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Original Research Article

Sperm characteristics following freezing in extenders supplemented with whole egg yolk and different concentrations of low-density lipoproteins in the collared peccary (*Pecari tajacu*)

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ABSTRACT

The aim of the current study was to compare sperm quality characteristics of the collared peccary (*Pecari tajacu*) following freezing in extenders supplemented with whole egg yolk and different concentrations of low-density lipoproteins (LDL). Semen from 11 adult males was obtained by electroejaculation and evaluated for sperm motility, vigor, morphology as well as membrane integrity analyzed by the hypo-osmotic swelling (HOS) test and a fluorescent staining. Moreover, the semen was diluted in a Tris-based extender containing 20% egg yolk (control group) or 5, 10 or 20% LDL (treatment groups). The semen samples were frozen in liquid nitrogen and thawed in a water bath for 60 s at 37 °C. The treatments did not affect ($p > 0.05$) sperm vigor, morphology or membrane integrity analyzed by the HOS test. However, post-thaw sperm motility was significantly higher ($p < 0.05$) in the extender supplemented with 20% LDL ($36.4 \pm 5.3\%$) compared with the egg yolk extender and extender supplemented with 10% LDL. Furthermore, the percentage of membrane-intact frozen-thawed spermatozoa analyzed by the fluorescent staining was significantly higher ($p < 0.05$) in the extender supplemented with 20% LDL ($27.4 \pm 6.5\%$) than in the other groups. In conclusion, 20% LDL can be used to substitute the whole egg yolk as a cryoprotective additive for freezing semen of the collared peccary.

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1. Introduction

As one of the most hunted species in tropical areas, the collared peccary (*Pecari tajacu*) represents a major source of meat and income for local inhabitants [1]. Due to the growing interest for the meat and leather of the collared peccary in the international market, efforts have been made to increase its reproduction under captivity [2], and to develop protocols for its germ plasma conservation [3,4].

Even though some components of the egg yolk have negative effects on sperm cryo-survival [5], egg yolk is one of the most important cryoprotective additives used in the freezing extenders. On the other hand, egg yolk, as a component of animal origin, may represent a potential risk of bacterial contamination for artificial insemination (AI) doses [6], thus reducing its widespread use in the commercial swine industry [7]. Aiming to overcome these challenges, various studies demonstrated the possibility to remove some of the whole egg yolk components by centrifugation, isolating the beneficial ones such as low density lipoproteins (LDL) [8–11]. The LDL increases sperm resistance against cold shock, resulting in improved sperm motility after semen storage [11].

Numerous studies have reported the beneficial effects of LDL on semen cryopreservation in the boar [12–14], which is closely related to the collared peccary [15]. Even though collared peccaries look like pigs, they belong to the Tayassuidae family [16]. Recent studies have demonstrated that standardized tests used in boar semen processing technology cannot be applied directly in collared peccary semen technology [4,17]. Presently, the protocols that have been developed for cryopreservation of the collared peccary semen include Tri-based extender [3,18] or coconut water-based extender [17,19] supplemented with egg yolk. Therefore, the current study aimed to compare sperm quality characteristics of the collared peccary (*P. tajacu*) following freezing of semen in the Tri-based extender supplemented with whole egg yolk and different concentrations of low-density lipoproteins.

2. Materials and methods

The ethics committee of the Universidade Federal Rural do Semi-Árido – UFERSA approved the experimental protocols and the animal care procedures used in the current study (Process No. 23091.0253/114). The reagents used in the study were obtained from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise stated.

2.1. Animals

Eleven sexually matured male collared peccaries, aged 40.7 ± 1.6 months and weighted 22.5 ± 2.8 kg, were used in the present study. The animals were obtained from the Centre of Multiplication of Wild Animals from UFERSA, located in the northeast Brazil (Mossoró, RN, Brazil; S: 5°10′, W: 37°10′). The climate of this region is typically semi-arid, with an average annual temperature of 27 °C. Experiment was conducted during the dry season (February–March 2013), and the animals were kept under a 12-h natural photoperiod. They were kept outdoors in groups of five and six animals in paddocks (20 m × 3 m) with a covered area (3 m × 3 m). The animals were fed with sow food and fruits, and water was available *ad libitum*.

2.2. Semen collection

Twelve hours before starting the experiment, the animals were not fed. Each animal was physically restrained using a hand net and anesthetized as described previously [20]. The animals were kept in lateral recumbency and semen was collected using an electroejaculator (Autojac®, Neovet, Campinas, SP, Brazil) according to a previously described procedure [3]. Semen was collected in plastic tubes and immediately evaluated. A total of 11 ejaculates were obtained from 11 different males.

2.3. Semen quality analysis

Each fresh semen sample was subjected to macroscopic examination. Following the initial assessment, a 10 μL semen aliquot was diluted with 10% buffered formalin (2 mL), and sperm concentration was determined using a Neubauer counting chamber (Bright-Line, Precicolor [HBG], Germany) [21]. Sperm motility and vigor (sperm progressive motion on a 0–5 scale) were subjectively assessed using a light microscope (Eclipse E-200, Nikon, Tokyo, Japan) at 100× and 400× magnification, respectively. Bengal Rose-stained smears were prepared with 5 μL of semen to evaluate sperm morphology using light microscopy at 1000× magnification [22].

The functional integrity of the sperm membrane was evaluated by the hypo-osmotic swelling (HOS) test [23]. Semen aliquots (10 μL) were added to 90 μL of distilled water (0 mOsm/L) and incubated for 40 min at 37 °C. Two hundred spermatozoa were counted under a phase contrast microscope at 1000× magnification (ABM-200, Alltion, China) in at least five fields and classified as osmotically reactive or non-reactive based on the presence or absence of coiled (swelled) tails. The percentage of spermatozoa with tail defects (based on sperm morphology evaluation) was subtracted from the percentage of osmotically reactive spermatozoa. The sperm plasma membrane integrity was also assessed using fluorescent staining assay with 6-carboxyfluorescein diacetate (CFDA) and propidium iodide (PI) [19]. Approximately 200 sperm cells were examined by epifluorescence microscopy (Eclipse TS 100, Nikon). Sperm cells displaying only green fluorescence over the head, mid-piece and tail regions were classified as membrane-intact spermatozoa (CFDA), whereas membrane-damaged spermatozoa emitted red fluorescence (PI).

2.4. Extraction of low-density lipoproteins

The LDL were extracted from hen egg yolk according to a previously described method [24], which yielded 97% purity of LDL. The extraction method is protected under the patent no. 0100292 held by the ENVN and INRA of Nantes. Briefly, the yolks derived from fresh hen eggs were separated from the albumen, diluted with an isotonic saline solution (0.17 M NaCl) (w/w) and centrifuged at 11,400 × *g* for 45 min at 4 °C. The

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