

FEASIBILITY OF REFREEZING HUMAN SPERMATOZOA THROUGH THE TECHNIQUE OF LIQUID NITROGEN VAPOR

SIDNEY VERZA JR, SANDRO C. ESTEVES

Center for Male Infertility, ANDROFERT, Campinas, São Paulo, Brazil

ABSTRACT

Objective: To assess the feasibility of refreezing human semen using the technique of liquid nitrogen vapor with static phases.

Materials and Methods: Twenty samples from 16 subjects who required disposal of their cryopreserved semen were thawed, corresponding to 6 cancer patients and 10 participants in the assisted reproduction (AR) program. Samples were refrozen using the technique of liquid nitrogen vapor with static phases, identical to the one used for the initial freezing, and thawed again after 72 hours. We assessed the concentration of motile spermatozoa, total and progressive percent motility and spermatic vitality, according to criteria of the World Health Organization (WHO), as well as spermatic morphology according to the strict Kruger criterion, after the first and after the second thawing.

Results: We observed a significant decrease in all the parameters evaluated between the first and the second thawing. Median values for the concentration of motile spermatozoa decreased from $2.0 \times 10^6/\text{mL}$ to $0.1 \times 10^6/\text{mL}$ ($p < 0.01$); total percent motility from 42% to 22.5% ($p < 0.01$); progressive percent motility from 34% to 9.5% ($p < 0.01$); vitality from 45% to 20% ($p < 0.01$); and morphology from 5% to 5% ($p = 0.03$). There was no significant difference in the spermatic parameters between the cancer and assisted reproduction groups, both after the first and after the second thawing. We observed that in 100% of cases there was retrieval of motile spermatozoa after the second thawing.

Conclusion: Refreezing of human semen by the technique of liquid nitrogen vapor allows the retrieval of viable spermatozoa after thawing.

Key words: fertility; in vitro fertilization; sperm; freezing; nitrogen
Int Braz J Urol. 2004; 30: 487-93

INTRODUCTION

The cryopreservation technique for human spermatozoa and the use of semen bank have been employed for more than 40 years (1). Since then, efforts have been made in order to improve the technique and obtain better results after thawing, concerning both quantity and quality of spermatozoa, since generally between 25 and 75% of spermatozoa are lethally or sub-lethally damaged during the freezing-thawing process (2).

It is well known that the process of freezing and thawing human spermatozoa affects their fertile potential under several aspects, such as the decrease of spermatic motility (2), decreased penetration into the cervical mucus (3), changes in the plasmatic membrane (4), making it less fluid, as well as in the acrosomal integrity (5), in addition to changing the activity of protease acrosin (6). For these reasons, lowest fertilization and pregnancy rates are achieved when thawed spermatozoa are used for intra-uterine insemination (7) and conventional in-vitro fertilization

(8). However, after the development of intracytoplasmic sperm injection (ICSI), it has been shown that similar fertilization and pregnancy rates are achieved with this technique using both frozen-thawed and fresh motile spermatozoa (9).

The cryopreservation of spermatozoa is indicated in situation where there is risk of fertility loss and/or decrease in the future fertility. Moreover, the cryopreservation of human semen is used in assisted reproduction programs, both for preserving exceeding spermatozoa obtained from the testis or epididymis or in cases of azoospermia, and for cases where it is impossible to conciliate semen collection and aspiration of oocytes. Among indications for semen cryopreservation, the group of male cancer patients deserves special attention, and several works have alerted to the importance of semen cryopreservation in these individuals (10). Some types of cancer, such as testicular cancer, affect mainly men in reproductive age. Due to advancements in its management, currently cure and survival rates are quite high, sometimes reaching more than 90% (11).

The objective of the present study was to assess the feasibility of refreezing-thawing of human spermatozoa using the technique of liquid nitrogen vapor with static phases.

MATERIALS AND METHODS

Twenty semen samples were obtained from 16 individuals who required the disposal of cryopreserved semen that was stored in the therapeutic semen bank of a tertiary care institution.

Among those, 6 men with mean age 26.5 ± 7.2 years had their semen cryopreserved due to cancer, and other 10 men with mean age 39.6 ± 4.7 years had their semen cryopreserved for use in an assisted reproduction program involving in vitro fertilization. All individuals or their legally responsible person signed a document authorizing the disposal and the utilization of samples for performing this study, which was approved by the Institutional Research Ethics Committee. The reasons for disposal in the cancer group were death in 3 cases and successful treatment with subsequent recovery of spermatogenesis in the remaining three. In the assisted reproduction group,

6 individuals required disposal due to successful treatment (pregnancy), 1 due to financial difficulty for maintaining the cryopreserved samples, and 3 did not state the reason for disposal.

Cryopreservation Protocol

On the day of cryopreservation, samples were collected by masturbation in sterile vials, remaining on a heating plate (Labline, USA) for 30 minutes until complete liquefaction. An aliquot was reserved for performing complete seminal analysis, according to the WHO criteria (12). Next, freezing was performed under aseptic conditions in a biological safety cabinet (Veco, Brazil). In short, the procedure consisted in conditioning the liquefied semen inside a 15-mL conical tube (Falcon, USA), adding aliquots of cryoprotector medium, corresponding to 25% of the semen volume to be frozen, each 5 minutes. This procedure was repeated until equal volumes of diluent medium and ejaculate were obtained (proportion 1:1, v/v). The cryoprotector agent employed was a ready-to-use preparation, containing 20% yolk egg (v/v), 12% glycerol (v/v), 85 mM of Tris ([hydroximetyl] amino methane), 189 mM of TES (n-Tris [hydroximetyl] metyl-2-amino-etano-sulphonic acid), 11 mM of glucose, 0.25 mg/mL of streptomycin sulfate, 0.15 mg/mL of penicillin and pH = 7.5 (Test yolk-buffer, Irvine Scientific, Santa Ana, USA). The final mix was distributed in sterile plastic, cylindrical tubes with conical base, with capacity for conditioning 1.0 mL of mixture each (Nunc, Denmark). Cryopreservation was performed by the technique of liquid nitrogen vapor with static phases (5). Freezing itself consisted of 3 consecutive steps: 1) cooling phase – the metallic racks containing the cylindrical tubes with the sample were put inside a freezer, with temperature set to minus 20°C, on horizontal position, and were then maintained in this environment for 8 minutes, in order to reach a temperature of +4°C; 2) freezing phase – the metallic racks containing the cylindrical tubes were transferred to the tankard of the liquid nitrogen barrel (N₂L), with each rack vertically positioned, and the 2 cylindrical tubes located in upper positions. The tankard was then transferred to the barrel containing liquid nitrogen only at the base, so that the lower cylindrical tube

was located at 15 cm from the N₂L level and the upper tube at 18 cm. Temperature at the place occupied by the cylindrical tubes was around minus 79°C, as measured by an appropriate thermometer, and those were maintained in this closed environment of N₂L vapor for a period 2 hours. Freezing rate during this phase is estimated in 10°C / minute; 3) storage phase - after 2 hours in N₂L vapor, the metallic racks containing the cylindrical tubes were transferred to the storage barrel, and were then immersed in N₂L at -196°C.

Thawing, Assessment and Refreezing of Samples

Samples were thawed by removing the cylindrical tubes from the storage barrel with liquid nitrogen, which were maintained at room temperature for 5 minutes (5). Next, the tubes were taken to water-bath (Fanem, Brazil) at 37°C, where they remained for 20 minutes. Samples were then homogenized, and an aliquot was removed for assessing the following parameters: concentration of motile spermatozoa, percentage of motile spermatozoa, percentage of spermatozoa with progressive motility (grades A and B), vitality and spermatid morphology. The parameters were assessed in accordance to the instructions in the WHO procedure manual (12), with exception of spermatid morphology, which was assessed according to Kruger's strict criteria (13). For the eosin-nigrosin test, a 1% eosin solution was used as spermatid stain and a 10% nigrosin solution was used as background stain, in order to make reading easier. For morphologic assessment, thin 5- μ L smears of thawed semen were prepared on dry slides that were previously cleaned with 70% alcohol. The smears were dried on fresh air, and subsequently fixed and stained using an appropriate kit (Laborclin, PR, Brazil) as follows: the dry slide was immersed in fixation solution for 5 times during 1 second at each time, with a 1-second interval between each immersion. Once the slide was completely dry, it was immersed in the solution I, for 5 times during 1 second at each time, with a 1-second interval between immersions. Excessive stain was removed, and the slide was finally immersed in the solution II, for 2 times, during 1 second at each time, with a 1-second interval between immersions. The

slide was rinsed with deionized water, in order to remove excessive staining, and was left to dry naturally. At least 200 spermatozoa were evaluated per smear in order to measure the percentage of live and morphologically normal spermatozoa, using bright field light microscopy under immersion with a magnification of 1000 times (Nikon Alphaphot, Japan).

The remaining sample of thawed semen was kept in the cylindrical tubes on the heating plate at 37°C during the assessment of concentration and motility parameters, that is, approximately 1 hour, and underwent cryopreservation again, according to the method described above, however without adding the cryoprotector diluent, since it was not removed during thawing. After 72 hours, samples were thawed again and the same spermatid parameters were assessed according to the method described above.

Statistical Analysis

Kolmogorov-Smirnov test was used to verify the type of data distribution. Wilcoxon and Mann-Whitney non-parametric tests were used to compare the spermatid parameters after the first and the second thawing and to compare both subgroups of patients (cancer and assisted reproduction), respectively. Data were expressed in median and 25% and 75% percentile. P values < 0.05 were considered statistically significant. The statistical analysis was performed using the StatSoft software, Tulsa, United Kingdom.

RESULTS

The results for spermatid parameters after the first and the second thawing are expressed in Table-1. We observed a significant decrease between the first and the second thawing in the number of motile spermatozoa (from 2.0x10⁶/mL to 0.1x10⁶/mL, p < 0.01), in total motility (from 42% to 22.5%, p < 0.01) and progressive motility (from 34% to 9.5%, p < 0.01), in the percentage of live spermatozoa (from 45% to 20%, p < 0.01) and spermatozoa with normal morphology (median values of 5% for the first and the second thawing, but with p = 0.03). Nevertheless, despite the decrease in the quality of all the analyzed

REFREEZING OF HUMAN SPERMATOZOA

Table 1 – Assessment of spermatic parameters after the first and the second thawing of 20 seminal samples. The values are expressed as median and 25 and 75 percentiles.

	After the First Thawing	After the Second Thawing	p value
Concentration of Motile spermatozoa (x 10 ⁶ /mL)	2 (0.1 - 11)	0.1 (0.05 - 3)	< 0.01
Total Motility (%)	42 (17.5 - 54)	22.5 (6.5 - 34.5)	< 0.01
Progressive Motility (grade A + B) (%)	34 (0.5 - 38)	9.5 (2 - 18)	< 0.01
Vitality (%)	45 (20 - 54)	20 (10 - 24)	< 0.01
Morphology (%)	5 (3 - 8)	5 (2.5 - 5)	= 0.03

parameters, live and motile spermatozoa were found after the second thawing in all cases.

We also compared the 2 subgroups of individuals, that is, those who had semen cryopreservation due to cancer and those who cryopreserved their semen for subsequent use in the assisted reproduction program, aiming to assess if there was any difference in the retrieval and survival rates relative to the reason for freezing. Deleterious effects of cryopreservation on spermatic parameters were observed in both groups between the first and the

second thawing (Table-2), with no difference between them in the magnitude of changes (Table-3).

COMMENTS

Semen cryopreservation has allowed many men to guarantee their future fertility and generate their own children. Among them, cancer patients and those who will undergo chemotherapy and/or radiotherapy deserve special attention, since many are in their reproductive years and do not have children

Table 2 – Spermatic parameters from 16 individuals divided into Assisted Reproduction and Cancer subgroups, after the first and the second thawing. The values are expressed as median and 25 and 75 percentiles.

	First Thawing			Second Thawing		
	Assisted Reproduction (n = 10)	Cancer (n = 6)	p value	Assisted Reproduction (n = 10)	Cancer (n = 6)	p value
Concentration of Motile Spermatozoa (x 10 ⁶ /mL)	4.7 (0.1 - 16)	2.5 (0.1 - 10)	NS	0.5 (0.01 - 4)	0.5 (0.1 - 3)	NS
Total Motility (%)	47 (26 - 56)	47.5 (17 - 55)	NS	23.5 (7 - 39)	27 (6 - 34)	NS
Progressive Motility (grade A + B) (%)	34 (19 - 51)	37.5 (11 - 38)	NS	14.5 (2 - 25)	12.5 (2 - 18)	NS
Vitality (%)	47.5 (42 - 55)	47 (24 - 54)	NS	20 (12 - 22)	23.5 (18 - 25)	NS
Morphology (%)	5 (4 - 8)	7.5 (3.5 - 8)	NS	5 (4 - 6)	5 (5 - 5)	NS

NS = non significant

REFREEZING OF HUMAN SPERMATOZOA

Table 3 – Percent difference between the first and the second thawing on spermiatic parameters from 16 individuals in the Assisted Reproduction and Cancer subgroups. The values are expressed as median and 25 and 75 percentiles.

	Assisted Reproduction (n = 10)	Cancer (n = 6)	p value
Concentration of Motile Spermatozoa (x 10 ⁶ /mL)	66.6 (41.2 – 80)	78.5 (70 – 96.6)	NS
Total Motility (%)	48.5 (42.8 – 61.1)	50.0 (43.7 – 65)	NS
Progressive Motility (grades A + B) (%)	62.7 (49.1 – 80)	72.2 (56.7 – 81.8)	NS
Vitality (%)	56.9 (27.3 – 64.5)	48.9 (43.2 – 66.7)	NS
Morphology (%)	12.5 (-25 – 33)	28.5 (0.0 – 37.5)	NS

NS = non significant

yet. Thanks to the advances in oncologic treatments, increasingly higher survival rates have been reached (11). However, treatment can lead to germinative aplasia, and it is impossible to predict which individuals will recover normal spermatogenesis. Consequently, a concern with future fertility arises, and many studies have alerted about the importance of semen cryopreservation before starting chemotherapy and/or radiotherapy (10,14). Probably due to the stress generated by the disease, in addition to the increase in the circulating levels of cytokines and tumoral markers, as well as orchiectomy in cases of testicular cancer, we observe that approximately 50% of individuals present a significant decrease in the quality of semen at the moment of freezing (14). This reduction in the initial quality, associated with the small number of collected samples due to the urgency in initiating treatment, can limit success at the moment when these samples are used, especially when low-complexity techniques for assisted reproduction, such as intra-uterine insemination, are used for obtaining pregnancy. However, with the advent of ICSI, just one single spermatozoon per oocyte is required to possibly obtain pregnancy. ICSI can be performed even with non-motile spermatozoa, provided they are alive, with good results (15), though the use of specific tests, such as the hypo-osmotic test for selecting live spermatozoa, even if non-

motile, can optimize the process efficacy (16). Thus, even in very unfavorable conditions concerning number and quality of spermatozoa, it is possible to offer satisfactory chances of paternity, lying around 20-40% per treatment cycle (15). However, due to the limited success per trial that is inherent to the technique, multiple trials can be required in order to obtain pregnancy.

Cryopreserved spermatozoa are used in association with different techniques for assisted reproduction, depending on their number and quality after thawing. When techniques requiring few spermatozoa are employed, such as conventional in vitro fertilization or ICSI, commonly there are exceeding spermatozoa that are not used and thus are discarded. Refreezing of these exceeding spermatozoa would enable new trials of assisted reproduction, increasing the chances of pregnancy, particularly for individuals who have only one or few cryopreserved samples. Studies focusing on this aspect have been developed, and results are as encouraging as those found in the present study are. Polcz et al. (17) demonstrated that human spermatozoa can resist to 5 repeated freezing-thawing cycles, though significant reductions in the spermiatic parameters have been observed (decrease in motility from 70.1% before freezing to 24.4; 8.0; 3.5; 1.5 and

1.8% after each thawing, respectively), confirming our findings. Rofeim et al. (18) also demonstrated that human spermatozoa resist to refreezing, and suggest that they can be used for ICSI.

In this study, we assessed the feasibility of refreezing human spermatozoa as well, but using a simple and low-cost technique, instead of computerized protocols, which are more complex and expensive. Other studies indicate that there is no significant difference in spermatid survival when freezing by liquid nitrogen vapor with static phases is compared with automated techniques (19,20). Bandularatne & Bongso (20) assessed the fertilization rates obtained with refrozen and thawed spermatozoa through the ICSI test in hamster oocytes, which is a functional test designed to assess the fertile potential of human spermatozoon, and obtained similar rates when refrozen and fresh spermatozoa were compared (22.2 versus 27.3% respectively, non significant). These authors also assessed the survival of refrozen spermatozoa in relation to the type of samples from which they were derived, normozoospermic and oligozoospermic, and observed that there was no significant difference in survival rates and in the decrease of spermatid parameters between the 2 groups (20). Such fact motivated us to compare in the present study 2 subgroups of individuals that had their semen frozen for different reasons, aiming to assess if the subgroup of men who froze their semen due to cancer would have lower performance following refreezing and thawing, however no significant difference was observed. Such findings enable us to suggest that these individuals could benefit from refreezing as well.

Though the results from this and other mentioned studies are promising, other studies are required in order to assess the fertile potential of refrozen human semen in cycles of assisted reproduction, with emphasis not only on rates of term pregnancy, but also on rates of miscarriage, complications and malformations.

CONCLUSION

There was a significant reduction in all the spermatid parameters under evaluation between the first and the second freezing-thawing cycle. However,

refreezing of human semen through the technique of liquid nitrogen vapor with static phases enables the recovery of viable spermatozoa. We observed that in 100% of cases there was retrieval of motile spermatozoa after the second thawing.

Work presented at the Meeting of the American Society for Reproductive Medicine, San Antonio, USA, September 2003.

REFERENCES

1. Sherman JK: Low temperature research on spermatozoa and eggs. *Cryobiology*. 1964; 1: 103-29.
2. Centola GM, Raubertas RF, Mattox JH: Cryopreservation of human semen. Comparison of cryopreservatives, sources of variability, and prediction of post-thaw survival. *J Androl*. 1992; 13: 283-8.
3. Fjallbrant B, Ackerman DR: Cervical mucus penetration in vitro by fresh and frozen-preserved human semen specimens. *J Reprod Fertil*. 1969; 20: 515-7.
4. Esteves SC, Sharma RK, Thomas AJ Jr, Agarwal A: Suitability of the hypo-osmotic swelling test for assessing the viability of cryopreserved sperm. *Fertil Steril*. 1996; 66: 798-804.
5. Cross NL, Hanks SE: Effects of cryopreservation on human sperm acrosomes. *Hum Reprod*. 1991; 6: 1279-83.
6. Mack SR, Zaneveld LJ: Acrosomal enzymes and ultrastructure of unfrozen and cryotreated human spermatozoa. *Gamete Res*. 1987; 18: 375-83.
7. Peterson EP, Alexander NJ, Moghissi KS: A.I.D. and AIDS – too close for comfort. *Fertil Steril*. 1988; 49: 209-10.
8. Szczygiel MA, Kusakabe H, Yanagimachi R, Whittingham DG: Intracytoplasmic sperm injection is more efficient than in vitro fertilization for generating mouse embryos from cryopreserved spermatozoa. *Biol Reprod*. 2002; 67: 1278-84.
9. Kuczynski W, Dhont M, Grygoruk C, Grochowski D, Wolczynski S, Szamatowicz M: The outcome of intracytoplasmic injection of fresh and cryopreserved ejaculated spermatozoa – a prospective randomized study. *Hum Reprod*. 2001; 16: 2109-13.
10. Hallak J, Kolettis PN, Sekhon VS, Thomas AJ Jr, Agarwal A: Cryopreservation of sperm from patients

- with leukemia: is it worth the effort? *Cancer*. 1999; 85: 1973-8.
11. Srougi M, Simon SD: *Urological Cancer*. São Paulo, Platina. 1990; vol 1, p. 377. [in Portuguese].
 12. World Health Organization (WHO): *Laboratory Manual for the examination of human semen and sperm-cervical mucus interaction*. 4th ed., Cambridge, Cambridge University Press, 1999.
 13. Kruger TF, Menkveld R, Stander FS, Lombard CJ, Van der Merwe JP, Van Zyl JA, et al.: Sperm morphologic features as a prognostic factor in-vitro fertilization. *Fertil Steril*. 1986; 46: 1118-23.
 14. Padron OF, Sharma RK, Thomas AJ Jr, Agarwal A: Effects of cancer on spermatozoa quality after cryopreservation: a 12-year experience. *Fertil Steril*. 1997; 67: 326-31.
 15. Shulman A, Feldman B, Madgar I, Levron J, Mashlach S, Dor J: In-vitro fertilization treatment for severe male factor: the fertilization potential of immotile spermatozoa obtained by testicular extraction. *Hum Reprod*. 1999; 14: 749-52.
 16. Liu J, Tsai YL, Katz E, Compton G, Garcia JE, Baramki TA: High fertilization rate obtained after intracytoplasmic sperm injection with 100% nonmotile spermatozoa selected by using a simple modified hypo-osmotic swelling test. *Fertil Steril*. 1997; 68: 373-5.
 17. Polcz TE, Stronk J, Xiong C, Jones EE, Olive DL, Huszar G: Optimal utilization of cryopreserved human semen for assisted reproduction: recovery and maintenance of sperm motility and viability. *J Assist Reprod Genet*. 1998; 15: 504-12.
 18. Rofeim O, Brown TA, Gilbert BR: Effects of serial thaw-refreeze cycles on human sperm motility and viability. *Fertil Steril*. 2001; 75:1242-3.
 19. Esteves SC, Spaine DM, Cedenho AP, Srougi M: Effects of the technique of cryopreservation and dilution/centrifugation after thawing on the motility and vitality of spermatozoa of oligoasthenozoospermic men. *Int Braz J Urol*. 2003; 29: 133-40.
 20. Bandularatne E & Bongso A: Evaluation of the human sperm function after repeated freezing and thawing. *J Androl*. 2002; 23: 242-9.

Received: September 13, 2004

Accepted after revision: November 23, 2004

Correspondence address:

Dr. Sandro C. Esteves
 Av. Dr. Heitor Penteado, 1464
 Campinas, SP, 13075-460
 Fax: + 55 19 3294-6992
 E-mail: s.esteves@androfert.com.br