J Assist Reprod Genet (2009) 26:151–157
DOI 10.1007/s10815-008-9288-0

ANDROLOGY

Effect of leukocytospermia and processing by discontinuous density gradient on sperm nuclear DNA fragmentation and mitochondrial activity

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Received: 8 October 2008 / Accepted: 18 December 2008 / Published online: 29 January 2009

Abstract
Purpose To assess the effect of leukocytospermia and semen processing on sperm DNA and mitochondria.
Methods Twenty-two patients with and 41 without leukocytospermia were included. Sperm DNA fragmentation was assessed by the Comet assay, and mitochondrial activity by a colorimetric method for active mitochondria. Semen was processed using Percoll, and motility, DNA fragmentation, and mitochondrial activity were analyzed pre- and post-processing.
Results No differences were observed in age, abstinence, volume, sperm morphology, progressive motility, concentration, and vitality ($p>0.10$). Variables were grouped according to time (pre- vs post-processing) and group (leukocytospermia vs non-leukocytospermia) because no interactions could be observed. Leukocytospermia was associated to increased DNA fragmentation, while semen processing led to a decrease in DNA fragmentation and to increased mitochondrial activity.
Conclusion While semen processing selects sperm with higher rates of DNA integrity independent of the presence or absence of leukocytes in semen, samples without leukocytospermia present more sperm without DNA fragmentation. Semen processing also selects sperm with higher mitochondrial activity.

Keywords DNA damage · Mitochondria/metabolism · Leukocytospermia · Semen processing · Sperm

Capsule Leukocytospermia leads to an increase in DNA fragmentation, while semen processing leads to a decrease in DNA fragmentation and to an increase in mitochondrial activity.

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Introduction

Infertility, classically defined as the inability to achieve pregnancy after 12 months of sexual relations without the use of contraceptives [1], affects around 15% of couples in reproductive age, and the male partner is responsible for up to 50% of these cases [1, 2]. Male factor may occur due to a number of different factors, such as varicocele, genetic alterations, and systemic diseases. Assessment of male fertility potential usually initiates with observing semen analysis values. The World Health Organization defines cutoff values for sperm concentration, motility, morphology, and concentration of round cells and leukocytes. Presence of excessive leukocytes ($\geq 1\times10^6$/mL) in semen is a known cause of decreased male fertility [1, 3].

Round cells may be normally found in semen (up to 5×10^6 million round cells/mL of semen is considered normal), and it is important to differentiate these cells from leukocytes because of the alterations associated to leukocytospermia [1]. Some different techniques for this differentiation are available [1], of which peroxidase staining of neutrophils is one of the simplest. The traditional method for counting leukocytes in human semen is to use a histochemical procedure to identify the peroxidase enzyme that characterizes polymorphonuclear granulocytes. All classes of human leukocytes express a specific antigen (CD45) that can be detected using an appropriate monoclonal antibody [1].

The mechanism by which leukocytes may determine decreased fertility potential is due to the release of reactive oxygen species (ROS), oxygen molecules with an unpaired electron [2, 4–6]. While baseline ROS concentration is essential during sperm capacitation and acrosome reaction [7, 8], excessive ROS production will lead to oxidative stress [9, 10], which in turn will decrease sperm functional integrity (mitochondrial activity, sperm-egg binding, and DNA integrity, for example) [11, 12].

Seminal oxidative stress (OS) takes place when antioxidants are not able to counteract the excessive ROS [13]. Sperm are especially susceptible to OS because they present high plasma membrane concentrations of polyunsaturated fatty acids (PUFA)—methylene groups present between double bonds in the carbon chain are sites where hydrogen molecules may easily be removed by ROS [14, 15]—and lipid peroxidation will eventually lead to loss of mitochondrial activity [16] and increased DNA fragmentation [17].

Among the many therapeutic options offered to infertile couples, assisted reproductive techniques (intra-uterine insemination, in vitro fertilization, and intracytoplasmic sperm injection) have greatly increased their odds of achieving a successful viable pregnancy [18]. In order to select a better population of sperm for these techniques, semen is usually processed, either through a swim-up or a density gradient centrifugation technique, and these have both proven to be efficient methods for selecting motile and morphologically normal sperm [19]. Centrifugation through a density gradient also removes leukocytes, cellular debris, and dead or immotile sperm [20]. On the other hand, centrifugation removes the antioxidant-rich seminal plasma and increases ROS production and release, both in sperm and in leukocytes [21]. Activation of leukocytes by centrifugation may increase ROS production in up to 100-fold [22].

Thus, because assisted reproductive techniques require semen processing and excessive leukocytes may increase ROS, it is important to assess possible interactions between the effect of leukocytes and semen processing on sperm DNA integrity and mitochondrial activity.

Materials and methods

Study design

A prospective controlled study was employed, involving two groups of patients presenting to the Human Reproduction Section of the Sao Paulo Federal University. The study group included 22 patients with leukocytospermia. A group of patients without leukocytospermia (n=41) was included as a control group. Inclusion criteria were the presence of leukocytospermia (over one million leukocytes/milliliter of semen) for the study group and absence of leukocytospermia for the control group. Exclusion criteria were a history of previous urogenital diseases (such as cryptorchidism), not considering those which lead to increased neutrophil count in semen, current or previous systemic diseases which would lead to testicular alterations, such as cancer (and chemotherapy), endocrinopathies and low ejaculate volume (<1.0 mL). Institutional Review Board approval was obtained from the Sao Paulo Federal University Research Ethics Committee. All reagents were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, USA), unless otherwise stated.

Semen analysis and processing

Semen samples were collected by masturbation following 2 to 4 days of ejaculatory abstinence. After semen liquefaction, seminal analysis was performed according to World Health Organization criteria [1] and sperm morphology was evaluated by Kruger's strict criteria [23]. An aliquot was used for evaluation of sperm DNA fragmentation and mitochondrial activity, and semen was processed.

When samples presented at least 1×10^6 round cells/mL of semen, cells were stained with peroxidase to verify the number of neutrophils in the sample, according to Mortimer [24]. The percentage of stained (peroxidase positive) round cells was determined, and the total number of round cells/milliliter multiplied by this percentage to determine the number of neutrophils/milliliter of semen.

Semen was processed by discontinuous density gradient centrifugation (Percoll®, GE Healthcare, Amersham Place, UK). Briefly, 1.0 mL of semen was layered on top of a discontinuous two-layer (45% to 90%) Percoll gradient in a 15-mL conical tube and centrifuged at 300×g for 30 min. Spermatozoa collected from the bottom layer (90% layer) were washed twice by resuspension in 5 mL of BWW (Biggers Whitten Whittingan) media supplemented with 10% BSA (bovine serum albumin) and centrifugation at 300×g for 10 min. After the second centrifugation. The supernatant was removed and the pellet resuspended in 1 mL of BWW. After 10 min sperm motility and concentration were assessed, and an aliquot was used for the evaluation of sperm DNA fragmentation and mitochondrial activity.

Determination of DNA integrity

To evaluate sperm nuclear DNA integrity, a modified alkaline single-cell gel electrophoresis, or Comet assay, adapted from Donnelly et al. [25] was performed on semen samples (pre- and post-semen processing) from each study subject. Slides were pre-coated with 1% normal melting point agarose (GE Healthcare, Amersham Place, UK) in

TBE (Tris, borate, EDTA) buffer (0.089 M Tris base, 0.089 M borate, and 0.002 M EDTA) overnight. A 100 μL aliquot of fresh semen diluted to a final concentration of 1×10^6/mL in 0.75% low melting point agarose (GE Healthcare, Amersham Place, UK) in TBE was added to each slide. This was covered with a coverslip and kept for 10 min at 4°C to solidify. Following gel solidification, the coverslips were gently removed and 300 μL of 0.75% low melting point agarose in TBE were added. After 10 min the slides were immersed in cold lysis solution (100 mM Na_2-EDTA, 10 mM Tris, 2.5M NaCl, pH=11, 4 mM dithiothreitol, 2% Triton X-100) for 2 h.

The slides were then immersed in Milli-Q water for 10 min, in order to remove the excess salts, and subsequently immersed in alkaline electrophoresis solution (300 mM NaOH, 1 mM Na_2-EDTA, pH>13) for 20 min. Electrophoresis was performed for 20 min at 3 V/cm, 150–300mAmp. The slides were then washed twice with TBE (pH=7.4) for 15 min and covered with ethanol 100% twice for 5 min each. After drying, the slides were stained with ethidium bromide (7 μg/mL) (Invitrogen, Carlsbad, California) for 15 min. The slides were washed three times with TBE (5 min each wash) to remove background staining and evaluated using an Olympus BX51 epifluorescence microscope equipped with a rhodamine/TRITC filter and a 100-W. A total of 200 sperm cells were scored according to intensity of DNA damage as assessed by Comet tail and nuclear intensity and visually classified as I (high DNA integrity) to IV (high DNA fragmentation). Class I cells presented a nucleus with intense fluorescence and did not present a Comet tail. Class II cells still presented an evident nucleus but also a Comet tail, indicating either early DNA fragmentation or DNA decondensation. Class III cells presented a weak nucleus and a strong tail, indicating already important DNA fragmentation, and Class IV cells did not present a nucleus, only a Comet tail.

Evaluation of mitochondrial activity

To determine sperm mitochondrial activity, the method proposed by Hrudka [26] was used, based on the oxidation, polymerization, and deposition of 3,3′-diaminobenzidine (DAB) by mitochondrial cytochrome c-oxidase. Briefly, semen was diluted 1:1 to 1:3 in a solution containing 1 mg/mL of 3-3′-diaminobenzidine (DAB) in PBS (phosphate-buffered saline—137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH=7.4) and incubated at 37°C for 1 h in the dark. Two 10 μL smears were then prepared on microscope slides and air dried. The slides were fixed in 10% formaldehyde for 10 min, washed and air dried again. A total of 200 sperm were counted under a ×1,000 magnification differential interference contrast (DIC) Olympus BX51 microscope, and cells were classified as: class I (100% of the midpiece was stained), class II (more than 50% of the midpiece was stained), class III (less than 50% of the midpiece was stained), and class IV (absence of staining in the midpiece). Class I cells indicate cells that maintain complete mitochondrial activity, while class II cells indicate some loss of mitochondrial activity which does not lead to severe impairment of motility and fertilization capacity. Class III cells are cells that present extensive loss of mitochondrial activity, unlikely to achieve hypermotility during capacitation, and class IV cells are either dead cells or cells which maintain minimal energy production through glycolysis.

Statistical analysis

Statistical analysis was performed using SPSS 13.0 for Windows. All variables were initially tested in order to determine variance homogeneity and data normality, and heteroscedastic data were transformed to their square roots. Groups were compared using Student's *T*-test for unpaired samples, in order to verify differences in age and semen variables. A Repeated Measures Analysis of Variance was used to verify a possible interaction between group (presence or absence of leukocytospermia) and time (pre- and post-processing). Because no interactions were observed, variables were grouped when comparing each of these effects. For correlation analysis, Spearman's ranked test was chosen so we could work with untransformed values. An alpha of 5% was adopted. Data are presented as Mean; Standard Deviation [95% confidence interval of the mean].

Results

Routine semen analysis

No differences were observed in age, ejaculatory abstinence, ejaculate volume, and sperm concentration, morphology, and vitality ($p>0.10$). As expected, round cell ($p=0.003$) and neutrophil ($p<0.0001$) counts were higher in the study group (Table 1).

Although semen processing increased progressive (a + b) sperm motility ($p<0.00001$), this increase was not different between samples with and without leukocytospermia (Table 2).

Determination of DNA integrity and mitochondrial activity

No interactions between the effects of leukocytospermia and semen processing were observed. Thus, each effect was analyzed individually. Leukocytospermia was associated to an increase in DNA fragmentation (decrease in DNA

Table 1 Age, ejaculatory abstinence and semen analysis results for men with normal semen analysis (control group) and men with leukocytospermia (study group)

	Control group	Study group	*P* value
Age (years)			
Mean; SD	36.78; 5.34	37.36; 8.33	0.73
95% CI	[35.10; 38.46]	[33.67; 41.06]	
Abstinence (days)			
Mean; SD	4.30; 2.78	4.82; 2.34	0.46
95% CI	[3.43; 5.18]	[3.78; 5.86]	
Volume (mL)			
Mean; SD	2.86; 0.99	3.35; 2.01	0.19
95% CI	[2.55; 3.17]	[2.46; 4.24]	
Concentration ($x10^6$/mL)			
Mean; SD	108.10; 87.90	78.50; 52.63	0.154
95% CI	[80.34; 135.80]	[55.13; 101.80]	
Morphology (% normal)			
Mean; SD	11.29; 4.92	11.59; 5.27	0.82
95% CI	[9.74; 12.84]	[9.25; 13.93]	
Vitality (% live)			
Mean; SD	71.43; 9.65	72.71; 13.72	0.80
95% CI	[65.86; 77.00]	[60.03; 85.40]	
Round cell ($x10^6$/mL)			
Mean; SD	2.53; 3.14	5.21; 3.60	0.003*
95% CI	[1.54; 3.52]	[3.62; 6.80]	
Neutrophiles ($x10^6$/mL)			
Mean; SD	0.15; 0.19	3.56; 3.00	<0.0001*
95% CI	[0.09; 0.21]	[2.23; 4.90]	

SD standard deviation, *95% CI* 95% confidence interval of the mean
*$p<\alpha$

integrity—Comet grade II—$p=0.034$), while semen processing led to a decrease in sperm with important DNA fragmentation (Comet grades III and IV) and to an increase in mitochondrial activity (increase in DAB classes I and II, decrease in DAB class III and IV; Table 2).

In men without leukocytospermia, a negative correlation could be observed between post-processing Comet assay grade IV cells (high sperm nuclear DNA fragmentation) and progressive motility ($r=-0.432$, $p=0.005$), and this could not be observed in men with leukocytospermia ($p>0.05$). A positive correlation was observed between pre-processing sperm progressive motility and morphology ($r=0.698$, $p<0.001$) in men with leukocytospermia but not in men without leukocytospermia. When considering neutrophil count as a continuous variable, the only (expected) correlation observed was between neutrophil count and round cell count ($r=0.479$; $p=0.0001$)

Discussion

Leukocytospermia is an important indicator of male urogenital tract infections and/or inflammations [27], and is considered a contributing cause of male infertility [1] because of excessive ROS production and release [3]. Imbalances between ROS production and semen antioxidant capacity will lead to oxidative stress [9, 10], which will ultimately result in oxidation of membrane polyunsaturated fatty acids [14, 15], loss of mitochondrial membrane potential [16], and single and double strand DNA fragmentation [17].

There is a strong association between decreased sperm motility and oxidative stress [11, 12, 28, 29]. Sperm motility is generated through constant ATP production by mitochondria localized in the sperm midpiece. In order to produce ATP through oxidative phosphorylation, the mitochondrial membrane must present selective permeability to maintain an electrolytical gradient between the inner and outer mitochondrial environments. Thus, excessive ROS will (1) alter phospholipid membranes and thus dysrupt membrane selectivity and will also (2) inhibit oxidative phosphorylation, ultimately leading to decreased ATP production [30, 31].

In our study, semen processing had an expected positive impact on sperm motility and mitochondrial activity. It is interesting to note that leukocytospermia did not affect alterations on sperm progressive motility. However, although semen processing does present positive results independent of the number of leukocytes when considering only progressive motility, this may not hold true for other aspects of sperm analysis, such as mitochondrial activity or DNA integrity.

Excessive production of ROS may also lead to increased DNA damage, through the production of lipid degradation by-products which bind to DNA, through oxidation of DNA bases (mainly guanosine), or through direct interaction with the DNA strand, leading to non-specific single- and double-strand breaks [17, 29, 32]. Although the other mechanisms are important, in our study we selected a test which assessed the third stage, when excess ROS will directly interact with DNA and cause single and double-strand non-specific breaks in the DNA molecule [28]. Also, as a more sensitive test, the Comet assay will allow analysis of very initial lesions to the DNA. Although these lesions are usually not considered as important as double-strand DNA damage, evaluation of initial alterations is very useful to understand how ROS may cause damage which eventually builds up to double-strand damage.

In our study, leukocytospermia led to a decrease in the percentage of Comet assay class II cells, which demonstrates higher DNA integrity. There are many different tests currently used for determination of sperm nuclear DNA fragmentation or damage. The alkalyne Comet assay is considered to possess higher sensitivity and lower specificity because it is capable of detecting damage to the DNA single strand but does not differentiate types of DNA fragmentation (single, double or apoptotic) [33]. However, these tests are also limited in that they are not able to determine other forms

Table 2 Sperm progressive motility, mitochondrial activity, and DNA integrity before and after seminal processing in samples with and without leukocytospermia (mean; standard deviation)

	Pre-processing		Post-processing		Repeated measures ANOVA effects		
	Control group	Study group	Control group	Study group	Interaction	Leukocytospermia	Semen processing
Motility (a + b, %)							
Mean; SD	56.0; 12.2	56.0; 13.8	86.0; 5.8	79.3; 19.8	0.069	0.236	<0.00001*
95% CI	[52.2; 59.8]	[49.9; 62.1]	[84.1; 87.8]	[70.5; 88.1]			
Comet I (%)							
Mean; SD	30; 16.5	38; 17.4	33.7; 22.5	39.4; 20.5	0.693	0.116	0.367
95% CI	[24.8; 35.2]	[30.3; 45.6]	[26.6; 40.8]	[30.3; 48.5]			
Comet II (%)							
Mean; SD	55.2; 16.5	46.3; 14.9	59.6; 22.2	50.5; 18.3	0.971	0.034*	0.111
95% CI	[50.0; 60.4]	[39.7; 52.9]	[52.6; 66.5]	[42.3; 58.6]			
Comet III (%)							
Mean; SD	11.1; 5.6	11.4; 6.4	5.2; 5.9	7.7; 6.6	0.159	0.309	<0.00001*
95% CI	[9.3; 12.8]	[8.5; 14.2]	[3.3; 7.0]	[4.8; 10.7]			
Comet IV (%)							
Mean; SD	3.8; 2.9	4.4; 2.7	1.6; 3	2.4; 3.3	0.817	0.289	<0.00001*
95% CI	[2.9; 4.7]	[3.2; 5.6]	[0.7; 2.5]	[0.9; 3.9]			
DAB I (%)							
Mean; SD	3.1; 4.9	2.1; 3.4	5.4; 8	4.1; 6.1	0.656	0.707	<0.00001*
95% CI	[1.5; 4.6]	[0.6; 3.6]	[2.8; 8.0]	[1.4; 6.8]			
DAB II (%)							
Mean; SD	78.3; 8.2	81.5; 8.9	90; 8.3	89.7; 8.1	0.212	0.416	<0.00001*
95% CI	[75.7; 80.9]	[77.5; 85.4]	[87.3; 92.6]	[86.1; 93.3]			
DAB III (%)							
Mean; SD	11.4; 6.2	10.1; 4.8	2.8; 2.7	3.4; 3.5	0.390	0.765	<0.00001*
95% CI	[9.4; 13.4]	[8.0; 12.3]	[2.0; 3.7]	[1.8; 4.9]			
DAB IV (%)							
Mean; SD	7.3; 5.3	6.3; 5.3	1.8; 1.9	2.8; 2.8	0.100	0.573	<0.00001*
95% CI	[5.5; 8.9]	[3.9; 8.7]	[1.2; 2.4]	[1.6; 4.1]			

SD standard deviation, *95% CI* 95% confidence interval of the mean
*$p<\alpha$

of DNA damage, such as binding of DNA by MDA, a subproduct of lipid peroxidation.

Also, leukocytospermia was not associated with a decrease in Comet class I cells. Although unexpected, one possible explanation for these results is the fact that leukocyte-derived ROS are not as important as those derived from morphologically abnormal or dead sperm [34]. Therefore, it would be expected that ROS generated by leukocytes will only determine damage to sperm with DNA more sensitive to initial damage (class II cells), but not those with high integrity (class I).

Specifically regarding DNA fragmentation, results are still contradictory. Lewis and Aitken (2005) demonstrated that DNA damage will decrease in vivo and in vitro fertilization rates [35], and presence of excess leukocytes is cited as a cause for DNA fragmentation, but other authors suggest that leukocytospermia is not a cause of decreased in vitro fertilization results [36, 37].

Because sperm is processed for assisted reproduction, we evaluated the effect of semen processing through density gradient centrifugation on sperm with excess leukocytes. Semen processing is routinely used in assisted reproductive technologies in order to select sperm with progressive motility [20]. However, current techniques require centrifugation, which may lead to production and release of ROS by sperm and leukocytes [21]. Our study indicates that semen processing does indeed decrease the percentage of sperm with high DNA fragmentation (Comet grades III and IV).

Some studies have shown that after discontinuous density gradient centrifugation some seminal samples show an increase in sperm with cromatin alterations when compared with samples before processing, evaluated by SCSA [38]. Zini et al., however, demonstrated a significant increase in sperm with undamaged DNA after seminal processing performed by swim-up. This increase was not observed when density gradient discontinous centrifugation was performed in the same samples [39]. A study by our group also demonstrated that semen processing by a discontinuous density gradient did not improve apoptotic

DNA fragmentation, evaluated by the TUNEL technique [40]. The use of a more sensitive method for analysis of DNA fragmentation may explain the fact that differences were found in our study.

Interestingly, in men without leukocytospermia, decreasing values of post-processing progressive motility led to higher values of Comet class IV. Because the same could not be observed in men without leukocytospermia, one possible assumption is that, in men without leukocytospermia, a balance between ROS and antioxidants exists, in that oxidative stress-induced decreases in progressive motility are accompanied by increases in DNA fragmentation. In men with leukocytospermia, excessive ROS from leukocytes lead to loss of this balance, and the increase in DNA fragmentation is not dependent on oxidative stress-induced loss of motility.

Conclusion

In conclusion, while semen processing selects sperm with higher rates of DNA integrity independent of the presence or absence of leukocytes in semen, samples without leukocytospermia present more sperm without DNA fragmentation. Semen processing also selects sperm with higher mitochondrial activity.

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