Assessment of sperm DNA in patients submitted the assisted reproduction technology procedures

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ABSTRACT

Objective: This study aimed to produce data on sperm quality while maintaining the integrity of sperm DNA samples taken from patients submitted to in vitro fertilization (IVF) procedures at our center, and determine whether increased levels of histones were associated with sperm DNA damage and decreased fertilization, cleavage, and pregnancy rates. Such findings might shed light on the physiology and outcomes of pregnancy.

Methods: Semen samples from 27 patients divided into two groups were analyzed. The case group included individuals offered IVF; the control group had subjects with normal spermograms. Sperm DNA structure was assessed through phosphorylated histone H2AX analysis by flow cytometry.

Results: The patients with altered sperm parameters had more histones in sperm chromatin than the individuals with normal sperm parameters.

Conclusion: Results indicated that increased levels of histone in sperm chromatin do not affect embryo production, but affect the cleavage rate, embryo quality, and might thus reduce pregnancy rates. The integrity of the paternal genome is of paramount importance in the initiation and maintenance of a viable pregnancy in patients treated with assisted reproduction technology procedures. Further studies on sperm diagnostic tests at a nuclear level might improve the treatment offered to infertile couples.

Keywords: Sperm DNA, Histones, Pregnancy, Fertilization rate, ICSI.

INTRODUCTION

The Sperm DNA integrity is essential for the accurate transmission of the genetic code, in addition to serving as an indicator of the quality of spermatogenesis and male fertility (Smith *et al.*, 2007). Disturbances in the integrity of chromatin are characterized by the presence of breakages in single or double strands of sperm DNA, which lead to the formation of denatured segments (Rybar *et al.*, 2004).

Increased susceptibility to denaturation is suggestive of chromatin structure heterogeneity and has been linked to disturbances in spermatogenesis, abnormal morphology (Enciso *et al.*, 2011), decreased sperm concentration and motility (Benchai *et al.*, 2003), hampered embryonic development, and decreased fertility (Hallap, 2005). Although many authors have reported high DNA fragmentation levels in infertile patients with abnormal spermatozoa parameters (Aitken & De Luliis, 2007; Henkel, 2007), some patients with normal semen parameters and idiopathic infertility have been shown to present higher levels of damaged sperm DNA (Saleh *et al.*, 2003; Cohan *et al.*, 2006). More than 30% of these patients require longer times to establish a normal pregnancy, more assisted reproduction cycles, and are at a higher risk of

experiencing a miscarriage (Giwerzman *et al.*, 2010).

Sperm DNA damage can occur within the testes, the epididymis or in ejaculates. Its multifactorial etiology may include extrinsic and intrinsic factors (Zini & Sigman, 2009; Sakkas & Alvarez, 2010). Different hypotheses have been proposed to explain the origin of DNA damage in mature sperm of infertile men, including abnormalities in chromatin condensation, microdeletions, aneuploidies, chromosome rearrangements, breakages in single or double strand DNA, apoptosis, and oxidative stress (Gorcza *et al.*, 1993; Manicardi *et al.*, 1995; Sakkas *et al.*, 1999; Barroso *et al.*, 2000; Cho *et al.*, 2001; Souza *et al.*, 2010). In addition to biological factors such as infection, sexually transmitted diseases, obesity, diabetes, age, lifestyle factors including smoking (Pacey, 2010) may also affect chromatin integrity.

Sperm chromatin is organized within a highly condensed and compressed structure that significantly protects the DNA against damage while making it promptly available to the ooplasm (Bjorndahl & Kvist, 2010). Sperm chromatin undergoes a series of changes during spermatogenesis, with the first important event being the hyperacetylation of histones to facilitate the disassembly of the nucleosome. During this process, sperm DNA is extensively complexed with transition proteins. At the end of spermiogenesis these transition proteins are replaced by protamines, leaving the DNA in human sperm chromatin a fraction in nucleohistones and a fraction in nucleoprotamines, with 15% of the DNA bound to histones and 85% bound to protamines (Bjorndahl & Kvist, 2010; Oliva, 2006). Infertile men have increased histone to protamine ratios when compared to fertile controls. This change in the ratio, which consists of abnormal chromatin packaging, increases the susceptibility of sperm DNA to external stress, due to the lower compaction of chromatin (Zini & Sigman, 2009; Ozmen *et al.*, 2007).

In recent years, rapid advances in molecular biology have led to the development of numerous tests to assess various aspects of DNA and chromatin quality. The tests are divided into direct and indirect assays. Direct assays include TUNEL, the neutral comet assay, and in situ nick translation; indirect assays include the alkaline comet assay, the sperm chromatin structure assay (SCSA), and the sperm chromatin dispersion (SCD) test (Lewis *et al.*, 2008). These methods might detect cases of infertility missed in semen analysis. Studies have shown that levels of DNA damage in mature spermatozoa are significantly correlated with sperm fertilization potential. DNA damage analysis may help predict the success of pregnancy and birth produced by natural and assisted reproduction technology (ART) procedures. Moreover, characterizing normal levels of DNA damage in normal sperm might help prevent the use of samples with high levels of DNA damage in ART procedures (Tesarik *et al.*, 2004).

This study aimed to produce data on sperm quality while maintaining the integrity of sperm DNA samples taken from patients submitted to in vitro fertilization (IVF) procedures at our center, and determine whether increased levels of histones were associated with sperm DNA damage and decreased fertilization, cleavage, and pregnancy rates. Such findings might shed light on the physiology and outcomes of pregnancy.

MATERIALS AND METHODS

Twenty-one couples were offered intracytoplasmic sperm injections (ICSI) at the Endogin Clinic in Bauru, Brazil. Individuals who smoked, drank alcohol, or had an infertile partner were excluded. Ejaculated semen samples from six individuals with normal findings on physical examination – normal volume, absence of varicocele, and history of pregnancy with a partner without using ART procedures within the last 12 months – were used as controls. Only the samples and data of the individuals who gave consent to participating in the study were used.

The patients referred to ICSI underwent controlled ovarian stimulation along with suppression of the hypothalamic-pituitary axis. Pituitary suppression was carried out between days 18 and 22 of the menstrual cycle with a gonadotropin-releasing hormone analogue (Lupron TM Kit, Abbott SA, Paris, France). Fourteen to twenty days later, the patients were started on ovarian stimulation with follicle-stimulating hormone (TM Gonal-F, Serono, Geneva, Switzerland) at dosages ranging between 150 and 300 IU depending on the patient's age. Induction was followed with serial transvaginal ultrasound scans. When the dominant follicle reached 18 mm or at least two follicles reached 16 to 18 mm in diameter, the patients were administered 250 µg of recombinant human chorionic gonadotropin (rhCG) (Ovidrel, Serono, Geneva, Switzerland) to enable final follicular maturation. Follicular aspiration was performed approximately 34 to 36 hours after the administration of rhCG. The recovered oocytes were denuded and incubated in the initial culture medium (G1Plus, Vitrolife, Gothenburg, Sweden), covered with paraffin oil (Ovoil, Vitrolife) previously equilibrated at pH 7.2-7.4 and kept at 37°C.

Oocyte morphology was assessed and patients with most of their oocytes showing a perivitelline space, extrusion of the first polar body (PB) MII, and an intact oolemma were selected, administered microinjections, and included in the case group.

Semen samples were obtained by masturbation after a period of three to five days of abstinence from the day of oocyte aspiration. The collected material was kept for 30 minutes at room temperature and after liquefaction the samples were macroscopically assessed for color, volume, viscosity, and liquefaction. Then microscopic analysis was performed to determine sperm concentration and motility. Normal values were defined as a concentration >15 million/ml or 39x10⁶/ejaculate and motility >32% (progressive motility) and >40% (total motility). Morphology was considered normal when >4% according to the Tygerberg criteria.

Portions of the semen samples were centrifuged at 300 g for seven minutes and were then frozen. Seminal plasma was discarded and the seminal pellet was washed twice in PBS at a pH of 7.4, resuspended at a final concentration of 5x10⁶/ml, and stored at -196°C for sperm chromatin analysis. The same procedure was performed with the semen samples of the control group.

Intracytoplasmic sperm injection (ICSI) was performed according to the standard technique described by Palermo *et al.*, (1992). Embryo quality was assessed based on the modified morphological criteria described by Veeck (1991),

including items such as number and symmetry of blastomeres and degree of fragmentation. Samples assigned to Grade I had no fragmentation and equal blastomeres; samples on Grade II had <20% fragmentation and equal blastomeres; samples on Grade III had <20% fragmentation and unequal blastomeres; samples on Grade IV had >20% fragmentation and unequal blastomeres; and samples on Grade V had > 50% fragmentation.

Three days after oocyte aspiration, the embryos were selected for transfer. Fourteen days after embryo transfer the patients were tested for beta hCG levels to verify whether embryo implantation was successful. Ultrasound scans were performed in the 6th week of pregnancy to confirm fetus viability. Clinical pregnancy was defined when a gestational sac was detected in ultrasound scans. Clinically pregnant individuals who lost their fetuses before the 12th week of pregnancy were categorized as cases of miscarriage.

Tests to determine the presence of histones in sperm chromatin

A fraction of the semen samples was tested with the Apoptosis, DNA Damage and Cell Proliferation Kit (BD Biosciences, catalog number 562253). Analysis by flow cytometry was performed according to the manufacturer's instructions. Sperm samples were thawed and diluted in 1ml buffer (1x10⁶ sperm) and centrifuged at 250g for five minutes. The supernatant was discarded and the pellet resuspended in 100 µl BD Cytotfix for fastening and Cytosperm (permeabilizing solution) and kept at room temperature for 30 minutes. Then it was washed with 1 ml 1x BD Perm / Wash Buffer, and the supernatant was again removed and the pellet resuspended in 20µl BDPerm / Wash Buffer plus antibody Alexa Fluor 647Mouse anti-H2AX (pS139.5µl / test). The samples were incubated for 20 minutes at room temperature. After 60 minutes, the samples were washed and resuspended in 1ml of 1 x BD Perm / Wash Buffer and evaluated in the flow cytometer (FACScalibur, Becton Dickinson, San Jose, California, USA). A minimum of 100,000 cells per sample were assessed.

Statistical Analysis

Statistical analysis was performed using software package GraphPad Prism Version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). The Kolmogorov-Smirnov test was used to verify the normality and homogeneity of variance when the samples had parametric data. The t-test was used the samples had non-parametric data. The Mann-Whitney test or the Kruskal-Wallis test were performed followed by Dunn's test. Significance was attributed to events with a $P < 0.05$. Fisher's exact test was used to assess embryo quality and percentage of histones in relation to embryo quality.

RESULTS

The semen samples of 27 subjects were analyzed and divided into two groups separating patients offered IVF and healthy controls with normal spermograms. No statistically significant differences were found (Figure 1A) between case and control group subjects. A trend was observed ($P = 0.0808$) in which IVF patients ($n = 21$; 3.9 ± 1.4) had a higher incidence of histones in sperm DNA chromatin than controls ($n = 6$; 0.48 ± 0.11). The impact of the level of histones in male fertility was further analyzed in terms of positive and negative cases of clinical pregnancy (Figure 1B). Again, no statistically significant difference was found in clinical pregnancy rates ($P = 0.2486$). This may have occurred because one patient with high levels of histones was positive for clinical pregnancy. Patients were also assessed for semen quality before freezing (Figure 1C), but no sta-

Figure 1: Representation of Whisker plots (min to max) of the data obtained from the analysis of histones in the sperm chromatin of patients undergoing in vitro fertilization procedures versus controls – males whose partners have had spontaneous pregnancies within less than 12 months (A). Patients positive for pregnancy versus individuals negative for pregnancy versus controls (B); Patients grouped according to sperm quality into abnormal, normal, and controls (C).

Figure 2: Representation of Whisker plots (min to max) of the data obtained from the analysis of histones in the sperm chromatin of patients undergoing in vitro fertilization procedures grouped into subjects positive for pregnancy versus individuals negative for pregnancy in relation to cleavage rates (Fig A); and rate of transferable embryos (Fig B).

Figure 3: Graphs representing the analysis of embryo quality versus pregnancy using Fisher's exact test (fig A). Thirty-two embryos of Grades I and II (good quality) resulted in pregnancy, while 40 embryos – 17 in Grades I and II and 23 in Grades III and IV (poor quality) – did not result in pregnancy. Concerning the level of histones in sperm chromatin, 22 transferred embryos (10 of good and 12 of poor quality) had high histone levels ($> 1\%$) and 50 (39 good and 11 poor quality embryos) had low histone levels ($< 0.99\%$).

tistically significant differences were found ($P = 0.3757$). However, patients considered to have normal quality semen ($n = 13$) had higher histone levels (5.4 ± 2.2) than controls ($n = 6$; $00:48 \pm 00:11$), while individuals with altered semen quality had only a slight increase in histone levels ($n = 8$; 1.8 ± 0.8).

Cleavage rate $P = 0.0506$ (Figure 2A) and the percentage of histones in sperm chromatin influenced the results. Patients with positive pregnancies (98.61 ± 1.38) had higher cleavage rates than individuals negative for pregnancy (87.98 ± 4.0). No statistically significance difference

($P = 0.1245$) was observed in terms of the percentage of transferable embryos (64.57 ± 13.6 versus 57.6 ± 8.0 , for individuals positive and negative for pregnancy, respectively), as shown in Figure 2B.

Embryo quality was also assessed. As expected, clinical pregnancy rates were higher among individuals with higher quality embryos (Grades I and II) than in subjects with poorer quality embryos (Grades III and IV), as shown in Figure 3A ($P < 0.001$). The presence of histones in sperm chromatin significantly interfered with the quality of the transferred embryos (Figure 3B). Better quality embryos

were produced from patients with lower sperm chromatin histone levels ($P = 0.0123$).

DISCUSSION

Oocytes fertilized by sperm with DNA alterations may significantly impair embryonic development and fetal health (Evenson *et al.*, 2002), in addition to transmitting mutations that might affect the fertility of the next generation (Aitken & Krausz, 2001). Thus, chromatin integrity plays a vital role in the paternal contribution to the production of a healthy fetus.

This study compiled data on chromatin alterations that may compromise the viability of sperm used in IVF. These changes are basically caused by apoptosis during spermatogenesis, breaks during chromatin remodeling, and fragmentation induced by reactive oxygen species (Tavalee *et al.*, 2009; Sakkas & Alvarez, 2010).

In recent years, the evaluation of sperm DNA fragmentation was recognized as a predictor of the likelihood of conception. The capacity for natural conception decreases in sperm with abnormal chromatin (Spano *et al.*, 2000; Sergerie *et al.*, 2005). Henkell *et al.* (2004) and Simon *et al.* (2011) found that patients treated repeatedly with conventional IVF experienced negative effects on fertilization and blastocyst development in the course of pregnancy. Reports on ICSI have published contradictory results. Some groups found (statistically significant) negative associations between the percentage of sperm with fragmented DNA and fertilization rates with ICSI (Lopes *et al.*, 1998; Simon *et al.*, 2013), while other authors did not observe differences in the fertilization rate but reported negative effects upon pregnancy rates (Benchaib *et al.*, 2003) or embryo quality (Muriel *et al.*, 2006). Higher levels of DNA fragmentation have been described as a cause for low quality embryo implantation and miscarriage (Tesarik *et al.*, 2004; Evenson *et al.*, 2002; Borini *et al.*, 2006). Conversely, some studies did not describe sperm DNA fragmentation as having a role in ICSI results (Bungum *et al.*, 2004; Nicopoullos *et al.*, 2008). These authors looked into DNA fragmentation in the total population of sperm (morphologically abnormal and normal), but during the ICSI procedure, only sperm with good motility and morphology was selected for injection. The inadvertent selection of sperm cells based solely on normal motility and morphology without considering DNA integrity may yield undesired outcomes in ICSI procedures, which might impair not only embryonic development, but also the resulting offspring. Greco *et al.* (2005) showed that microinjection of sperm with fragmentation above 15% as analyzed using TUNEL resulted in a pregnancy rate of 5.6% versus 44.4% when sperm DNA fragmentation was below 6%.

The results analyzed in this study showed that even men with normal sperm counts may have high levels of alteration in the compaction of chromatin, which means it might take longer to have a successful pregnancy, thus increasing the chances of having more ART cycles and the risk of having a miscarriage. Tests showed that these alterations were not related to fertilization rates, but to significant reductions in embryo quality and consequently in the pregnancy rates attained with ICSI cycles.

Although contradictory, no associations were found between embryo quality and sperm DNA damage measured by sperm chromatin structure assay (SCSA) and TUNEL (Irvine *et al.*, 2000; Larson *et al.*, 2000; Benchaib *et al.*, 2003; Huang *et al.*, 2005; Benchaib *et al.*, 2007; Frydman *et al.*, 2008), despite the different test sensitivities and the types of DNA damage measured specifically in each test.

There is currently a threshold assigned to percent DNA fragmentation in a sample above which pregnancy becomes unlikely. This threshold varies between 12%

(Duran *et al.*, 2002) and 36.5% (Henkel *et al.*, 2003), and has been described as 15% (Benchaib *et al.*, 2007) and 20% (Sergerie *et al.*, 2005). Many are the possible reasons for the discrepant thresholds: different study populations (intrauterine insemination (IUI), in vitro fertilization (IVF) and/or intracytoplasmic sperm microinjection (ICSI); type of sperm used (obtained after washing or selected); or the type of count (operator or by flow cytometry).

Despite these observations, sperm with damaged DNA can preserve the potential for fertilization and produce viable embryos (Tesarik *et al.*, 2004). While the oocyte might repair the damaged paternal DNA, this ability is limited and depends on the level of DNA damage (Fatehi *et al.*, 2006). DNA damage may predispose the developing embryo to mutations and induce disease in the offspring (Aitken & Krausz, 2001). Sperm DNA integrity is an important factor in the overall success of ART procedures. Although several studies have demonstrated the importance of assessing chromatin integrity and thresholds have been set to assess fertility levels, this parameter has not been evaluated routinely in andrology laboratories. This is possibly due to the complex protocols and expensive equipment – the likes of fluorescence microscopes and flow cytometers – required to run such tests.

Successful ART procedures rely on many factors other than semen quality and the ability of sperm to merge with the oolemma, such as the oocyte, uterine receptivity, and maternal immune status.

CONCLUSIONS

Changes in chromatin structure negatively affect the clinical outcomes of ICSI procedures. Clinical evidence suggests that these changes may curtail the effect of ART procedures even in men with normal sperm. The impact of chromatin structure alterations on fertilization rates is still controversial, but there is agreement over the negative effects it produces on embryonic development and pregnancy rates.

The predictive value of a DNA fragmentation test consists of a sum of factors, such as: 1) whether fragmentation occurred in single or double-stranded DNA (single offers better prognosis); 2) percentage of sperm with damaged DNA; higher percentages mean the likelihood of the egg being fertilized by sperm with intact DNA is reduced; 3) degree of DNA fragmentation in sperm (more intense fragmentation means DNA is less likely to be repaired); 4) primary or secondary damage; 5) type of test used (direct or indirect); 6) whether the damage affects encoding sequences (relatively low likelihood, as more than 90% of the DNA consists of non-encoding sequences); 7) the ability of the oocyte to repair the damage; 8) ability of the embryo to repair the damage; 9) number of oocytes in metaphase II; and 10) sample processing. It is also important to remember that although the 2006 Practice Committee Guidelines of the American Society for Reproductive Medicine (ASRM) accepted that sperm DNA fragmentation is more common in infertile men and that it may contribute to poor reproductive performance, there is no proven role for routine testing of DNA integrity in the evaluation of infertility, as current findings are enough only to suggest, rather than confirm, an association between sperm DNA testing and ART procedure outcomes.

The lack of an indication for routine testing was supported in a review published by Collins *et al.* (2008). More studies are needed to verify the association of DNA damage test results and the clinical outcomes of ART procedures. Oocyte quality may also be a major determinant on the negative effects introduced by sperm DNA damage.

CONFLICT OF INTERESTS

No conflict of interest have been declared.

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