Lipid peroxidation assays in canine fresh semen

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Abstract

Gavazza, M.; Gutiérrez, A.M.; Marmunti, M.; Palacios, A.: ***Lipid peroxidation assays in canine fresh semen.*** *Rev. vet. 20: 2, 97–102, 2009.* Lipid peroxidation (LP) is a potential cause of infertility in males of numerous species. Levels in which the spermatozoa loses mobility *in vitro* is correlated with the rate of LP that it suffers. The objective of this work was to determine the fatty acid composition and analyze the sensitivity to LP (ascorbate–Fe^{++} dependent) in spermatozoa obtained from different samples of canine fresh semen. LP was evaluated using chemiluminescence (CL) (cpm/mg of protein) and fatty acid (FA) profile by means of gas chromatography. The saturated FA content found in the analyzed spermatozoa was approximately 40%, whereas the total unsaturated FA content was approximately 55% with a prevalence of docosapentaenoic acid (C22:5 n6). When the control and ascorbate–Fe^{++} dependent samples were compared, it was observed a significant increase in the light emission (CL). Consequently, significant decrease in the percentage of the polyunsaturated fatty acids (PUFA) was obtained, being more affected: C18: 2 n6, C20: 4 n6, C22: 4 n6, C22: 5 n6 and C22: 6 n3. The unsaturation index used to evaluate the alterations generated during the LP was correlated with the data mentioned before. Our results indicate that dog semen contains great amounts of PUFA, which were vulnerable to the LP. The alteration in the PUFA composition could be the common base of different degenerative processes.

Key words: dog, spermatozoa, lipid peroxidation, polyunsaturated fatty acids.

Resumen

Gavazza, M.; Gutiérrez, A.M.; Marmunti, M.; Palacios, A.: ***Ensayos de peroxidación lipídica en semen fresco canino.*** *Rev. vet. 20: 2, 97–102, 2009.* La peroxidación lipídica (PL) es causa potencial de infertilidad en machos de numerosas especies. Los niveles en los cuales los espermatozoides pierden movilidad *in vitro* se correlaciona con el grado de peroxidación lipídica que sufren. El objetivo de este trabajo fue conocer la composición de ácidos grasos y analizar la sensibilidad a la PL (ascorbato–Fe^{++} dependiente) en espermatozoides obtenidos a partir de diferentes muestras de semen fresco canino. La PL fue evaluada utilizando quimioluminiscencia (QL) (cpm/mg de proteína) y perfil de ácidos grasos (AG) medidos por cromatografía gaseosa. El porcentaje de AG saturados hallado en los espermatozoides analizados fue de 40% aproximadamente, mientras que el porcentaje total de AG insaturados fue de 55% con predominio del ácido graso docosapentaenoico (22:5 n6). Cuando se compararon las muestras control (sin el agregado de ascorbato–Fe^{++}) con las muestras ascorbato–Fe^{++} dependientes, se observó un incremento significativo en la emisión lumínica (QL). Consecuentemente se obtuvo una disminución significativa en el porcentaje de los ácidos grasos polininsaturados (AGPI), siendo los más afectados C18: 2 n6, C20: 4 n6, C22: 4 n6, C22: 5 n6 y C22: 6 n3. El índice de insaturación utilizado para evaluar las alteraciones generadas durante la PL estuvo correlacionado con los datos antes mencionados. Nuestros resultados indican que el semen de perro contiene grandes cantidades de AGPI, los cuales fueron vulnerables a la PL. Las alteraciones en la composición de los AGPI podrían ser la base común de diferentes procesos degenerativos.

Palabras clave: perro, espermatozoides, peroxidación lipídica, acidos grasos poliinsaturados.

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INTRODUCTION

The role of lipids in the structure and function of the male reproductive system continues to be an interesting and important area of investigation [11]. Lipid peroxidation (LP) damage to the sperm cell membrane results from the generation of reactive oxygen species, such as the superoxide anion, hydroxyl radical, and hydrogen peroxide. LP can cause the loss of membrane integrity, which increases cell membrane permeability leading to enzymes inactivation, structural damage of DNA, and cell death [18].

Mammal spermatozoa are particularly susceptible to LP–induced damage because they contain high levels of polyunsaturated fatty acids (PUFAs) and lack antioxidant enzymes, such as superoxide dismutase, glutathion peroxidase, and catalase [25]. As a result of oxidative stress, the polyunsaturated fatty acid membrane undergoes peroxidation, and the spermatozoa lose function [29]. Because LP can cause irreversible loss of sperm motility, it can serve as a biochemical index of semen quality [7, 28].

Among cellular macromolecules, PUFAs exhibit the highest sensitivity to oxidative damage. It is accepted that the sensitivity increases as a power function of the number of double bonds per fatty acid molecules [37]. The measurement of LP is one of the most commonly used assays for induced radical damage [22, 36, 37].

A combination of ascorbate plus iron can trigger a Fenton–reaction with formation of highly reactive hydroxyl radicals, which can cause chain–initiation reaction of LP and secondary protein oxidation [39]. Ascorbate may enhance the process by keeping iron in the reduced state. In crude tissue fractions iron in reduced form can degrade preformed lipid hydroperoxides forming radicals that can catalyze the chain propagation phase of LP, without involving directly the hydroxyl radicals or other active oxygen species [10].

LP termination involves the reaction of $LOO^{\cdot}$ to form non–radical products or the reaction of one $LOO^{\cdot}$ with another terminating radical to generate non–propagating radical species [14]. Some LP products are light–emitting species and their spontaneous chemiluminescence can be used as an internal marker of oxidative stress [20].

The aim of this study was to determine the fatty acid composition and analyze the sensitivity to LP (ascorbate–Fe^{++} dependent) in spermatozoa obtained from different samples of canine fresh semen. For this purpose, the potential relationships between the fatty acid composition of the food and any changes in spermatozoa samples, were considered. Light emission chemiluminescence and fatty acid composition were used as an index of the oxidative destruction of lipids. The unsaturation index (UI), a parameter based on the maximal rate of oxidation of specific fatty acids, was used to evaluate the fatty acid alterations observed during the process [30].

MATERIAL AND METHODS

Chemicals. Butylated hydroxytoluene (BHT) and phenyl–methyl–sulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO, USA). Bovine serum albumin (BSA) (Fraction V) was obtained from Wako Pure Chemical Industries, Japan. L(+) ascorbic acid and boron–trifluoride– methanol complex were from Merck. Standards of fatty acids methyl esters were from Nu Check Prep Inc, Elysian, MN, USA. All other reagents and chemicals were of analytical grade from Sigma.

Animals. The fresh canine semen samples were obtained from three Yellow Labrador Retriever young male dogs from Penitential Service, Olmos, Provincia de Buenos Aires, Argentina. The semen samples were collected by masturbation after 15 days of sexual abstinence. The ejaculate was collected in a sterile container and allowed to liquefy at 37°C for 30 min [27]. The fresh semen canine samples were donated by Cátedra de Reproducción Animal, Facultad de Ciencias Veterinarias, UNLP, Argentina. The dogs were fed commercial chow and water was provided *ad libitum*.

Preparation of fresh canine semen samples. An aliquot (1 ml) of semen from each sample was centrifuged at 800x g for 10 min, sperm pellets were separated, and washed by resuspending in PBS and recentrifuging (three times). After the last centrifugation, 1 ml of deionized water was added to spermatozoa [12] and they were snap–frozen and stored at –83°C and used within a week of preparation, after one cycle of freezing and thawing. All operations were performed at 4°C.

Non–enzymatic lipid peroxidation of spermatozoa. Chemiluminescence and LP were initiated by adding ascorbate–Fe^{++} to spermatozoa preparations [45]. Spermatozoa samples (1 mg of protein) were incubated at 37°C with 0,01 M phosphate buffer (pH 7.4), 0.4 mM ascorbate, final volume 1 ml. Phosphate buffer is contaminated with sufficient iron to provide the necessary ferrous or ferric iron for lipid peroxidation, (final concentration in the incubation mixture was 2.15 uM) [40]. Spermatozoa preparations which lacked ascorbate–Fe^{++} (control) were carried out simultaneously. Chemiluminescence was measured as counts per min in a liquid scintillation analyzer Packard 1900 TR. Membrane light emission was determined over 120 min period, and recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein.

LP is a branching chain reaction which can be considered as taking place in four main stages: (1) chain initiation, (2) chain propagation, (3) chain branching and (4) chain termination. At least three reactions are known to break the chains: (a) interaction of two radicals leading the chains, (b) interaction of one radical with changing valency metal, and (c) reaction between such a radical and a molecule of "antioxidant". $LO_2^{*} + LO_2^{*}$ (k)$\rightarrow$ P* $\rightarrow$ P + φhν (chemiluminescence). Reac-

tion (a) is particularly interesting since it is accompanied by chemiluminescence which intensity (I) may serve as a measure of peroxide free radical (LO_2^*) concentration according to the following equation: $I = K \varphi k [LO_2^*]^2$. Where φ represents the chemiluminescence quantum yield and k the coefficient depending on the net sensitivity of the instrument. LO_2^* is a free radical produced from lipids molecules [42].

Measurement of fatty acid composition. Spermatozoa lipids were extracted with chloroform/methanol (2:1 v/v containing 0.01% BHT as antioxidant) from peroxidized membranes [15]. Fatty acids were transmethylated with 10% F_3B in methanol at 60°C for 3 h. Fatty acid methyl esters were analyzed with a GC–14A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a packed column (1.80 m x 4 mm i.d.) GP 10% DEGS–PS on 80/100 Supelcoport. Nitrogen was used as the carrier gas. The injector and detector temperatures were maintained at 250°C, the column temperature was held at 200°C. Fatty acid methyl ester peaks were identified by comparison of the retention times with those of standards. All compositions were expressed as % by area of total fatty acids.

Unsaturation index (UI). UI was calculated according to the formula, UI = sum (fatty acid percent) x (number of double bonds) [30]. *Protein determination*. Proteins were determined by the method of Lowry using BSA as standard [33].

Statistical analysis. Saturated fatty acids were calculated as SFA = Σ% (16:0 + 18:0). Unsaturated fatty acids were calculated as UFA = Σ% (MUFA + PUFA). The saturated/unsaturated ratio was also calculated. Monounsaturated fatty acids were calculated as MUFA = Σ% (16:1 + 18:1). Polyunsaturated fatty acids were calculated as PUFA = Σ% (PUFAn3 + PUFAn6). The double bond index was calculated as UI = Σ (fatty acid percent) x (number of double bonds). The data were subjected to the Student's t–test. Data were expressed as mean ± SD. The 0.05 level was selected as the point of minimal statistical significance. Statistical criterion for significance was selected at different p values and indicated in each case.

RESULTS

The fatty acid composition of total lipids isolated from spermatozoa samples, native, control and peroxidized is shown in Table 1. The saturated long chain fatty acids present in spermatozoa were mainly palmitic acid (C16:0) and stearic acid (C18:0) in a percentage of approximately 31 and 8% respectively. The concentration of total unsaturated fatty acids of spermatozoa was approximately 55%, with a high percentage of long chain polyunsaturated fatty acids as docosapentaenoic acid (C22:5 n6) and linoleic acid (18:2 n6) and in minor proportion the linoleic acid (18:3 n3), arachidonic acid (C20:4 n6) and docosahexaenoic acid (C22:6 n3).

Table 1. Fatty acid composition of total lipids of dog spermatozoa control (without ascorbate–Fe^{++}) and peroxidized (with ascorbate–Fe^{++}).

fatty acid	native	control	peroxidized
16:0	31.67 ± 7.09	27.22 ± 5.58	40.73 ± 3.59 [b]
18:0	8.57 ± 2.61	7.22 ± 0.78	14.80 ± 4.69
18:1 n9	13.29 ± 2.21	14.73 ± 4.30	25.90 ± 4.97 [b]
18:2 n6	6.64 ± 3.72	7.22 ± 0.67	2.15 ± 1.29 [a]
18:3 n3	3.30 ± 1.31	4.54 ± 1.04	3.32 ± 1.11
20:4 n6	3.27 ± 1.08	5.39 ± 1.15	1.00 ± 0.61 [a]
22:4 n6	1.87 ± 0.48	2.31 ± 0.61	0.04 ± 0.06 [a]
22:5 n6	27.11 ± 2.34	21.67 ± 3.72	5.23 ± 1.51 [a]
22:6 n3	3.35 ± 0.45	2.16 ± 0.70	0 [a]
saturated	40.23 ± 9.67	34.43 ± 6.17	55.53 ± 3.02 [b]
monounsaturated	13.29 ± 2.21	14.73 ± 4.30	25.90 ± 4.97 [b]
polyunsaturated	42.19 ± 6.64	41.12 ± 4.08	11.73 ± 3.60 [a]
total unsaturated	55.48 ± 8.77	55.85 ± 5.85	37.64 ± 2.16 [b]
saturated/unsaturated	0.76 ± 0.28	0.63 ± 0.6	1.48 ± 0.17 [a]
UI	192.58 ± 22.72	181.91 ± 17.86	70.46 ± 9.87 [a]

UI: unsaturation index. Data are given in percentages of total fatty acid content are mean ± SD of six experiments independent. The UI was calculated as the sum of the percentage by weight of each fatty acid x the number of olefinic bonds. Data were evaluated statistically by Student–t test. Statistically significant differences between control vs. peroxidized groups are indicated by [a] $p<0.005$, and [b] $p<0.05$.

Spermatozoa membranes incubation in the presence of ascorbate–Fe^{++} led to membrane phospholipids peroxidation as evidenced by light emission (chemiluminescence) and PUFAs diminution, mainly C22:5 n6. Changes in the fatty acid profiles were used as an index of the oxidative damage to lipids.

Figure 1 shows the total chemiluminescence (sum of all the readings obtained every 10 min for 120 min a 37°C). Total chemiluminescence increased from 172.4 ± 19.23 cpm in control (without ascorbate) up to 670.5 ± 66.39 cpm in peroxidized (with ascorbate–Fe^{++}) groups. In consequence, the value of the LP process, measured as total light emission during non enzymatic LP was 4 fold higher in ascorbate–Fe^{++} than control group.

The fatty acid profile was markedly modified by peroxidation, PUFA content decreased and concomitantly saturated fatty acid percentage increased. A substantial difference was found in the content of the main peroxidable fatty acids C18:2 n6, C20:4 n6, C22:4 n6, C22:5 n6 and C22:6 n3 that showed significant decreases as expected when the LP process occurs.

The UI shows significant differences between control and peroxidized samples 181,91 ± 17,86 and 70.46 ± 9,87, respectively. The unsaturated FA that prevailed in the diet was C18:1 n9, whereas the rest were found only in traces (Figure 2).

In the diet the saturated fatty acid content was 2 times greater than that found in spermatozoa whereas the unsaturated FA content was 3 times and the UI was seven times lower than that in spermatozoa (Figure 3).

DISCUSSION

The phospholipids of spermatozoa of all species contain large amounts of PUFA. The n–3 and n–6 PUFA´s are considered to be essential due to the inability of vertebrates to synthesize them. The n–3 series docoxahexaenoic acid dominates the lipid make up of mammalian spermatozoa [26, 31].

Even though the levels and combinations for fatty acids of both n–6 and n–3 series within the phospholipids fractions demonstrate distinct species specificity, the total level of unsaturation of the male gamete lipid is always high.

In this study, we demonstrated that the fatty acids profiles of spermatozoa of dog (Yellow Labrador Retriever) exhibit extremely low levels of C22:6 n3 which contrast most strongly with the situation of the bull and ram, in which levels of C22:6 n3 are extremely high [38]. The spermatozoa of the dog displays an interesting fatty acid profile with high levels of n–6 counterpart docosapentaenoic acid (C22:5 n6).

The fatty acids C20:4 n6, C22:4 n6, C22:5 n6 and C22: 6 n3 are synthesized from their dietary precursors 18:3 n6 and 18:2 n4, respectively [35]. Dog spermatozoa exhibited a high total unsaturated fatty acid content and UI when spermatozoa and diet samples were compared.

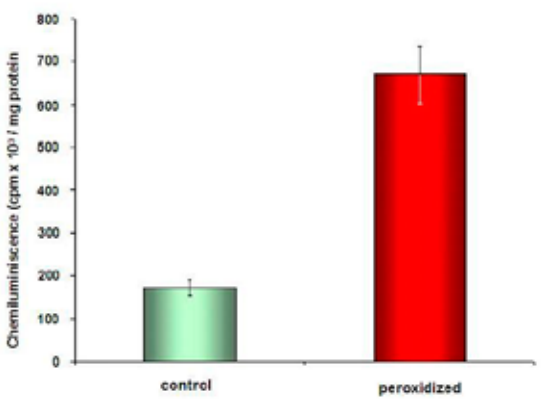

Figure 1. Lipid peroxidation ascorbate–Fe^{2+} of dog spermatozoa. Chemiluminescence was determined over a 120 min period and recorded as cpm every 10 min and the sum of the total chemiluminescence was used to calculate cpm/mg protein. Results are expressed as mean ± SD of three independent experiments. Statistically significant differences between control and peroxidized are indicated by p<0.0001.

These results indicate that the level of unsaturated fatty acids is homeostatically regulated in tissues [34]. The control of membrane fatty acid unsaturation has been attributed to negative feed back regulation of transcription of desaturase genes dependent on lipid composition [34, 41] and to the modulation of desaturases by the metabolic–hormonal status [17]. Taking this observation into account we considered that the high content of double bonds observed in dog spermatozoa would be independent from diet.

LP is recognized as a damaging process to spermatozoa leading to motility loss and reduced fertilizing ability in spermatozoa of many species including man [1, 4, 6, 13, 16, 19, 43]. LP occurs spontaneously in mammalian spermatozoa [8] and is greatly enhanced in human sub–fertile ejaculates [2] or fowl stored semen [9, 44]. The mechanisms by which reactive oxygen species (ROS) disrupt the sperm function is believed to involve the peroxidation of the polyunsaturated fatty acids present in the sperm plasma membrane [5] and this process plays an important role in the pathophysiology of male infertility [3]. ROS increases DNA fragmentations [32], modifies

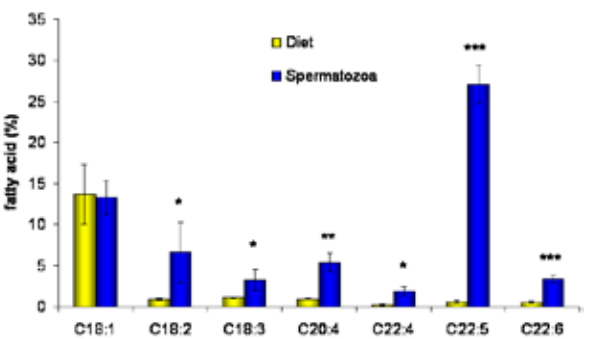

Figure 2. Fatty acid composition of fatty acids present in the diet and spermatozoa lipids of dog. Results are expressed as mean ± SD of three independent experiments. Statistically significant differences between diet and spermatozoa are indicated by * p<0.05, ** p<0.001, *** p<0.0001.

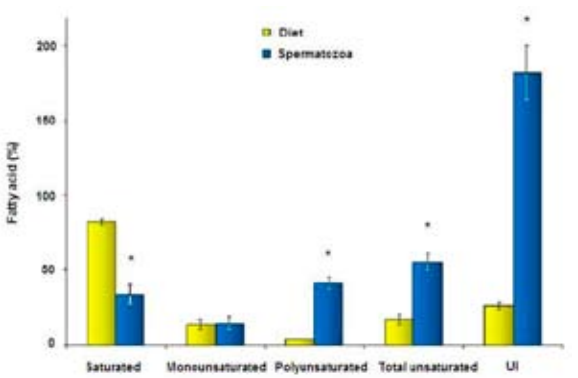

Figure 3. General index fatty acid present in the diet and spermatozoa lipids of dog. Results are expressed as mean ± SD of three independent experiments. Statistically significant differences between diet and spermatozoa are indicated by * p<0.0001.

the cytoskeleton [21], affects the sperm axoneme development [13], and inhibits sperm–oocyte fusion [2].

Individual acyl chains differ greatly in their chemical propensity for oxidative damage [23]. The n–3 PUFA are more peroxidation–prone than n–6 PUFA and within each PUFA class there is a 4–fold increase in peroxidizability between the short and long–chain fats. C22:6 n3 is 320–fold more susceptible to peroxidation than 18:1 n9 [24]. The high content of unsaturated fatty acids of the dog spermatozoa is correlated with the vulnerability to LP observed in this semen, which showed a positive correlation with the level of long–chain polyunsaturated fatty acid and higher values of light emission.

In conclusion, our results indicate that 1) semen contains great amounts of PUFA, which were vulnerable to the lipid peroxidation, 2) the alteration in PUFA composition will be the common base of different degenerative processes, 3) canine spermatozoa FA profile was not correlated with the diet composition.

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