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Cryopreservation of Limited Numbers of Ejaculated and Surgically Retrieved Human Spermatozoae within Empty Human Zona Pellucida

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Abstract

Introduction: Cryopreservation of single human spermatozoa is a new technique designed to improve the management of male infertility, it was first introduced in 1997 and was reported by others latter. The aim of this research was to evaluate the efficiency of this method in preserving either ejaculated or surgically retrieved spermatozoae.

Materials and Methods: Spermatozoae were obtained from patients attending the ICSI program at Koassar Fertility Center, Tehran, Iran, and the cases were divided into three groups according to the method of sperm retrieval : a-ejaculated sperm, b-PESA and c-TESE (both with shaking sperm).

For each group, 10 empty zonae were prepared using degenerated, immature or unfertilized human oocytes. Spermatozoa (8-10) were transferred inside each empty zona. The zonae were put in a droplet of follicular fluid + 15% glycerol (V/V) for five minutes before it was loaded in 0.25 ml straws and plunged in liquid nitrogen. After thawing, spermatozoa were extracted from the zonae and sperm viability and motility were recorded and analyzed statistically.

Results: The results showed that less than 12% of the sperms were lost prior to cryopreservation and that there was no significant difference among the groups. Post -thaw viability rate was 80-84% and it was the same for all the groups. Motility rate was 70-74%, again with no significant difference among the groups.

Conclusion: This sperm cryopreservation method is suitable for oligozoospermia patients because of high recovery and motility rates. It can also help azoospermic patients by preventing repeated surgeries.

Key Words: Sperm, Cryopreservation, Empty zona pellucida

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Introduction

Semen cryopreservation offers several advantages over the use of fresh semen in donor insemination programs, including: ease of use; assurance of viability over an extended period of time and the possibility of ruling out infection with human immunodeficiency virus. Cryopreservation of semen can be performed before radiation therapy, chemotherapy, vasectomy or other events that may cause irreversible damage to the male gonads.

Cryopreservation is currently used as a standard procedure to preserve ejaculated, epididymal and testicular sperms which can be recovered at the time of urologic interventions to be used latter on for intracytoplasmic sperm injection (ICSI) (1).

The cut-off level for conventional freezing in mini straw is 1200 spermatozoa, but any men have even less cells, therefore freezing of multiple samples of very few permatozoa would nevertheless guarantee several attempts of ICSI and avoid repeated testicular aspiration if further treatments are required (2).

In the other hand, it is well known that men with non-obstructive azoospermia occasionally show few spermatozoa in the ejaculate (3). For these cases, and also for men with successful surgical extraction of spermatozoa, it would be beneficial to cryopreserve all available spermatozoa for future ICSI treatment cycle (4). Often spermatozoae are only present in very low numbers and cannot be cryopreserved successfully using standard cryopreservation protocols (4).

Some investigators offer very simple methods by placing small droplets of the medium on a dry ice surface before freezing in liquid nitrogen (5). Although, these techniques are elegant and extremely useful, its application falters when levels drop below one thousand spermatozoa per ejaculate (2).

Special methods are necessary to store spermatozoa in case of extreme oligozoospermia. This circumstance constantly arises during ICSI when epididymal or testicular spermatozoa are very rare (2). In these cases, Cohen et al (6) recommended using an empty zona pellucida for storage and cryopreservation of single human spermatozoon. By this method, spermatozoa suitable for ICSI were recovered after thawing, and fertilization with normal embryonic development were achieved.

Others made further improvements in the method based on the use of a laser system for drilling a single hole into the zona pellucida (7). Walmsley et al (4) compared the above mentioned cryopreservation method between patients diagnosed as zoospermic, extreme oligozoospermic or oligoasthenozoospermic and could obtain in average fertilization rate for post-thaw injected spermatozoa around 65% which as comparable with the regular fertilization rate. Other investigators (8) compared the use of human with the use of mouse empty zona pellucida and observed no differences.

Our purpose was to evaluate the efficiency of this method for preservation of either jaculated or surgically retrieved spermatozoae.

Materials and Methods

Spermatozoae were obtained from patients attending the ICSI program at Koassar Fertility Center, Tehran, Iran. They were divided into three groups: a- ejaculated sperms (with motile sperms) b- PESA (with shaking sperms) c- TESE (with shaking sperms).

Ejaculated sperms and biopsy preparations were performed, and aliquots of the suspended collected sperms were diluted into 50μl droplets of Hepes-buffered medium (Ham's-F10+Hepes) under mineral oil in a culture dish. In the same dish, degenerated or immature, sperm-free human oocytes and also excessive embryos, which were not suitable for freezing, were transferred. All micromanipulation steps were carried out on a heated stage of an inverted microscope equipped with modulation contrast optics (Leica) and micromanipulation tools (Narishige, Tokyo, Japan).Ten empty zonae were prepared for each group. First, the oocytes or embryos were positioned with a holding capillary. A micropipette (with 7-9 μm diameter) was inserted through the zona and the ooplasm was completely removed. Several penetrations of the zona was prevented, and it was tried to remove the ooplasm completely in one step. After the preparation of the empty zonae, 8-10 spermatozoa were transferred into empty zonae using

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4μm diameter injecting pipette.

In the first group, we slightly immobilized the sperm to inhibit losing them during the process, using a mild strike on the sperm tail which could cause a temporary arrest of sperm motility, and then transferred them into the zona.

* *Cryopreservation method*

The zona, with the sperms inside, was transferred in a droplet of F.F. (Follicular Fluid), supplemented with 15% glycerol (V/V) for five minutes and then was loaded in a 0.25μl straw. For this purpose, the straw was loaded with a column of F.F., followed by a bubble of air, then a column of F.F. + glycerol containing zona, followed by another air bubble, then a short column of F.F. was loaded, and then the straw was sealed. The straw was kept on nitrogen vapor for 3-5 minutes and then immersed into liquid nitrogen for at least two days.

* *Thawing procedure*

For spermatozoa recovery, the zonae were thawed rapidly. The straw was removed from the liquid nitrogen, in air for 20 sec, then plunged into a 37°C water bath until the columns melted, then the recovered zonae were washed twice in the medium and placed into a droplet of the medium.

The zona was positioned with a holding pipette so that spermatozoa were located next to the holding capillary opposite the initial opening. The micropipette was carefully pushed through the zona, and the spermatozoa were removed and transferred to another droplet of the medium. Post-thaw viability was assessed by Eosin-B stain. For statistical analysis we used Chi-square test.

Results

The results are summarized in Table 1. One of the zonae of group c (TESE) was damaged and it´s sperms could not be found. In the first group, (ejaculated sperms) 82 spermatozoae were recovered (88.1%). Meanwhile 85 and 72 spermatozoae from the other two groups were found, respectively (88.6% vs 88%). So, we lost around 12% of the spermatozoa of all groups, and there was no meaningful difference among groups.

Membrane integrity as it was indicated by Eosin staining was normal in 82.9% (68 sperms out of 82 recovered sperms) for the ejaculated group, 83.5% (71 out of 85 recovered sperms) for the second group, and 80.5% (58 out of 72 sperms) for the TESE group.

The viability rate after using this cryopreservation method was around 80-84 percent and there was no significant difference between groups. The results showed that 73.5 percent of the first group spermatozoa, were motile (50 sperms) and this rate for PESA and TESE groups were 74.3% and 70.6%, respectively (55 and 41 spermatozoae of 74 and 58 spermatozoae, respectively), and again there was no significant difference, although the motility rate was lower in the last group.

Discussion

Cryopreservation of single human spermatozoa is a new technique used to improve the management of male infertility, it was first introduced by Cohen et al (6), then it underwent some changes and attempts improve the results (7, 8, 9).

We tried to refine the procedure by using a narrower micropipette to evacuate the eggs and embryos and to immobilize sperm slightly so that loss of spermatozoa was prevented during the subsequent freezing procedure. Cohen et al. (6) used acid Tyrod´s solution to make an opening in the zona but this study showed that there was no need to make a hole and then removing the ooplasm. Besides we tried to evacuate the zonae immediately and prevent repeated

Table 1: Cryopreservation of a few motile human spermatozoae recovered from ejaculation, PESA and/or TESE

Type of sperm	No. of zonae	No. of sperm	No. of zonae recovered	No. of sperm recovered	No. of sperm lost prior to freezing	No. of viable sperm	No. of motile sperm
Ejaculated	10	93	10	82/93(88.1%)	8/93(11.9%)	68/82(82.9%)	50/68(73.5%)
PESA	10	96	10	85/96(88.6%)	11/96(11.4%)	71/85(83.5%)	55.74(74.3%)
TESE	10	82	9	72/82(88%)	10/82(12%)	58/72(80.5%)	41/58(70.6%)

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return inside the zona to prevent sperm loss during freezing-thawing procedures.

Montage et al. (7) believed that the presence of only a single hole compared to the double-hole technique presented by Cohen et al. (6) insured that the spermatozoa would not suck accidentally into the holding capillary as was reported by Cohen et al. (6). In addition to that, this study concluded that using an injecting micropipette with 7-10μm diameter and removing the ooplasm immediately would reduce the chance of sperm loss. Montage et al (4) recommended using laser to make a hole in the zona, it seemed to be suitable but if it is not available, using a micropipette with 7-10μm diameter can help.

Cohen et al. (6) immobilized the spermatozoa before transferring it into the zonae as Palermo et al. (10) had suggested and gained low fertilization rate. Montage et al. (7) used laser to immobilize sperm and assumed that damaging of the sperm tail with the injection pipette could be detrimental if it was applied to subsequently cryopreserved spermatozoa. The use of the injection micropipette could immobilize the sperm very slightly and was applied to inhibit sperm escape from micropipette or empty zona.

After thawing, most of the viable sperms showed motility and it can help to choose the best sperms in order to do ICSI. To get successful results during ICSI, we need a very specific criteria of viable sperm and the best is motility.

The other important factor for successful freezing procedure is to choose a useful cryoprotectant where 15 percentage reported as a suitable cryoprotectant (9), moreover, follicular fluid (F.F.) was used as a basic medium because it can preserve the viability and motility of spermatozoa very well and it can probably help us to obtain high viability and motility rates after thawing, however, a higher concentration of glycerol was used of (sixty percent) and it was toxic (data was not shown).

One zona was damaged during the procedure. Some investigators recorded the same results about zona damage during embryo cryopreservation (11). There should be more attempts to avoid this problem.

It is concluded that cryopreservation of a few viable spermatozoae is a valuable addition to any ICSI procedure and can be offered to men who only occasionally show few spermatozoa in the ejaculate, and also in cases of PESA or TESE to avoid another surgical procedure for sperm retrieval. Besides, when spermatozoae are motile, there is no difference from where it was retrieved.

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