1995). Besides that, semen banks are used to supply donors to couples with infertility disorders (Byrd *et al*., 1990). More recently, cryopreservation is been employed to store exceeding spermatozoa, aspirated from the epididymis or the testicle, for eventual use in procedures of high complexity assisted reproduction. Furthermore, the exceeded spermatozoa obtained during reconstructive microsurgeries of male genital tract will store which later on can use for further curing cycles (Sharma *et al*., 1997). But, conventional sperm suspension cryopreservation is not suitable for patients with sever oligospermia, azoospermia and in cases with small volume of semen. Because after the traditional freezing and thawing procedure, using centrifuge techniques dilute the volume that cause loose of the spermatozoa.

Cryopreservation is a widely practiced procedure for storing donor sperm prior to intrauterine insemination or *in vitro* fertilization (Kuczynski *et al*., 2001). The critical point is that each cryo-bank determines the optimal rate of freezing and thawing for its preparation of specimens. Conventional slow-rate freezing requires approximately 1.5 h (Keller *et al*., 2002). Recently the technique of ultra-rapid freezing of a small number of sperm using cryo-loops has been developed, decreasing freezing time to only 5 min (Keller *et al*., 2002; Schuster *et al*., 2002). Many researches were conducted to solve this problem, such as Cohen *et al*. (1997), first demonstrated that the zona pellucida is an ideal vehicle for sperm cryopreservation. They removed the cellular material from oocyte or embryos and provided empty ZP that used as a suitable vehicle for preserving the few spermatozoa which can obtain from patient with sever male infertility.

In another research Levi Setti *et al*. (2003) modified this procedure by filling empty human zona pellucida with test yolk buffer. After filling the ZP they injected 750 motile spermatozoa in every empty zona. Their results were show 73% recovery rate of spermatozoa. Later on, Just *et al*. (2004) suggested the single algae cell (Volvox globator), also could use as a vehicle for storage. These procedures have some drawbacks, for example if the ZP is not evacuated completely the number of trapped sperm increases that lead to sperm recovery rate decrease, furthermore the technique itself will be time consuming. However, the empty zona pellucida requires immature ova to remove from their zona pellucida, while use of non human material is not acceptable in an IVF environment, because the tools which used are inappropriate. Another method using small volumes and vitrification also maybe valuable for initial samples but loading individual sperm into cryoloops may not be feasible within the time limits associated with vitrification (Schuster *et al*., 2003). Recently, Ottolini *et al*. (2006) cryopreserved individual motile sperm in 2-3 μL of media contained within 1 cm lengths of polycarbonate embryo handling pipettes. In this study only thirty eight percent of transferred sperms were recovered. The cause of poor recovery was unexplained (Ottolini *et al*., 2006). The open pulled straws vitrification method (Vajta *et al*., 1997) has been successfully applied to the cryopreservation of matured bovine oocytes, precompaction and preimplantation stage bovine embryos (Vajta *et al*., 1998), mature mouse as well as human oocytes (Chen *et al*., 2000a, b), but this method did not used for cryopreservation of normal human sperm. For the mentioned reason the aim of the present study was use open pulled straw as a carrier for small volume human normal sperm cryopreservation.

MATERIALS AND METHODS

Semen samples: Semen samples were obtained from 10 men undergoing evaluation for infertility after 3-4 days of abstinence. The study was conducted from 12th Jan 2007 to 13th Oct 2008. The study was approved by the Institutional Ethics Committee of Ahwaz Jondishapour University of Medical Sciences (AJUMS). Written consents were taken from all the participants of this study.

Samples were allowed to liquefy for 30 min at room temperature prior to analysis. Standard semen analysis were performed manually by a single individual and consisted of assessment of semen volume, PH, viscosity, liquefaction, sperm count, sperm motility, sperm agglutination and sperm morphology. Semen sample with normal parameters according to the world Health Organization criteria (World Health Organization, 2001) were selected for experimental use. Each semen sample was prepared by density gradient separation using Isotone. The supernatant was removed after centrifugation at 300 g for 20 min and the pellet collected. Each sample was divided into three aliquots as follows: (1) Fresh semen; (2) cryopreservation semen in open pulled straw and (3) crypreservation in conventional straw. Initial sperm motility was assessed at 37°C and scored under x40 Hoffman optics with an inverted microscope. Grade-based classifications of spermatozoa were done as: (1) progressive (a + b); (2) no progressive (c) and (3) immotile spermatozoa (d). Initial sperm motility

Fig. 1: The removed plugs of 0.25 mL plastic straws

Fig. 2: Heat-softened straw over a hot plate

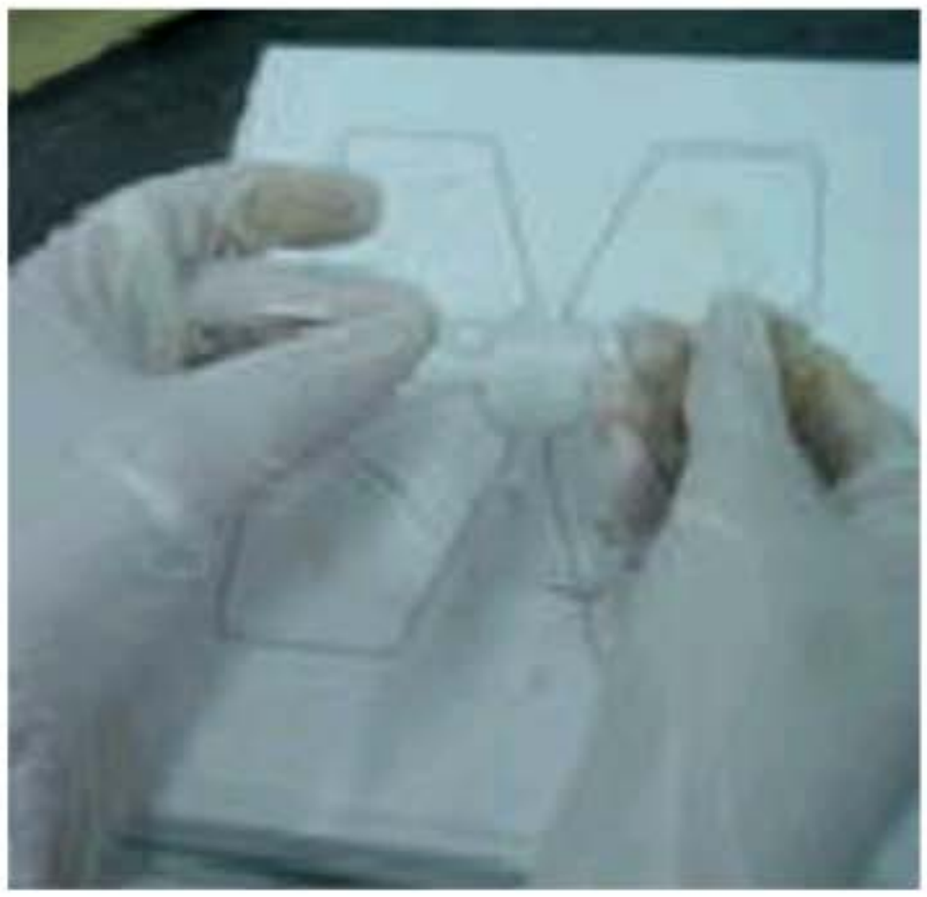

Fig. 3: Manually pulled straw

Fig. 4: Loading open pulled straw

was manually assessed by a single individual in duplicate for each sample by evaluating 100 sperms (Orief *et al*., 2005).

Manufacture of the open pulled straws: Pulled straws were manufactured as described by Vajta *et al*. (1998). The plugs of 0.25 mL plastic straws (IMV, L'Aigle, France) were removed, (Fig. 1), then the straws were heat-softened of the midpoint over a hot plate (Fig. 2) and pulled manually (Fig. 3). The pulled straws were cooled in air and then cut at the tapered end with a razor blade. The inner diameter of the tip was ~0.8 mm, with a wall thickness of about 0.07 mm. The thin part of each open pulled straw was approximately 2.5 cm long.

Cryopreservation of sperm in open pulled straw: After mixing of sperm with equal volume of test yolk buffer and 12% v/v glycerol, each specimen was allowed to equilibrate for 15 min prior to freezing. After that time the 3-4 µL of solution were loaded into the narrow end of the each pulled straw by using syringe (Fig. 4). Then the straw was immediately plunged into liquid nitrogen (LN2)

and stored up to 3 months. In this study a total of 10 samples were frozen in more than 200 open pulled straws (Each sample in more than 20 straws). The straws were taken out of liquid nitrogen for thawing and then the tip of OPS was put into a drop of 10 µL drop of Human Tubal Fluid (HTF) medium. The cryoprotectant was expelled from the straws into medium. The sperm recovered were cultured in HTF medium + 1% Human Serum Albumin (HSA) at 37°C in 5% CO_2 in humidified air for 1 h. Later on sperm motility was assessed and classified as mentioned earlier.

Cryopreservation of sperm in conventional straw: For cryopreservation of sperm in conventional straw first spermatozoa mixed 1:1 with test yolk buffer which added drop wise with gentle swirling then stored at 4°C but was allowed to equilibrate to room temperature before use. The mixture was left at room temperature for 10 min and then sealed in 0.25 mL straw. The straws were suspended in liquid nitrogen vapour (10 cm above the level of liquid nitrogen) for 15 min. Then the straws were plunged into liquid nitrogen and stored until up 3 months. For thawing the straws were removed from liquid nitrogen and left to thaw at room temperature for 15-20 min. When samples were totally thawed the content of every straw was pipetted into 1 mL tube and equal volume of HTF medium +1% human serum albumin (HSA was added to each tube and then the mixture was centrifuged at 200 g for 6 min to remove cryoprotectant. The supernatant was removed the sperm recovered were cultured in HTF medium+1% Human Serum Albumin (HSA) at 37°C in 5% CO_2 in humidified air for one hour. Later on sperm motility was assessed and classified as mentioned in open pulled straw method.

Statistical analysis: The percentage of progress motility, non-progress motility and immotile sperm pre and post cryopreservation, were calculated in both open pulled straw and conventional straw methods individually. The statistical difference between the fresh and two cryopreserved groups was determined using χ^2-test. $p<0.05$ was considered statistically significant. The results expressed as percent progress motility±SD.

RESULTS

Progress motility of sperm in fresh group was evaluated as 59.2±7.6 comparing to 37.5±8.2 in cryopreserved sperm in open pulled straws group and value of 26.3±6.4 in case of conventional straw group.

Table 1: Percentage of sperm motility pre- and post-cryopreservation

Group of experiment	Progress motility±SD (%)	Non progress motility±SD (%)	immotile±SD (%)
Fresh semen	59.2±7.6	22.0±6.3	9.80±2.50
Cryopreserved in ops	37.5±8.2	42.8±3.2	20.02±6.60
Cryopreserved in conventional straw	26.3±6.4	45.4±2.8	28.30±4.20

Non progress motility of sperm in fresh group show the value of 22.0±6.3 comparing to 42.8±3.2 in cryopreserved sperm in open pulled straws group and value of 45.4±2.8 in case of conventional straw group. Immotile sperm value in fresh group observed as 9.8±2.5 comparing to 20.02±6.6 in cryopreserved sperm in open pulled straws group and value of 28.3±4.2 in case of conventional straw group (Table 1). The statistical analysis show that the difference between progressive motility of fresh sperm and experimental groups was significant ($p<0.05$) and the difference of progressive motility between two experimental groups was highly significant ($p = 0.001$), respectively. In addition, the percentage of non progressive sperm motility and also immotile sperm were significantly increased after cryopreservation ($p<0.05$).

DISCUSSION

Vitrification as a cryopreservation method has many primary advantage and benefits, such as no ice crystal formation through increased speed of temperature conduction, which provides a significant increase in cooling rates. This permits the use of less concentrated cryoprotectant agents so that the toxic effect will decrease. Additionally, chilling injuries are considerably reduced. Compared to the slow-freezing method, vitrification has economic advantages and its cost benefit, because there is no need for freezing instruments and vitrification/warning requires only a few seconds (Palermo *et al*., 1992). The indication for sperm cryobanking have been greatly expanded by recent breakthroughs in assisted reproduction, in which immotile but viable sperm can be used successfully for oocyte fertilization through intra-cytoplasmic injection (ICSI) (Anjer *et al*., 2003). This procedure has enabled men who have few sperm in ejaculated semen or even with only rare sperm retrieved from the testes to be able to fertilize partner oocytes (Arav *et al*., 2002). Techniques of human sperm vitrification are diverse. In most of the centers that working in infertility treatment current techniques, commonly use plastic straws or vials. In this technique after mixing of semen sample with cryoprotectant the sample loaded in straw or cryovial. In order to do thawing, the straw or vial is brought to room temperature or to 37°C. It is further processed for use by diluting it with a suitable buffer or media and then centrifuging into a pellet

slowly to remove the cryoprotectant. But this procedure is not suitable if the volume of semen is low because after thawing and diluting most sperm are lost due to centrifuge. The results of present study show that with use of open pulled straws, multiple vial containing clinical enough sperm for ICSI can be individually thawed without the need to centrifuge and also without to refreeze unused portion. This procedure has several advantages for example: (a) the technique is easier and quicker than Cohen *et al*. (1997) and Levi Setti *et al*. (2003) techniques and (b) in conventional straw with high wall thickness during cryopreservation heat transfer from the sample into the LN leads to the evaporation gas layer, which acts as an insulator. This insulator maybe reduces the heat transfer and makes it impossible to achieve uniform and rapid cooling rates (Arav *et al*., 2002). Surprisingly the open pulled straw with low wall thickness, the cooling rate and heat transfer will increases. For this reason the percentage of recovered progress motile sperm from open pulled straws was more comparing to conventional straw. Also maybe the cell membrane sperm integrity which cryopreserved in open pulled straw remain unchanged after the initial cellular damage induced by the freezing process. Additionally, the unused sperm obtained from testicular biopsies for ICSI on the day of isolation, may be simply stored by using open pulled straw for future use and this method will eliminate the need for the patient to undergo a repeat biopsy. In addition, this technology could also allow the surgeon to rapidly cryopreserve the few sperm obtained from the epididymal fluid at the time of vasoepididymostomy.

In conclusion, these preliminary experiments indicate that cryopreservation of small volume of normal human spermatozoa is possible using open pulled straw. We believe that the convenience of this sperm carrier will push the development of this technique to higher level of clinical efficiency and utilization especially in sperm banking.

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