ORIGINAL ARTICLE

Vol. 38 (1): 108-115, January - February, 2012

Rapid thawing human sperm does not affect basic parameters in normozoospermic men: a double-blind prospective study

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ABSTRACT

Purpose: To compare sperm recovery from slow versus rapid thawing technique using thirty-eight normozoospermic human sperm samples, as follows. Twenty-one samples from men taking part in routine infertility screening exams (infertile group) and seventeen from proven fertile volunteer men with at least one child (fertile group).

Materials and Methods: After analysis of motility, concentration, strict morphology and functional integrity of membranes, sperm was divided into two aliquots of 0.5 mL each and frozen in TyB-G medium. Samples were thawed at room temperature (25 ± 2º C) for 25 minutes (slow thaw) or in a water bath at 75º C for 20 seconds followed by water bath at 37º C for 3 minutes (rapid thaw). After thawing, motility, strict morphology and functional integrity of membranes were evaluated by a blinded investigator. The results were expressed as mean ± standard deviation for parametric variables and analyzed using Student's t-test. Data with unpaired non-parametric variables were expressed as median (interquartile range) and analyzed by the Mann-Whitney test. Wilcoxon test was used to analyze non-parametric paired variables.

Results: There was no significant difference between techniques for total and progressive motility, percentage of normal morphological forms, hypoosmotic swelling test.

Conclusions: Although the rapid thawing protocol was completed in a shorter time (three minutes and 20 seconds versus 25 minutes, respectively), it wasn't harmful since both techniques showed comparable spermatozoa recovery. Additional research is needed to confirm its safety in clinical research before introducing this methodology in routine assisted reproduction.

ARTICLE INFO

Key words:
sperm; cryopreservation; sperm bank; semen preservation; spermatozoa

Int Braz J Urol. 2012; 38: 108-115

Submitted for publication: August 17, 2011

Accepted after revision: November 29, 2011

INTRODUCTION

Sperm quality of thawed samples is still considered unsatisfactory due to relatively low recovery rate of viable sperm after freezing and thawing processes, as less than 60% of them regain motility after thawing (1).

Several studies have been published about animal sperm cryopreservation showing better results regarding the viability of the sperm after thawing. Many sperm thawing protocols for animals use high heating levels (above 50º C) and obtain good motility recovery rates (2-4).

There is not a consensus protocol for thawing human semen, which can be done in several ways. Semen can be thawed at room temperature for about 5-60 minutes or using a water bath at 37º C for 5-10 minutes or with both techniques (5-10). Accordingly to our protocol, cryogenic vial samples are removed from the liquid nitrogen and allowed to thaw at room temperature (25º ± 2º C) for 25 minutes to achieve a complete thaw before being processed (5,8). This procedure has been performed for several years with good clinical results.

We aim to investigate whether the use of a higher thawing temperature (75º C) would be harmful to human spermatozoa when compared with room temperature (25º ± 2º C) thawing regarding motility, morphology and integrity of sperm membranes, and if it would be feasible to introduce this technique in routine assisted reproduction.

MATERIALS AND METHODS

This study was carried out from September 25 to November 4, 2009, at Professor Aroldo Fernando Camargos Laboratory of Human Reproduction, Federal University of Minas Gerais. All participants signed a consent form and completed a questionnaire about reproductive history. This research has been approved by The Ethics Committee under protocol number COEP-UFMG 348/08.

Only subjects who provided semen within the standard parameters (normozoospermic, semen volume ≥ 2 mL) set by the 1999 World Health Organization were included (11). Thirty-eight men provided sperm samples. Twenty-one of them from routine infertility screening exams (infertile group) and seventeen were proven fertile volunteers with at least one child (fertile group).

Sperm was obtained by masturbation into a non-toxic sterile collector, after two to five days from last intercourse or ejaculation. Once collected, sperm was maintained at 37º C on a warm plate until total liquefaction. Samples were analyzed within 60 minutes after ejaculation for concentration, motility, morphology and functional sperm membrane integrity and basic macroscopic parameters (liquefaction, volume, color, viscosity, pH). Rapid progressive spermatozoa (type a), slow progressive spermatozoa (type b), non progressive (type c) and immotile (type d) were defined in conformity with WHO criteria (1999). All counts were done in a Neubauer chamber after appropriate dilutions of semen aliquots.

Morphology was evaluated by the hematoxylin staining technique and slides were analyzed by the Kruger strict criteria (12). Morphology of two hundred randomly chosen spermatozoa were assessed using a Carl Zeiss® optical microscope with 1,000 X millimetered ocular lens under oil immersion.

Functional sperm membrane integrity was evaluated by the hypoosmotic swelling test. Briefly, a 0.1 mL semen sample was diluted into 1.0 mL of hypoosmotic solution at 37º C inside an Eppendorf® tube and incubated for one hour in a 5% CO^2 incubator, at 37º C. After homogenization, a small drop was observed under 400X magnification. One hundred spermatozoa were analyzed in each sample and the percentage of typical morphology changes was calculated.

Test Yolk Buffer with Gentamicine (TyB-G) and 12% Glycerol (Irvine Scientific, USA) freezing medium was used (proportion 1:1) for a total volume of 1 mL. Just before cryopreservation, this mixture was divided into 2 equal samples. Before storing in liquid nitrogen (- 196º C), samples were maintained in nitrogen vapor for 10 minutes at a 10 cm height from liquid nitrogen surface. Cryogenic tubes were stored for 14 to 45 days. Samples were thawed at room temperature (25º ± 2º C) by a different investigator for 25 minutes (slow thaw) or in a water bath at 75º C for 20 seconds and then maintained at 37º C for three minutes in another water bath (rapid thaw) and then analyzed for motility, morphology and functional integrity of sperm membrane. The investigator who analyzed the thawed semen was blinded to the technique used for the thawing.

The minimal sample size was estimated to be 17 patients, using a standard deviation of 10%, minimum difference to be detected of 10% and a statistical power of 81%. Data were ex-

pressed as mean ± standard deviation for parametric variables and analyzed by Student's t-test. Non-parametric non-paired variables were expressed as median (interquartile range) and analyzed by the Mann-Whitney test. Wilcoxon test was used to analyze non-parametric paired variables. The significance level was $p < 0.05$.

RESULTS

Fertile and infertile subjects had similar age and comparable fresh sperm parameters, including total fresh spermatozoa and spermatozoa per mL count, normal morphology, head or middle piece or tail morphology defects, or cytoplasmic droplets (Table-1).

Fertile and infertile men also showed similar semen results after both thaw techniques (slow and rapid thaw). Likewise, thawed sperm didn't show differences in progressive motility (type a + b), reactive sperm percentage after hypoosmotic swelling test or strict morphology, defects in the middle piece and tail (Table-2).

Fertile thawed samples showed similar percentage of normal spermatozoa morphology after thawing by both techniques, compared with fresh samples; however there was an increase of morphological head defects ($p \leq 0.01$) and a decrease of cytoplasmic droplets ($p \leq 0.004$), and percentages of spermatozoa reactive to the hypoosmotic swelling test ($p < 0.0001$) (Table-3). There was a decrease of progressive sperm motility ($p < 0.0001$) (Table-4).

Thawed infertile samples didn't show difference of normal spermatozoa morphology percentage regarding slow thaw technique; nevertheless rapid thaw method demonstrated a reduction ($p = 0.0034$). There was a significant increase of morphological head defects only for rapid thaw protocol ($p = 0.0362$) and a decrease in the percentages of spermatozoa reactive to the hypoosmotic swelling test ($p < 0.0001$) by both

Table 1 - Fresh sperm evaluation.

Variable	Fertile	Infertile	p Value
Age (years + SD)	37.12 + 8.23	36.19 + 5.81	0.6867
Counting			
Total count (x 10^6)	160 (80 - 300)	260 (145 - 368)	0.2774
Count (x 10^6/ mL)	52 (28 - 120)	58 (47 - 82)	0.5472
Progressive motility	65 (60 - 75)	60 (55 - 70)	0.4531
Morphology (Krüger)			
Normal	17 (16 - 22)	17 (16 - 22)	0.8255
Head defect	55 (50 - 60)	54 (52 - 55)	0.7351
Middle piece defect	12 (9 - 15)	13 (12 - 15)	0.1580
Cytoplasmic droplets	4 (4 - 5)	4 (3 - 5)	0.1992
Tail defect	8 (6 - 14)	9 (6 - 10)	0.6805
Hypoosmotic test	70 (65 - 75)	70 (62 - 75)	0.7575

Student's t-test for age. Data expressed as mean ± standard deviation

Mann-Whitney's test for counting, motility, morphology and hypoosmotic test.

Data are expressed as median and interquartile interval.

Table 2 - Sperm evaluation according to thawing protocol.

Variable	Slow thaw	Rapid thaw	P Value
Fertile			
Progressive motility	35 (5 - 50)	30 (15 - 55)	0.2706
Morphology (Krüger)			
Normal	18 (13 - 20)	17 (14 - 20)	0.7045
Head defect	60 (57 - 62)	61 (60 - 63)	0.3344
Middle piece defect	10 (8 - 14)	10 (9 - 12)	0.5558
Cytoplasmic droplets	3 (3 - 4)	3 (3 - 4)	0.8705
Tail defect	9 (7 - 10)	9 (7 - 12)	0.6915
Hypoosmotic test	45 (36 - 48)	42 (34 - 49)	0.8486
Infertile			
Progressive motility	35 (25 - 45)	40 (25 – 55)	0.3139
Morphology (Krüger)			
Normal	15 (13 - 21)	15 (11 - 18)	0.5458
Head defect	60 (50 - 63)	57 (53 - 65)	0.8800
Middle piece defect	11 (8 - 14)	12 (10 - 15)	0.2678
Cytoplasmic droplets	3 (3 - 4)	3 (2 - 4)	0.4784
Tail defect	9 (8 - 11)	10 (7 - 14)	0.7624
Hypoosmotic test	47 (42 - 51)	50 (39 - 54)	0.7340

Mann-Whitney's test for motility, morphology and hypoosmotic test.
Data are expressed as median and interquartile interval.

methods (Table-3). The two analyzed techniques demonstrated a decrease of progressive sperm motility ($p < 0.0001$) with recovery rates ranging from 46% to 67% (Table-4) for the fertile and infertile groups.

DISCUSSION

Many sperm thawing methods for animals use high heating temperatures (above 50° C) (2-4). In contrast, the majority human cryopreservation protocols use thawing temperatures ranging from 20 to 37° C (5-10), requiring a longer thawing time. This fact, coupled with the need to refine the protocols of freezing and thawing of human semen, sparked interest in an unprecedented way to check how human sperm undergoing this type of procedure reacts.

Our hypothesis was that if the sperms of many animal species recovered so well after fast thawing at higher temperatures, it could also be the same with human sperm. So we decided to investigate whether the use of higher heating curves would be as safe to human spermatozoa as conventional thawing, and if it would be feasible to introduce this technique to routine procedures for cryopreservation of human semen in assisted reproduction.

Table 3 – Sperm parametere before and after cryopreservation.

Sperm parameter	Before freezing	After thawing	p Value
Fertile			
Normal morphology			
Slow thaw	17 (16 - 22)	18 (13 - 20)	0.5966
Rapid thaw	17 (16 - 22)	17 (14 - 20)	0.3289
Morphological head defects			
Slow thaw	55 (50 - 60)	60 (57 - 62)	0.0107
Rapid thaw	55 (50 - 60)	61 (60 - 63)	0.0026
Cytoplasmic droplets			
Slow thaw	4 (4 - 5)	3 (4 - 5)	0.0040
Rapid thaw	4 (4 - 5)	3 (3 - 4)	0.0034
Hypoosmotic test			
Slow thaw	70 (65 - 75)	45 (36 - 48)	< 0.0001
Rapid thaw	70 (65 - 75)	47 (34 - 50)	< 0.0001
Infertile			
Normal morphology			
Slow thaw	17 (16 - 22)	15 (13 - 21)	0.2428
Rapid thaw	17 (16 - 22)	15 (11 - 18)	0.0034
Morphological head defects			
Slow thaw	54 (52 - 55)	60 (50 - 63)	0.1232
Rapid thaw	54 (52 - 55)	57 (53 - 65)	0.0362
Cytoplasmic droplets			
Slow thaw	4 (3 - 5)	3 (3 - 4)	0.0674
Rapid thaw	4 (3 - 5)	3 (2 - 4)	0.0665
Hypoosmotic test			
Slow thaw	70 (62 - 75)	47 (42 - 51)	< 0.0001
Rapid thaw	70 (62 - 75)	50 (39 - 54)	< 0.0001

Wilcoxon test was used to analyze non-parametric paired variables.

Data are expressed as median and interquartile interval.

Table 4 – Recovery rates after cryopreservation in fertile and infertile men.

Sperm parameter	Before freezing	After thawing	Recovery rate (%) (final/initial motility)	p Value
Fertile				
Progressive motility (grade A + B)				
Slow thaw	65 (60 - 75)	35 (5- 50)	54	< 0.0001
Rapid thaw	65 (60 - 75)	30 (15 - 55)	46	< 0.0001
Infertile				
Progressive motility (grade A + B)				
Slow thaw	60 (55 - 70)	35 (25 - 45)	58	< 0.0001
Rapid thaw	60 (55 - 70)	40 (25 - 55)	67	< 0.0001

Wilcoxon test was used to analyze non-parametric paired variables progressive motility

Data are expressed as median and interquartile interval.

This research was able to evaluate and compare the viability of human sperm after thawing at temperatures of 75º C and room temperature using the parameters of motility, morphology and integrity of sperm membranes. The temperature of thawing in a water bath at 75º C for 20 seconds followed by immersion in water bath at 37º C for three minutes was adapted from a study that has thawed equine semen at this temperature (4).

A pre-warm (37º C) was not performed in slow thaw group before sperm motility measurement. Moreover, with this lower temperature used in the slow thaw group it would be expected a higher probability of finding a difference, but our study did not demonstrate any difference. Interesting, Calamera et al. (2010) did not show any influence of room temperature (20º C) versus 40º C on sperm motility.

Sperm recovery from fertile and infertile patients submitted to a higher thawing temperature did not diverge from slow thawing protocol, suggesting that the rapid thaw protocol appears not to be harmful for the analyzed variables. Furthermore, it was performed in a shorter time (three minutes and 20 seconds vs. 25 minutes, respectively).

Even though there would be a risk of DNA-damage once a high temperature is involved (75º C), this study did not have the intention of evaluating sperm DNA fragmentation. As we couldn't find any study that has used 75º C for human semen thawing, more studies should be performed in this field.

Several studies have confirmed the decline in sperm motility after thawing (11,13,14). In a recent prospective study, the motility of spermatozoa thawed in water bath at 20, 37, 38, 39 and 40º C decreased significantly (15). In our study, both thawing techniques resulted in decrease of sperm progressive motility (a + b) in fertile and infertile groups compared to fresh samples ($p < 0.0001$) (Table-4). However there wasn't a significant difference between techniques ($p = 0.2706$ and $p = 0.3139$, respectively, Table-2).

Regarding the sperm morphology, the most commonly used staining methods for sperm morphology evaluation include hematoxylin stain, the Papanicolaou method, the Shorr method, the Spermac method or the Diff-Quik method. Some published papers have used Kruger morphological evaluation with other dyes (8,16). The best method should be the most beneficial to the laboratory as each method has limitations (17). Therefore, hematoxylin was chosen as a routine staining procedure for Kruger strict criteria sperm evaluation.

The percentage of morphologically normal thawed sperm didn't reveal a significant difference when comparing fertile and infertile groups or slow and rapid thawing methods, which is in agreement with other studies (1,13).

However, there was a decrease in the percentages of morphologically normal sperm from infertile group when using the rapid thawing protocol. This decrease is in agreement with some published papers (10,14). A possible explanation for this result can be due to lower resistance of sperm from infertile men to damage caused by the cryopreservation process (18).

The hypoosmotic swelling test is widely used in various studies involving frozen semen in the veterinary field (4,19). In human reproduction, despite having a questionable validity in procedures that involve semen freezing and thawing, data was published using this test to evaluate the integrity of the sperm membrane (1,15). Therefore in our study, we also chose to include this test to evaluate this parameter before and after sperm thawing.

Cellular membrane damage could be demonstrated by a decrease in the percentage of sperm cells reactive to the hypoosmotic swelling test when comparing fresh to thawed samples ($p < 0.001$). This decline is similar to another study that demonstrated reduced spermatozoa membrane integrity (1).

To our knowledge, we couldn't find any study that had done the same comparison (slow versus fast human sperm thawing). This is the first study to do this experiment. Additional analogous basic research is needed to confirm our results with non normozoospermic sperm samples and in clinical research to access normal birth rates before introducing this methodology in routine assisted reproduction.

CONCLUSIONS

The techniques of fast and slow thawing showed the same recovery of spermatozoa in normozospermic men. Sperm from fertile and infertile patients submitted to higher thawing temperature did not diverge from slow thawing protocol, suggesting that the rapid thaw protocol seems not to be harmful for the analyzed variables. Furthermore, it was performed in a shorter time (three minutes and 20 seconds vs. 25 minutes, respectively). Additional research is needed to confirm its safety in clinical research before introducing this methodology in routine assisted reproduction.

ACKNOWLEDGEMENTS

This research received financial support from Coordenação de Aperfeiçoamento do Pessoal de Nível Superior (CAPES) and The Postgraduate Program in Woman's Health, Faculty of Medicine, Federal University of Minas Gerais.

Financial support has been provided by The Postgraduate Course in Women´s Health – Faculty of Medicine – Federal University of Minas Gerais and Coordenação de Aperfeiçoamento do Pessoal de Nível Superior (CAPES).

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Vutyavanich T, Piromlertamorn W, Nunta S: Rapid freezing versus slow programmable freezing of human spermatozoa. Fertil Steril. 2010; 93: 1921-8.
2. Barbas JP, Mascarenhas RD: Cryopreservation of domestic animal sperm cells. Cell Tissue Bank. 2009; 10: 49-62.
3. Peña AI, Barrio F, Quintela LA, Herradón PG: Effect of different glycerol treatments on frozen-thawed dog sperm longevity and acrosomal integrity. Theriogenology. 1998; 50: 163-74.
4. Snoeck PPN: Aspects of equine sperm cryopreservation: dilutor medium composition, cryopreservation curves and fertility [dissertation]. Belo Horizonte (MG): Federal University of Minas Gerais, 2003; p. 116.
5. Hossain A, Nagamani M: Cryopreservation of Male Gamets. In: Botros R, Juan GV, Antonis M (ed.), Infertility and Assisted Reproduction. New York: Cambridge University Press, 2008; pp. 466-78.
6. Verza S Jr, Esteves SC: Feasibility of refreezing human spermatozoa through the technique of liquid nitrogen vapor. Int Braz J Urol. 2004; 30: 487-93.
7. Punyatanasakchai P, Sophonsritsuk A, Weerakiet S, Wansumrit S, Chompurat D: Comparison of cryopreserved human sperm in vapor and liquid phases of liquid nitrogen: effect on motility parameters, morphology, and sperm function. Fertil Steril. 2008; 90: 1978-82.

8. Jackson RE, Bormann CL, Hassun PA, Rocha AM, Motta EL, Serafini PC, et al.: Effects of semen storage and separation techniques on sperm DNA fragmentation. Fertil Steril. 2010; 94: 2626-30.
9. Thomson LK, Fleming SD, Barone K, Zieschang JA, Clark AM: The effect of repeated freezing and thawing on human sperm DNA fragmentation. Fertil Steril. 2010; 93: 1147-56.
10. Ozkavukcu S, Erdemli E, Isik A, Oztuna D, Karahuseyinoglu S: Effects of cryopreservation on sperm parameters and ultrastructural morphology of human spermatozoa. J Assist Reprod Genet. 2008; 25: 403-11.
11. World Health Organisation. WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction, 4th edn. Cambridge: Cambridge University Press. 1999; pp. 128.
12. Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S: Predictive value of abnormal sperm morphology in in vitro fertilization. Fertil Steril. 1988; 49: 112-7.
13. Ngamwuttiwong T, Kunathikom S: Evaluation of cryoinjury of sperm chromatin according to liquid nitrogen vapour method (I). J Med Assoc Thai. 2007; 90: 224-8.
14. Dejarkom S, Kunathikom S: Evaluation of cryo-injury of sperm chromatin according to computer controlled rate freezing method part 2. J Med Assoc Thai. 2007; 90: 852-6.
15. Calamera JC, Buffone MG, Doncel GF, Brugo-Olmedo S, de Vincentiis S, Calamera MM, et al.: Effect of thawing temperature on the motility recovery of cryopreserved human spermatozoa. Fertil Steril. 2010; 93: 789-94.
16. Zini A, Phillips S, Courchesne A, Boman JM, Baazeem A, Bissonnette F, et al.: Sperm head morphology is related to high deoxyribonucleic acid stainability assessed by sperm chromatin structure assay. Fertil Steril. 2009; 91: 2495-500.
17. Graves JE, Higdon HL 3rd, Boone WR, Blackhurst DW: Developing techniques for determining sperm morphology in today's andrology laboratory. J Assist Reprod Genet. 2005; 22: 219-25.
18. Donnelly ET, Steele EK, McClure N, Lewis SE: Assessment of DNA integrity and morphology of ejaculated spermatozoa from fertile and infertile men before and after cryopreservation. Hum Reprod. 2001; 16: 1191-9.
19. Pinto CR, Kozink DM: Simplified hypoosmotic swelling testing (HOST) of fresh and frozen-thawed canine spermatozoa. Anim Reprod Sci. 2008; 104: 450-5.

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