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Impact of induced levels of specific free radicals and malondialdehyde on chicken semen quality and fertility

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ABSTRACT

Over the past decades, scientists endeavored to comprehend oxidative stress in poultry spermatozoa and its relationship with fertilizing ability, lipid peroxidation (LPO), free-radical scavenging systems, and antioxidant therapy. Although considerable progress has been made, further improvement is needed in understanding how specific reactive oxygen species (ROS) and malondialdehyde (MDA, a toxic byproduct of LPO) disrupt organelles in avian spermatozoon. Hence, this study examined functional changes in chicken spermatozoa after incubation with different ROS, and their implications for the fertility. First, semen samples from 14 roosters were individually diluted and aliquoted into five equal parts: control, superoxide anion, hydrogen peroxide (H_2O_2), hydroxyl radicals, and MDA. After incubation with these molecules, aliquots were analyzed for motility, plasma membrane and acrosome integrity, mitochondrial activity, and LPO and DNA damage. Hydrogen peroxide was more detrimental for sperm motility than hydroxyl radicals, whereas the superoxide anion and MDA exhibited no differences compared with controls. In turn, plasma membrane and acrosome integrity, mitochondrial activity, LPO and DNA integrity rates were only affected by hydroxyl radicals. Thereafter, semen aliquots were incubated under the same conditions and used for artificial insemination. In accordance to our *in vitro* observations, H_2O_2 and hydroxyl radicals sharply reduced egg fertility, whereas superoxide anion and MDA only induced slight declines. Thus, chicken sperm function was severely impaired by H_2O_2 and hydroxyl radicals, but their mechanisms of action seemingly comprise different pathways. Further analysis regarding susceptibility of spermatozoon organelles to specific radicals in other poultry will help us to understand the development of interspecific differences in scavenging systems and to outline more oriented antioxidant approaches.

1. Introduction

Reactive oxygen species (ROS) are natural byproducts in a variety of biochemical reactions, such as mitochondrial respiratory chain, which at controlled levels play important roles in sperm function (e.g., acquisition of motility and fertilizing ability) [1]. Notwithstanding, whenever generation of ROS surpasses cell antioxidant capacity, these molecules exert detrimental effects in a process called oxidative stress (OS) [2,3]. Due to their need for fluidity and flexibility, spermatozoa have an abundance of polyunsaturated fatty acids (PUFAs) in their membranes, making them highly vulnerable to free-radical attack [1,4]. Consequently, overexposure of spermatozoa to ROS results in lipid peroxidation of the plasma membrane, an autocatalytic self-propagating reaction that leads to impaired motility, reduced acrosomal reaction, DNA damage, and ultimately death [1,3,4]. That is

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why both seminal plasma and spermatozoa possess scavenging systems through enzymatic (superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase, and ceruloplasmin) and nonenzymatic pathways (vitamins C and E, albumin, β-carotenes, L-carnitine, glutathione, pyruvate, taurine, hypotaurine, ubiquinol, and zinc) to prevent free radical formation [4]. However, the dramatic loss of most of the cytoplasm during spermatogenesis confers to spermatozoa a reduced antioxidant capacity compared with somatic cells, making them more vulnerable to oxidative insult [5].

Previous studies have shown that avian spermatozoa differ from their mammalian counterparts not only in membrane lipid composition but also in resistance to cold shock damage (with rooster sperm being more resistant to injuries during rapid cooling) [6,7]. For instance, docosahexaenoic acid (22: 6n-3) is the most important PUFA in spermatozoa of several mammals, whereas docosatetraenoic and arachidonic acids (DTA; 22: 4n-6 and AA; 20: 4n-6, respectively) are the major PUFAs in avian species [7,8]. Mammalian spermatozoa also contain much higher amounts of PUFAs, thereby providing greater membrane fluidity [7,9]. Even among poultry, interspecific differences in susceptibility of sperm membrane to oxidation of PUFAs are found, as revealed by Surai et al. [7] through peroxidizability indexes. Therefore, it seems reasonable to assume that avian and mammal spermatozoa may react distinctly to the presence of different ROS as they do in face of other adverse circumstances such as cooling and cryopreservation. Evidence that human, mouse, rabbit, dog, and ram can be 5 to 100 times more resistant than chicken to the effects of hydrogen peroxide (H_2O_2) on sperm motility [10], support this hypothesis.

Details regarding the mechanisms by which each of these molecules disrupts avian sperm function are of utmost importance for the understanding of interspecific differences in scavenging systems and the design of antioxidant strategies. To date, however, most research on ROS in poultry has focused on lipid components of sperm membranes, lipid peroxidation, antioxidant enzymes activity, and antioxidant supplementation through nutrition or directly into semen diluents [7,11–15]. Despite the existing knowledge, there is still a gap toward the effects of each one of the oxygen-centered radicals on different organelles of the spermatozoon and how they impact semen quality and fertilizing ability. Thus, we became interested in investigating the *in vitro* and *in vivo* consequences associated with incubation of fowl spermatozoa with superoxide anion (O_2^-), H_2O_2, hydroxyl radicals (OH^-), and malondialdehyde (MDA). *In vitro* analysis comprised sperm motility and motion parameters, acrosome and plasma membrane integrity, mitochondrial activity, lipid peroxidation, and DNA damage, whereas *in vivo* assessment consisted of artificial insemination (AI) using incubated samples followed by egg fertility checking.

2. Materials and methods

2.1. Animals and experimental design

Fourteen roosters and 40 hens (Lohmann LSL-Classic, 50 wk of age) were housed at the Poultry Research Facility (College of Veterinary Medicine and Animal Science—University of São Paulo) under standard management practices, which included individual cages (25 × 45 × 44 cm), 14L: 10D photoperiod, water *ad libitum*, and twice-a-day feeding regimen (120 and 90 g/day for males and females, respectively). A standard commercial diet was fed to the animals throughout the study (2750 kcal ME/Kg, 17% CP, and 3% Ca–Presence Postura 17, Presence Nutrição Animal, Brazil). In experiment 1, individual samples of semen were diluted, aliquoted, and separately incubated with all of the above mentioned molecules (O_2^-, H_2O_2, OH^-, and MDA groups), and then spermatozoa were assessed for motility, plasma membrane and acrosome integrity, mitochondrial activity, lipid peroxidation, and DNA damage. In experiment 2, pooled samples (2–3 males) were processed as in experiment 1, briefly evaluated for motility and concentration, and subsequently used for AI. Mean percent fertility of the eggs following inseminations was determined through breakout analysis. All animal procedures were performed with approval and under the guidance of the Institutional Animal Care and Use Committee at the University of São Paulo.

2.2. Semen collection and processing

Semen was collected from roosters once in a week using the dorso-abdominal massage method [16]. Sperm concentration of clean ejaculates (i.e., without feces or urine contamination) was immediately determined by spectrophotometry (Accuread rooster and turkey photometer, IMV, France), and final concentration (100×10^6 spermatozoa/mL) was achieved by diluting each sample with Biggers-Whitten-Whittingham medium. After dilution, samples were divided into five microtubes in accordance with treatment groups (control, O_2^-, H_2O_2, OH^-, and MDA—400 μL/microtube).

2.3. Reactive oxygen species (ROS)

Semen aliquots (400 μL) were incubated with three different systems of ROS production and a highly toxic byproduct of lipid peroxidation, known to also lead to oxidative damage. The xanthine-xanthine oxidase generating system (X 0.5 mM; XO 0.05 IU/mL, [17]) was used for production of superoxide anion, since Amstrong et al. [18] verified that O_2^- is the major species produced by this reaction, along with reduced amounts of O_2^--dependent H_2O_2 and negligible quantities of OH^-. Hydroxyl radicals were induced through incubation of diluted samples with ferrous sulfate (Fe_2SO_4, 4 mM) and sodium ascorbate (20 mM) [19]. Hydrogen peroxide and malondialdehyde were added directly to aliquots (both at a concentration of 20 mM). Incubations for all treatments were performed at 37 °C for 30 minutes.

2.4. Computer-assisted sperm analysis

Evaluation of sperm motility parameters was conducted using a Hamilton Thorne Version 12.3K IVOS (Hamilton Thorne Biosciences, Beverly, MA, USA). For each measurement, 6-μL aliquots of each semen sample were loaded onto a