Reduced Senescence and Retained Nuclear DNA Integrity in Human Spermatozoa Prepared by Density Gradient Centrifugation¹

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Purpose: To investigate whether removal of extraneous cells and immotile spermatozoa from a sperm preparation by density gradient centrifugation could help to maintain normal spermatozoa in a viable state and retain their deoxyribonucleic acid integrity.

Methods: Sperm motility was assessed on a daily basis in aliquots of neat semen, extended semen, and spermatozoa prepared on a *PureSperm*[®] density gradient. At the same time, aliquots of each sperm sample were preserved for TUNEL assay and nick translation.

Results: Spermatozoa prepared using density gradient centrifugation survived three times as long as spermatozoa in neat semen or in extended semen. Both deoxyribonucleic acid integrity and sperm motility were retained in the gradient preparations.

Conclusions: Preparing spermatozoa by density gradient centrifugation is advantageous in prolonging sperm survival and maintaining deoxyribonucleic acid integrity, presumably by removing sources of reactive oxygen species. Stored spermatozoa could be used for a second attempt at fertilization if oocyte immaturity was suspected.

KEY WORDS: Oocyte immaturity; *PureSperm*; reduced senescence; sperm DNA integrity.

INTRODUCTION

Oocytes may fail to fertilize on the first day of in vitro fertilization (IVF) because of either oocyte immaturity or sperm abnormality. In some cases related to oocyte immaturity, the male partner may be unavailable or unable to provide a second semen sample and no frozen spermatozoa are available to attempt to fertilize the oocytes. The clinician is faced with the dilemma of either abandoning the IVF attempt or using the semen sample from the previous day.

There is evidence that human spermatozoa retain good motility over a period of several days when prepared appropriately. For example, in one study, human spermatozoa prepared using the swim-up method and cultured in medium alone remained motile for up to 2 days while after 12 days of coincubation, $15 \pm 4\%$ of spermatozoa in direct contact with epithelial cells remained motile (1), although their ability to fertilize was not determined. A consequent problem with maintaining spermatozoa in vitro is that the prolonged culture causes damage at both the nuclear and organelle level. One particular problem is damage to the nuclear DNA (2). This has further implications as it may hinder embryonic development or cause pregnancy failure (3–8). Recently, spermatozoal DNA integrity was found to be closely associated with time to pregnancy or with fertilization in IVF and subsequent pregnancy (9–12).

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Sperm nuclear DNA damage, however, may be caused by factors other than sperm senescence, for example reactive oxygen species (ROS) from contaminating neutrophils and immotile spermatozoa (13–17). Therefore, a sperm preparation technique that removes the sources of ROS may help to retain chromatin integrity and stability, thus contributing to a successful outcome from (assisted reproduction technologies, ART) with “ageing” spermatozoa. Density gradient centrifugation is a sperm preparation technique which enables highly motile spermatozoa to be separated from the seminal plasma, while removing dead or damaged spermatozoa, leukocytes, and cellular debris from the ejaculate, all of which may be sources of ROS. *PureSperm*[®] is one such density gradient product which has been commercially available since 1996. Spermatozoa processed on *PureSperm*[®] gradients are widely used in fertility clinics, giving good fertilization results (e.g., 12,18,19).

The objective of the present study was to investigate whether preparation of spermatozoa using density gradient centrifugation followed by storage at room temperature in a sperm maintenance medium could provide a source of spermatozoa with retained nuclear DNA integrity, potentially for use in subsequent IVF attempts. The study addressed the following topics: (i) do human spermatozoa show increased nuclear DNA damage as they age while maintained in a sperm medium; (ii) could preparation by density gradient centrifugation extend the useable life of spermatozoa by delaying their senescence.

MATERIALS AND METHODS

Preparation of Semen Samples

Ejaculates were obtained from donors aged 20–35 whose fertility status was unknown. Liquefied ejaculates were subjected to routine semen analysis and aliquots were either fixed in paraformaldehyde for TUNEL assay or frozen for subsequent nick translation (raw ejaculate = Group A).

Experiment 1

Sperm samples were prepared as follows: Half of each of nine ejaculates (up to 1.5 mL) was carefully layered on density gradients made up of *PureSperm*[®] 40 and *PureSperm*[®] 80 (2 mL per layer), which were then centrifuged at 300g at room temperature for 20 min. The sperm pellets were resus-

pended in 5-mL *PureSperm*[®] wash and centrifuged at 500g for 10 min before resuspending the resulting sperm pellet in 1-mL sperm maintenance medium, *SpermAssist*[™]. All sperm preparation materials were purchased from NidaCon International, Gothenburg, Sweden. Gradient-prepared spermatozoa constituted Group B.

The remaining half of each ejaculate was diluted in *SpermAssist*[™] to give the same sperm concentration as in the gradient-prepared samples: this group constituted Group C. Sperm motility assessments were made on both gradient and extended sperm preparations. The sperm samples were stored at room temperature (approximately 21°C). Subjective assessments of the proportion of motile sperm were made on a daily basis until less than 20% of the spermatozoa were motile. Aliquots (50 µL) of the sperm preparations were fixed in paraformaldehyde (3.7%) for subsequent assessment using the TUNEL assay (20). The samples were coded and the TUNEL assessment was performed by an operator who was not aware of the sample coding.

Briefly, TUNEL labelling was carried out using a “Cell Death Detection” kit from Boehringer-Roche (East Sussex, UK) and was performed according to the manufacturer’s instructions. Samples were washed in PBS before being resuspended for 2 min in 200 µL of 0.1% Triton X-100 (Sigma) in 0.1% sodium citrate (Sigma). After a further wash, 50-µL TUNEL reagent was added to each sample, except the negative control where reagent without DNA polymerase was added, and incubated for 1 h at 37°C. Samples were washed with PBS and analyzed immediately by flow cytometer.

Experiment 2

A further 10 ejaculates were prepared as described above. An aliquot (50 µL) of each sperm sample was frozen on dry ice and stored for subsequent nick translation analysis using the methods described previously (21,22).

STATISTICS

Analysis of variance was used to compare the mean percentage of TUNEL positivity on the first day for the different treatment groups, and also sperm survival for sperm in Groups B (gradient-prepared) and C (extended) (23).

Table I. Comparison of the Mean ($\pm$ SD) Values of Sperm Motility, Survival, and TUNEL Positivity in Sperm Samples Prepared in Different Ways in Experiment 1 ($n = 8$)

Parameter	A: Neat ejaculate	B: Gradient prepared	C: Extended semen	Statistics
Proportion of motile sperm on day 1 (%) ^a	54.0 $\pm$ 23.7	92.6 $\pm$ 5.4	61.25 $\pm$ 18.7	
Sperm survival (h) ^b (range)	<24	72.0 $\pm$ 49.6 (24–120)	25.3 $\pm$ 12.7 (24–48)	$P < 0.001$
TUNEL positivity day 1 (%)	20.9 $\pm$ 8.1	12.8 $\pm$ 8.1	32.1 $\pm$ 13.3	$P < 0.001$

^a Subjective estimation, immediately after preparation.

^b Defined as the time over which >20% spermatozoa remained motile; since readings were made daily, values are given to the nearest whole day.

RESULTS

Experiment 1

Mean values for the proportion of motile spermatozoa, sperm survival, and percentage of TUNEL positivity in the different treatment groups on the day of preparation (day 1) are shown in Table I. Group B (gradient prepared) spermatozoa showed significantly less ($P < 0.001$) TUNEL positivity than Group A (raw ejaculate) and Group C (extended semen) samples. This observation confirms previous results indicating that density gradient centrifugation has the ability to improve the quality of the sperm preparation by reducing the number of spermatozoa with DNA strand breaks (24).

Sperm motility was not retained overnight in the untreated semen samples, all Group A spermatozoa having less than 20% sperm motility at 24 h after collection. Group C (extended semen) spermatozoa survived better than Group A spermatozoa, with eight of the nine samples having more than 20% motile spermatozoa after 24-h storage. However, only two sperm samples had retained more than 20% motility at 48 h after collection. TUNEL positivity rose to approximately 40% after 24 h in Group C spermatozoa, compared to Group B spermatozoa where TUNEL positivity had risen to only 16%. Furthermore, in Group B spermatozoa, TUNEL positivity remained less than or equal to 20% in five out of nine samples for a further 48 h of storage (Fig. 1).

Fig. 1. Effect of sperm preparation technique on mean TUNEL positivity over time for gradient-prepared and nongradient prepared, extended semen.

Table II. Comparison of the Mean ($\pm$ SD) Values of Sperm Motility, Survival, and Nick Translation Positivity in Sperm Samples Prepared in Different Ways in Experiment 2 ($n = 10$)

Parameter	A: Neat ejaculate	B: Gradient prepared	C: Extended semen	Statistics
Proportion of motile sperm on day 1 (%) ^a	51.4 $\pm$ 19.4	71.6 $\pm$ 22.8	50.1 $\pm$ 16.6	$P < 0.05$
Sperm survival (h) ^b (range)	<24	85.5 $\pm$ 27.9 (24–96)	35.1 $\pm$ 17.6 (24–72)	$P < 0.001$
Nick translation on day 1 (%)	17.6 $\pm$ 12.2	6.5 $\pm$ 13.3	15.9 $\pm$ 15.8	$P < 0.05$

^a Subjective estimation, immediately after preparation.^b Defined as the time over which >20% spermatozoa remained motile.

For Group B (gradient-prepared) spermatozoa, mean survival time (72 h) was significantly longer ($P < 0.001$) than in Groups A and C (<24 and 25.3 h, respectively).

Experiment 2

Group B (gradient-prepared) spermatozoa showed only 6.5% positivity for nick translation, significantly lower ($P < 0.05$) than 16% for Group C spermatozoa (Table II). Positivity for nick translation of Group B spermatozoa increased marginally with storage time, while positivity of Group C spermatozoa did not change significantly.

DISCUSSION

The study was designed to answer specific questions on sperm ageing, namely whether spermatozoa show increased nuclear DNA damage as they age and whether spermatozoa, prepared on density gradients and maintained in an optimized sperm maintenance medium, senesce more slowly than spermatozoa which are not prepared in this manner. The results showed that spermatozoa from human ejaculates extended in a commercially available sperm maintenance medium and stored at room temperature showed an increase in DNA damage over a 24-h period. Levels of DNA damage greater than 20% TUNEL positivity may affect fertilization (7,25). The extended semen samples in this study showed a high level of DNA damage on the first day, which increased further on storage for 24 h. Therefore, it would have been inadvisable to use the extended sperm sample at all, and certainly not after 24-h storage. In contrast, according to the DNA integrity criteria reported by Lopes *et al.* (7) and Sun *et al.* (25), it would have been possible to use the gradient-prepared spermatozoa for up to 72 h after collection, since the level of

DNA damage remained low while sperm motility was retained.

Since the sperm concentration was similar in both gradient-prepared and extended semen samples, the differences in survival time and level of DNA damage were not due to differences in sperm numbers. Therefore, preparing spermatozoa on density gradients appeared to be beneficial in extending sperm survival and maintaining DNA integrity. One explanation for the increased survival time seen here could be the removal of sources of ROS by the density gradient. Although ROS exert important physiological effects on spermatozoa at low concentrations, they have pathological effects at high concentrations (26), including both structural damage to sperm chromatin and sperm functional abnormalities (27,28). Cells, particularly leukocytes, and immature or dying sperm are known to be sources of ROS which damage sperm DNA (13–17). A further source of ROS are spermatozoa with retained spermatid cytoplasm, i.e. immature spermatozoa. Since such sperm are less dense than normal spermatozoa, they are removed by the density gradient centrifugation (29,30).

In fertile men, ROS generation is controlled by antioxidants present in seminal plasma, for example, superoxide dismutase, catalase, and glutathione peroxidase/reductase (31), which catalyze the degradation of hydrogen peroxide to water and oxygen. In situations where spermatozoa are removed from seminal plasma, as in centrifugation, it is important also to remove sources of ROS to prevent chromatin damage of the unprotected spermatozoa, or to provide antioxidants in the sperm medium. Density gradient centrifugation enables a highly motile population of sperm to be separated from the seminal plasma, and from immature, dead or damaged sperm, leukocytes, and cellular debris in the ejaculate. At the same time antioxidants are supplied in the form of HEPES (32) and EDTA. Therefore, this preparation method should, theoretically,

separate out normal spermatozoa from potential sources of ROS. In fact, levels of ROS in *PureSperm*[®] sperm preparations are reported to be negligible (33), indicating absence of oxidative stress in such preparations.

DNA-damaged spermatozoa retain the ability to fertilize oocytes and produce apparently high-quality embryos (34). Although mammalian sperm lack an effective DNA-repair system (35), the oocyte has the capability to repair the damaged DNA of sperm to some extent (36,37). However, there is evidence of a direct relationship between the extent of sperm DNA damage and subsequent embryonic development, resulting in pregnancy failure (38). Evenson *et al.* (34) proposed that the biological impact of abnormal chromatin structure depends on a combination of the level of chromatin damage and the capacity of the oocyte to repair that preexisting damage. Therefore, use of senescing spermatozoa for IVF may result in a low rate of embryonic development and high early pregnancy loss (34).

The results reported here support the findings of previous studies in which density gradient centrifugation was found to enrich the sperm population by separating out spermatozoa with nicked DNA and those with poorly condensed chromatin (12,24,39). However, in contrast, Zini *et al.* (40) reported an increase in denatured DNA following preparation of spermatozoa on Percoll density gradients. These conflicting results may reflect the different methods used to detect DNA damage, since Zini *et al.* (40) used acridine orange to determine the proportion of DNA-damaged spermatozoa in the population. Alternatively, the type of gradient itself and the centrifugal force employed may be responsible for some DNA damage, since centrifuging spermatozoa at 600g on Percoll gradients has been reported previously to cause an increase in DNA denaturation (41). The recommendations for preparing spermatozoa on *PureSperm*[®] gradients include using a centrifugal force of 300g. Therefore, to obtain high quality sperm preparations, it is vitally important to use only gradient material which is intended for clinical use, such as *PureSperm*[®] (Percoll is sold for research purposes only), and to follow the instructions for use developed by the manufacturer.

In conclusion, preparing spermatozoa by density gradient centrifugation and storing them in an optimized sperm maintenance medium is advantageous in prolonging sperm survival and maintaining DNA integrity. The technique could be of benefit to prepare and store spermatozoa for use in subsequent attempts

to fertilize oocytes if no fertilization has been achieved on the first day of IVF.

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