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Original Research

Addition of Glutathione to an Extender for Frozen Equine Semen

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ABSTRACT

The manipulation of equine semen during cryopreservation reduces sperm viability and fertility because of, among other factors, membrane lipid peroxidation that makes cells highly susceptible to free radicals and reactive oxygen species (ROS). The oxidative effect caused by the generation of ROS can be reduced by the addition of antioxidants to the seminal plasma or to the extenders used for freezing. The current study was performed to test the in vitro effect of exogenous glutathione added in five different concentrations (control, 2.5 mM, 5.0 mM, 7.5 mM, and 10 mM [treatments 1-5, respectively]) to the extender for 12 stallions. Analyzed parameters were sperm motility, viability, and acrosome and plasmatic membrane integrity. Total motility was higher in treatments 1 and 2 ($P < .05$); viability, progressive motility, and plasmatic membrane integrity were higher in treatment 2 ($P < .001$). As for acrosome membrane integrity, treatment 3 showed the best results ($P < .05$). The addition of 2.5 mM glutathione to the freezing extender preserves total motility and increases sperm viability, progressive motility, and plasmatic membrane integrity. Concentrations above 2.5 mM were deleterious to spermatozoa.

1. Introduction

Fifty-four years have passed since the first foal was born with the use of frozen semen [1]. Since then, important advances have been observed with the use of this biotechnique; however, pregnancy rates continue to be low and are affected by several factors.

The use of frozen semen created a new dimension for the horse breeding industry by making possible the preservation of this biological material for unlimited time and its worldwide distribution. This optimizes the use of stallions with superior genetics and reduces the costs with transport and diseases. Geographic barriers are abolished, and one can use frozen semen from stallions that are in competition or are recovering from pathologies that would prevent them from mating, and even from dead stallions [2].

The manipulation of equine semen during these processes reduces sperm viability and fertility because of, among others, membrane lipid peroxidation, because of its high polyunsaturated fatty acids content, which makes the cells highly susceptible to free radicals and reactive oxygen species (ROS) [3].

ROS scavengers are present in seminal plasma, and the primary ROS scavengers described in equine semen are glutathione peroxidase, superoxide dismutase, and catalase. Sperm centrifugation used to remove seminal plasma and concentrate spermatozoa before freezing removes antioxidants present in semen, exposing spermatozoa to excessive ROS damage [4].

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A way to improve sperm viability and, consequently, fertilizing capacity would be the addition of antioxidants to the freezing medium. Although most studies that examined the addition of antioxidants to cryopreserved equine semen did not show positive effects on post-thaw parameters and fertility [4], it still depends on the antioxidant type and/or concentration, as well as on the mechanism of action regarding sperm protection [5].

Glutathione is one of the antioxidants added to different semen specimens. It is a thiol tripeptide (ɣ-glutamyl cysteinyl glycine) with several biological functions found widely in the animal body, not only in somatic cells but also in gametes as well. This thiol has an important role in the antioxidation process of endogenous and exogenous composts, as well as in the maintenance of intracellular redox conditions. Glutathione is a natural reservoir of redox force, which can be quickly used by defend cells against oxidative stress [6]. It is synthesized from glutamate, cysteine, and glycine amino acids. Its reductive power is used to maintain thiol groups in intracellular proteins and other molecules. It acts as a cysteine physiological reservoir and is involved in the regulation of protein synthesis, cellular detoxification, and leukotriene synthesis. The protection by glutathione against oxidative damage is provided by its sulphydryl group (SH), which can be presented in reduced glutathione (GSH) and oxidized glutathione (GSSG) forms. The GSH's attack against ROS is favored by the interaction with enzymes, such as glutathione reductase and glutathione peroxidase (GPx) [6].

The thiol antioxidant system is represented mainly by glutathione, the primary antioxidant in equine semen, abundant in seminal plasma. The amount of GSH in equine seminal plasma is 10 times higher than that in swine [6].

Based on this evidence, the hypothesis of the present study was that the addition of different glutathione concentrations favors equine cryopreserved sperm viability. Lack of information regarding fertility parameters and data discrepancies about the effects of the addition of antioxidants to equine cryopreserved semen indicate the need for more study. Therefore, the objective of the present study was to evaluate the in vitro effect of glutathione addition in five different concentrations to equine spermatozoa subjected to cryopreservation.

2. Materials and Methods

2.1. Semen Collection and Processing

Stallions semen were collected once a day for 7 days to stabilize extragonadal reserve and daily sperm output. Afterward, ejaculates were collected three times per week from 12 fertile light-horse stallions between the ages of 5 and 15 years, using the Colorado model (Equine Artificial Vagina; ARS, Chino-CA, USA) artificial vagina, with a total of 36 ejaculates obtained. Semen samples were collected in a plastic bottle and filtered immediately after collection to create gel-free semen. Only ejaculates with more than 60% motility were used for cryopreservation [7].

The sperm-rich fraction (gel-free) was diluted in a 1:1 ratio using a skim milk-glucose extender with penicillin G procaine [8].

2.2. Addition of Antioxidant and Sample Freezing

Samples were centrifuged at 600 × *g* for 10 minutes, and sperm pellets were resuspended in a freezing extender (Botucrio; Botupharma, Botucatu, Brazil) to a concentration of 200 × 10^6 cells/mL and placed in five (15-mL) sterile centrifuge tubes. These aliquots were used for the treatment groups with the addition of glutathione (G6013; Sigma Chemical Co.) in different treatment concentrations, as follows: treatment 1, control; treatment 2, 2.5 mM; treatment 3, 5 mM; treatment 4, 7.5 mM; and treatment 5, 10 mM. Aliquots were packed into 0.5-mL straws placed in an automated freezing system (TK 3000 SE; TK Tecnologia em Congelação LTDA, Uberaba, Brazil) to stabilize the cooling and freezing rates.

For stabilization, straws were placed on a straw holder inside the cooling tube until it reached 5°C at a rate of −0.25°C/min, remaining 20 minutes in this temperature. After this time, the straw holder was moved to a thermal box containing liquid nitrogen, at a freezing rate of 15°C/min from 5°C until reaching −80°C and from 10°C until reaching −120°C. Once this temperature was reached, the straws were plunged into liquid nitrogen (−196°C) and stored in a liquid nitrogen holding tank.

2.3. Post-Thawing Analysis

Two straws from each treatment, from the same stallion, were thawed in a water bath at 37°C for 30 seconds, 24 hours after storage in the cryogenic container.

The computerized analysis of sperm movement characteristics was performed with an Ivos-Ultimate 12 unit (Hamilton Thorne Biosciences), previously adjusted for equine semen. Three fields were selected for analysis. Measured variables were total motility (% of TM), progressive motility (% of PM), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat-cross frequency (BCF, Hz), linearity (% of LIN), and straightness (% of STR).

For the evaluation of viability, the supravital eosin-nigrosin staining technique (LIVE/DEAD) was used, where equal volumes (20 μL) of semen and stain were mixed and transferred to a preheated (37°C) labeled microscope slide and smeared by sliding a cover slip in front of it. The smears were air dried and examined directly. Samples were evaluated by microscopy (magnification ×1,000). Five hundred sperm cells were counted per sample, and unstained cells were classified as those that were viable [9].

To evaluate sperm plasmatic membrane integrity, the hypo-osmotic swelling test (HOST) was used by incubation of 100 μL of semen in 1.0 mL of a sucrose solution of 100 mOsm/L in a water bath at 37°C for 30 minutes. After this time, 20 μL of this solution was analyzed in a humidity chamber, using phase-contrast microscopy at ×1,000 magnification. A total of 200 spermatozoa were counted, and those considered swollen (coiled) were determined to possess membrane integrity after the subtraction of the percentage of tail alterations found in the morphologic evaluation [10].