Influence of bovine sperm DNA fragmentation and oxidative stress on early embryo in vitro development outcome

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Abstract

Sperm chromatin fragmentation may be caused by a number of factors, the most significant of which is reactive oxygen species. However, little is known about the effect of sperm oxidative stress (OS) on DNA integrity, fertilization, and embryonic development in cattle. Therefore, the goal of this study was to evaluate the influence of sperm OS susceptibility on the DNA fragmentation rate and in vitro embryo production (IVP) in a population of bulls. Groups of cryopreserved sperm samples were divided into four groups, based on their susceptibility to OS (G1, low OS; G2, average OS; G3, high OS; and G4, highest OS). Our results demonstrated that the sperm DNA integrity was compromised in response to increased OS susceptibility. Furthermore, semen samples with lower susceptibility to OS were also less susceptible to DNA damage (G1, 4.06%; G2, 6.09%; G3, 6.19%; and G4, 6.20%). In addition, embryo IVP provided evidence that the embryo cleavage rate decreased as the OS increased (G1, 70.18%; G2, 62.24%; G3, 55.85%; and G4, 50.93%), but no significant difference in the blastocyst rate or the number of blastomeres was observed among the groups. The groups with greater sensitivity to OS were also associated with a greater percentage of apoptotic cells (G1, 2.6%; G2, 2.76%; G3, 5.59%; and G4, 4.49%). In conclusion, we demonstrated that an increased susceptibility to OS compromises sperm DNA integrity and consequently reduces embryo quality.

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Introduction

In vitro production (IVP) of bovine embryos with frozen/thawed semen is used worldwide for commercial purposes. However, a high variability in the outcome of bovine embryo IVP has been reported (Palma & Sinowatz 2004, Alomar et al. 2008). One of the reasons proposed to explain such variation is the impaired integrity of sperm chromatin (Boe-Hansen et al. 2005).

The primary cause of sperm chromatin damage is reactive oxygen species (ROS)-induced oxidative stress (OS; Sakkas & Alvarez 2010). ROS are molecules or atoms with one or more unpaired electrons (Halliwell 1991); the unpaired electron(s) makes such substances extremely reactive with other molecules (Makker et al. 2009). ROS are important in the regulation of normal cellular functions. In sperm cells, they also play a key role in essential events such as sperm capacitation, acrosome reaction, hyperactivation, and oocyte interaction (Agarwal & Allamaneni 2004). Despite the beneficial role of ROS in sperm physiology, an imbalance between ROS production and scavenger systems can be extremely detrimental to spermatozoa. The male germ cells are especially susceptible to OS because of the high content of polyunsaturated fatty acids in the sperm membrane. These fatty acids contain more than two carbon–carbon double bonds that are essential for conferring the fluidity necessary for the plasma membrane to participate in the membrane fusion events associated with fertilization (Aitken & Krausz 2001, Makker et al. 2009). However, when ROS attack the double bonds, a lipid peroxidation chain reaction is initiated that, if not arrested, may lead to mitochondrial...
disruption, chromatin fragmentation by increasing the frequency of single- and double-strand DNA breaks, and the impairment of sperm fertilizing capacity and embryo development (Aitken & Krausz 2001, Makker et al. 2009).

Studies have demonstrated the role of sperm DNA in embryo IVP (Esterhuizen et al. 2000, Fernandez et al. 2003, Enciso et al. 2006). According to Virro et al. (2004), paternal genome alterations can influence embryo development even when a sperm is injected directly into the oocyte via ICSI. Another study has suggested that human infertility disturbances in the absence of an etiological diagnosis can be related to sperm DNA fragmentation (Tesarik et al. 2002). Consistent with these data, Evenson et al. (1999) reported that a normal-shaped sperm head may contain chromosomes with microdeletions, aneuploidy, DNA strand breaks, and abnormal sperm chromatin structures and still fertilize an oocyte.

The goal of this study was to correlate the oxidative status and sperm DNA fragmentation rate in bulls and to verify the influence of OS on IVP embryo development and DNA integrity.

Subjects and methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical. Tissue culture media (TCM; Hepes and Bicarbonate) and FCS were obtained from Gibco. The experiments were approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Sciences, University of São Paulo (protocol number 1110/2007).

Initial semen processing

Commercial semen straws from 45 bulls were used. Two straws from each animal were thawed in a 37 °C water bath for 30 s and then washed twice in DPBS (200 g) for 5 min. After the second wash, the sperm motility and concentration were evaluated, and the spermatozoa were diluted to a final concentration of 1×10⁶ cells/ml. The sperm cells were then subjected to sperm evaluation (a thiobarbituric acid reactive substances (TBARS) assay, a comet assay, and an assessment of sperm DNA integrity) and IVP of bovine embryos.

Thiobarbituric acid reactive substances

The TBARS assay is used to estimate the resistance of sperm to OS (Nichi et al. 2007). Briefly, lipid peroxidation was induced by adding ferrous sulfate (250 ml, 4 mM) and sodium ascorbate (250 ml, 20 mM) to 1 ml of the sperm suspension. This mixture produces a hydroxyl radical-generating system based on the Fenton reaction (Lloyd & Phillips 1999). The mixture was then incubated for 1.5 h at 37 °C. Subsequently, 500 µl of the incubation mixture and 1000 µl of a 10% solution (v:v) of trichloroacetic acid (10%) were mixed and centrifuged (18 000 g, 15 min, 15 °C) to precipitate protein. A 500 µl aliquot of the supernatant was then mixed with 500 µl 1% (v:v) thiobarbituric acid (1% TBA, diluted in 0.05 M sodium hydroxide) in a cryotube vial, placed into a boiling water bath (100 °C) for 15 min and then immediately cooled in an ice bath (0 °C) to stop the chemical reaction. The TBARS were then quantified using a spectrophotometer (u.v.-vis Spectrophotometer Ultrospec 3300 Pro; Biochrom Ltd., Cambridge, UK) at a wavelength of 532 nm. The TBARS concentration was determined using the value of 1.56×10⁷ M⁻¹ cm⁻¹ as the malondialdehyde (MDA) extinction coefficient (Buege & Aust 1978, Donnelly et al. 2000). The lipid peroxidation index was described as nanograms of TBARS/10⁸ sperm.

Experimental design

The samples were divided into four groups based on their level of TBARS. The median and quartiles were calculated and used to determine the limit values for each group as follows: Group 1 included the minimum value to the lower quartile (low TBARS Group, G1); Group 2 included the lower quartile to the median (average TBARS Group, G2); Group 3 included the median to the upper quartile (high TBARS Group, G3); and Group 4 included the upper quartile to the maximum value (highest TBARS Group, G4).

Assessment of DNA integrity by a modified alkaline comet assay

The sperm nuclear DNA integrity was evaluated by modified alkaline single-cell gel electrophoresis (comet assay) as described by Donnelly et al. (2000), with modifications. The slides were pre-coated with 1% normal-melting-point agarose in TBE (0.089 M Tris base, 0.089 M borate, and 0.002 M EDTA) overnight. A 100 µl aliquot of fresh semen diluted in 0.75% low-melting-point agarose in TBE to a final concentration of 1×10⁶/ml was added to each slide, covered with a coverslip and cooled for 5 min at 4 °C to solidify. Following gel solidification, the coverslips were gently removed, and the slides were covered with 300 µl 0.75% low-melting-point agarose in TBE and cooled for 10 min at 4 °C to solidify. After solidification, the coverslips were gently removed, and the slides were covered with 1000 µl of lysis solution 1 (100 mM Na₂–EDTA, 10 mM Tris, 2.5 M NaCl, pH 11.0, and 20 µg proteinase K) for 1 h at 56 °C. The slides were then washed three times in Milli-Q water to remove lysis solution 1, covered with 1000 µl lysis solution II (100 mM Na₂–EDTA, 10 mM Tris, 2.5 M NaCl, pH 11.0, 40 mM dithiothreitol, and 2% Triton X-100), and cooled for 2 h at 4 °C. The slides were then washed three times in Milli-Q water for 5 min to remove the excess salts and immediately immersed in cold alkaline electrophoresis solution (300 mM NaOH, 1 mM Na₂–EDTA, pH > 13.0) for 20 min. Electrophoresis was then performed for 25 min at 3 V/cm and 270 mAmp. Following electrophoresis, the slides were washed three times with TBE (pH 7.4) and fixed with ethanol (70, 92.8, and 100%) for 5 min each. After the slides were air dried, they were stained with 0.5 µl ethidium bromide (10 mg/ml) and diluted in 1500 µl TBE for 15 min. The slides were rehydrated with TBE for 15 min to remove background staining and evaluated using a fluorescence Olympus IX80 microscope (Olympus Corporation, Tokyo, Japan) equipped with an excitation filter of 515–560 nm from a 100-W mercury lamp and a barrier filter of 600 nm. A total of
200 sperm cells were evaluated using Comet Score Software (Sumrduck, VA, USA), which was freely provided by TriTek-Corp (http://autocomet.com/products_cometscore.php).

The software provides a rectangular measurement frame to evaluate comets and assumes that the amount of DNA at a location is proportional to the pixel intensity at that position. The cells were scored for the comet tail length (the number of pixels in the horizontal direction in the comet), the comet area (the number of pixels in the comet), the mean comet intensity (the mean intensity of pixels in the comet), the olive moment (the sum of the tail intensity profile values multiplied by their relative distances to the head center and divided by the total comet intensity), the mean head intensity (the mean intensity of pixels in the head), the percentage of DNA in the head (the total head intensity divided by the total comet intensity and multiplied by 100), the tail length (the head diameter subtracted from the comet length), the mean comet intensity (the mean intensity of pixels in the comet), and the tail moment (the percentage of DNA in the tail multiplied by the tail length).

**Assessment of sperm DNA integrity**

To further analyze the sperm DNA, chromatin susceptibility to acid-induced denaturation in situ was assessed. The chromatin instability was then quantified by flow cytometric measurement of the metachromatic shift from green (double-strand DNA) to red (denatured single-strand DNA) of acridine orange (AO) fluorescence, as described by Boe-Hansen (2005). The samples were thawed in a water bath and diluted with 100 µl TNE buffer (0.01 M Tris–HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) mixed with 400 µl of acidified detergent solution (0.08 M HCl, 0.1% Triton X-100, 0.15 M NaCl, pH 1.2), and after 30 s, the spermatozoa were stained by adding 600 µl of AO staining solution (0.037 M citric acid, 0.126 M Na2HPO4, 0.0011 M disodium EDTA, 0.15 M NaCl, pH 6.0). After 3–5 min of staining, the samples were examined in a flow cytometer (Guava EasyCyte, Guava Technologies, Hayward, CA, USA); 5000 spermatozoa were evaluated in each sample.

**IVP of embryos**

Embryos were produced according to Yamada et al. (2007). Briefly, ovaries obtained from a slaughterhouse were transported to the laboratory in saline solution at 30 °C within 2–3 h. Cumulus–oocyte complexes (COCs) were aspirated with an 18-gauge needle from 2 to 8 mm follicles. The oocytes with homogeneous ooplasm surrounded by multilayer compacted cumulus cells were selected for in vitro maturation (IVM). For IVM, COCs were washed three times in the holding medium (TCM 199 Hepes supplemented with 10% FCS, 22 µg/ml pyruvate, and 50 µg/ml gentamycin) and three times in the maturation medium (TCM 199 Bacarbonate supplemented with 10% FCS, 22 µg/ml pyruvate, 50 µg/ml gentamycin, 0.5 µg/ml FSH–Follitropin-V (Vetrepharm, Inc., Belleville, ON, Canada), 50 µg/ml human chorionic gonadotrophin (Vetecor Laboratories, Calier, Spain), and 1 µg/ml 17β-estradiol) and placed in 90 µl microdroplets of maturation medium covered with mineral oil at 38.5 °C under 5% (v/v) CO2 in air and high humidity. Selected oocytes (30 oocytes/group per replicate; a total of seven replicates with approximately seven bulls per replicate, depending on the number of oocytes available) were matured for ~22–24 h and then inseminated with 1×106 spermatozoa/ml (all groups). At 18 h post-insemination (hpi), putative zygotes were mechanically denuded of cumulus cells by pipetting. Presumptive zygotes were cocultured in a granulose cell monolayer in 90 µl microdroplets of SOFAa (Terovit et al. 1972) supplemented with 5% (v/v) FCS for 9 days. Cleavage and blastocyst rates were recorded at 48 hpi and at day 9 of embryo culture respectively. Blastocysts were collected at day 9 of in vitro culture and washed individually in PBS–PVP for the TUNEL assay. Initial droplets with 30 oocytes were considered experimental units.

**TUNEL assay**

The TUNEL assay was performed as described by Paula-Lopes & Hansen (2002) with small modifications. Embryos fixed in 4% (w/v) paraformaldehyde were subjected to TUNEL analysis using an In Situ Cell Death Detection Kit, Fluorescein (Boehringer Mannheim/Roche Diagnostics) and following the manufacturer’s instructions. Briefly, embryos were washed once in PBS (pH 7.4) containing 1 mg/ml PVP (PBS–PVP) and permeabilized for 2 h in four-well plates containing 500 µl 0.5% (w/v) Triton X-100 and 0.1% (w/v) sodium citrate at room temperature. The positive and negative controls were incubated in RQ1 RNase–free DNase (50 U/ml) at 37 °C for 1 h. The embryos were washed in PBS–PVP and incubated in a 25 µl drop of the TUNEL reaction mixture (containing FITC-conjugated dUTP and terminal deoxynucleotidyl transferase) for 1 h at 37 °C in the dark. The negative control was incubated in the absence of terminal deoxynucleotidyl transferase. The embryos were then incubated in four-well plates containing 5 µg/ml Hoechst 33342, for 10 min at room temperature. The embryos were washed three times in PBS–PVP to remove excess Hoechst 33342, placed on microscope slides, and mounted with 10 µl Antifade (1,4-diazabicyclo [2.2.2]octane (DABCO)). The TUNEL labeling was observed using an Olympus IX80 epifluorescence microscope (Olympus Corporation) with fluorescence detection filters appropriate for blue (Hoechst 33342) and green (fluorescein) wavelengths. The total number of nuclei and the number of TUNEL-positive (TUNEL+) nuclei were determined for each embryo.

**Statistical analysis**

All data were evaluated using the SAS System for Windows (SAS Institute, Inc., Cary, NC, USA). To calculate the median and quartiles, the PROC MEANS was used. The effect of the TBARS groups was determined using parametric (general linear model procedure–PROC GLM; least significant difference (LSD) test) and non-parametric (NPAR1WAY; Mann–Whitney U test) tests, according to the residue normality (Gaussian distribution) and the variance homogeneity of each variable. A P value <0.05 was considered statistically significant. The results are reported as untransformed means ± S.E.M. Pearson and Spearman correlations were used for parametric and non-parametric variables respectively.
Results

**Thiobarbituric acid reactive substances**

The TBARS assay was used to divide the population into four groups. The quartiles (lower and upper) and the median were used as limit values (Table 1). Group 1 included animals with TBARS values below 86.7 (low TBARS Group, G1), Group 2 included animals with TBARS values between 86.7 and 131.9 (average TBARS Group, G2), Group 3 included animals with TBARS values between 132 and 211.4 (high TBARS Group, G3), and Group 4 included animals with TBARS values > 211.4 (highest TBARS Group, G4).

**Evaluation of DNA integrity assessed by the modified alkaline comet assay**

The sperm DNA integrity was negatively influenced by an increase in the OS susceptibility of these cells. Table 2 shows the results of the comet assay. A large amount of DNA in the tail of the comet was observed for Groups 1, 2, and 4 compared with that observed for Group 3 (P < 0.05). No differences were found between groups for the other variables evaluated by the comet assay (P > 0.05).

**Assessment of DNA integrity by the sperm chromatin structure assay**

The assessment of the sperm chromatin structure integrity demonstrated that the Group 1 samples, which had the lowest TBARS values, were also less susceptible to DNA damage compared with samples from the other groups (P < 0.05; Fig. 1).

**In vitro production**

Sperm motility, which was evaluated prior to IVF, ranged from 45.45 ± 5.62% to 58.18 ± 5.85% (mean ± S.E.M.) with no differences observed among the groups (G1, 52.5 ± 5.52; G2, 58.18 ± 5.85; G3, 51.82 ± 5.36; and G4, 45.45; P > 0.05). However, variations among groups were observed for the embryo IVP results. The cleavage rate (cleaved embryos/oocytes) decreased as the OS increased, but no significant difference in the blastocyst rates (blastocyst/oocytes) was observed among the groups (Fig. 2).

**TUNEL assay**

No significant difference in the total number of embryo cells (blastomeres) was found among the groups (P > 0.05). However, the number of TUNEL+ cells significantly increased as the index for OS increased (P < 0.05; Fig. 3).

**Correlations**

The correlation rates among the sperm motility, the cleavage rate, the blastocyst rate, the number of blastomeres (BLAST), the number of positive cells for the TUNEL assay (T+), the TBARS value, the sperm chromatin structure assay (SCSA), and the comet assay (the comet length, the comet area, the comet mean intensity, the comet head mean intensity, the percentage of DNA in the comet head, the comet tail length, the comet tail mean intensity, the percentage of DNA in comet tail, the tail moment, and the olive moment) are shown in Table 3. Only correlation rates with a significance level of P < 0.05 were included in the table.

### Table 1

<table>
<thead>
<tr>
<th>Mean</th>
<th>Median</th>
<th>Upper quartile</th>
<th>Lower quartile</th>
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<tr>
<td>169.2</td>
<td>131.9</td>
<td>211.4</td>
<td>86.7</td>
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### Table 2

<table>
<thead>
<tr>
<th>Variables (mean ± S.E.M.)</th>
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<tr>
<td></td>
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<tr>
<td>Total comet</td>
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<tr>
<td>Length (px)</td>
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<tr>
<td>Area (px)</td>
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<tr>
<td>Mean intensity (px)</td>
<td>49.91±2.30</td>
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<tr>
<td>Olive moment (px)</td>
<td>2.26±0.88</td>
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<tr>
<td>Comet head</td>
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<tr>
<td>Mean intensity (px)</td>
<td>50.48±2.37</td>
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<tr>
<td>DNA% (px)</td>
<td>93.03±1.60</td>
</tr>
<tr>
<td>Comet tail</td>
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</tr>
<tr>
<td>Length (px)</td>
<td>6.57±2.29</td>
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<tr>
<td>Mean intensity (px)</td>
<td>90.59±16.54</td>
</tr>
<tr>
<td>DNA% (px)</td>
<td>6.97±1.60±4</td>
</tr>
<tr>
<td>Tail moment (px)</td>
<td>2.37±1.20</td>
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Different symbols (* and †) in each line represent significant differences (P < 0.05).
A negative correlation was found between the comet mean intensity and the blastocyst rate. There was also a negative correlation between the blastocyst rate and the comet head mean intensity. The sperm susceptibility to OS (TBARS) was negatively correlated with the cleavage and blastocyst rates. In addition, the TBARS value was positively correlated with the sperm susceptibility to DNA denaturation (SCSA) and the number of TUNEL + blastomeres.

Discussion

ROS are physiologically produced during sperm metabolism (Marti et al. 2008). These free radicals are necessary for sperm physiological processes such as the maintenance of membrane fluidity, sperm capacitation, acrosome reaction, sperm–oocyte interactions, and the signaling pathways involved in the fertilization, implantation, and early embryo development process (de Lamirande et al. 1997, Sakkas et al. 1998, Sanocka & Kurpisz 2004, Bansal & Bilaspuri 2010). However, an excessive level of free radicals can cause deleterious effects on sperm function, fertilization, preimplantation embryo development, and the fate of subsequent offspring (Agarwal & Allamaneni 2011).

In our study, semen samples from bulls selected for embryo IVP were divided into four groups based on their susceptibility to OS as measured by the TBARS assay and then evaluated for sperm DNA fragmentation by both the SCSA and the comet assay. A positive correlation between susceptibility to OS (TBARS) and sperm DNA fragmentation was observed in both the SCSA and the comet assay. However, no correlation between the SCSA and the comet assay was observed. It is important to emphasize that even with a high degree of chromatin condensation, sperm DNA remains susceptible to damage because some histones remain in the mature spermatozoon chromatin (Miller & Paradowska 2013). This is important because histone-bound DNA is much more susceptible to damage than protamine-bound DNA. According to Shaman & Ward (2006), most of the DNA breaks identified by the SCSA are located in the toroid linker regions, which are chromatin regions that are more sensitive to damaging agents. By contrast, in the comet assay, all protamines and histones were extracted; therefore, it may detect more DNA breaks per sperm cell than the SCSA. However, the DNA fragmentation detection in the comet assay cannot distinguish the chromosomal localization of the damage. Therefore, the SCSA maybe more effective at identifying possible cell chromatin impairment that will be intensified during IVF by the considerable stress caused by incubation at physiological temperature with a lack of suitable antioxidants.

The present work was performed using cryopreserved semen. According to Amirat et al. (2005), deleterious effects may occur during the initial dilution in the sperm freezing process, contributing to ROS formation. However, an increased susceptibility to OS immediately after thawing may not necessarily indicate a higher level of sperm DNA fragmentation. Indeed, the SCSA results showed here demonstrate that even a small increase in spermatozoon susceptibility to OS can significantly increase spermatozoon susceptibility to chromatin fragmentation (Fig. 1). In addition, the positive correlation between the SCSA and the TBARS assay ($r = 0.416$, $P = 0.009$; Table 3) may support this hypothesis.

Routine semen analysis includes an evaluation of sperm motility, sperm concentration, and the number of morphologically normal cells. Many studies have demonstrated that these assessments are insufficient because it is not the absolute number of sperm but the sperm’s functional competence that determines
fertility (Liu & Baker 2002, Aitken 2006, Bungum et al. 2011, Shamsi et al. 2011). Interestingly, sperm motility in this study was not affected by the increase in OS. This result may be because the technique used to evaluate OS was based on a challenge with an ROS-generating system. Thus, the TBARS assay demonstrated the susceptibility of sperm to OS, not that sperm function was already impaired.

Several studies in humans have verified the influence of OS on IVF results (Ruder et al. 2009, Mahfouz et al. 2010, Agarwal et al. 2012). Moreover, all cellular components including lipids, proteins, nucleic acids, and sugars are potential targets of OS (Agarwal et al. 2008). Therefore, OS is considered one of the most important factors contributing to poor semen quality. In addition, when manipulated in vitro during assisted reproductive techniques, spermatozoa may be exposed to high levels of ROS (Bansal & Bilaspuri 2010). This imbalance between ROS and antioxidants (Desai et al. 2010) may induce DNA damage and decrease the sperm fertility during storage.

It is known that sperm may affect embryonic development. For example, embryos produced by high fertility bulls enter the first S-phase of the cell cycle faster and are greater in length than embryos produced with sperm from low fertility bulls (Eid et al. 1994). In our study, a lower cleavage rate was observed in embryos from sperm groups with high susceptibility to OS (TBARS). Furthermore, there was a negative correlation between the susceptibility to OS and the cleavage rate. While OS negatively affected the embryonic cleavage, the blastocyst rate was not significantly different among the experimental groups. Therefore, we can infer that independent of their susceptibility to OS, embryos that bypass the embryonic blocking phase are able to reach the blastocyst stage. Corroborating this hypothesis, no

**Figure 3** Role of sperm oxidative stress susceptibility on the average number of embryo blastomeres and the number of embryonic cells undergoing apoptosis. The results are means ± s.e.m. Different superscript letters in each bar represent significant differences (P < 0.05). (G1: low susceptibility to oxidative stress; G2: average susceptibility to oxidative stress; G3: high susceptibility to oxidative stress; and G4: highest susceptibility to oxidative stress).

<table>
<thead>
<tr>
<th>TBARS groups</th>
<th>Average number of blastomeres</th>
<th>TUNEL-positive cells</th>
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<tbody>
<tr>
<td>G1</td>
<td>80 ± 10</td>
<td>3 ± 0.5</td>
</tr>
<tr>
<td>G2</td>
<td>90 ± 15</td>
<td>4 ± 0.7</td>
</tr>
<tr>
<td>G3</td>
<td>100 ± 15</td>
<td>5 ± 0.8</td>
</tr>
<tr>
<td>G4</td>
<td>110 ± 20</td>
<td>6 ± 1.0</td>
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<tr>
<th>Cleavage</th>
<th>Blastocyst</th>
<th>SCSA</th>
<th>BLAST</th>
<th>TUNEL+</th>
<th>Not significant (P &gt; 0.05)</th>
</tr>
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<tbody>
<tr>
<td>1.00</td>
<td>1.00</td>
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Table 3 Correlations (r) and significance levels (P) among the measured variables.
difference in the blastocyst cell number was observed among the TBARS groups.

Although we observed that an increased susceptibility to OS did not affect the number of blastomeres, it significantly increased the number of embryonic cells undergoing apoptosis. Therefore, an increased susceptibility to OS can induce developmental abnormalities in embryos and, thus, lead to apoptosis in the embryo. No trend or pattern related to the localization of TUNEL+ cells (trophectoderm or inner cell mass) was observed when embryos were evaluated by the TUNEL assay (Fig. 4). It has been reported that although DNA damage does not influence the spermatozoon ability to fertilize an oocyte, it has a negative effect on embryonic development (Fatehi et al. 2006). Thus, as the susceptibility to OS in this study was correlated with spermatozoon DNA damage, it is plausible that the high apoptosis incidence in blastocysts derived from fertilization with spermatozoa with high susceptibility to OS could be the consequence of high DNA fragmentation in this cell.

The blastocyst apoptosis results can also be explained by the spermatozoa sensibility to oxygen-induced damage mediated by lipid peroxidation (Sikka 1996, Desai et al. 2009). Lipid peroxidation in spermatozoa promotes a progressive accumulation of lipid hydroperoxides in the plasma membrane of these cells; the accumulated lipid hydroperoxides subsequently decompose to form MDA (Voitkun & Zhitkovich 1999). As MDA is a mutagenic substance, it is possible that fertilization using spermatozoa with high susceptibility to OS may lead to embryonic apoptosis regardless of the existence of DNA fragmentation (Zribi et al. 2011). Moreover, the TUNEL assay results were positively correlated with the TBARS assay results, demonstrating that the greater the susceptibility to OS, the greater the number of embryonic cells undergoing apoptosis.

The quality of a blastocyst predicts its ability to implant and its future development. Apoptosis that occurs during this stage of embryonic development can be beneficial or detrimental, depending on its incidence (Fabian et al. 2005). For example, the presence of massive cell death can damage embryo homeostasis; the resulting damaged embryos can arrest or die (Brad et al. 2007). Therefore, sperm evaluation and its susceptibility to OS is an important tool for guaranteeing the quality of embryo development.

The fate of a fertilized oocyte is dependent on the rate of sperm DNA fragmentation. The first and less deleterious possibility is fertilization by a sperm with low DNA fragmentation. Often minor damage in sperm chromatin can be repaired by the oocyte or even by the embryo (Wyrobek et al. 2006), leading to the birth of a normal individual. Another possible outcome is oocyte fertilization by a sperm with a large amount of DNA fragmentation. In this scenario, zygote development begins, but paternal chromatin damage can lead to early embryonic death or abortion, even in later stages of pregnancy (Tesarik et al. 2004). The worst outcome for the latter scenario would be the fertilization of an oocyte by a sperm with high levels of DNA fragmentation that is still capable of supporting the developing embryo and fetus to term. It is clear from the literature that excessive damage to sperm DNA can affect offspring (Acharyya et al. 2005) and lead to childhood cancer (Aitken 2006), congenital diseases (Hazout et al. 2008), and even infertility (Aitken & De Iuliis 2007). Because OS is the most significant cause of sperm DNA fragmentation, evaluation of ROS in spermatozoa is an important step in preventing the problems described above. From our study, we conclude that susceptibility to OS compromises the integrity of sperm DNA and reduces embryo quality because it reduces the rate of cleavage and increases the number of apoptotic cells in embryos that reach the blastocyst stage.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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