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Sperm swim-up techniques and DNA fragmentation

E.V.Younglai[1,2,3], D.Holt[1,2], P.Brown[1,2], A.Jurisicova[1,2] and R.F.Casper[1,2]

[1]Division of Reproductive Sciences, The Toronto General Hospital, The Samuel Lunenfeld Research Institute, and [2]Department of Obstetrics and Gynecology, University of Toronto, Toronto, Ontario, Canada

[3]To whom correspondence should be addressed at: Department of Obstetrics and Gynaecology, McMaster University Medical Centre, Hamilton, Ontario, Canada L8N 3Z5. E-mail: younglai@mcmaster.ca

BACKGROUND: Swim-up techniques for sperm separation may have detrimental effects on sperm DNA. We wished to determine whether the normal swim-up method with centrifugation used in our laboratory, which involves a centrifugation step, was harmful to sperm compared with swim-up without centrifugation. METHODS: Semen samples were obtained from patients undergoing IVF or andrology assessment. An aliquot was removed for fixation and subsequent DNA fragmentation determination. The remaining sample was divided into two equal parts, which were subjected to swim-up either with (normal swim-up) or without (direct-swim-up) centrifugation. Semen analysis was performed both before and after swim-up. DNA fragmentation, in spermatozoa previously fixed in 4% paraformaldehyde, was assessed by the terminal transferase-mediated DNA end-labelling procedure (TUNEL). The percentage of spermatozoa with DNA damage after each swim-up technique was compared with that in the original semen sample. RESULTS: DNA damage was <5% in most samples. No significant change in DNA fragmentation was observed between the two swim-up procedures, although the 'normal' swim-up sample had significantly less DNA fragmentation than the pre-swim-up sample. CONCLUSIONS: We conclude that our normal swim-up technique caused no more DNA damage to spermatozoa from normal semen samples than a direct swim-up technique that involved no centrifugation step.

Key words: DNA fragmentation/semen analysis/spermatozoa/swim-up

Introduction

Sperm preparation techniques involving centrifugal pelleting of unselected spermatozoa for intrauterine insemination or IVF have been considered to result in sperm damage and subsequent iatrogenic failure of pregnancy in some cases (Mortimer, 1991). A suggested mechanism for this effect comes from the observation that centrifugal pelleting of spermatozoa may result in the generation of reactive oxygen species (ROS), 2–5-fold above baseline, within 5 min (Aitken and Clarkson, 1988). We have also demonstrated that exposure of normal spermatozoa to ROS can cause a 2-fold increase in sperm DNA damage within 60 min and a 4-fold increase by 2 h (Lopes *et al*., 1998a). Low levels of ROS, may enhance sperm function and rates of sperm–oocyte binding, by compacting DNA (Aitken, 1999). As the level of oxidative stress increased, spermatozoa exhibited significantly elevated levels of DNA damage and yet continued to express an enhanced capacity for sperm–oocyte fusion (Aitken *et al*., 1998). However, at a critical high level (Twigg *et al*., 1998b) ROS induces irreversible damage to the sperm membrane by lipid peroxidation, causes DNA damage and a dramatic decline in sperm motility, thereby impairing the fertilizing ability of the spermatozoa. In spite of such damage, similar spermatozoa used for intracytoplasmic sperm injection can lead to pronucleus formation (Twigg *et al*., 1998a).

In an attempt to prevent damage by centrifugation and generation of ROS, other methods of sperm preparation have been developed using density gradient media or direct swim-up from the original sperm sample (Mortimer, 1991). The latter method has recently been recommended for sperm preparation (World Health Organization, 1999). Some studies have found that Percoll did not improve sperm quality as measured by motility, velocity, percentage normal morphology and intact acrosomes (Ng *et al*., 1992) whereas others have found improved performance as judged by the hamster egg penetration assay (Chan and Tucker, 1992). Assessment of DNA integrity following any preparation method has been limited to date. It has recently been reported that Percoll preparation of spermatozoa can lead to a 2-fold increase in denatured spermatozoa (Zini *et al*., 1999). A double wash centrifugation followed by a swim-up from the pellet actually improved sperm chromatin structure properties (Spano *et al*., 1999). It was therefore of interest to determine whether the normal swim-up technique involving centrifugation, as used in our IVF programme, could lead to sperm DNA damage, and to compare this with one that does not use a centrifugation step.

Materials and methods

Twenty-nine semen samples were collected from men undergoing semen analysis in the Andrology and IVF clinics at the Toronto

General Hospital. Twenty-four of these samples were subsequently used for IVF. Of these, it was possible to recover 16 sperm samples from the culture media 24 h after insemination. These 16 samples were processed further for DNA fragmentation. Informed consent for the use of the spermatozoa was obtained according to the institutional ethics and research review board. The mean ± SEM age was 37.4 ± 0.9 years with a range of 29–50, the median was 37 years with an interquartile range of 34–40.

Semen samples were collected after 48 h of abstinence. A small aliquot (~50 µl) of the original semen sample was fixed in 4% paraformaldehyde to determine the percentage DNA fragmentation. Semen analysis was performed according to published methods (World Health Organization, 1987). Motility was assessed qualitatively since most of the samples were used for insemination in IVF.

Normal swim-up technique

Sperm preparation using a double wash and centrifugation procedure was carried out as previously described (Lopes *et al*., 1998a) and at the time this was the normal method used in our laboratories. Briefly, one of the two portions of semen was diluted with 2×volume of Ham's F10 (Gibco, Life Technologies, Grand Island, NY, USA) containing 10% synthetic serum substitute (SSS) (Irvine Scientific, Irvine, CA, USA) and then centrifuged at 220 *g* for 10 min. The supernatant was transferred to another tube. The pellet was resuspended in 2 ml of Ham's F10 with 10% SSS. Both supernatant and pellet were centrifuged at 220 *g* for 10 min and the supernatants discarded. The pellets were combined and resuspended in 0.5 ml of Ham's F10 with 10% SSS. This suspension was layered gently under 1 ml of Ham's F10 with 10% SSS. The tube was slanted and incubated for 1 h at 37°C in a 5% CO_2 incubator. The swim-up sperm fraction was then fixed in 4% paraformaldehyde for later determination of DNA fragmentation.

Direct swim-up technique

The second portion of each semen sample was used for the direct semen swim-up procedure without centrifugation. The semen sample, 0.10–0.15 ml, was layered gently under 0.3 ml of Ham's F10 with 10% SSS and incubated for 1 h at 37°C in a 5% CO_2 incubator. The swim-up portion was then fixed in 4% paraformaldehyde as above.

Sperm DNA fragmentation

DNA fragmentation in the spermatozoa was measured using a modification of the method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin end-labelling (TUNEL) as we previously described (Lopes *et al*., 1998a). The air-dried slides were washed in phosphate-buffered saline (PBS, pH 7.4 prepared in house), then spermatozoa were permeabilized with Triton X-100 (Caledon Laboratories Ltd, ON, Canada). A buffer containing 10 U of TdT enzyme (Pharmacia LKB Biotech, Piscataway, NJ, USA), 3 µmol/l biotin-16-dUTP (Boehringer Mannheim, Laval, PQ, Canada), 12 µmol/l dATP Pharmacia LKB Biotech), and 0.1% Triton X-100 was added to the slide and allowed to incubate at 37°C for 60 min. Following TdT exposure, spermatozoa were treated with a staining buffer containing 1% streptavidin/Texas red anti-biotin (Calbiochem-Novabiochem Corporation, La Jolla, CA, USA) and incubated at 4°C in the dark for 30 min. The stained cells were washed in PBS and counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Sigma, St Louis, MO, USA), which stains all chromatin, prior to analysis. Percentage of spermatozoa with DNA damage was determined by dividing TUNEL positive spermatozoa by total spermatozoa analysed as determined by DAPI staining. At least 200 cells were counted to determine the percentage.

Statistical analysis

Statistical analyses were done using the SPSS statistical package version 10 for windows. One-way analysis of variance with Tukey's post hoc testing and, where appropriate, linear regression were applied to determine the correlation between the DNA fragmentation in the original semen sample and semen parameters. The Wilcoxon signed ranks test was also used to compare differences between groups for DNA fragmentation.

Results

The results of the various analyses are shown in Table I. Compared with original semen, there was a significant (~2-fold, $P < 0.05$) decrease in sperm concentration after standard swim-up and a 10-fold decrease with the direct swim-up ($P < 0.01$). In contrast, motility and morphology of spermatozoa after both swim-up methods were significantly increased ($P < 0.05$). DNA fragmentation was ~2% in the original semen, and no increase was observed after either method of sperm preparation. Two of the original semen samples had DNA fragmentation of 30.8 and 26.7% respectively. These fell to 1.65 and 2.45% after the normal swim-up technique, but were still 21 and 6.2% after the direct swim-up procedure. Spermatozoa from these two samples were used after the normal swim-up method and the fertilization was >80% in both cases. In fact, the fertilization rate was 76 ± 5.3% for the 24 samples used in IVF. Moreover, in the 16 samples where processed spermatozoa were obtained on the day after insemination of oocytes, there was no significant increase in the degree of DNA fragmentation (1.51 ± 0.40%). In none of these samples was the DNA fragmentation >10%.

When the percentage DNA fragmentation was correlated with concentration, motility and morphology by linear regression, there was a significant interaction between DNA fragmentation in the original semen and the morphology of the same sample ($P = 0.045$). Analysis of the DNA fragmentation with the Wilcoxon signed ranks tests for non-parametric data showed that there was a significant difference between the original semen and spermatozoa prepared by the normal swim-up technique ($P = 0.045$). There was no significant difference between DNA fragmentation in the original sample and spermatozoa prepared by the direct swim-up method. Although the DNA fragmentation in the spermatozoa processed by the direct method was lower than that in the original semen, it was higher than that seen in the normal swim-up procedure.

Discussion

The results of this study support the notion that the double centrifugation steps of swim-up, as practised in our laboratory, do not induce DNA damage to normal human spermatozoa as assessed by the TUNEL assay. Further support for the lack of damage to spermatozoa prepared by the swim-up procedure, similar to that described here, has been reported (Spano *et al*., 1999). Using a sperm chromatin structure assay on 19 normozoospermic subjects, they demonstrated that the post-rise sample contained a subpopulation of spermatozoa characterized by a general improvement of biochemical, morphological and kinetic parameters. This subpopulation also exhibited improved

Table I. Semen analyses in original semen and after two different swim-up techniques

	Original semen	Normal swim-up	Direct swim-up
Concentration ($\times 10^6$/ml) (mean ± SEM)	58.61 ± 8.31	35.18 ± 6.92[a]	6.45 ± 1.29[b]
Motility (%) (mean ± SEM)	61.38 ± 2.27	96.38 ± 0.45[a]	93.26 ± 1.57[a]
Morphology (% normal) (mean ± SEM)	68.52 ± 1.49	91.38 ± 0.42[a]	90.56 ± 0.55[a]
DNA fragmentation (%) (mean ± SEM)	2.36 ± 1.37	1.08 ± 0.50[c]	1.78 ± 0.80
Concentration ($\times 10^6$/ml) [median (IQR)]	50.50 (25.7–78.5)	23.20 (6.8–53.5)	4.10 (3.2–5.8)
Motility (%) [median (IQR)]	60.00 (55.0–71.5)	97.00 (95.0–98.0)	95.00 (91.0–98.0)
Morphology (% normal) [median (IQR)]	69.00 (64.5–75.0)	90.00 (90.0–95.0)	90.00 (90.0–90.0)
DNA fragmentation (%) [median (IQR)]	0.010 (0.01–0.4)	0.010 (0.01–1.1)	0.010 (0.01–1.5)

A total of 29 semen samples were split into different aliquots and processed.
[a]$P < 0.05$, [b]$P < 0.01$, versus original semen sample (one-way ANOVA), $n = 29$.
[c]$P < 0.05$, versus original semen sample (Wilcoxon's signed rank test).
IQR = interquartile range.

chromatin structure properties. The improvements in motility and proportion of normal cells noted by Spano *et al*. (1999) are similar to those of our study (Table I). Although not significantly different, the DNA fragmentation in the spermatozoa prepared by the normal swim-up method was lower than that prepared by the direct swim-up method. Analysis of the DNA fragmentation showed that the mean percentages were not significantly different between the different preparation methods and the original sample. This lack of significance is probably due to the high levels of DNA damage in two samples. However, the non-parametric test showed there was an improvement in DNA fragmentation between the original sample and the normal swim-up method.

Another study (Zini *et al*., 1999), using the same chromatin structure assay of Evenson (Evenson *et al*., 1980), found in 25 non-azoospermic men that with two- or four-layered Percoll density-gradient gradient centrifugation, there was a 2-fold increase in denatured sperm DNA when compared to whole semen. They did not compare any other sperm preparation method. These latter results call into question whether Percoll density preparation of spermatozoa actually improves sperm quality. After our study was completed, a new edition of the *Laboratory Manual* (World Health Organization, 1999) appeared in which it was recommended that the standard method for sperm preparation should avoid centrifugation. The results of our study, however, suggests that there is little to be gained in terms of DNA fragmentation but much is lost in terms of recovery when centrifugation is avoided.

Given the speed at which ROS can cause sperm damage (Aitken and Clarkson, 1988; Lopes *et al*., 1998a) one would have expected that DNA fragmentation would be increased after the normal (centrifugation) swim-up, and especially at 24 h after insemination. In fact, the normal swim-up procedure for preparing spermatozoa appears to result in very low rates of sperm DNA damage and appears to be free of any detrimental effect on the fertilizing ability of the spermatozoa as noted in the DNA data from samples used for IVF. Even the two samples with high DNA fragmentation in the original semen improved after the normal swim-up procedure and were effective in fertilizing oocytes. It would have been interesting to compare the fertilizing ability of sperm prepared by the two methods but this was not the original objective of the study.

Notwithstanding the fact that DNA repair is feasible, it is possible that the damage noted (Mortimer, 1991) may be due to the three centrifugation steps normally carried out by some laboratories (Aitken and Clarkson, 1988). Centrifugation removes the antioxidant properties (Aitken, 1999) of seminal plasma and induces iatrogenic DNA damage in spermatozoa (Twigg *et al*., 1998c). This is more pronounced with poor quality sperm samples with numerous leukocytes. In fact, 55% of oligozoospermic patients were found to have defective sperm function as assessed by the hamster egg penetration assay, and elevated production of ROS (Aitken *et al*., 1989). Semen samples with poor semen analysis parameters and poor fertilization and cleavage rates in IVF, were found to have increased proportion of spermatozoa with DNA fragmentation (Sun *et al*., 1997). Such sperm samples with high DNA fragmentation, when used for ICSI, were correlated with failed fertilization (Lopes *et al*., 1998b). In the current study >90% of the original samples were normal as judged by the semen parameters. This fact may have precluded the observation of higher percentage of sperm DNA damage.

Linear regression analysis of the data in this study indicated that there was a significant relationship between DNA fragmentation and sperm morphology. This is in contrast to other data (Irvine *et al*., 2000) showing that sperm concentration most accurately reflected the incidence of sperm DNA damage and (Hughes *et al*., 1996) that spermatozoa from normospermic men, in contrast to that from infertile men, were more susceptible to DNA damage by irradiation. A significant negative correlation between semen quality as assessed by motility, morphology and concentration and DNA damage has been demonstrated (Sun *et al*., 1997). Using a TUNEL-coupled flow cytometry method, it was shown that the extent of sperm DNA fragmentation in unselected and swim-up spermatozoa was positively related to abnormal morphology and associated with defects of the sperm tail (Muratori *et al*., 2000). A negative correlation was found between DNA breakage and progressive motility. In a similar study, using a TUNEL assay, with spermatozoa from infertile men (Barroso *et al*., 2000), low sperm motility was associated with high DNA damage. These variations in correlations among the different studies may be a reflection of the variations in the methods used for semen analyses. The morphological analysis employed in our

study can be criticized in view of the stricter criteria being employed in many laboratories. However, there is a common observation among all the studies that DNA damage is related to sperm parameters.

In summary, our results suggest that swim-up separation of motile spermatozoa from normal semen, either with or without centrifugation, does not increase the level of DNA damage. In addition, double centrifugation of normal spermatozoa does not impair the fertilizing ability of the recovered spermatozoa.

References

Aitken, R.J. (1999) The Amoroso lecture. The human spermatozoon — a cell in crisis? *J. Reprod. Fertil*., **115**, 1–7.

Aitken, R. J. and Clarkson, J. S. (1988) Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J. Androl*., **9**, 367–376.

Aitken, R.J., Clarkson, J.S., Hargreave, T.B. *et al*. (1989) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligozoospermia. *J. Androl*., **10**, 214–220.

Aitken, R.J., Gordon, E., Harkiss, D. *et al*. (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod*., **59**, 1037–1046.

Barroso, G., Morshedi, M. and Oehninger, S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum. Reprod*., **15**, 1338–1344.

Chan, S.Y.W. and Tucker, M.J. (1992) Differential sperm performance as judged by the zona-free hamster egg penetration test relative to differing sperm penetration techniques. *Hum. Reprod*., **7**, 255–260.

Evenson, D.P., Darzynkiewics, Z. and Melamed, M.R. (1980) Comparison of human and mouse sperm chromatin heterogeneity to fertility. *Science*, **240**, 1131–1133.

Hughes, C.M., Lewsi, S.E.M., McKelvey-martin, V.J. *et al*. (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol. Hum. Reprod*., **2**, 613–619.

Irvine, D.S., Twigg, J.P., Gordon, E.L. *et al*. (2000) DNA integrity in human spermatozoa: relationships with semen quality. *J. Androl*., **21**, 33–44.

Lopes, S., Jurisicova, A., Sun, J-G. and Casper R.F. (1998a) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Hum. Reprod*., **13**, 896–900.

Lopes, S., Sun, J-G., Jurisicova, A., *et al*. (1998b) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil. Steril*., **69**, 528–532.

Mortimer, D. (1991) Sperm preparation techniques and iatrogenic failures of in-vitro fertilization. *Hum. Reprod.*, **6**, 173–176.

Muratori, M., Piomboni, P., Baldi, E. *et al*. (2000) Functional and ultrastructural features of DNA-fragmented human sperm. *J. Androl*., **21**, 903–912.

Ng, F.L.H., Liu, D.Y. and Baker, H.W.G. (1992) Comparison of Percoll, mini-Percoll and swim-up methods for sperm preparation from abnormal semen samples. *Hum. Reprod*., **7**, 261–266.

Spano, M., Cordelli, E., Leter, G. *et al*. (1999) Nuclear chromatin variations in human spermatozoa undergoing swim-up and cryopreservation evaluated by the flow cytometric sperm chromatin structure assay. *Mol. Hum. Reprod*., **5**, 29–37.

Sun, J-G., Jurisicova, A. and Casper, R.F. (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. *Biol. Reprod*., **56**, 602–607.

Twigg, J.P., Irvine, D.S. and Aitken, R.J. (1998a) Oxidative damage to DNA in human spermatozoa does not preclude pronucleus formation at intracytoplasmic sperm injection. *Hum. Reprod*., **13**, 1864–1871.

Twigg, J., Fulton, N., Gomez, E. *et al*. (1998b) Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Hum. Reprod*., **13**, 1429–1436.

Twigg, J., Irvine, D.S., Houston, P. *et al*. (1998c) Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol. Hum. Reprod*., **4**, 439–445.

World Health Organization (1987) *Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 2nd edn. Cambridge University Press, Cambridge.

World Health Organization (1999) *Laboratory Manual for the Examination of Human Semen and Semen–Cervical Mucus Interaction*, 4th edn. Cambridge University Press, Cambridge.

Zini, A., Mak, V., Phang, D. and Jarvi, K. (1999) Potential adverse effect of semen processing on human sperm deoxyribonucleic acid integrity. *Fertil. Steril*., **72**, 496–499.

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