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Andrology

Vitality of Oligozoospermic Semen Samples Is Improved by Both Swim-Up and Density Gradient Centrifugation Before Cryopreservation

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***Purpose*:** To ascertain whether washing sperm from oligozoospermic and normozoospermic samples before cryopreservation improves post-thaw vitality.
***Methods*:** Normozoospermic ($n = 18$) and oligozoospermic ($n = 16$) samples were divided into three aliquots. The first aliquot remained untreated and the second and third aliquots were subjected to the swim-up and discontinuous density gradient sperm washing techniques respectively. Vitality staining was performed, samples mixed with cryopreservation media and frozen. Spermatozoa were thawed, stained, and vitality quantified and expressed as the percentage of live spermatozoa present.
***Results*:** Post-thaw vitality in untreated aliquots from normozoospermic samples (24.9% ± 2.3; mean ± SEM) was significantly higher (unpaired t-tests; $P < 0.01$) than untreated oligozoospermic samples (11.9% ± 2.3). Post-thaw vitality was significantly higher after swim-up in normozoospermic samples (35.6% ± 2.1; $P < 0.001$; one-way ANOVA) and oligozoospermic samples (27.7% ± 1.7; $P < 0.01$). Density gradient centrifugation significantly improved post-thaw vitality in oligozoospermic (22.4% ± 1.0; $P < 0.01$) but not normozoospermic (30.8% ± 1.8) samples.
***Conclusions*:** Sperm vitality in cryopreserved oligozoospermic samples was improved by both the swim-up and density gradient centrifugation washing techniques prior to freezing.

KEY WORDS: Cryopreservation; human spermatozoa; spermatozoa vitality; spermatozoa washing.

INTRODUCTION

Cryopreservation of human spermatozoa has been used for assisted reproductive techniques for 50 years since the first births from intrauterine insemination using previously cryopreserved human spermatozoa were reported in 1953 by Bunge and Sherman (1). Cryopreservation of human sperm is now routinely practiced in Assisted Reproductive Technology and Andrology laboratories for a range of reasons including, the quarantine of donor sperm, long-term storage for men who are undergoing chemotherapy or vasectomy, and short-term storage for men who will be absent during their partner's assisted reproductive treatment.

Over the last 15 years considerable research has been undertaken to improve semen-freezing protocols (reviewed in 2,3). A great deal of work has been directed at investigating the effects of semen preparation techniques pre- and post-thaw, the use of various cryoprotectants and manipulation of freezing and thawing conditions (2,3). Although many recent advances in cryobiology have been made, freezing human sperm still results in significant loss of motility and viability (4,5). Furthermore, post-thaw survival rates vary considerably between

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different individuals and survival in ejaculates from the same individual can vary markedly (6,7). Survival rates of cryopreserved human sperm appear to be further compromised in semen samples from oligozoospermic men (8,9). Indeed, the survival rate of sperm from oligozoospermic samples was 29% and 48% less than that of the normozoospermic samples (8,9). In a clinical setting, increasing the survival of sperm from subfertile men is of considerable importance in order to maximize their partner's chance of achieving a successful pregnancy outcome, when the use of frozen–thawed sperm is necessary.

Studies examining semen preparation techniques have shown that separating the sperm from the seminal plasma using the swim-up washing procedure before freezing can significantly improve sperm motility and acrosome status (10–12), while improvement in motility was reported after using a Percoll sperm separation technique prior to freezing (13,14). Whether these sperm preparation techniques improve post-thaw survival in oligozoospermic samples has not been examined. The aim of this study therefore, was to ascertain whether sperm sample preparation by washing prior to freezing improves post-thaw vitality in oligozoospermic samples.

MATERIALS AND METHODS

Written approval for this study was obtained from the King Edward Memorial and Princess Margaret Hospital Institutional Ethics Committee and all subjects gave informed consent.Thirty-four semen samples were collected by masturbation into sterile specimen jars and allowed to liquefy for 30 min at room temperature. Sperm concentration and motility was measured using a Makler Chamber (Sefi-Medical Instruments, Isreal) and morphology assessed following WHO guidelines (15). Using the WHO criteria for normality, semen samples were deemed to be normozoospermic ($n = 18$) or oligozoospermic ($n = 16$).

Semen samples were separated into three aliquots. The first aliquot remained untreated and the second and third aliquots were subjected to the swim-up procedure and discontinuous density gradient centrifugation respectively. For the swim-up procedure, 1 mL of semen was placed underneath 3 mL of culture media (Earles Balanced Salt Solution; Australian Biosearch, Australia; containing 5 mg/L phenol red, 96,000 IU Penicillin, 38,000 IU Streptomycin, 945 g/L sodium hydrogen carbonate, and 5% HSA, Sage Pharamcia) in a 10-mL Falcon tube (Becton Dickinson, USA) and incubated at 37°C for up to 2 h. Culture media containing sperm was then removed and centrifuged at 400*g* for 7 min. Supernatant was removed and the sperm pellet reconstituted in 3 mL of culture media and centrifuged again at 400*g* for 7 min after which the supernatant was removed and the sperm pellet resuspended in 0. 5 mL of culture media.

Discontinuous density gradients were prepared by sequentially placing 1 mL of 90% and 45% Isolate solution (Irvine Scientific, USA) into Falcon 15 mL conical tubes (Becton Dickinson, USA). Semen (1 mL) was placed on top of the density gradient and the tubes centrifuged at 300*g* for 20 min. The sperm pellet was removed and placed into 3 mL of culture media, centrifuged at 400*g* for 7 min and then reconstituted in 3 mL of media. After a second centrifugation the sperm pellet was reconstituted in 0.5 mL of culture media.

After the swim-up and discontinuous density gradient preparation sperm concentration and motility was assessed (15). Sperm vitality was also assessed using eosin–nigrosin staining as previously described (15). Briefly, one drop of untreated semen or washed semen was mixed with one drop of 1% eosin stain (BDH, UK). After 30 s three drops of 10% nigrosin (BDH, UK) was added and the solutions mixed. A drop of this mixture was placed onto a microscope slide and a smear made. The smears were allowed to air dry and were viewed under oil immersion with light microscopy at a magnification of 1000×. Live sperm appear white while dead sperm with disrupted membranes have taken in the eosin stain and appear red. Vitality was quantified according to WHO guidelines by counting 200 sperm and expressed as percentage live sperm (15).

Immediately prior to cryopreservation each aliquot of untreated and washed sperm samples (swim-up and discontinuous density gradient) were mixed (1:1) sequentially with sperm cryopreservation media (in house egg yolk media) containing glucose (8 g/L), sodium citrate (5 g/L), and glycine (3 g/L) and drawn into 0.5 mL semen freezing straws (Genetics Australia, Australia). Samples were then frozen in a Planar Kryo 10 control rate-freezing machine (Planar, UK) using a single freezing ramp of −0.10°C/min to −80°C. Samples were then removed from the machine and plunged into liquid nitrogen. To assess post-thaw vitality straws were removed from liquid nitrogen storage, thawed at room temperature for 30 min and subjected to eosin–nigrosin staining as described above.

Table I. Semen Parameters for Samples Used in This Analysis

	Age (years)	Ejaculate volume (mL)	Concentration (million/mL)	Motility (% forward progression)	Morphology (% normal)
Normozoospermic	33.3 ± 3.7	3.9 ± 1.2	79.7 ± 21.4	73.3 ± 14.1	31.3 ± 13.6
Range	27–37.5	1.7–5.1	51–111	45–91	15–46
Oligozoospermic	36.6 ± 5.5[a]	4.6 ± 1.9	10.6 ± 5.4[b]	51.9 ± 17.0[b]	20.5 14.4[b]
Range	27.5–43	2.5–9	1.5–20	23–80	2–46

Note. Values are the mean ± SD and the range.
[a] $P < 0.05$ compared to normozoospermic.
[b] $P < 0.001$ compared to normozoospermic.

One-way analysis of variance and least significant difference tests were used to assess differences in post-thaw vitality among the untreated and washed samples (swim-up and discontinuous density gradient) and unpaired t-tests were used to assess differences between normozoospermic and oligozoospermic samples.

RESULTS

Semen parameters from the samples used in this study are presented in Table I. Individual samples were classified as normozoospermic ($n = 18$) or oligozoospermic ($n = 16$) according to WHO guidelines. For the oligozoospermic group, three of the samples were classified as oligoasthenoteratozoospermic. There was an age difference between the participants with those men with oligozoospermia significantly older than those with normal semen parameters ($P < 0.05$; unpaired t-test). Differences were also observed between total sperm concentration, motility, and normal morphology with normozoospermic samples having higher values for these parameters (Table I; $P < 0.001$; unpaired t test).

Post-thaw vitality in untreated normozoospermic samples was significantly higher ($P < 0.01$; unpaired t-tests) than that observed in the untreated oligozoospermic samples (Table II). Post-thaw vitality was also significantly higher in normozoospermic samples when treated by swim-up or density gradient centrifugation when compared to oligozoospermic samples in the same groups ($P < 0.01$; Table II). Vitality was not different between these groups prior to freezing (data not shown). After the swim-up washing procedure, post-thaw vitality was significantly higher in both the normozoospermic samples ($P < 0.001$; one-way ANOVA) and oligozoospermic samples ($P < 0.01$) compared to untreated aliquots (Table II). When compared to post-thaw vitality in untreated aliquots, density gradient centrifugation significantly improved post-thaw vitality in oligozoospermic samples ($P < 0.01$), but this improvement was not observed in the normozoospermic samples (Table II).

A further analysis was carried out to ascertain whether post-thaw vitality as measured by eosin–nigrosin staining was related to the prefreezing motile sperm count. Positive correlations were observed when comparing post-thaw vitality with the prefreeze concentration of motile sperm in untreated ($r^2 = 0.698$, $P < 0.001$) and washed ($r^2 = 0.512$, $P < 0.01$ for swim-up; $r^2 = 0.534$, $P < 0.01$ for discontinuous density gradient) aliquots (Fig. 1).

DISCUSSION

Cryopreservation induces lethal and sublethal damage that leads to loss of around 50% of live sperm (16,17). Damage can cause loss of sperm membrane integrity (18), loss of acrosome function (7), DNA fragmentation (19), loss of motility (16,17), and poor fertilization (17). Freezing damage to the cell membrane is usually attributed to rupture by intracellular or extracellular ice crystal formation, osmotic effects (20), or the introduction of cryoprotectant (21). Damage to sperm can also occur during thawing (7,20,22).

Table II. Post-Thaw Vitality in Normozoospermic and Oligozoospermic Samples

	Untreated	Swim-up	Discontinuous density gradient
Normozoospermic	24.9 ± 2.3	35.6 ± 2.1[a]	30.8 ± 1.8
Oligozoospermic	11.9 ± 2.3[b]	27.7 ± 1.7[b,c]	22.4 ± 1.0[b,c,d]

Note. Vitality is expressed as the percentage live sperm observed. Values are the mean ± SEM ($n = 18$ for normozoospermic; $n = 16$ for oligozoospermic).
[a] $P < 0.001$ compared to untreated.
[b] $P < 0.01$ compared to normozoospermic.
[c] $P < 0.01$ compared to untreated.
[d] $P < 0.05$ compared to swim-up.

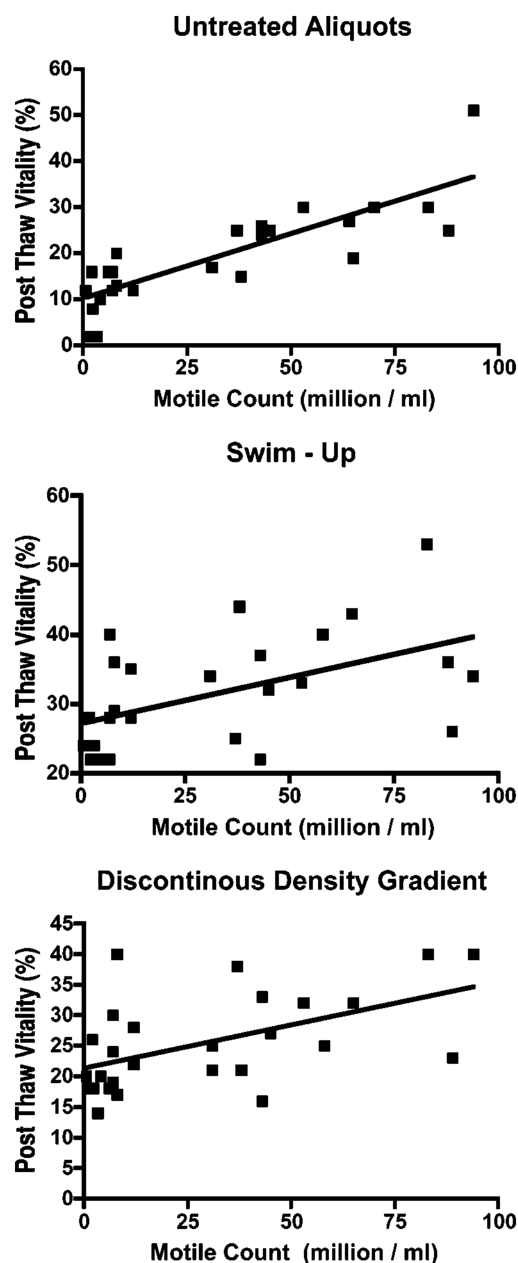

Fig. 1. Correlation of the concentration of motile sperm (million/mL semen) in aliquots of fresh semen compared to post-thaw vitality (% live sperm) in untreated and washed aliquots.

The severity of damage during the freeze–thaw cycle varies considerably between different individuals and different samples from the same individual. Furthermore, samples that are oligozoospermic are more susceptible to damage than normozoospermic samples (8,9,16). The aim of the present study was to examine whether sperm washing techniques such as the swim-up procedure or density gradient centrifugation could be used to improve the vitality of cryopreserved human sperm from oligozoospermic men.

The main finding of our study was that both the swim-up and density gradient centrifugation washing procedures improved the post-thaw vitality in oligozoospermic samples, whereas only the swim-up procedure was of benefit to normozoospermic samples. Previous studies have shown that continuous percoll gradient centrifugation of normozoospermic samples resulted in significantly higher values for motility characteristics and normal morphology (14) and that percoll washed sperm retained their post-thaw motility longer than sperm not separated from the semen prior to freezing (13). In the present study post-thaw vitality in normozoospermic samples was not improved by discontinuous gradient (isolate) washing. The difference between this and the previous findings might be related to differences in the percoll and isolate preparations or to the different type of gradient used. The improved post-thaw vitality observed in normozoospermic samples in the present study confirms earlier reports that swim-up washing before freezing significantly enhances overall post-thaw sperm quality (11,12). Specifically, samples subjected to the swim up washing procedure before freezing have more normal morphology (11), a faster velocity and progression, and more intact acrosomes (12) than those samples that were not treated. Although both sperm washing procedures improved the post-thaw vitality of oligozoospermic samples in the present study, the swim-up procedure was superior to the discontinuous gradient, consistent with the finding that this procedure can be used effectively for washing fresh oligozoospermic samples (23). Although the swim-up washing procedure might not usually be the washing method of choice for severely oligozoospermic samples due to the low sperm numbers, adjustments to the procedure such as multiple tube swim-up or increasing the swim-up time can be made to maximize the numbers of sperm obtained for freezing. The success of sperm washing techniques in improving post-thaw sperm vitality might be related to the removal of the seminal plasma which contains nonviable sperm and other cellular components that can be a source of reactive oxygen species known to cause oxidative damage during the freeze–thaw process (24). As previously discussed oxidative damage can affect both the sperm membrane and DNA with lethal and sublethal consequences (17,19,25). Other potential advantages of prefreeze washing include producing a more even dilution of the sample

with cryoprotectant, especially in those samples that are highly viscous, and freezing lower numbers of higher quality sperm is more efficient use of storage space in the sperm bank. Whether prefreeze sperm washing improves fertilization, implantation, and pregnancy rates following assisted reproductive technology procedures remains to be determined.

Consistent with other workers (8,9), the post-thaw vitality observed in oligozoospermic samples in the present study was markedly reduced compared to the normozoospermic samples. The reasons for this discrepancy are not clear but might be related to the higher frequency of head membrane defects observed in oligozoospermic samples. Lin *et al.* (8) observed a higher incidence of head membrane defects in fresh oligozoospermic semen compared to fresh normozoospermic samples, suggesting a possible relationship between compromised head membrane and a greater intolerance to freezing of oligozoospermic semen.

To compare the effects of cryopreservation on washed and unwashed semen samples, vitality staining using the eosin–nigrosin technique was used. This technique uses a supravital stain (eosin) that penetrates the membrane of the sperm head if the membrane has lost its integrity. Motility (another index used to assess cryo damage) has been directly related to membrane integrity (11,18). In our study and others (11) a strong positive correlation was found between the number of progressive sperm and those with intact membranes. Although Lin *et al.* (8) did not observe a correlation between disruption of the head membrane and motility, they did suggest that other membrane damage could be a major factor contributing to a decline in motility. In conclusion, this study has shown that sperm vitality in cryopreserved oligozoospermic samples was improved by both the swim-up and density gradient centrifugation washing techniques prior to freezing.

REFERENCES

1. Bunge RG, Sherman JK: Fertilization capacity of frozen human spermatozoa. Nature 1953;172:767–768
2. McLaughlin EA: Cryopreservation and storage of sperm. *In* Textbook of Assisted Reproductive Techniques: Laboratory and Clinical Perspectives, DK Gardner, A Weissman, CM Howles, Z Shoham (eds), United Kingdom, Martin Dunitz, 2001, pp 261–272
3. Royere D, Barthelemy C, Hamamah S, Lansac J: Cryopreservation of spermatozoa: A 1996 review. Hum Reprod Update 1996;2:553–559
4. Critser JK, Arneson BW, Aaker DV, Huse-Benda AR, Ball GD: Cryopreservation of human spermatozoa. II Post thaw chronology of motility and of zona-free hamster ova penetration. Fertil Steril 1987;47:980–984
5. McLaughlin EA, Ford WCL, Hull MGR: The effect of cryopreservation on the motility characteristics and membrane integrity of human spermatozoa. J Reprod Fertil 1992;95:527–534
6. Heuchel V, Schwartz D, Czyglik F: Between and within subject correlations and variations for certain semen characteristics in fertile men. Andrology 1983;15:171–176
7. McLaughlin EA, Ford WCL, Hull MGR: Effects of cryopreservation on the human sperm acrosome and its response to A23187. J Reprod Fertil 1993;99:71–76
8. Lin MH, Morshedi M, Srisombut C, Nassar A, Oehninger S: Plasma membrane integrity of cryopreserved human sperm: An investigation of the results of the hypoosmotic swelling test, the water test, and eosin—Y staining. Fertil Steril 1998;70:1148–1155
9. Hughes R, Zuvela E, Mableson J, Bruce N, Dharmarajan A, Matson P: The survival of human spermatozoa frozen by three different protocols. *In* Proceedings 11th World Congress on In Vitro Fertilization & Human Reproduction, 1999, pp 349–352
10. Kobayashi T, Kameko S, Hara I, Park YJ, Sato H, Nozawa S: Concentrating human sperm before cryopreservation. Andrology 1991;23:25–28
11. Perez-Sanchez F, Cooper TG, Yeung CH, Nieschlag E: Improvement in quality of cryopreserved human spermatozoa by swim-up before freezing. Int J Androl 1994;17:115–120
12. Esteves SC, Sharma RK, Thomas AJ, Agarwal A: Improvement in motion characteristics and acrosome status in cryopreserved human spermatozoa by swim-up processing before freezing. Hum Reprod 2000;15:2173–2179
13. Sharma RK, Agarwal A: Sperm quality improvement in cryopreserved human semen. J Urol 1996;156:1008–1012
14. Yogev L, Gamzu R, Paz G, Kleiman S, Botchan A, Hauser R, Yavetz H: Pre-freezing sperm preparation does not impair thawed spermatozoa binding to the zona pellucida. Hum Reprod 1999;14:114–117
15. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Semen—Cervical Mucus Interactions, 4th edn. Cambridge, UK, Cambridge University Press, 1999.
16. Grizard G, Chevalier V, Griveau JF, Le Lannou D, Boucher D: Influence of seminal plasma on cryopreservation of human spermatozoa in a biological material-free medium: Study of normal and low quality semen. Int J Androl 1999;22:190–196
17. Oehninger S, Duru NK, Srisombut C, Morshedi M: Assessment of sperm cryodamage and strategies to improve outcome. Mol Cell Endocrinol 2000;169:3–10
18. Bell M, Wang R, Hellstrom WJG, Sikka SC: Effect of cryoprotective additives and cryopreservation protocol on sperm membrane lipid peroxidation and recovery of motile human sperm. J Androl 1993;14:472–478
19. Medeiros CMO, Forell F, Oliveira ATD, Rodrigues JL: Current status of sperm cryopreservation: Why isn't it better? Therology 2002;57:327–344
20. Mohammad SN, Barratt CL, Cooke ID, Moore HD: Continuous assessment of human spermatozoa viability during cryopreservation. J Androl 1997;18:43–50
21. Mahadevan MM, Trounson AO: Effect of cryoprotective media and dilution methods on the preservation of human spermatozoa. Andrology 1984;16:52–60

22. Wolley DM, Richardson DW: Ultrastructural injury to human spermatozoa after freezing and thawing. J Reprod Fertil 1978;53:389–394
23. Adiga SK, Kumar P: Influence of swim-up method on the recovery of spermatozoa from different types of semen samples. J Assist Reprod Genet 2001;18:160–164
24. Wang AW, Zhang H, Ikemoto I, Anderson DJ, Loughlin KR: Reactive oxygen species generation by seminal cells during cryopreservation. Urology 1997;49:921–925
25. Alvarez JG, Storey BT: Evidence of increased lipid peroxidative damage and loss of superoxide dismutase activity as a mode of sublethal cryodamage to human sperm during cryopreservation. J Androl 1992;13:232–241