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Semen processing by density gradient centrifugation is useful in selecting sperm with higher double-strand DNA integrity

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Summary

The objective of this study was to determine the effects of density gradient centrifugation on sperm cell DNA integrity and to correlate any detected DNA damage with semen analysis parameter. A total of 40 semen samples were collected from nonazoospermic men presenting for infertility evaluation at our department. Individual samples were divided into two parts: one part of the semen was washed and the remainder was prepared using the PureSperm density gradient centrifugation. Sperm DNA fragmentation as evaluated by the terminal desoxynucleotidyl transferase-mediated deoxyuridine triphosphate biotin nick-end labelling assay, was monitored in the initially washed sample and in the different layers of the density gradient centrifugation. No significant correlations were observed between sperm DNA fragmentation, age of patient, concentration and motility. However, a significant correlation existed with strict spermatic morphology. Following density gradient centrifugation, the proportion of spermatozoa with DNA fragmentation decreased significantly when compared with whole semen. In addition, we found that spermatozoa isolated in the 90% layer possessed a significantly lower percentage of DNA damage when compared with those remaining in the 70% and 50% layers. These results demonstrate that semen processing by the PureSperm gradient is useful in selecting sperm with higher double-strand DNA integrity.

Introduction

Male infertility has traditionally been diagnosed by microscopic assessment of concentration, motility and morphology of sperm in the ejaculate (WHO, 1999). However this routine examination of semen is not able to assess alterations in sperm chromatin organisation, such as irregular condensation or DNA damage (Bianchi *et al.*, 1996; Sakkas *et al.*, 1998). The presence of spermatozoa with such anomaly in the insemination medium may have consequences during the fertilisation process and for the developing embryo (Sakkas *et al.*, 1996; Sun *et al.*, 1997; Lopes *et al.*, 1998). Many studies have shown that sperm nuclear DNA fragmentation correlated positively with lower fertilisation rates, impaired implantation rates, increased incidence of abortion and disease in offspring, including childhood cancer (Sun *et al.*, 1997; Lopes *et al.*, 1998; Larson *et al.*, 2000; Morris *et al.*, 2002; Benchaib *et al.*, 2003). Therefore, the ability of sperm preparation technique to remove spermatozoa with such anomalies has important implication for assisted reproductive technique (ART) outcomes. Density gradient centrifugation is commonly used for sperm preparation that leads to an increased conception rate (Allamaneni *et al.*, 2005). This technique separates superior motile spermatozoa with normal morphology from the total sperm population, leaving behind immature and morphologically abnormal spermatozoa (Mortimer, 1999; Henkel & Schill, 2003; Mousset-Simeon *et al.*, 2004; Canto *et al.*, 2006; Morrell, 2006; Marchesi & Feng, 2007). However, there is still controversy as to the effects of this sperm separation technique on sperm cell DNA integrity. Several studies have shown that this sperm separation method is quite effective in sorting out spermatozoa with nicked DNA

and poorly condensed chromatin as evaluated by a variety of sperm DNA integrity assays (Golan *et al.*, 1996; Larson *et al.*, 1999; Spano *et al.*, 1999; Larson *et al.*, 2000; Sakkas *et al.*, 2000; Hammadeh *et al.*, 2001; Tomlinson *et al.*, 2001; Gandini *et al.*, 2004). However, others studies have found that this semen preparation is not useful in selecting sperm with high double-strand DNA integrity (Zini *et al.*, 2000a,b; Stevanato *et al.*, 2008). Because of the contradictory and confusing data, we have designed a prospective study to evaluate the efficiency of density gradient centrifugation in the removal of spermatozoa with nuclear DNA damage particularly with DNA fragmentation.

Materials and methods

Semen collection and analysis

Semen samples were obtained from men consulting for infertility evaluation at our laboratory of Cytogenetics and Reproductive Biology, Farhat Hached University Teaching Hospital, Sousse (Tunisia). Samples were produced by masturbation after 3 days of sexual abstinence. After liquefaction of semen at room temperature, standard semen parameters were obtained according to World Health Organization (WHO) guidelines (1999). None of the semen samples, included in our study had leucocytospermia or a significant percentage of dead spermatozoa. All of the selected patients ($n = 40$) had no history of radiotherapy, chemotherapy, chronic illness or medication. This protocol was approved by the local ethics committee and all patients had previously given informed consent for the study.

Semen preparation

After semen analysis, individual samples were divided into two parts: one part of the semen was washed and the remainder was prepared using the PureSperm density gradient centrifugation.

Whole semen

Approximately 1 ml of unprocessed semen was suspended in 8 ml of phosphate buffered saline (PBS, pH 7.4) (Sigma: St Louis, MO, USA) and centrifuged at 400 ***g*** for 5 min. Then the final pellets were fixed with 5 ml of acetic acid/methanol mixture (Merck, Darmstadt, Germany) for at least 30 min at 4 °C.

PureSperm density gradient centrifugation

The sperm samples were prepared using a PureSperm discontinuous density gradient (Nidacon, Gothenburg, Sweden). This discontinuous density gradient consisted of three 1 ml layers of pure sperm: 90%, 70% and 50%. On the 50% layer, 1 ml of semen was deposited. The gradient was then centrifuged at 300 ***g*** for 20 min. After centrifugation, the seminal plasma supernatant was discarded and each layer of the gradient was collected separately and washed with 5 ml of RPMI (GIBCO™, Grand Island, USA) at 650 ***g*** for 10 min. The sperm pellet obtained after each centrifugation was fixed with 8 ml of acetic acid/methanol mixture.

Measurement of DNA fragmentation by TUNEL assay

The presence of DNA strand breaks in spermatozoa was evaluated by the TUNEL assay, using the ApopTag® Apoptosis Detection Kit (QBiogene, Paris, France). The previously fixed cells from the whole semen, the 90% layer, the 70% layer and the 50% layer were spread out over sialinised slides and allowed to air dry. The slides were permeabilised with PBS with 1% of Triton X100 (Sigma) then equilibrated with the equilibration buffer (provided in the kit ApopTag®) at room temperature for 10 s and incubated in a dark moist chamber at 37 °C, for 1 h, with the terminal desoxynucleotidyl transferase solution in order to allow DNA elongation. After stopping the enzyme reaction, the slides were washed twice in PBS and the DNA elongation was revealed by incubating the cells with anti-digoxigenin antibody coupled to peroxidase, during 30 min in a dark moist chamber. The peroxidase was revealed with diaminobenzidine. The sperm nucleus was counterstained with Harris' haematoxylin and finally observed under a microscope (Zeiss, Oberkochen, Germany) equipped with a 100 magnification lens. Spermatozoa with fragmented DNA had brown-coloured nuclei, whereas the other cells were blue-grey. On each slide, approximately 500 cells were counted, and the DNA fragmented index (DFI) was calculated.

Data analysis

Statistical analysis was performed using SPSS 13.0 (SPSS, chicago, IL, USA) for Windows. All variables were initially tested in order to determine variance homogeneity and data normality. The values are expressed as mean ± one standard error of the mean (SEM). To identify differences in the mean values of DFI between fresh semen and processed semen for the same patient, a paired *t*-test was utilised. Pearson's correlation was performed to examine the relationship between the percentage of sperm with DNA fragmentation and standard semen parameters. All hypothesis testing was two-sided with a probability value of 0.05 deemed as significant.

Results

DNA fragmentation and sperm characteristics

Our semen samples tested, before semen processing, had a mean (±SEM) sperm volume of 3.14 ± 1.09 ml (range 2–6 ml), a mean sperm vitality of 76.75 ± 3.24% (range 77–97%), a mean sperm concentration of 67.51 ± 46.17 × 10^6/ml (range 20–194 × 10^6/ml), a mean total sperm motility of 33.85 ± 8.69% (range 5–48%), a mean sperm abnormal morphology of 86.69 ± 6.01% (range 80–100%), and a mean percentage of sperm exhibiting fragmented DNA of 32.82 ± 11.86% (range 18–72%).

The age of our patients ranged from 27 to 54 years with a mean of 38.48 years, no statistically significant correlation was found between the age of the patients and the DFI ($P > 0.05$). In this study, we evaluated the relationship between the different sperm parameters, measured on semen before discontinuous gradient centrifugation and sperm DNA fragmentation index. The results of correlation analysis are listed in Table 1. No significant correlations were observed between sperm DNA fragmentation, concentration and motility ($P > 0.05$). However, a positive and significant correlation exists with abnormal morphology ($P < 0.01$) (Fig. 1).

Efficiency of PureSperm density gradient centrifugation on human sperm DNA fragmentation

Fig. 2 summarises the results of percentage of spermatozoa with DNA fragmentation before and after discontinuous gradient centrifugation.

The proportion of spermatozoa with DNA fragmentation as assessed by TUNEL assay was significantly higher in the initial washed sample (32.82 ± 11.86%). The value of DFI expected 30% in most of the samples. However, this percentage decreased significantly after semen processing with PureSperm density gradient centrifugation.

Table 1 Correlation analysis between sperm parameters and sperm DNA fragmentation before density gradient centrifugation

Sperm parameter	Mean ± SEM	Correlation coefficient (*r*)	*P*-value
Volume (ml)	3.14 ± 1.09	0.022	0.894
Sperm concentration (×10^6/ml)	67.51 ± 46.17	0.168	0.300
Total sperm motility (%)	33.85 ± 8.69	0.051	0.741
Abnormal sperm morphology (%)	86.68 ± 6.01	0.420	0.007[a]
Necrozoospermia (%)	23.25 ± 5.79	0.545	0.001[a]

[a]Statistically significant.

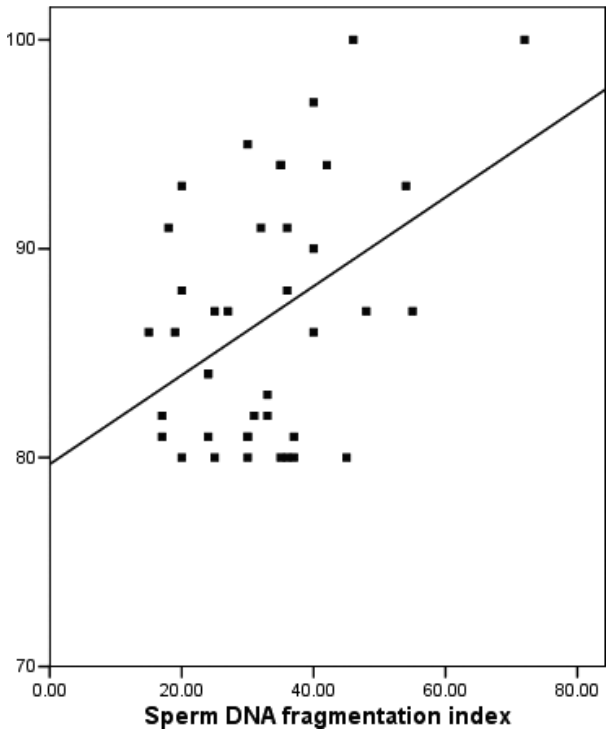

Fig. 1 Correlation between the percentage of spermatozoa with abnormal sperm morphology and the sperm DNA fragmentation index.

A significant difference was found between the percentages of spermatozoa with DNA fragmentation recovered from the 90% fraction when compared with those spermatozoa in the initial wash sample ($P < 0.0001$).

When examining the different fractions prepared after using the PureSperm technique, we found that spermatozoa isolated in the 90% layer possessed a significantly lower percentage of DNA damage compared with those remaining in the 70% and 50% layers. A significant difference was found between the different fractions ($P < 0.001$ in all comparisons).

Discussion

Results of the present study point first to a significant relationship between DNA damage, detected by TUNEL assay in semen samples before semen processing, and traditional sperm evaluation parameters. In particular, a highly significant negative correlation was found between the degree of DNA fragmentation and sperm morphology. Similarly, using a TUNEL-coupled flow cytometry method, Muratori *et al.* (2000) have shown that the extent of sperm DNA fragmentation in unselected spermatozoa was positively related to abnormal morphology. Using the alkaline Comet assay, Trisini *et al.* (2004)

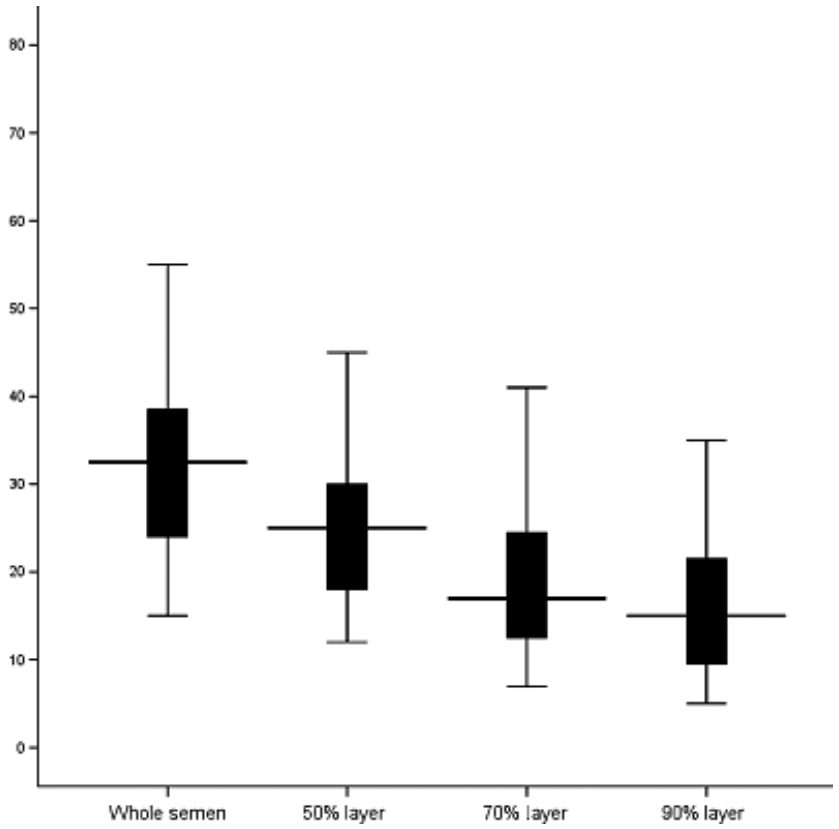

Fig. 2 Percentage of spermatozoa with DNA fragmentation in the initial sample and various fractions after isolating using Pure Sperm® technique. Values are medians (—) with inter-quartile ranges (25–75%) and minimum–maximum values (*I*).

reported significant correlations between sperm strict morphology and sperm DNA damage. Using non-strict sperm morphologic assessment, several investigators have observed significant relationships between sperm DNA damage and sperm morphology defects (Sun *et al.*, 1997; Morris *et al.*, 2002; Borini *et al.*, 2006). Despite the use of different techniques for DNA damage and for sperm morphology analysis, all these observations are in concordance with our observation. Therefore, the possibility of a causal link between DNA integrity and spermatozoa morphology should be suspected.

Many studies have shown that high sperm DNA fragmentation might affect the outcome of assisted reproduction in multiple ways, including effects on the fertilisation rates (Lopes *et al.*, 1998; Chan *et al.*, 2001; Benchaib *et al.*, 2003), embryo cleavage (Virant-Klun *et al.*, 2002; Saleh *et al.*, 2003) and pregnancy rates (Lewis & Aitken, 2005). For the TUNEL technique, the range of the pathological threshold of the percentage sperm DNA fragmentation seems to be between 15% and 20% (Benchaib *et al.*, 2003). So it seems reasonable to bring the DFI under its pathological threshold before undertaking an ART procedure. Density gradient centrifugation is the most commonly applied method in assisted reproductive technology. PureSperm® is one such density gradient product which has been commercially available since 1996. The reported advantages of this technique are that it is rapid, requiring 20 min of centrifugation versus an average of 1 h incubation for swim-up, relatively simple to prepare and easy to carry out under sterile conditions, thus making it readily applicable for use in routine clinical work. Spermatozoa separated on a discontinuous PureSperm gradient form distinct bands at the interfaces between the different PureSperm concentrations. The fractions in the region between 85% and 100% are supposed to contain optimal yields of motile spermatozoa, with abnormal spermatozoa and seminal debris largely eliminated. However, there is still controversy as to the effect of this density gradient centrifugation on sperm cell DNA integrity. In this study, we have shown that selecting sperm using a discontinuous density gradient can decrease significantly the proportion of spermatozoa with fragmented DNA. All samples having a DFI above 30% in neat semen DFI decreased to below 30% after density gradient centrifugation, which means that this preparation had removed a significant amount of spermatozoa with DNA breaks as measured by TUNEL assay. We found that with three layered PureSperm density gradient centrifugation; there was a 2-fold decrease in fragmented sperm DNA compared with neat semen.

In addition, when examining the different fractions prepared after semen processing, we found a significant difference between the percentages of spermatozoa with DNA fragmentation recovered from the 90% fraction compared with those remaining in the 70% fraction and in the 50% fraction. It appears that in addition to isolating cells with the best motility and morphology (Canto *et al.*, 2006; Morrell, 2006), sperm preparation by density centrifugation also isolates the spermatozoa with best DNA integrity for use in assisted reproduction techniques. Our data are in agreement with previous results (Golan *et al.*, 1997; Larson *et al.*, 1999, 2000; Spano *et al.*, 1999; Tomlinson *et al.*, 2001; Younglai *et al.*, 2001; Gandini *et al.*, 2004; Lachaud *et al.*, 2004; McVicar *et al.*, 2004; Morrell *et al.*, 2004; Piomboni *et al.*, 2006) which showed that sperm preparation procedures can decrease the fraction of sperm DNA fragmentation initially present in the whole semen. Donnelly *et al.* (2000) suggested that Percoll density gradient centrifugation was useful in isolating a subpopulation of sperm exhibiting a lower proportion of fragmented DNA than in the original semen, using both the Comet and the TUNEL assays. Sakkas *et al.*, (2000) and Hammadeh *et al.*, (2001) demonstrated that the density gradient centrifugation not only improves the motility but also results in the isolation of sperm with normal morphology and chromatin integrity. Spano *et al.* (1999) had also reported that the double wash centrifugation followed by a swim-up from the pellet improved sperm chromatin structure properties. This notion was also supported by Younglai *et al.* (2001) who suggested that the swim-up separation of motile spermatozoa from normal semen, either with or without centrifugation, does not increase the level of DNA damage. In contrast to those results, unchanged or worse results have been reported by Zini *et al.* (1999, 2000a,b). In two separate studies Zini *et al.* (1999) demonstrated that density gradient centrifugation may lead to an increase in sperm cells with altered chromatin stability, as evaluated by the sperm chromatin structure assay. They found that with two or four layered Percoll density gradient centrifugation; there was a 2-fold increase in denatured sperm DNA when compared with whole semen. In the other separate study; they demonstrated that density gradient centrifugation is comparable to swim-up technique in terms of recovering spermatozoa with enhanced motility. However, spermatozoa recovered after swim-up but not density gradient centrifugation possesses higher DNA integrity (Zini *et al.*, 2000b). This discrepancy could be explained by the heterogeneity of the different methods used to detect DNA damage, since Zini *et al.* used acridine orange to determine the proportion of DNA-damaged spermatozoa in the population. An additional explanation for this discordance is related to the choice of the type of gradient itself and the centrifugal force employed may be responsible for some DNA damage. The centrifugal force used in the preparation of the spermatozoa was considerably greater than that used in our current study (600 ***g*** compared with 300 ***g***). Recently, Stevanato *et al.* (2008) using the TUNEL assay, demonstrated that semen processing by density gradient centrifugation is not generally useful in selecting sperm with higher double-strand DNA integrity, and for patients with high underlying DNA fragmentation other semen processing techniques had to be considered. The authors quote previous research that spermatozoa were exposed to oxidative stress during the centrifugation steps of the preparation process as a possible explanation of their results.

In conclusion, our data support a significant correlation between sperm DNA fragmentation and strict spermatic morphology. In addition, we have shown that semen processing by the PureSperm gradient with a centrifugal force of 300 ***g*** for 20 min is useful in selecting sperm with higher double-strand DNA integrity, and recommended to be used in sperm preparation for assisted reproduction.

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