



University of Groningen

Selection of non-apoptotic, DNA intact spermatozoa

Mahmoud, Tamer Mahmoud Said

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version Publisher's PDF, also known as Version of record

Publication date: 2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA): Mahmoud, T. M. S. (2009). *Selection of non-apoptotic, DNA intact spermatozoa: an approach to improve* sperm fertilization potential. [s.n.].

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: https://www.rug.nl/library/open-access/self-archiving-pure/taverneamendment.

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): http://www.rug.nl/research/portal. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

CHAPTER 7

Effects of Magnetic-Activated Cell Sorting on Sperm Motility and Cryosurvival Rates

Said TM, Grunewald S, Paasch U, Rasch M, Agarwal A, Glander H-J Fertil Steril, 2005, 83 (5), 1442-1446

ABSTRACT

Objective: Superparamagnetic annexin V-conjugated microbeads can separate spermatozoa with externalized phosphatidylserine, which is considered one of the early features of late apoptosis. Our objective was to evaluate the effect of magnetic-activated cell sorting in cryopreservation-thawing protocols on sperm motility and cryosurvival rate.

Design: Prospective-controlled study.

Setting: Andrology department at a University based medical institution.

Patients: Ten healthy volunteer sperm donors.

Interventions: Sperm populations were separated using annexin-V magnetic-activated cell sorting before and after the cryopreservation-thawing process.

Main outcome measures: Sperm motility and cryosurvival rate.

Results: Annexin-negative sperm separated by magnetic-activated cell sorting had significantly higher motility following cryopreservation-thawing than sperm that were not separated. Similarly, annexin-negative spermatozoa also had higher cryosurvival rate than sperm cryopreserved without magnetic-activated cell sorting and sperm that were annexin-positive.

Conclusions: The separation of a distinctive population of non-apoptotic spermatozoa with intact membranes may optimize the cryopreservation-thawing outcome. Magnetic-activated cell sorting using annexin-V microbeads enhances sperm motility and cryosurvival rates following cryopreservation.

Key words: annexin; cryosurvival rate; magnetic-activated cell sorting; mitochondrial membrane potential; sperm

INTRODUCTION

Cryopreservation of human semen is the most commonly accepted method of preserving male reproductive capacity. Cryopreserved spermatozoa may be used in assisted reproductive techniques (ART) (1), especially in cases where the patient elects to undergo vasectomy for contraception or, most importantly, when a patient is diagnosed with cancer and the treatment may render him infertile (2). The indications for sperm cryobanking have been greatly expanded by recent breakthroughs in ART, in which immotile but viable sperm can be used successfully for oocyte fertilization through intracytoplasmic sperm injection (ICSI).

Despite recent methodological advances, cryopreservation exerts detrimental effects on spermatozoa that lead to significant decreases in sperm viability and motility and ultimately in cryosurvival rates (CSR) (3). The fertility potential of cryopreserved mammalian spermatozoa is lower than that of fresh sperm. The reduction arises from both a lower post-thaw viability and sublethal dysfunction in a proportion of the surviving subpopulation (4). Programmed cell death (apoptosis) most likely contributes to the decrease in sperm quality after cryopreservation (5).

The sperm plasma membrane is one of the key structures affected by cryopreservation that displays apoptotic features (6). Early phases of disturbed membrane functions are associated with asymmetry of the membrane phospholipids. The phospholipid phosphatidylserine (PS), which is normally present on the inner leaflet of the plasma membrane, becomes externalized to the outer leaflet (7). The externalization of PS is a known early marker for apoptosis (8). Because annexin-V has a high affinity for PS, it cannot pass through an intact sperm membrane. Therefore, when annexin-V binds to spermatozoa, it signifies that the integrity of the membrane has been disturbed (9).

Colloidal super-paramagnetic microbeads (~50 nm in diameter) conjugated with annexin-V may be used to separate dead and apoptotic spermatozoa by magnetic-activated cell sorting (MACS). Cells with PS that has externalized to the outer leaflet will bind to these microbeads. When placed into a column containing iron balls and passed through a strong magnetic field, those cells remain in the separation column. On the other hand, cells with intact membranes remain unlabelled and pass freely through the column (10, 11).

The process of cryopreservation increases the amount of apoptotic spermatozoa, which in turn decreases the success rates of ARTs. The binding of superparamagnetic annexin-V microbeads (ANMB) can effectively eliminate spermatozoa in early apoptotic stages from cryopreserved samples (12). Therefore, ANMB-negative spermatozoa may have higher survival potential after cryopreservation. The objective of our study was to determine if the inclusion of MACS in cryopreservation-thawing protocols improves sperm motility and the CSR.

MATERIALS AND METHODS

Sample preparation

This study was approved by our Institution Review Board. Semen samples were collected from 10 healthy donors, and semen parameters exceeded the World Health Organization (WHO, 1999) (13) reference ranges for the normal fertile population. To separate the predominantly mature spermatozoa, the liquefied semen was loaded onto a 55% and 80% discontinuous SupraSperm gradient (MediCult, Jyllinge, Denmark) and centrifuged at 500*g* for 20 minutes. The resulting 80% pellet (mature spermatozoa) was aspirated and re-suspended in human tubal fluid media (HTF, Irvine Scientific, Santa Ana, CA).

The sperm cell suspension was divided into 2 separate fractions. The first was subjected to MACS followed by cryopreservation and thawing whereas the second was cryopreserved-thawed first and then subjected to MACS. Sperm motility was assessed in all fractions at each step of the experiment. The different steps of our experiment design are illustrated in Figure 1.



Figure 1. Flow diagram of overall experiment design: spermatozoa from same sample were subjected to cryopreservation-thawing before and after MACS. Number in parenthesis represents the aliquot number. MACS = magnetic activated cell separation; ANMB = annexin-V magnetic microbead; CSR = cryosurvival rate.

Isolation of spermatozoa with deteriorated membranes by MACS

The sperm suspensions were passed through a magnetic field (MiniMACS; Miltenyi Biotec, Bergisch Gladbach, Germany), and the spermatozoa were classified as either ANMB-positive or ANMB-negative based on the binding of the microbeads to their outer surface (14).

Briefly, the washed spermatozoa were incubated with 100 μ L ANMB at room temperature for 15 minutes, placed on top of the separation column containing iron balls, which was placed in a magnet. The apoptotic spermatozoa were retained in the separation column and labeled as ANMB-positive whereas the spermatozoa with intact membranes passed through the column and were labeled ANMB-negative. The power of the magnetic field was measured as 0.5 tesla between the poles of the magnet and up to 1.5 tesla within the iron globes of the column. After the column was removed from the magnetic field, the retained fraction was eluted using an annexin-binding buffer (15). The number of cells collected in each column exceeded 1 X 10^6 /mL.

Cryopreservation – thawing protocol

All specimens were cryopreserved using TEST-Yolk Buffer (TYB, 20% egg yolk and 12% glycerol, Irvine Scientific, Santa Ana, CA), (16). TYB was added to the sperm samples at room temperature. An aliquot of the freezing medium equal to 25% of sperm sample volume was added to the specimen and gently mixed for 5 minutes using a Hema-Tek aliquot mixer (Miles Scientific, Elkhart, IN). This was repeated to give a final 1:1 (v/v) ratio of freezing medium to the sperm samples. Cryovials (1.5 mL, Corning, Pittsburg, PA) containing the specimens were placed in the freezer at -20°C for 8 minutes and thereafter in liquid nitrogen vapor at -80°C for 2 hours. The vials were finally transferred to liquid nitrogen tanks at -196°C. Twenty-four hours after the samples were frozen, a vial was removed and thawed by incubating it at 37°C for 20 minutes. Spermatozoa were washed and re-suspended in HTF media immediately after thawing, and sperm motility was re-assessed.

Statistical analysis

Student's paired *t*-test was used to calculate the difference between samples. Hypothesis testing was two-tailed, and P values 0<0.05 were considered statistically significant. All values are given as mean \pm SD. All calculations were performed with Statistical 6.0 software (StatSoft; Tulsa, OK).

RESULTS

Raw semen samples collected from the donors had a sperm concentration of 89.5 \pm 22.3 X 10⁶/mL and percentage motility of 64.5 \pm 6.43. The percentage motility of the different aliquots

are listed in Table 1. After MACS separation pre-freeze, the ANMB-negative sperm had significantly higher motility values (76 \pm 15.06, P = 0.03) than the raw samples (64.5 \pm 6.43) whereas the ANMB-positive sperm had significantly lower values (41 \pm 29.61, P < 0.0001). The ANMB-negative sperm consistently had higher motility than the ANMB-positive sperm (P = 0.006).

The cryopreservation-thawing process significantly decreased the motility of the spermatozoa that were not subjected to MACS separation compared with the pre-freeze control (20 ± 12.02 vs. 62.5 ± 20.45 , P = 0.0001). Similarly, the cryopreservation-thawing process decreased sperm motility in the ANMB-negative spermatozoa (38.5 ± 11.31 , P = 0.01) and in the ANMB-positive spermatozoa (4 ± 7 , P < 0.0001). However, the ANMB-negative sperm were the least affected by the cryopreservation-thawing process. The ANMB-negative sperm had higher motility following cryopreservation-thawing than the ANMB-positive sperm (P < 0.0001) and the sperm that were not separated by MACS (P = 0.003).

The use of TYB did not lead to any decrease in sperm motility. Motility values obtained after density gradient centrifugation were comparable to those obtained when TYB was added prior to cryopreservation in the samples that were not separated by MACS (62.5 ± 20.45 vs. 68.00 ± 10.33). When MACS was performed on the samples after the cryopreservation-thawing process, sperm motility was severely affected. Motility in the cryopreserved-thawed spermatozoa pre-MACS was significantly higher than that in the post-MACS ANMB-positive cells (20.00 ± 12.02 vs. 0.00 ± 0.00 , P = 0.0005) and that of the ANMB-negative cells (20.00 ± 12.02 vs. 2.58 ± 0.81 , P = 0.0005).

The percentage of sperm that survived the cryopreservation process (percentage CSR) was calculated using the following formula: 100 X post-thaw total motile sperm/pre-freeze total motile sperm. The CSR was highest in the ANMB-negative spermatozoa that were separated prior to freezing. This sperm aliquot had a significantly higher CSR than the sperm cryopreserved without MACS (76.6 \pm 59.75 vs. 30.29 \pm 16.06, P = 0.04) and the sperm that were ANMB-positive (76.6 \pm 59.75 vs. 12.7 \pm 31.19, P = 0.04).

Aliquot	Aliquot content	MACS timing	CPT	Motility (%)
Number	(n=10)			
1	Rawsemen	N/P	No	64.5 ± 6.43
2	Control (post density gradient)	N/P	No	62.5 ± 20.45
3	Control (post density gradient)	N/P	Yes	$20\pm12.02^{\text{b,c}}$
4	ANMB-negative	Pre-freeze	No	76 ± 15.06^{a}
5	ANMB-positive	Pre-freeze	No	41 ± 29.61^{a}
6	ANMB-negative	Pre-freeze	Yes	$38.5 \pm 11.31^{\text{b}}$
7	ANMB-positive	Pre-freeze	Yes	$4\pm7^{b,c}$
8	ANMB-negative	Post-thaw	Yes	$2\pm2.58^{\text{b,c}}$
9	ANMB-positive	Post-thaw	Yes	$0.00\pm0.00^{\text{b,c}}$

Table 1. Motility values obtained from spermatozoa separated and non separated by MACS. N/P = MACS was not performed; ANMB = fraction separated by annexin-V magnetic beads; CPT = cryopreservation-thawing. Values presented as mean \pm standard deviation; P < 0.05considered significant compared to: a) raw semen, b) non-cryopreserved control, and c) ANMBnegative spermatozoa separated by MACS and cryopreserved.

DISCUSSION

Despite various advances in cryopreservation methodology, the recovery rate of functional postthaw spermatozoa remains unsatisfactory. Specifically, sperm motility significantly decreases after freezing (17). In our study, the cryopreservation-thawing process significantly decreased the percentage of motile spermatozoa. Sperm motility may have decreased because of a change in temperature and the formation and dissolution of ice in the extracellular environment (4). On the other hand, TYB may be excluded as a potentially damaging factor since sperm motility was maintained after it was added. The cryopreservation-thawing process induces many changes in mammalian spermatozoa (18, 19). The lipid components of cell membranes tend to reorganize after being subjected to cooling, which may decrease the stability of the lipid bilayer (20, 21). In turn, sperm membrane integrity becomes impaired as PS translocates from the inner to the outer leaflet of the sperm plasma membrane (6, 22, 23), which is considered one of the early signs of apoptosis (7, 8). Annexin-V is a 35-36 KDa phospholipid-binding protein that specifically binds to PS (6, 8, 9) and therefore can be used to detect early deleterious changes in the sperm plasma membrane that follow cryopreservation (24).

Superparamagnetic microbeads coupled with the use of specific antibodies can effectively separate cells. Based on the antibody used, leukocytes may be extracted from ejaculates or an immature germ cell population may be separated from testicular tissue (25, 26). The beads may also be used for immunomagnetic separation of membrane-intact and non-apoptotic spermatozoa (9, 12, 14, 27). The superparamagnetic annexin V-conjugated microbeads are able to eliminate spermatozoa with externalized PS (apoptotic cells) and disintegrated plasma membranes from cryopreserved semen samples (12, 27).

In the current study, we assessed the integration of MACS coupled with annexin V-conjugated microbeads in our cryopreservation protocol. The procedure delivers 2 sperm fractions: ANMB-positive (labeled apoptotic spermatozoa) and ANMB-negative (unlabeled with intact membranes). Prior to cryopreservation, ANMB-negative spermatozoa separated by MACS had the highest motility compared with the ANMB-positive and non-separated spermatozoa. Following cryopreservation and thawing, ANMB-negative spermatozoa still had the highest motility and CSR compared with the other fractions. Therefore, it appears that the elimination of spermatozoa with early apoptotic changes positively affects motility and CSR after cryopreservation.

Sperm cryopreservation and thawing is associated with increased reactive oxygen species (ROS) production and decreased antioxidant levels (28, 29). In our earlier study, we described that ROS levels have a positive correlation with the extent of apoptotic sperm (30). Therefore, the higher cryosurvival rates in ANMB-negative sperm following cryopreservation-thawing could be related, at least in part, to the exclusion of the ROS producing ANMB-positive sperm. In support, superoxide dismutase and catalase that act as selective scavengers for ROS, were able to improve sperm recovery after cryopreservation-thawing (31).

In general, MACS is a feasible and safe method that may be used to produce a high-quality sperm fraction (9, 12, 14, 27). Although the separation columns and their magnetic field do not exert any detectable effect on the spermatozoa (12), sperm motility decreased dramatically in our current study when MACS was performed on cryopreserved-thawed samples. Because the deleterious effect of MACS was manifested only in the cryopreserved-thawed samples and not

114

in the fresh samples, we therefore believe that this procedure can be integrated in cryopreservation protocols provided it is conducted on pre-freeze specimens.

MACS, if used, should be performed before cryopreservation. In our present study, sperm motility deteriorated when MACS was performed after cryopreservation, possibly because the cryopreservation procedures (eg, centrifugation re-suspension) made the cells vulnerable to damage. Moreover, ANMB-negative spermatozoa may still display a very early phase of PS translocation. In that case, only a limited number of beads would bind to the sperm—too few to be retained in the column.

Cryopreservation of human sperm is a fundamental tool for the preservation of male fertility. However, the process of cryopreservation impairs sperm fertility. Separating a distinctive population of non-apoptotic spermatozoa with intact membranes before subjecting it to the cryopreservation-thawing process may optimize the outcomes. Our findings suggest that MACS coupled with ANMB not only can be used to perform this separation but that it enhances sperm motility and CSR following cryopreservation.

REFERENCES

- 1. Hovatta O. Cryobiology of ovarian and testicular tissue. Best Pract Res Clin Obstet Gynaecol 2003;17:331-342.
- Agarwal A, Ranganathan P, Kattal N, Pasqualotto F, Hallak J, Khayal S et al. Fertility after cancer: a prospective review of assisted reproductive outcome with banked semen specimens. Fertil Steril 2004;81:342-348.
- 3. Anger JT, Gilbert BR, Goldstein M. Cryopreservation of sperm: indications, methods and results. J Urol 2003;170:1079-1084.
- 4. Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000;60-61:481-492.
- 5. Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 2002;66:354-360.
- Glander HJ, Schaller J. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Hum Reprod 1999;5:109-115.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Methods 1995;184:39-51.
- Martin SJ, Reutelingsperger CP, McGahon AJ, Rader JA, van Schie RC, LaFace DM et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995;182:1545-1556.
- 9. Glander HJ, Schiller J, Suss R, Paasch U, Grunewald S, Arnhold J. Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines. Andrologia 2002;34:360-366.
- 10. Miltenyi S, Muller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry 1990;11:231-238.
- 11. von Schonfeldt V, Krishnamurthy H, Foppiani L, Schlatt S. Magnetic cell sorting is a fast and effective method of enriching viable spermatogonia from Djungarian hamster, mouse, and marmoset monkey testes. Biol Reprod 1999;61:582-589.
- 12. Grunewald S, Paasch U, Glander HJ. Enrichment of non-apoptotic human spermatozoa after cryopreservation by immunomagnetic cell sorting. Cell Tissue Bank 2001;2:127-133.
- 13. World Health Organization Laboratory Manual for the Examination of Human Semen and Sperm -Cervical Mucus Interaction. 4th ed. Cambridge University Press, Cambridge, New York, 1999.
- 14. Paasch U, Grunewald S, Fitzl G, Glander HJ. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. J Androl 2003;24:246-252.
- 15. Margolis LB, Namiot VA, Kljukin LM. Magnetoliposomes: another principle of cell sorting. Biochim Biophys Acta 1983;735:193-195.
- 16. Hallak J, Sharma R, Wellstead C, Agarwal A. Cryopreservation of human spermatozoa: comparison of TEST-yolk buffer and glycerol. Int J Fertil Womens Med 2000;45:38-42.
- 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;16:1191-1199.

- 18. Medeiros CM, Forell F, Oliveira AT, Rodrigues JL. Current status of sperm cryopreservation: why isn't it better? Theriogenology 2002;57:327-344.
- Martin G, Sabido O, Durand P, Levy R. Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 2004;71:28-37.
- 20. Schiller J, Arnhold J, Glander HJ, Arnold K. Lipid analysis of human spermatozoa and seminal plasma by MALDI-TOF mass spectrometry and NMR spectroscopy effects of freezing and thawing. Chem Phys Lipids 2000;106:145-156.
- 21. Fadok VA, Savill JS, Haslett C, Bratton DL, Doherty DE, Campbell PA et al. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. J Immunol 1992;149:4029-4035.
- 22. Schuffner A, Morshedi M, Oehninger S. Cryopreservation of fractionated, highly motile human spermatozoa: effect on membrane phosphatidylserine externalization and lipid peroxidation. Hum Reprod 2001;16:2148-2153.
- 23. Duru NK, Morshedi MS, Schuffner A, Oehninger S. Cryopreservation-Thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. J Androl 2001;22:646-651.
- 24. Pena FJ, Johannisson A, Wallgren M, Rodriguez-Martinez H. Assessment of fresh and frozen-thawed boar semen using an Annexin-V assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003;60:677-689.
- 25. Hipler UC, Schreiber G, Wollina U. Reactive oxygen species in human semen: investigations and measurements . Arch Androl 1998;40:67-78.
- 26. van der Wee KS, Johnson EW, Dirami G, Dym TM, Hofmann MC. Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. J Androl 2001;22:696-704.
- 27. Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. Fertil Steril 2004;81 Suppl 1:802-809.
- 28. Chatterjee S, Gagnon C. Production of reactive oxygen species by spermatozoa undergoing cooling, freezing, and thawing. Mol Reprod Dev 2001;59:451-458.
- 29. Bilodeau JF, Chatterjee S, Sirard MA, Gagnon C. Levels of antioxidant defenses are decreased in bovine spermatozoa after a cycle of freezing and thawing. Mol Reprod Dev 2000;55:282-288.
- 30. Moustafa MH, Sharma RK, Thornton J, Mascha E, Abdel-Hafez MA, Thomas AJ, Jr. et al. Relationship between ROS production, apoptosis and DNA denaturation in spermatozoa from patients examined for infertility. Hum Reprod 2004;19:129-138.
- Rossi T, Mazzilli F, Delfino M, Dondero F. Improved human sperm recovery using superoxide dismutase and catalase supplementation in semen cryopreservation procedure. Cell Tissue Bank 2001;2:9-13.