Does centrifugation and semen processing with swim up at 37°C yield sperm with better DNA integrity compared to centrifugation and processing at room temperature?

Deepthi Repalle, Priya Bhave Chittawar, Shilpa Bhandari, Geetanjali Joshi, Mansi Paranjape, Charudutta Joshi

Department of Reproductive Medicine, Mohak Hi-tech Speciality Laparoscopy and Infertility Centre, Sri Aurobindo Institute of Medical Sciences, Indore, Madhya Pradesh, India

Address for correspondence:
Ms. Deepthi Repalle, Senior Embryologist, Mohak Hi-tech Specialty Laparoscopy and Infertility center, SAIMS campus, Indore-Ujjain State Highway, M.R.-10, Indore, Madhya Pradesh.452010, India.
E-mail: deepthisarshika@gmail.com

Received: 05.05.2012
Review completed: 14.07.2012
Accepted: 07.02.2013

Access this article online

Quick Response Code:

Website: www.jhrsonline.org

DOI: 10.4103/0974-1208.112375

ABSTRACT

AIM: To evaluate whether semen processing at 37°C yield sperm with better DNA integrity compared to centrifugation and processing at room temperature (RT) by swim-up method. **SETTINGS:** This study was done at tertiary care center attached to Reproductive Medicine Unit and Medical College. **DESIGN:** Prospective pilot study. **PATIENTS:** Normozoospermic men (n = 50) undergoing diagnostic semen analysis. **MATERIALS AND METHODS:** Normozoospermic samples (World Health Organization, 2010 criteria) after analysis was divided into two aliquots (0.5 mL each); one was processed at 37°C and the other at RT by swim-up method. DNA fragmentation of both samples post wash was calculated by acridine orange method. **STATISTICAL ANALYSIS USED:** The values of sperm DNA fragmentation were represented as mean and standard error (mean ± SEM) of the mean. Paired t-test was used for calculating the sperm DNA integrity difference between post wash at RT and 37°C. **RESULTS:** Statistically significant difference was not observed in post wash sperm DNA fragmentation values at 37°C compared to RT. **CONCLUSION:** Our data represents that there was no significant difference in sperm DNA fragmentation values of samples processed at 37°C and at RT. Hence, sperm processing at 37°C does not yield sperm with better DNA integrity compared to centrifugation and processing at RT.

KEY WORDS: Acridine orange, semen preparation, sperm DNA integrity, temperature

INTRODUCTION

Sperm DNA fragmentation is being increasingly recognized as an important though controversial topic in male infertility.[1] The sperm DNA fragmentation can be induced by various factors like apoptosis, alterations in chromatin remodeling during the process of spermiogenesis, reactive oxygen species, activation of caspases and endonucleases, chemotherapy and radiotherapy and also by environmental toxicants.[1]

Sperm DNA damage can be assessed by different methods like TUNEL, comet, CMA3, *in-situ* nick translation, DBD-FISH (DNA breakage detection fluorescence *in-situ* hybridization, sperm chromatin dispersion test (SCD) and the acridine orange (AO) fluorescence staining, assessed either by microscopy or flow cytometry (sperm chromatin structure assay, SCSA).[1] AO fluoresces green when it intercalates into native DNA (double stranded and normal) as a monomer and red when it binds to denatured (single stranded) DNA as an aggregate.

Sperms used for ART (Assisted reproductive technology) are obtained after processing by one of the different methods of semen processing like density gradient, swim-up, or washing. These methods are aimed at obtaining a fraction of highly motile morphologically normal sperms with no debris. With increasing recognition of DNA fragmentation, the focus is on finding methods of sperm processing which yield sperms with normal DNA integrity.[2-4]

There is controversy on which method yields sperms with normal DNA integrity, with some studies claiming that density gradient is better, others claiming that swim-up is better method.[3,5,6] Some studies claim that DNA integrity is comparable in different methods of semen preparation.[4] There is some evidence that yield of motile sperms is higher when centrifugation is carried out at temperature of 37°C.[7] However, the processing and incubation of sperms *in vitro* itself may cause ROS (Reactive oxygen species)-induced DNA damage.[8-11]

The incubation of prepared ejaculate sperm results in increase in DNA fragmentation index, a measure of DNA integrity. This change is more in samples incubated at 37°C compared to room temperature (RT).[10]

We conducted this study to explore whether centrifugation and processing of semen samples at 37°C resulted in better DNA integrity compared to samples centrifuged and processed at RT.

MATERIALS AND METHODS

Patients

This study was a prospective study done on 50 patients with mean age of 33.14 ± 0.93 years undergoing diagnostic semen analysis in our Reproductive Medicine Unit, whose samples were normozoospermic according to World Health Organization (WHO) 2010 criteria.[12] Sexual abstinence was between 2 and 7 days for the subjects undergoing the test.

Semen analysis

Patients were asked to collect semen samples in sterile nontoxic containers by masturbation. They were evaluated according to WHO 2010 criteria.[12] Sperm concentration was calculated by using Neubauer's chamber with 10 μL of diluted sample with semen diluting fluid in 1:20 ratio. Motility was assessed by placing a 10 μL drop of semen sample on a slide and covered with cover slip (22 × 22). Under ×40 magnification, 200 spermatozoa were evaluated for motility grading. Morphology was assessed using Diff-Quik staining method under oil immersion.[12]

Semen processing

Semen samples were processed by swim-up method, 1:2 ratio of semen sample and sperm washing medium with HEPES buffer (Quinn's medium, Sage, USA) with 5% Human Serum Albumin (HSA) were gently mixed in 15 mL conical tubes (BD falcon, 2095, NJ). Samples were centrifuged at 300xg for 10 min, pellet was resuspended in 1.0 mL of sperm wash medium and again centrifuged at 200xg for 5 min. 0.5 mL of the medium was layered over the final pellet and incubated for 30 min.

After the completion of semen analysis, the remaining semen sample was divided into two parts of 0.5 mL each. One part of the semen sample (0.5 mL) was used for semen preparation at RT and the other part (0.5 mL) was used for semen preparation at 37°C. In semen samples processed at RT, the sample was centrifuged at RT (REMI R-8C, India) and incubation after the wash was done at RT in air for 30 min to not more than 1 h.

For semen samples processed at 37°C the sample was centrifuged at 37°C (Spermfuge, Fornax, Shivani Scientific Pvt. Ltd., India) and postwash incubation was done at 37°C in test tube block heater in air (Ketan Digi block, Shivani Scientific Pvt. Ltd., India) for 30 min to not more than 1 h. After the assessment of sperm DNA fragmentation, all the semen samples were discarded.

Assessment of sperm DNA fragmentation

Smears with 10 μL of pre-and postwash samples at RT and 37°C were prepared and air dried at RT. Slides were fixed overnight in freshly prepared carnoy's solution (methanol: Glacial acetic acid, 3:1 vol/vol). After air drying, slides were stained with AO (0.19 mg/mL) prepared daily.[13] Briefly, a stock solution of AO was prepared (1 g/L in distilled water) and stored in the dark at 4°C. The staining solution consisted of 10 mL of stock solution, 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M $Na_2HPO_4 7H_2O$. The final pH of the solution was adjusted to 2.5. The staining solution (2-3 mL) was applied to the slides for 5 min. Slides were gently rinsed in a stream of deionized water and coverslips were applied.

Slides were evaluated for sperm DNA fragmentation with a fluorescence microscope (Olympus C × 31) under oil at ×1000. Normal, mature, and intact sperm fluoresces were green, whereas red indicates fragmented and denatured sperms. Orange or yellow heads, as well as those displaying green and red color simultaneously, were also considered as fragmented sperms.[13] A minimum of 200 spermatozoa were evaluated in each slide in two replicates to calculate the sperm DNA fragmentation. Slides were evaluated for sperm DNA fragmentation by same andrologist (D.R) for consistency.

Statistical analysis

The percentage of sperm DNA fragmentation values was represented as mean ± standard error of mean. Paired *t*-test was used to calculate the level of significance using SPSS software (version 16.0). A P of < 0.01 was considered statistically significant.

RESULTS

A total of 50 samples smear of prewash and postwash at RT and 37°C were analyzed for sperm DNA fragmentation. The mean percentage (±SEM) of sperm DNA fragmentation of

prewash samples was 20.16% ± 2.06%, whereas postwash at RT was 11.58 ± 1.36% and postwash at 37°C was 13.48% ± 1.69% [Table 1].

Statistically, extremely significant difference was observed between the prewash and postwash at RT sperm DNA fragmentation mean percentage values ($t = 5.169$, $P < 0.0001$), similarly statistically significant difference was observed between the prewash and postwash at 37°C sperm DNA fragmentation mean percentage values ($t = 5.090$, $P < 0.0001$).

In our study, statistically significant difference was not observed in sperm DNA fragmentation values in postwash at 37°C compared to RT ($t = 1.6151$, $P = 0.1127$) [Figure 1].

DISCUSSION

AO method has been used in many studies for the evaluation of sperm DNA integrity.[13-16] The principle of AO method was similar to sperm chromatin structure assay (SCSA) method except for the number of sperm cells counted. The fertility threshold value determined by Evenson and colleagues (1999) for SCSA method between infertile and fertile men was correlating with AO values.[14] AO values are also good predictors of fertilization[15,16] or pregnancy rates[16,17] and also a determing factor whether to go for *in vitro* fertilization or intracytoplasmic sperm injection.[18]

Table 1: Sperm DNA fragmentation percentage in prewash and postwash at room temperature and 37°C

Sample	Sperm DNA fragmentation percentage (Mean±SEM)
Prewash	20.16±2.06
Post wash at RT	11.58±1.36
Post wash at 37°C	13.48±1.69

SEM = Standard error of the mean, RT = Room temperature

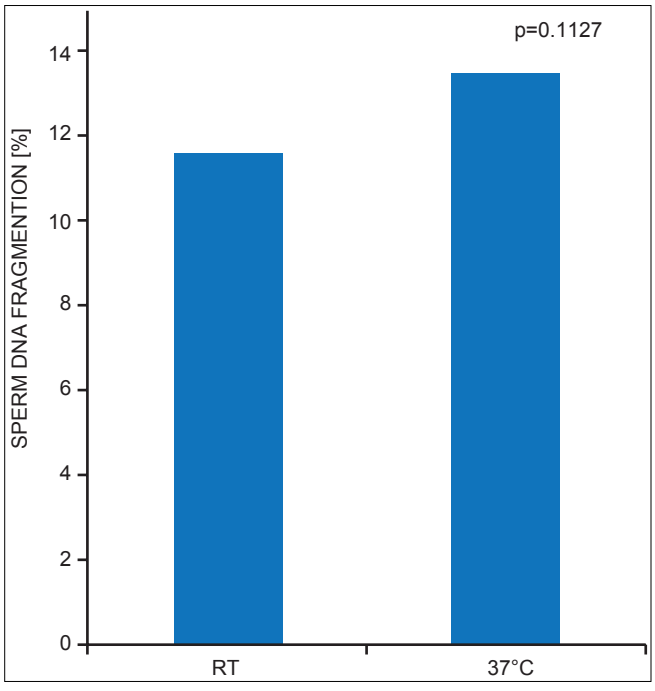

Figure 1: Effect of temperature on sperm DNA fragmentation in post wash at room temperature and 37°C

The proportion of sperm with nuclear chromatin maturity, as assessed by aniline blue,[19] AO staining,[19,20] or electrophoretic investigation,[21] is increased by selection of motile sperm by swim-up or density gradient centrifugation. In our study, when postwash (both at RT and 37°C) chromatin integrity values were compared to prewash, higher sperm chromatin integrity was observed in postwash samples (both at 37°C and at RT). Hence, washing procedure enhances the sperm chromatin integrity by eliminating the dead sperms and debris.[22,23]

Sperm DNA quality predicts the intrauterine insemination (IUI) outcome, >12% DNA fragmentation by AO method does not resulted in any pregnancies in IUI.[24] Out of the 50 samples assessed for sperm DNA fragmentation, 21 samples have >12% of sperm DNA fragmentation when processed at RT and 23 samples have >12% of sperm DNA fragmentation when processed at 37°C. Hence, the conventional semen processing at RT has equivalent results compared to the sperm processing at 37°C using a temperature-regulated centrifuge.

However, the average percentage of DNA fragmentation values were less than 12% in semen samples processed at RT (11.58% ± 1.36%), whereas more than 12% in semen samples processed at 37°C (13.48% ± 1.69%). The sperm DNA fragmentation values were elevated in samples processed at 37°C compared to RT. There was a trend toward better DNA integrity in samples processed at RT even though the difference was not statistically significant with this sample size.

Prospective studies with larger sample size are needed to verify this finding. Processing semen samples at 37°C requires expensive temperature-controlled centrifuges and test tube warmers. In our study, DNA fragmentation of processed sample is similar at both RT and 37°C irrespective of temperature during the processing. If the DNA fragmentation values of sample after processing are not related to temperature at which it is processed, we can do away with costly equipment needed for processing at 37°C.

CONCLUSION

Our data represents that there was no significant difference in sperm DNA fragmentation values of samples processed at 37°C and at RT, hence sperm processing at 37°C does not yield sperm with better DNA integrity compared to centrifugation and processing at RT.

REFERENCES

1. Sakkas D, Alvarez JG. Sperm DNA fragmentation: Mechanisms of origin, impact on reproductive outcome, and analysis. Fertil Steril 2010;93:1027-36.
2. Hammadeh ME, Nkemayim DC, Georg T, Rosenbaum P, Schmidt W. Sperm morphology and chromatin condensation before and after semen processing. Arch Androl 2000;44:221-6.
3. Zini A, Finelli A, Phang D, Javi k. Influence of semen processing technique on human sperm DNA integrity. Urology 2000;56:1081-4.
4. Jayaraman V, Upadhay D, Narayan PK, Adiga SK. Sperm processing by swim-up and density gradient is effective in elimination of sperm with DNA damage. J Assist Reprod Genet 2012;29:557-63.
5. Bormann CL, Rocha AM, Hassun PA, Motta ELA, Serafini P, Smith GD. The effect of sperm separation on sperm chromatin decondensation and motility at 0 and 24 hours of culture. Fertil Steril 2008;90:S452-3.
6. Sakkas D, Manicardi GC, Tomlinson M, Mandrioli M, Bizzaro D, Bianchi PG, *et al*. The use of two density gradient centrifugation techniques and the swim-up method to separate spermatozoa with chromatin and nuclear DNA anomalies. Hum Reprod 2000;15:1112-16.
7. Franken DR, van Wyk R, Stoumann C, Avari K. Temperature controlled centrifugation improves sperm retrieval. Andrologia 2011;43:217-21.
8. Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: Protective significance of seminal plasma. Mol Hum Reprod 1998;4:439-45.
9. Zini A, Mak V, Phang D, Jarvi K. Potential adverse effect of semen processing on human sperm deoxyribonucleic acid integrity. Fertil Steril 1999;72:496-9.
10. Matsuura R, Takeuchi T, Yoshida A. Preparation and incubation conditions affect the DNA integrity of ejaculated human spermatozoa. Asian J Androl 2010;12:753-9.
11. Zhang XD, Chen MY, Gao Y, Han W, Liu DY, Huang GN. The effects of different sperm preparation methods and incubation time on the sperm DNA fragmentation. Hum Fertil (Camb) 2011;14:187-91.
12. World Health Organization. WHO Manual for the Examination of Human Sperm and Sperm-Cervical Mucus Interaction. 5th ed. Switzerland: WHO press; 2010.
13. Erenpreiss J, Bars J, Lipatnikova V, Erenpreisa J, Zalkalns J. Comparative study of cytochemical tests for sperm chromatin integrity. J Androl 2001;22:45-53.
14. Evenson DP, Jost LK, Marshall D, Zinaman MJ, Clegg E, Purvis K, de Angelis P, Claussen OP. Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in human fertility clinic. Hum Reprod. 1999;14:1039-49.
15. Liu DY, Baker HW. Sperm nuclear chromatin normality: Relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates *in vitro*. Fertil Steril 1992;58:1178-84.
16. Hoshi K, Katayose H, Yanagida K, Kimura Y, Sato A. The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. Fertil Steril 1996;66:634-9.
17. Tejada RI, Mitchel JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril 1984;42:87-91.
18. Hammadeh ME, al-Hasani D, Stieber M, Rosembaum P, Kupker D, Diedrich K, *et al*. The effect of chromatin condensation (aniline blue staining) and morphology (strict criteria) of human spermatozoa on fertilization, cleavage and pregnancy rates in an intracytoplasmic sperm injection programme. Hum Reprod 1996;11:2468-71.
19. Le Lannou D, Blanchard Y. Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or swim-up procedure. J Reprod Fertil 1988;84:551-6.
20. Golan R, Schochat L, Weissenberg R, Soffer Y, Marcus Z, Oschry Y, *et al*. Evaluation of chromatin condensation in human spermatozoa: A flow cytometry assay using acridine orange staining. Mol Hum Reprod 1997;3:47-54.
21. Colleu D, Lescoat D, Gouranton J. Nuclear maturity of human spermatozoa selected by swim-up or by Percoll gradient centrifugation procedures. Fertil Steril 1996;65:160-4.
22. Angelopoulos T, Moshel YA, Lu L, Macanas E, Grifo JA, Krey LC. Simultaneous assessment of sperm chromatin condensation and morphology before and after separation procedures: Effect on the clinical outcome after *in vitro* fertilization. Fertil Steril 1998;69:740-7.
23. Fariello RM, Del Giudice PT, Spaine DM, Fraietta R, Bertolla RP, Cedenho AP. Effect of leukocytospermia and processing by discontinuous density gradient on sperm nuclear DNA fragmentation and mitochondrial activity. J AssistReprod Genet 2009;26:151-7.
24. Duran EH, Morshedi M, Taylor S, Oehninger S. Sperm DNA quality predicts intrauterine insemination outcome: A prospective cohort study. Hum Reprod 2002;17:3122-8.

How to cite this article: Repalle D, Chittawar PB, Bhandari S, Joshi G, Paranjape M, Joshi C. Does centrifugation and semen processing with swim up at 37°C yield sperm with better DNA integrity compared to centrifugation and processing at room temperature?. J Hum Reprod Sci 2013;6:23-6.

Source of Support: Nil, **Conflict of Interest:** None declared.