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The quantification of lipid and protein oxidation in stallion spermatozoa and seminal plasma: Seasonal distinctions and correlations with DNA strand breaks, classical seminal parameters and stallion fertility

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Abstract

The goal of this work was to correlate oxidative stress caused by reactive oxygen species (ROS) and DNA damage with classic semen parameters in spermatozoa and seminal plasma of fertile and subfertile stallions. Oxidation was measured in both lipids and proteins, using the thiobarbituric acid reactive species (TBARS) assay and the DNPH carbonyl groups assay, respectively. Sperm DNA damage was monitored using the TUNEL assay. These parameters were monitored in samples obtained during the breeding and the non-breeding seasons. In general, fertile stallions showed better classical semen parameters, and those parameters improved from the non-breeding to the breeding season, although an increase in sperm production was accompanied by a decrease in the semen quality from subfertile stallions in the breeding season. In terms of oxidation levels we found that there were clear differences whether lipids or proteins were considered. In the breeding season there seemed to be a tendency towards normalizing lipid oxidation in spermatozoa and seminal plasma, and protein oxidation in the seminal plasma, of both fertile and subfertile animals. Thus, differences monitored in the non-breeding season were no longer visible. Interestingly, a higher level of protein oxidation was found in the sperm of fertile animals in the breeding season. Considering that there were positive correlations between sperm protein oxidation and sperm motility and vitality, these results suggests that the oxidation of semen proteins may be important for sperm function. On the other hand, lipid oxidation in the seminal plasma seemed to be a general indicator for sperm damage. In the non-breeding

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season positive correlations between lipid and protein oxidation levels in both sperm and seminal plasma and several defects in sperm function were found, but only for subfertile animals, thus suggesting that lipid and protein oxidation may aid in the identification of subfertile stallions during the non-breeding season. Levels of ROS production never seemed to result in compromised sperm DNA integrity, indicating that measurements were within physiological levels and/or that there is an efficient antioxidant activity in stallion sperm cells.

Keywords: Sperm; Seminal plasma; Equine; Fertility; Seasonality; Lipid peroxidation; Protein oxidation; Oxidative stress

1. Introduction

The prediction of stallion fertility is one of the most subjective issues in horse breeding. Traditional semen parameters (e.g. progressive motility, morphology, membrane integrity) used to evaluate sperm quality, do not correlate with fertility (Magistrini et al., 1996). Biochemical criteria have been developed for equine sperm evaluation, based on methodologies described for other mammals. One of the most used biochemical criteria is the correlation between defective sperm function and oxidative stress (OS), due to the excessive generation of reactive oxygen species (ROS) and/or impairment of antioxidant protection within the male reproductive tract (Aitken and Clarkson, 1987; Aitken, 2006).

Excessive production of ROS is known to affect membrane lipids through lipid peroxidation (LPO; humans: Jones et al., 1979; boar: Johnson et al., 2000; stallion: Gadella et al., 2001), and also oxidized proteins and DNA. Although spermatozoa possess several potential targets for ROS, including a high content of polyunsaturated fatty acids (Sanocka and Kurpisz, 2004), little is known about the peroxidation of equine sperm cell membranes, although studies indicate that damage to equine sperm function is mainly due to hydrogen peroxide (H_2O_2; Baumber et al., 2000).

While the uncontrolled generation of ROS by defective spermatozoa can have detrimental effects on sperm function, controlled production of ROS plays physiologically relevant roles in signalling events controlling sperm capacitation, the acrosome reaction, hyperactivation and sperm–oocyte fusion (Baumber et al., 2000).

Oxidative stress can be assessed through the direct quantification of ROS, antioxidants, and by the measurement of oxidative stress end products. One product of lipid peroxidation is malondialdehyde (MDA), which can be determined by measuring the amount of thiobarbituric acid reactive species (Stradaioli et al., 2001; Sanocka and Kurpisz, 2004; Nichi et al., 2006). A high level of ROS may also result in protein oxidation, leading to the production of carbonyl groups. Consequently, determination of carbonyl content in proteins can be used as a biomarker of oxidative protein damage and several methods have been developed for determination and quantification of protein carbonyl groups (Levine et al., 1990; Reznick and Packer, 1994).

Oxidative stress may induce sperm DNA damage, possibly through an apoptotic mechanism (Blasco, 2003). However, the origin of DNA breaks in mature sperm is still unclear. Two of the hypotheses are that DNA breaks represent residual apoptotic cells not removed in the testis or epididymis, or apoptosis occurring in late mature sperm (Henkel et al., 2003). Evenson and Wixon (2006) suggested that DNA damage could be due to ROS, which may induce many kinds of DNA damage, including DNA base modifications. High levels of DNA damage and ROS production have been associated with spermatozoa with abnormal head morphology, and midpiece and tail defects (Aitken et al., 1989).

There are several studies in humans that have reported DNA damage associated with membrane lipid peroxidation (Chen et al., 1997; Twigg et al., 1998) and oxidative stress (Hughes et al., 1996; Aitken et al., 1998a). In the equine system previous work has demonstrated that ROS can promote LPO with an associated loss of motility (Baumber et al., 2000; Ball and Vo, 2002), but the influence of ROS on equine sperm DNA was only reported in cryopreserved spermatozoa (Baumber et al., 2003), and not mentioned in native semen. There are no reports on lipid oxidation in equine seminal plasma or protein oxidation in equine semen.

The purpose of this study was to quantify lipid and protein oxidation in both stallion spermatozoa and seminal plasma, and to correlate these measurements with DNA strand breaks, classical seminal parameters and stallion fertility.

2. Material and methods

2.1. Reagents

All chemicals were obtained from Sigma, St. Louis, MO, USA, unless stated otherwise.

2.2. Semen collection and assessment of semen quality

A total of eight stallions housed at the Agricultural School, Polytechnic Institute of Coimbra (Coimbra, Portugal), a region with a Mediterranean climate, were used in this study. The bulk of semen samples were collected in two breding seasons (March–July of 2005 and 2006) and in one non-breeding season (September 2005–February 2006).

These stallions were subject to semen collections of five daily ejaculates that were checked for seminal (qualitative and quantitative) and behavior parameters before use in the artificial insemination program, as described in Gamboa and Ramalho-Santos (2005). Behavior parameters included arousal time, mounting time and dismounting time. Qualitative seminal parameters included semen smell, color, opacity and viscosity, while quantitative parameters included semen volume and pH. Sperm quantitative parameters were sperm concentration (measured with a hemocytometer), sperm motility (determined with an inverted microscope), sperm morphology, sperm vitality and acrosomal status. Sperm morphology was determined after India ink contrast staining and sperm vitality was monitored using eosin/nigrosin (dead cells stain red). Acrosomal status was assessed after ethanol fixation and permeabilization of sperm using the lectin from *Pisum sativum* (PSA) coupled to fluorescein isothiocyanate (FITC-green). The lectin binds to acrosomal contents, thus staining intact sperm acrosomes with a homogeneous bright green pattern, which was viewed using a HUND H 600 AFL fluorescence microscope (Gamboa and Ramalho-Santos, 2005).

The stallions were classified as fertile (five animals) or subfertile (three animals) according to the protocol developed by Palmer and Fauquenot (1984). Fertility results at the end of each breeding season were calculated as previously described (Gamboa and Ramalho-Santos, 2005).

2.3. Protein concentration

The concentration of sperm and seminal plasma proteins was estimated with the Sedmak assay (Sedmak and Grossberg, 1977) using bovine serum albumin (BSA) as a standard. Care was taken to insure that all samples were read in the linear portion of the correlation curve.

2.4. Lipid peroxidation detected with the TBARS assay

The spectrophotometric tiobarbituric acid (TBA) test has been frequently used for many years as an indicator of the peroxidation of polyunsaturated fatty acids, and has been adapted to equine semen (Stradaioli et al., 2001). This test involves the reaction of aldehydes with TBA at 100 °C under acidic conditions to produce a pink colored chromogen, which strongly absorbs light at a wavelength of 532 nm (Sanocka and Kurpisz, 2004).

The extent of lipid peroxidation was determined in spermatozoa and seminal plasma by measuring the amount of thiobarbituric acid reactive species (TBARS) formed, according to a modified procedure (Serafini-Cessi and Cessi, 1968). To measure lipid peroxidation, 2 ml of TTH reagent [18.8 ml of cold 40% trichloroacetic acid (TCA); 28.7 ml of thiobarbituric acid (TBA); 0.25 N HCl] were added to 450 μl of the test material. The mixture was heated at 100 °C for 10 min, and was allowed to cool in ice before a 10 min centrifugation at 1500 × *g* in a Hettich centrifuge. The supernatant was collected and TBARS were spectrophotometrically quantified at 530 nm, against a blank prepared under similar conditions, but in the absence of sample. The amount of TBARS formed was calculated using a molar extinction coefficient of $1.56 \times 10^{-5}\ M^{-1}\ cm^{-1}$ for thiobarbituric acid and expressed as nmoles TBARS/mg protein (Buege and Aust, 1978).

2.5. Carbonyl group determination using the DNPH reaction

The accumulation of oxidized proteins, in either spermatozoa or seminal plasma, can be evaluated by the carbonyl group content, which can be spectrophotometrically quantified via reaction with DNPH (2,4-dinitrophenilhidrazine) at 360 nm (Fagan et al., 1999). All sperm samples were centrifuged and the pellet solubilized in 1 ml trichloroacetic acid (TCA) and centrifuged (1500 × *g*, 10 min). The sediments were incubated with 1 ml of 10 mM DNPH (freshly prepared in 2 M HCl, in the dark) for 1 h at room temperature, with vortex agitation every 10 min. Following this incubation, 1 ml of 20% TCA was added and samples were centrifuged at 20,000 × *g*, for 3 min. The supernatant was decanted and the pellet mixed with 1 ml of a 1:1 ethanol:ethyl acetate solution. The pellet was then incubated with 1 ml of 6 M guanidine (prepared in PBS, pH 6.5), for 15 min at 37 °C and centrifuged at 1500 × *g* for 10 min. The supernatant was collected and protein oxidation was estimated spectrophotometrically at 360 nm. For all samples a blank was prepared, which was incubated with 2 M HCl instead of DNPH. The carbonyl content was calculated using a molar extinction coefficient of 22 $mM^{-1}\ cm^{-1}$ for DNPH and was expressed as nmoles DNPH/mg protein.

The seminal plasma was separated from the pellet after the first centrifugation and then the process was carried out as described above.

2.6. TUNEL assay

The APO-BrdU^{TM} terminal transferase dUTP nick end labeling (TUNEL) Assay Kit (Molecular Probes; Eugene, OR, USA) was used to detect DNA damage. Sperm were allowed to adhere to poly-L-lysine coated coverslips, which were then fixed in 2 ml of a 2% (v/v) formaldehyde solution in PBS (200 mg/l KCl; 200 mg/l KH_2PO_4; 1150 mg/l Na_2HPO_4; 8000 mg/l NaCl; 500 mg/l NaN_3; pH 7,4) for 2 h at room temperature. Coverslips were then placed in a permeabilizing solution containing 1% (v/v) Triton X-100 in PBS 30 min. To store the samples, coverslips were placed in blocking solution (400 mM glycine; 1 mg/ml BSA in PBS) and kept at 4 °C.

To carry out the TUNEL assay coverslips were incubated with 50 μl of DNA-labeling solution [31.25 μl distilled water; 50 μl reaction buffer; 0.75 μl Tdt (terminal deoxynucleotidyl transferase); 8 μl BrdUTP] for 1 h at 37 °C in the dark. After a washing step with Rinse buffer; 2.5 μl Alexa Fluor 488 dye labeled anti-BrdUTP antibody were added and the sample incubated for 2 h at 37 °C in the dark. Following this incubation the sample was washed with 0.1% (v/v) Triton X-1000 for 30 min. TUNEL positive (TUNEL+) cells stain green, while propidium iodide was used as a DNA counterstain (red). The coverslips were mounted with antifade and observed with a HUND H 600 AFL fluorescense microscope. Both positive and negative controls were carried out before the analysis, as recommended by the manufacturer.

2.7. Statistical analysis

All statistical analyses were done using the Statistical Package for the Social Sciences Program (SPSS), Version 14.00, software for windows (SPSS Inc., Chicago, IL, USA). Given the non-normal distribution, non-parametic tests were used to compare the data concerning semen quality characteristics (concentration of spermatozoa, progressive motility, viability, pH, head abnormalities, midpiece abnormalities and tail abnormalities), acrosomal status, TUNEL positive sperm cells, TBARS and oxidized proteins. Spearman's correlation coefficients were calculated for all the variables in each group (fertile and subfertile) and season (breeding and non-breeding). Statistical significance was set at the 0.05 probability level. Results are expressed as medians, and include the range of monitored values.

3. Results

3.1. Classic semen parameters and structural integrity of the acrosome

Semen characteristics were monitored for each stallion, and the fertility rates determined at the end of the breeding season based on the pregnancies obtained. Accordingly, stallions were classified as fertile or subfertile (Gamboa and Ramalho-Santos, 2005).

Behavioral and qualitative semen parameters, as well as semen volume, were always within the normal range (data not shown, Gamboa and Ramalho-Santos, 2005). Data for classic semen analysis by fertility groups and seasons is shown in Table 1. Despite the fact that one of the fertile stallions presented poor semen quality, in general, fertile animals presented better seminal parameters, and statistical analyses showed significant differences ($p < 0.05$) between fertile and subfertile animals for sperm concentration, progressive motility, vitality, morphology and acrosomal status, especially in the breeding season. The low number of morphologically normal cells observed for subfertile animals was due to a high percentage of head abnormalities (data not show).

When comparing seasonal variations, in general semen parameters were better in the breeding season, and both groups of stallions showed significant differences ($p < 0.05$) between seasons for sperm concentration, vitality, morphology and acrosomal status (Table 1). Moreover, we also observed that a decrease in sperm concentration from the non-breeding to the breeding season, was accompanied by an increase in qualitative parameters, (morphology, acrosomal status, vitality), for both groups of animals (Table 1).

3.2. Biochemical parameters and DNA integrity

In terms of oxidized lipids (TBARS) and proteins (DNPH) in sperm (spz) and seminal plasma (sp) there was some variation between groups and seasons (Table 2). When we considered seasonal

Table 1
Classical semen parameters for semen samples from stallions of varying fertility

Season	Fertility	Seminal parameters					
		Concentration	Progressive motility[a] (%)	Vitality[b] (%)	Abnormal spermatozoa[c] (%)	pH	Intact acrosome[d] (%)
Breeding	Fertile	183.0a,A (42.0–803.0) *164*	40.0a (10.0–70.0) *164*	68.0a,A (17.0–95.0) *164*	27.0a,A (6.5–85.6) *158*	7.48 (4.00–8.50) *164*	77.7a,A (10.5–97.5) *76*
	Subfertile	169.0a,B (57.0–592.0) *69*	20.0b (10.0–60.0) *66*	47.5a,B (9.0–73.0) *67*	50.25a,B (13.5–89.5) *64*	7.50 (6.27–8.50) *69*	72.0a,B (45.0–90.0) *37*
Non-breeding	Fertile	204.5A (85.0–677.0) *46*	40.0a (5.0–75.0) *46*	61.5b,A (8.5–90.0) *46*	27.5b,A (16.0–69.3) *46*	7.43 (7.00–8.14) *46*	69.0A (44.0–87.0) *23*
	Subfertile	212.0B (57.0–719.0) *39*	25.0b (10.0–50.0) *39*	46.5b,B (10.0–80.0) *39*	52.0b,B (19.5–75.0) *39*	7.50 (7.00–8.50) *39*	65.75B (43.0–81.5) *26*

Values are medians, numbers in parenthesis indicate the minimum–maximum range. Numbers in italics indicate the number of ejaculates observed for each parameter. Within columns same letter (a-b) denotes significant differences between fertile and subfertile animals ($p < 0.05$), and (A-B) denotes significant differences between seasons ($p < 0.05$).

[a] Percentage of progressive motility observed after collection.

[b] Percentage of viable (non-eosin stained) cells. For each ejaculate, counts were performed on 200 cells.

[c] Percentage of cells that had abnormal morphology. For each ejaculate, counts were performed on 200 cells.

[d] Percentage of cells that had intact acrosome. For each ejaculate, counts were performed on 200 cells.

Table 2
Lipid and protein oxidation and DNA fragmentation observed in semen from stallions with varying fertility

Season	Fertility	Biochemical parameters				DNA damage
		nmoles $TBARS_{SPZ}$ (mg prot)	nmoles $TBARS_{SP}$ (mg prot)	nmoles $DNPH_{SPZ}$ (mg prot)	nmoles $DNPH_{SP}$ (mg prot)	TUNEL positive cells[a]
Breeding	Fertile	0.157 (0.005–1.767) *112*	0.189a (0.022–1.131) *112*	49.796a (2.087–138.609) *112*	2.521 (0.165–25.878) *112*	2.00 (0.00–30.00) *67*
	Subfertile	0.193A (0.014–1.907) *54*	0.218a (0.026–1.012) *54*	22.235a (0.829–77.540) *54*	3.403A (0.004–17.673) *54*	1.25 (0.00–29.00) *30*
Non-breeding	Fertile	0.201a (0.011–1.551) *68*	0.149b (0.051–0.711) *68*	38.816 (7.722–92.400) *68*	2.394a (0.008–11.806) *68*	2.50 (0.00–9.50) *30*
	Subfertile	0.099a,A (0.011–0.479) *52*	0.218b (0.103–0.506) *52*	28.026 (4.681–111.248) *52*	6.609a,A (0.243–20.903) *52*	2.50 (0.00–20.00) *23*

Values are medians. Numbers in parenthesis indicate the minimum–maximum range in each case and values in italics indicate the number of ejaculates observed for each parameter. Within columns same letter (a-b) denotes significant differences between fertile and subfertile animals ($p < 0.05$), and (A-B) denotes significant differences between seasons ($p < 0.05$). SPZ, spermatozoa; SP, seminal plasma.

[a] Percentage of TUNEL positive cells. For each ejaculate, counts were performed on 200 cells.

variations for all animals regardless of fertility status, we observed that both TBARS and DNPH levels in the seminal plasma differed significantly between seasons (data not shown). When fertility groups were considered we found that oxidized proteins in spermatozoa distinguished fertile from subfertile stallions in the breeding season, while all other parameters monitored were able to distinguish fertility groups in the non-breeding season. In the non-breeding season, fertile animals had a higher amount of oxidized sperm lipids, and lower amounts of oxidized seminal plasma lipids and proteins. It should be noted that although fertile stallions show a significantly lower level of oxidized lipids in the seminal plasma during the breeding season, a multivariate procedure that modeled the values of lipid and protein peroxidation, based on their relationships to fertility groups and breeding seasons, enabled us to determine that, unlike the other significant differences shown in Table 2, this was due to chance (data not shown). Therefore, in terms of biological oxidation, the breeding season is characterized by a higher level of oxidized sperm proteins in fertile animals. It should also be noted that fluctuations in oxidation levels in groups and seasons did not affect sperm DNA damage, as monitored by the TUNEL assay (Table 2).

When we analyzed the data for Spearman's correlation coefficients without separating fertility groups we found that DNPHspz presented correlations with motility ($r = 0.20$, $p = 0.001$) and vitality ($r = 0.23$, $p < 0.001$), stressing its biological significance in terms of fertility. Correlations were also found between biochemical parameters and seminal characteristics in both fertility groups and seasons. The most relevant coefficients found in the breeding season were observed for TBARS in the seminal plasma (TBARSsp) and DNPH in sperm cells (DNPHspz). For fertile stallions highest correlations were between TBARSsp and midpiece abnormalities ($r = 0.45$; $p < 0.001$) and DNPHspz and vitality ($r = 0.39$; $p < 0.001$). For subfertile animals significant correlations were between tail and midpiece abnormalities and TBARSsp ($r = 0.58$ and 0.48, $p < 0.001$, respectively) and motility and DNPHspz ($r = 0.34$; $p < 0.05$). Again, this suggests that while high levels of lipid oxidation in the seminal plasma seem detrimental, protein oxidation in sperm may be related to sperm function during the breeding season.

On the other hand, during the non-breeding season TBARSspz in fertile animals were correlated with tail abnormalities ($r = 0.34$, $p < 0.01$). However, biochemical parameters in subfertile animals showed a wide variety of correlations with classical semen parameters, mostly for DNPHspz and TBARSsp. For DNPHspz there were correlations with sperm concentration ($r = 0.82$, $p < 0.001$), tail abnormalities ($r = 0.37$, $p < 0.01$), TUNEL positive cells ($r = 0.34$, $p < 0.05$) and reacted acrosomes ($r = 0.49$, $p < 0.001$). For DNPHsp there were correlations with sperm concentration ($r = 0.41$, $p < 0.01$) and with TUNEL positive cells ($r = 0.57$, $p < 0.001$). For TBARSspz the only significant correlation was with tail abnormalities ($r = 0.34$, $p < 0.05$), while for TBARSsp correlations were found for concentration ($r = 0.48$, $p < 0.001$), tail abnormalities ($r = 0.37$, $p < 0.01$) and reacted acrosomes ($r = 0.39$, $p < 0.01$).

4. Discussion

The purpose of this study was to assess the level of oxidative stress, monitored both by lipid and protein oxidation, in spermatozoa and seminal plasma from semen collected from fertile and subfertile stallions during the breeding and non-breeding seasons. Oxidative stress in equine semen has been investigated using lipid peroxidation end-products (e.g. MDA; Stradaioli et al., 2001; Ball et al., 2001), and more recently employing a fluorescent assay involving the labeling of stallion sperm cells with the reporter probe C11-BODIPY$^{581/591}$ (Baumber et al., 2000; Neild et al., 2005). No previous observations on oxidized proteins in equine semen were uncovered in the literature, and to our knowledge this is the first such study.

As expected, we noted significant differences in classical semen parameters between seasons as well as between fertile and subfertile stallions, namely with the latter clearly showing poorer motility, vitality and acrosomal status levels (Table 1). It should be noted that a lower sperm concentration in the breeding season is likely due to the production of a higher volume of seminal fluid (Janett et al., 2003a,b). In contrast to what was reported by Blottner et al. (2001), the average percentage of morphological normal sperm during autumn and winter was lower than during spring and summer for both groups of animals. Interestingly, during the breeding season we noted that increased sperm production was accompanied by a decrease in the semen quality from subfertile stallions. Thus, despite the fact that seminal characteristics are better in the breeding season for both groups of animals, subfertile stallions revealed inverted correlations between sperm concentration and vitality, morphologically normal cells and intact acrosomes. It seems that subfertile animals have an upper limit in terms of capacity to produce normal sperm, which, when topped, results in poor(er) semen quality. Daily sperm production in stallions is also seasonally modulated by a mechanism of germ cell degeneration that occurs throughout spermatogenesis. However, the greatest seasonal impact on horses occurs during spermatocytogenesis in the breeding season (Johnson, 1985, 1991; Johnson et al., 1997) and this process may be affected in subfertile animals.

In terms of oxidation levels we found that there were clear differences whether lipids (TBARS) or proteins (DNPH) were considered (Table 2). Thus, in the breeding season there seemed to be a tendency towards normalizing lipid oxidation in spermatozoa and seminal plasma and protein oxidation in the seminal plasma of both fertile and subfertile animals (values were remarkably similar within fertility groups), and differences monitored in the non-breeding season at this level were no longer visible. Several issues must be considered when addressing these results, in terms both of possible susceptibility to oxidation, differential production of ROS and the presence of antioxidant defenses.

In this study, we found differences between fertility groups for TBARSsp in the non-breeding season (differences in the breeding season were due to chance), despite the reported fact that molar amounts of cholesterol and phospholipids in whole sperm and seminal plasma do not differ between fertile and subfertile stallions (Brinsko et al., 2006). It has been established that sperm possess a high amount of polyunsaturated fatty acids (i.e. susceptible to peroxidation) and that sperm lipid composition and distribution may vary throughout processes such as maturation or capacitation (Flesch and Gadella, 2000). Furthermore, these variations can depend on individuals, and even individual ejaculates, so it is possible that differential changes in lipid compositions between fertile and subfertile animals could help explain these results. On the other hand, fertile stallions showed much higher levels of sperm protein oxidation in the breeding season. It is known that some lipids and proteins from the seminal plasma bind to sperm, and have a role in sperm function, and, conversely, that other components may also be released from the sperm (Töpfer-Petersen et al., 2005). However, we are not able to determine the exact origin of equine sperm oxidized lipids and proteins, and it is likely that a more complex interplay between seminal plasma and sperm is involved. For example the iron-binding protein, lactoferrin, is a major secretory protein in the equine epididymis (Fouchecourt et al., 2000) and it appears that it may associate with spermatozoa (Jin et al., 1997) and may act to decrease the amount of free iron available to initiate lipid peroxidation.

Semen characteristics change markedly throughout the year, depending on the season, on the individual stallion and on fluid secreted by the vesicular glands. Furthermore, seminal components can differ in different animals (Brandon et al., 1999), chemical makeup of semen may fluctuate from one ejaculate to another in the same individual, and is dependent on such variables as

hormonal concentrations (Mann, 1975; Gerlach and Aurich, 2000) and even on the ejaculatory process (Mann, 1975).

Carefully controlled biological oxidation can play several relevant roles, and it is known to be involved in processes related to normal sperm function, such as capacitation and hyperactivation (Aitken et al., 1995, 1998b; de Lamirande and Gagnon, 2003). However, above a certain threshold, oxidation can lead to deleterious effects, and has been positively correlated, for example, with high levels of sperm DNA damage (Hughes et al., 1996; Kodama et al., 1997). This may be especially relevant in circumstances when ROS production results from bacteria or leucocytes in the ejaculate, due to infection (Aitken and Clarkson, 1987). Furthermore, the increased incidence of sperm defects and the significant correlations between TBARS and abnormal spermatozoa have been suggested to affect sperm quality in the bovine system (Nichi et al., 2006), and a high correlation between TBARS and the percentage of sperm head defects in human semen was also observed (Aitken and Clarkson, 1987). Finally, sperm oxidative damage may also take place following semen conservation protocols (for example during flash-freezing), where large amounts of ROS can be generated by damaged and abnormal spermatozoa, thus contributing to lower fertility (Ball et al., 2001).

However, in our system changes in both lipid and protein oxidation seem to be within physiologically relevant values, as very low levels of DNA damage were observed, and no changes in sperm DNA status were registered in response to statistically relevant variations in both lipid and protein oxidation levels (Table 2). Blottner et al. (2001) have also observed that percentages of spermatozoa with denaturated chromatin were minimal and showed minimal differences between fresh and frozen, between stallions or between seasons. The importance of protein oxidation for sperm function is evidenced by the positive correlations found between DNPHspz and motility and vitality during the reproductive season, while lipid oxidation in the seminal plasma (TBARSsp) seemed to be an indicator for several levels of sperm damage. Interestingly, this last finding carried over to the non-breeding season, but only for subfertile animals. In fact, these same animals showed positive correlations between lipid and protein oxidation levels in both sperm and seminal plasma and several defects in sperm function, suggesting that lipid and protein oxidation may aid in the identification of subfertile stallions during the non-breeding season.

In conclusion, lipid and protein oxidation takes place with different extents in sperm and seminal plasma from animals with varying fertility, and further work will attempt to address if this is due to different susceptibility to oxidation, production of ROS or the availability of antioxidant defenses. In this particular study, while levels of oxidation could be correlated with certain semen characteristics, higher levels of ROS production never seemed to result in compromised sperm DNA integrity, suggesting that measurements were within physiological levels and/or that there is an efficient antioxidant activity in stallion sperm cells. When comparing seasons we note that, while subfertile stallions matched fertile stallions in terms of lipid oxidation in the breeding season, fertile animals showed a much higher level of oxidation in sperm proteins. During the reproductive period DNPHspz could distinguish fertility groups suggesting that protein oxidation in sperm may be play an important role in equine fertility.

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