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Induced sperm oxidative stress in dogs: Susceptibility against different reactive oxygen species and protective role of seminal plasma

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ABSTRACT

Oxidative stress (OS) is characterized by an unbalance between increased levels of reactive oxygen species (ROS) and/or impaired antioxidant protection. In this context, the composition of seminal plasma (SP) plays a key role in protecting sperm against OS. However, reproductive biotechnologies applied to dogs recommend the removal of SP. Thus, antioxidant therapy may be an important alternative when applying biotechniques such as semen cryopreservation in this specie. However, in order to be efficient, the choice of the ideal antioxidant in each condition is essential since each ROS is preferably neutralized by different antioxidant systems. Therefore, this study aims to evaluate the susceptibility of canine spermatozoa to different oxidative challenges (superoxide anion [O_2^-], hydrogen peroxide [H_2O_2], hydroxyl radical [OH^-] and malondialdehyde [MDA]) in the present or absence of SP. We used ejaculates of eight dogs and submitted to induce oxidative challenges (with or without SP). After incubations, samples were evaluated for the susceptibility to lipid peroxidation, motility, mitochondrial activity and function, DNA integrity, plasma membrane and acrosome integrity. Sperm with SP had mitochondrial function preserved against ROS. However, in the absence of SP, H_2O_2 reduced mitochondrial membrane potential. In addition, regardless on SP, H_2O_2 was deleterious to sperm kinetics and plasma/acrosomal membranes. Incubation with OH^- reduced mitochondrial activity and increased DNA fragmentation also independent on the absence of presence of SP. Furthermore, samples with SP were more resistant to lipid peroxidation (i.e., decreased concentration of TBARS). In conclusion, H_2O_2 and OH^- appears to be the most deleterious ROS to dog sperm and SP protects the spermatozoa against mitochondrial injuries and lipid peroxidation.

1. Introduction

Dogs represent a significant role in society, with considerable impact on global economy [1]. Moreover, physiological similarities with wild canines and humans feature the dog as ideal experimental model for these species [2,3]. In this context, studies focusing on new advances in reproductive biotechnologies in dogs are encouraged, such as, artificial insemination and cryopreservation [2]. Semen cryopreservation allows a long-term preservation of breeding lineages of livestock animals and dissemination of the genetic material even *post-mortem* [2].

However, during the cryopreservation process sperm is prone to several damages on membrane and organelles, impaired motility and increased DNA damage [4,5]. These effects are directly of indirectly related to the higher production of reactive oxygen species (ROS) by the sperm [4]. Despite the physiological role of ROS on major events, such as, capacitation, hyperactivation, acrosome reaction and sperm oocyte penetration [6], high amounts of ROS can cause damage to sperm structures such as DNA, lipids, carbohydrates and proteins [6,7]. In fact, spermatozoa are particularly susceptible to ROS attack due to the reduced cytoplasm and consequent limited content of enzymatic antioxidants [8].

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Furthermore, sperm membrane is rich in polyunsaturated fatty acid (PUFA), which makes this cells more susceptible to oxidative stress due to double bonds (i.e., unsaturation) that are more easily cleaved by ROS [9].

To counterattack the exacerbated oxidative damages caused by ROS, antioxidants produced physiologically by the organisms, prevent or minimize the oxidation effects [10]. Most of the antioxidant defense present in dog semen are from the seminal plasma, wherein were previously identified the reduced glutathione (GSH), glutathione peroxidase (GPx), phospholipid hydroperoxide glutathione peroxidase (PHGPx) and superoxide dismutase (SOD) [11,12]. However, such positive effects of seminal antioxidants may be no longer available due to the removal of seminal plasma during the cryopreservation process [13], which contribute to the high susceptible of dog sperm to the attack of ROS [14].

Nevertheless, the addition of antioxidants in extenders may contribute to protect canine sperm against this free-radical induced injury during cryopreservation [15,16]. Several studies were performed aiming to evaluate the effect of antioxidants on sperm cryopreservation with contradictory outcomes [15,17–20]. One of the possible reasons for such controversial results is the use of unfitted antioxidants. Each ROS is susceptible to specific antioxidant systems [21,22]. Therefore, in cases where inadequate antioxidant therapies were used, such treatment may lead to inefficacious of even deleterious results. Therefore, choosing the ideal antioxidant by identifying the most deleterious ROS may be an interesting approach.

In this context, the aim of our study was to compare the impact of different ROS and malondialdehyde on canine spermatozoa to identify the most deleterious compound. In addition, our aim was to verify the possible protector effect of seminal plasma against oxidative challenges. Consequently, this result will allow target a specific antioxidant therapy during sperm cryopreservation in further studies.

2. Material and methods

2.1. Animals

The present experiment was approved by the Bioethics Committee of the School of Veterinary Medicine and Animal Science – University of São Paulo (protocol number: 2277/2011). Unless otherwise stated, all chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA).

We used eight dogs (n = 8; 2–6 years old) of the breeds German shepherd, Belgian shepherd malinois and Labrador with 28.2–30.5 average weight. Animals were healthy and sexually mature, and belonged to the Center for Breeding and Distribution of Canines of the 2nd Army Police Battalion (Osasco, São Paulo - Brazil). In addition, semen of these animals was collected periodically (approximately once a week) to be used in the reproductive routine of the army battalion.

All animals were submitted to semen collection using the digital manipulation method. Macroscopic and microscopic analyses were performed; samples were rejected whenever contaminated (i.e., urine and blood) or sperm motility was found to be lower than 70%. Semen was diluted (1:200) in formal buffered saline for sperm count in a Neubauer chamber and sperm concentration was expressed in million spermatozoa per mL.

2.2. Experimental design

The eight ejaculates (n = 8; one replicate per animal) were submitted to a 2 X 5 factorial arrangement, in which we considered the effect of seminal plasma (presence or absence) and the incubation systems with different ROS (superoxide anion [O_2^-], hydrogen peroxide [H_2O_2], hydroxyl radical [OH^-]), the byproduct of lipid peroxidation (malondialdehyde [MDA]) and with TALP medium (Control) as experimental factors.

2.3. Semen processing

Each ejaculate was divided into 2 fractions, with (SP) or without seminal plasma (NSP), which were centrifuged at 600 *x* g during 10 min. Fraction NSP was considered without seminal plasma and fraction SP with seminal plasma. For this, fraction NSP supernatant was discarded and the pellet was resuspended in TALP medium heated at 37 °C. The pellet of fraction SP, which in turn was resuspended in the supernatant itself (seminal plasma). The two fractions (SP and NSP) were resuspended to the same concentration (100×10^6 spermatozoa/mL).

Then, the fractions (with or without seminal plasma) were subdivided into five aliquots and incubated for 30 min at 37° C with ROS generation systems (superoxide anion [O_2^-], hydrogen peroxide [H_2O_2], hydroxyl radical [OH^-]), a product of lipid peroxidation (MDA) and finally a control system.

2.4. System of ROS production in vitro and by-product of lipid peroxidation

Semen aliquots (400 μl) were incubated with different systems of ROS production and malondialdehyde according to Rui et al. [23], and Kawai et al., [24]. For all incubations we used a concentration of 20×10^6 spermatozoa per sample. In Control group, 200 μL of TALP medium was added to 400 μL of the diluted sample. The xanthine-xanthine oxidase system (Xanthine 0.5 mM; Xanthine Oxidase 0.05 IU/ml) was used to induce the superoxide anion (O_2^-) production. Hydroxyl radical (OH^-) production was induced through incubation with ferrous sulphate (Fe_2SO_4, 4 mM) and sodium ascorbate (20 mM). Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were added directly to aliquots at a concentration of 4 mM. Samples were incubated over a period of 30 min at 37 °C in a water bath, and immediately after incubation, sperm were subjected to analysis.

2.5. Sperm analysis

2.5.1. Computer analysis of sperm kinetics patterns

Semen kinetics patterns were assessed using the Computer Assisted Sperm Analysis (CASA; Hamilton-Thorne®, Ivos 12.3, USA). For the analysis, 10 μl were used in counting chamber and ten fields were randomly selected for analysis. The following variables were considered: motility (%), progressive motility (%), VAP (average path velocity, μm/s), VSL (straight-line velocity, μm/s), VCL (curvilinear velocity, μm/s) ALH (amplitude of lateral head displacement, μm), BCF (beat cross-frequency, Hz) STR (straightness, %) and LIN (linearity, %). In addition to these parameters, the sperm velocity was also divided into four groups: rapid (%), medium (%), slow (%) and static (%) [25].

2.5.2. Sperm functional tests

The sperm functional tests were performed according to the methodology developed by Lucio et al. [15], for dogs. Using a flow cytometry (Guava EasyCyteTM Mini System, Guava® Technologies, 190 Hayward, CA, USA) and the cytochemical assay 3′3 diaminobenzidine (DAB technique).

The flow cytometry equipment contains a blue laser, which operates at 488 nm and emits a 20 mW visible laser radiation. A total of 10,000 events per sample were analyzed and data corresponding to yellow (PM1 photodetector – 583 nm), red (PM2 photodetector – 680 nm) and green fluorescent signals (PM3