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Sperm fractions obtained following density gradient centrifugation in human ejaculates show differences in sperm DNA longevity

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ABSTRACT

Objective: To investigate the DNA longevity characteristics associated with each resultant fraction following density gradient centrifugation (DGC) in comparison to that of the original neat ejaculated sample. **Methods:** An aliquot of neat semen (NSS) collected from 7 patients was processed using DGC resulting in 3 fractions; Fraction 1: seminal plasma/40% gradient interface (GI); Fraction 2: 40%GI/80%GI; Fraction 3: 80%GI/pellet. An aliquot of each fraction and NSS was cryopreserved, thawed and incubated at 37 ℃ for 24h; the increase of sperm DNA fragmentation was assessed using the Dyn-Halosperm assay following 0, 3, 6 and 24h of incubation. **Results:** While there was a significant reduction in the incidence of baseline sperm DNA fragmentation following DGC in Fraction 3, sperm DNA longevity was shown to be higher in the NSS than in any other sub-population following incubation. The highest levels of baseline DNA damage were found in Fractions 1 and 2; these fractions also showed the highest rate DNA fragmentation following incubation, subsequently exhibiting the lowest DNA longevity. **Conclusion:** 1) Unnecessary incubation of spermatozoa prior to artificial insemination or *in vitro* fertilization, should be avoided, since sperm DNA longevity is significantly reduced after *ex vivo* sperm handling and 2) Although sperm selection by DCG significantly reduces the baseline levels of SDF of sperm in Fraction 3, sperm DNA longevity in this fraction was ultimately lower following 24 h incubation when compared to sperm recovered from non-centrifuged NSS.

1. Introduction

Sperm washing followed by sperm selection by density gradient centrifugation (DGC) or swim-up are techniques frequently used prior to insemination in assisted reproductive techniques (ART) in order to remove seminal plasma or cryoprotectants that might otherwise interfere with the process of sperm capacitation. Some cryoprotectants can cause adverse reactions in the reproductive tract following intrauterine insemination or accelerate the process of sperm damage. Sperm separation from the seminal plasma enhances the fertilizing capacity of the semen sample via the recruitment of a high quality sub-population of spermatozoa[1-2]. Nevertheless, the benefits of sperm selection might also be mitigated to some extent by the latent expression of iatrogenic sperm damage associated with ex vivo sperm handling[3-6]. Not all the techniques used

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for sperm selection are equally effective in terms of selecting the best subpopulation of spermatozoa for ART. For example, the elimination of apoptotic-like spermatozoa from semen is not very effective when DGC is performed [7-9]. In addition, DGC and swim-up techniques have also been shown to have different efficiencies in removing single or double-stranded DNA breaks[10]. Sperm selection using swim-up is quite effective in isolating spermatozoa with singular characteristics such as large size telomeres, an observation that appears to be consistent with obtaining the best spermatozoa for successful fertilization and normal embryo development[11].

The human ejaculate, rather than being a homogeneous and synchronized mixture of spermatozoa, is comprised of a mixture of mature and immature, motile and non-motile and DNA intact and DNA damaged spermatozoa, germ cells, epithelial cells, different types of microorganisms and non-specific debris, all suspended in seminal plasma and which presents as a biological fluid of variable composition[12-13]. Processing of the semen sample for ART using sperm selection techniques is directed to the isolation of highly motile, morphologically normal, DNA intact, and functionally competent spermatozoa. While this should be the ultimate goal of the reproductive specialist, the probability of obtaining such an elite population of spermatozoa after one pass through the specific selection technique is not always achievable; consequently, in some cases, fertility could be compromised due to the presence of a high proportion of damaged sperm.

We propose that the sperm sub-population within the ejaculate that is of most concern in terms of reproductive outcome is that with single and double stranded DNA damage; the latter being difficult to repair either during pronuclei formation or during the first steps of embryo cleavage. While some reports indicate that sperm selection techniques such as swim-up and DGC can be used to partially eliminate sperm with DNA damage[14], other authors have shown that the percentage of sperm with DNA damage can still remain relatively high[7,15-16]. On the other hand, it has also been reported that an effective reduction in sperm DNA damage is highly dependent on the initial quality of the sperm samples to be processed. Thus, in clinical situations of severe male factor infertility, micromanipulation-based techniques such as Magnetic Cell Sorting (MACS), Physiological ICSI (PICSI) or Intracytoplasmic Morphologically selected Sperm Injection (IMSI) may also be of benefit in the selection of single and competent spermatozoa for injection into the oocyte[8,17,18].

This investigation was conducted to test the hypothesis of whether spermatozoa isolated in the 80% gradient pellet, have the best DNA quality when assessed either immediately after sperm selection or following incubation associated with a dynamic assessment of sperm DNA quality[16] to gain information on DNA longevity associated with each segregated subpopulation obtained after DGC. Our working hypothesis is that after DGC, we are empirically selecting a sperm subpopulation containing that contains the lowest baseline levels of DNA damage and the highest DNA longevity. To this purpose, the variations of sperm DNA damage in the different fractions of spermatozoa selected by DGC and a comparison of the dynamic DNA damage increase following incubation at 37 ℃ for 24h of the sperm isolated in the different fractions of the ejaculate were investigated.

2. Materials and methods

Ejaculates from seven fertile sperm donors that had produced at least 3 newborns were processed using an 80/40 sperm filter gradient (Cryos International, Aarhus, Denmark). Samples were centrifuged at 300 g for 20 min and the different fractions aspirated, re-suspended in 2 mL of SpermWash medium (Cryos International, Aarhus, Denmark) and centrifuged at 400 g for 10 min. Three different fractions were isolated: Fraction 1: seminal plasma/40% gradient interface; Fraction 2: 40/ 80% gradient interface; Fraction 3: 80% pellet. The liquefied neat semen was used as a control and a subsample of spermatozoa was cryopreserved using the same conditions as that used for spermatozoa in each fraction. Aliquots of the fractions were mixed 1:1 (v/v) with the cryoprotectant Sperm CryoProtec II (Nidacon, International AB, Goteborg, Sweden), exposed to liquid nitrogen vapors for 15 min and finally plunged into liquid nitrogen. To assess sperm DNA fragmentation (SDF), all sperm samples were thawed at 37 ℃ for 5 min and incubated at 37 ℃ for 0, 3, 6 and 24 h and the SDF assessed each time interval using the Halosperm test (Halosperm DNA, Madrid, Spain) for the static SDF assessment. Dyn-Halosperm (Halosperm DNA; Madrid, Spain) was used to calculate the dynamic loss of sperm DNA quality. The efficiency for sperm DNA damage reduction after gradient centrifugation (e-value) was defined as the percentage of sperm DNA damage variation after comparing the SDF values obtained in the neat semen sample with those obtained in the different fractions.

SPSS v.15.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The Wilcoxon signed-rank test for a non-parametric analysis was used to compare two related samples, while the SDF values of the different fractions were compared using a Kendall′s W test. Comparison of the dynamic loss of sperm DNA longevity was determined using the nonparametric maximum likelihood Kaplan-Meier estimator. A log rank test statistic (Mantel-Cox) was used to compare estimates of the hazard functions between the two groups at each time interval.

3. Results

SDF values obtained from the neat semen samples and the corresponding sperm fractions following DGC from each

sperm donor are shown in Table 1, along with e-values achieved in the different fractions. Significant differences were found when the mean SDF values of the different fractions were compared (Kendall´s W:0.630; *Chi*-Square: 13.2; P = 0.004); the lowest mean ± SD for SDF corresponded to Fraction 3 (8.7 ± 8.1) and the highest (33.6 ± 12.0) was the Fraction 1 subpopulation. The efficiency for SDF reduction (e-values) was negative in all the fractions except in Fraction 3 (Table 1). There was a significant difference between the e-values of sperm DNA fragmentation reduction in each fraction (Kendall´s W 0.799; P=0.004).

Table 1

Sperm DNA fragmentation (values in %) of the neat semen sample (NSS) and corresponding fractions F1, F2 and F3 following the density gradient centrifugation procedure.

Donor	NSS	F1	e-value F1	F2	e-value F2	F3	e-value F3
1	32.0	25.7	19.7	13.0	59.4	3.3	89.7
2	16.3	19.7	-20.9	20.3	-24.5	4.7	71.2
3	11.7	33.3	-184.6	33.3	-184.6	4.0	65.8
4	23.7	52.7	-122.4	59.0	-149.0	25.0	-5.5
5	15.0	22.0	-46.7	7.3	51.3	3.0	80.0
6	15.0	39.3	-162.0	19.3	-28.7	14.0	-6.7
7	18.3	42.7	-133.0	23.0	-25.7	7.3	60.1
Mean	18.9	33.6	-92.9	25.0	-43.1	8.8	52.6

The efficiency for SDF reduction (e-value) associated to each fraction after being compared with the values obtained in NSS is also represented.

The dynamic behavior of sperm DNA fragmentation recovered from each fraction and incubated for 24h is shown in Figure 1. To further examine sperm DNA survival, the rates of SDF following incubation at 37 ℃, were compared between the different sperm fractions of each individual ejaculate (Figure 1a-g). A Kaplan-Meier test was applied to evaluate the differential survival rate and in all the cases except in one (Figure 3f, Log Rank:Mantel-Cox; χ^2 = 3.1; *df*=3; P = 0.365), there were significant differences between the rate of SDF for each fraction (Log Rank:Mantel-Cox; χ^2 values not shown; *df*=3; P = 0.000 for all other individuals). This statistic revealed a significant difference in the rate of SDF of spermatozoa isolated from Fraction 3 of different individuals (inter-ejaculate survival test for the Fraction 3; Figure 1h; Log Rank:Mantel-Cox; χ^2=23.2; *df*=3; P = 0.000). Surprisingly, the best SDF dynamic behavior was observed in the neat unprocessed semen sample (white dots in Figure 1) followed by the DGC pellet (Fraction 3 - black dots; Figures 1a-h). Although the baseline levels of SDF in the neat semen sample were higher than those observed in Fraction 3 (80% gradient pellet) immediately after DGC, this was not the case in terms of the dynamic of DNA damage following incubation. The baseline level of SDF observed at T0 was correlated with the rate of SDF observed after 24 h of incubation (Figure 2); this two-dimensional representation of the rate of SDF, clearly showed how spermatozoa from Fraction 3 differed from other fractions (Neat semen, Fraction 1 and Fraction 2). In particular, spermatozoa from Fraction 1 showed the highest levels of sperm DNA damage both at time 0h and at 24h post-incubation.

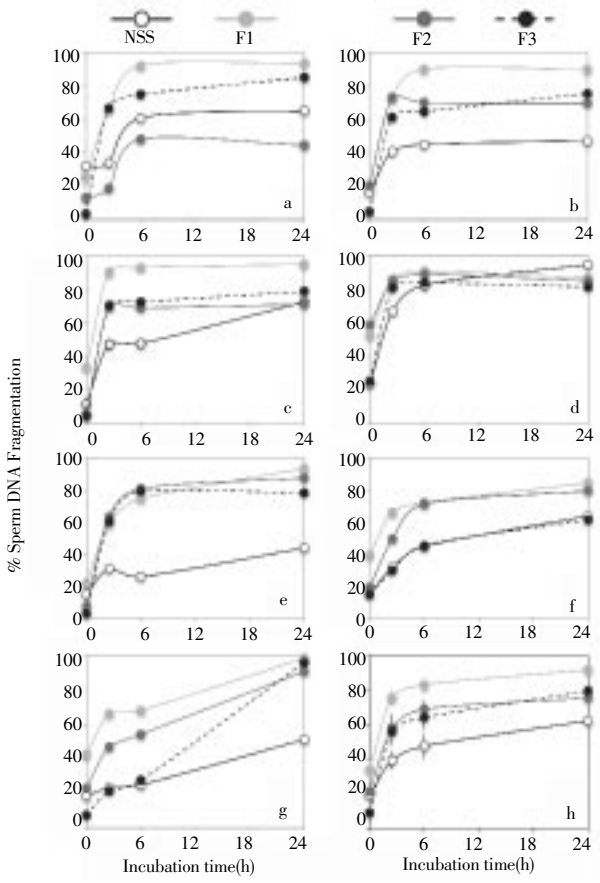

Figure 1. Dynamic behavior of sperm DNA fragmentation recovered from each fraction and incubated for 24h.

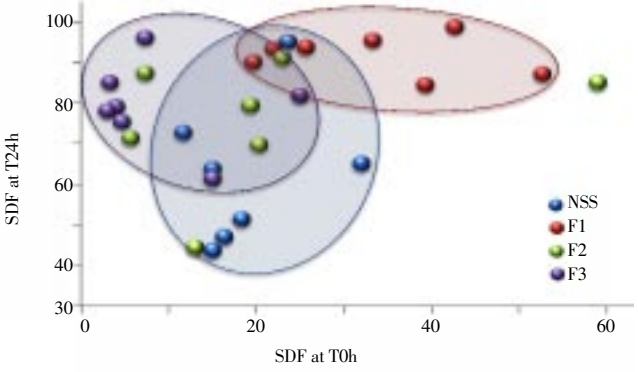

Figure 2. DNA damage following incubation.

4. Discussion

The main findings from this study were that following DGC, there was a significant reduction in the levels of baseline SDF in sperm isolated in the gradient pellet (Fraction 3). However, sperm DNA longevity was actually higher in

the neat semen sample than in any of the fractional sub-populations, including Fraction 3. The highest levels of baseline SDF were found in the low density Fraction 1 and Fraction 2. These fractions also showed the highest rate DNA fragmentation following incubation.

While a reduction in the baseline levels of sperm DNA damage in the gradient pellet is not a novel observation [16,19-20], the surprising finding of this experiment was that the sperm DNA longevity of the thawed neat semen samples were generally better than those obtained in all other fractions of the same ejaculate following DGC (see Figure 1). The effect of freezing and thawing appeared to have no impact on the behavior of each sperm fraction with respect to the DNA longevity, since all the samples within this experiment were cryopreserved, thawed and processed using the same protocol. Faced with this evidence, it can be concluded that sperm manipulation for DCG, although initially decreasing the baseline level of sperm DNA damage, in addition produced a certain level of deleterious damage that only became apparent after incubation at 37 ℃. Consequently for purposes of ICSI, we propose that a direct sperm washing using a standard semen extender and direct sperm isolation using PVP, thereby avoiding sperm centrifugation, may be less damaging to the resultant selected sperm. This suggestion runs directly against most rationale established for the protocols for sperm isolation, including sperm centrifugation, but also explains, in some instances, the negative effects of sperm centrifugation on sperm quality reported in majority of mammalian species[21-22]. In the particular case of human spermatozoa it has been shown that centrifugation using a dextran swim-up procedure induced sublethal damage but separation of the sperm fraction from seminal plasma avoiding centrifugation extends their motile life[23]. Using a similar reasoning, it has been proposed that certain variations on semen centrifugation are able to minimize the negative impact of iatrogenic sperm injuries caused by reactive oxygen species[24]. Recently, the use a specific sperm-selection chamber which avoids sperm centrifugation, has been shown to be efficient in improving the physiological characteristic of the spermatozoa by increasing the relative number of spermatozoa with a better DNA packing and morphological characteristics and diminishing the level of aneuploidies[25]. In general, all new sperm selection methodologies are aimed to diminish the impact of iatrogenic sperm damage and to obtain the best candidate sperm for an efficient fertilization[26].

While it has been previously reported that seminal plasma contains high levels of antioxidant enzymes that may decrease or at least compensate for the oxygen radical-induced sperm DNA damage that may occur during aerobic incubation at 37 ℃ after thawing[27-28], it is still nevertheless the case that seminal oxidative stress is a function of an imbalance between ROS-generating and scavenging activities[28-29]. While sperm diluted in seminal plasma may appear to be protected, at least in part against oxygen radical-induced DNA damage after thawing, once the seminal plasma is removed by DGC, spermatozoa are likely to be rendered more vulnerable to oxygen radical-induced DNA damage. We suggest that the effect of mechanical centrifugation and/or the removal of spermatozoa from the seminal plasma, rather than the cryopreservation procedure per se is likely to be the primary cause of the lose of sperm DNA longevity following post-thaw incubation, as both neat and fractionated spermatozoa were exposed to the same degree of iatrogenic damage via cryopreservation and thawing.

Another interesting finding was that the dynamics of sperm DNA damage showed significant inter-donor variation, even between fertile sperm donors. In fact, the sperm DNA longevity observed for Fraction 3 varied among different donors. This observation is consistent with the behavior of sperm DNA longevity observed, not only in human donors and patients[6,30], but also in the majority of mammalian species when the unfractionated sperm sample is analyzed to calculate the rate of SDF[6]. This variability in DNA longevity is possibly not detected or accounted for at the time of fertilization in most clinics, as the sample is typically assessed immediately post-thaw; however, it is possible that by the time the sperm are injected, in the case of ICSI, sperm DNA quality has significantly declined. Thus, sperm DNA longevity must be of relevance at the time of fertilization.

In conclusion, the primary "take-home" messages from this study are: (1) unnecessary incubation of processed spermatozoa, which is devoid of seminal plasma, prior to artificial insemination or *in vitro* fertilization, should be avoided since the sperm DNA longevity significantly decreases after *ex vivo* sperm handling; (2) although sperm selection by DCG significantly reduces the baseline levels of SDF in sperm from Fraction 3 compared to neat semen, sperm DNA longevity in Fraction 3 is ultimately lower following 24 h incubation than non-centrifuged neat semen. Our findings will hopefully generate a reassessment of the detrimental effects of centrifugation and the importance of iatrogenic damage prior to fertilization. Perhaps the next question to be answered should be: does the reduction in sperm DNA quality achieved after DGC compensate for the loss of DNA longevity? If we are correct, conventional IVF should be highly compromised when using cryopreserved sperm samples, since several hours are required for sperm preparation, oocyte insemination and fertilization; after this period, SDF would substantially increase, thus reducing the chance of a successful pregnancy.

Conflict of interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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