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Original Research

Dietary Polyunsaturated Fatty Acid Supplementation Improves the Quality of Stallion Cryopreserved Semen

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ABSTRACT

The objective was to assess the influence of polyunsaturated fatty acid supplementation on the quality of fresh, cooled, and frozen-thawed stallion semen. Ten stallions received their normal diet (control group) or normal diet plus 150 mL of polyunsaturated fatty acid (PUFA) linseed-based oil (PUFA group). Semen was collected every 15 days during 60 days. Stallions were reversed across the treatments after a sixty-day interval. Semen was evaluated at 2, 6, 12, and 24 hours after cooling and 24 hours after freezing. Motility (MOT), vigor, membrane viability, morphology, acrosome integrity, and osmotic tolerance test (OTT) were evaluated. In the frozen-thawed semen, sperm dynamic characteristics were analyzed by computer-assisted sperm analysis and thiobarbituric acid reactive substances (TBARs) determined. The effects of treatment, time, semen type, and their interactions were submitted to PROCMIX (SAS) and means compared by the Tukey test. There was no treatment effect on the quality of fresh and cooled semen. However, frozen-thawed semen MOT, vigor, and OTT were superior ($P < .05$) in control compared to PUFA group. An interactive effect of sample day by treatment was observed, such that, TBARs increased over time ($P = .002$) in the PUFA group after 15, 30, 45, and 60 days from the beginning of supplementation. Thus, sperm may become more susceptible to the reactive oxygen species, probably due to the incorporation of polyunsaturated fat in the cell membrane. The addition of PUFA-enriched oil may be an alternative for improving frozen-thawed semen quality by increasing its MOT and resistance to osmotic changes to which sperm cells are submitted during the freezing process.

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1. Introduction

The Mangalarga Marchador has been introduced to 17 countries, and Europe has its largest population outside Brazil. This international context underlines the relevance of cooled and frozen semen for transport and preservation purposes in horse reproduction [1].

The spermatozoa response to sudden temperature changes is different between equine breeds and also among individuals within the same breed [2]. This occurs due to specific differences in seminal metabolism between individuals, which make the development of customized cooling and freezing protocols an expensive and complex process [3,4]. This great variability between breeds and individuals is also related to genetic improvement programs that select animals based on their athletic performance and

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morphology and do not take into consideration semen quality and reproductive efficiency [5].

During semen cooling and freezing processes, the low temperature shock could cause irreversible damages on the sperm cell membrane. The reason why some species are more susceptible than others is directly related to the lipid composition of the cellular membrane [6]. Cholesterol increases the stability of the cell membrane, decreasing the temperature in which it transitions from the fluid to the gelatinous phase [7,8]. Phospholipids provide membrane flexibility, depending on their fatty acid composition, and are important for the permeability and functionality of the cell [5].

In horses, the physiological proportion of cholesterol in relation to phospholipids in the sperm plasma membrane may impair its ability to undergo cooling and especially freezing processes successfully [2]. Since the proportion of cholesterol to phospholipids cannot be altered in sperm [9] because it is determined genetically for each species and each individual, the studies have focused on the effect of the addition of polyunsaturated fatty acids to the diet in order to increase its proportion in relation to the sperm membrane phospholipids, improving its fluidity and resistance to thermal stress [10–12].

The objective of this study was to assess the effect of an polyunsaturated fatty acids (PUFAs)–enriched diet on the quality of fresh, cooled, and frozen-thawed semen of Mangalarga Marchador stallions. The hypothesis was that the incorporation of polyunsaturated fatty acids into the sperm cell membrane improves its fluidity and, consequently, the capacity of the semen to maintain its integrity and functionality during the cooling and frozen processes.

2. Material and Methods

This project is in agreement with the guidelines of the Federal University of Lavras Ethical Committee for the Use of Animals in Research (protocol number: 005/11).

2.1. Animals, Facilities, and Diet

Ten stallions of the Mangalarga Marchador breed (3.5–15 year-olds; 389.7 ± 39.7 kg body weight), from two stud farms, were used during a breeding season in southeastern Brazil (September 2011 to February 2012). Stallions were individually housed in 4 × 4 meters stalls and received a diet composed by: 6 kg of Tifton 85 hay (*Cynodon* spp); 4 kg of a pelleted concentrate with 12% CP (Equitage 12, Guabi Nutrição Animal, Campinas, São Paulo, Brazil), offered twice a day; plus free access to a mineral mix (Hiposal, Total Alimentos S.A., Três Corações, Brazil) and water. This diet had approximately 21 Mcal of DE and 850 g of CP, supplying the minimum daily requirements for breeding stallions [13].

2.2. Treatments

Stallions received their normal diet (control group), or their normal diet supplemented with 150 mL of PUFA linseed-based oil top dressed on the concentrate (PUFA group), offered twice a day. Since the oil increased daily energy intake to nearly 1.35 Mcal, the amount of concentrate for the PUFA group was adjusted, accordingly in the control group.

The fatty acid composition of the linseed-based oil was determined by gas chromatography [14]: 21.0% oleic acid (18:1), 14.8% linoleic acid (18:2), 45.1% alpha-linolenic acid (18:3), 2.6% eicosapentaenoic acid (EPA, 20:5), 2.7% docosahexaenoic acid (DHA, 22:6), and 13.8% saturated fatty acids. The antinutritional factors were deactivated and the supplement enriched with vitamin E.

Before the beginning of each trial, stallions went through a 14-day adaptation period, during which the oil amount was increased daily until the total intake proposed was reached. In a crossover design, animals were switched across the treatments in two replicates. In the first replicate, five stallions were randomly allocated to the PUFA group or control group for 60 days. Stallions were reversed across the treatments after a 60-day interval, in order to remove the possible PUFA residual effects on spermatogenesis and for adaptation [15].

2.3. Semen Collection and Processing

Three semen collections were performed, in alternate days, in order to exhaust extra-gonadal reserves [16]. The time necessary to reach exhaustion was shortened since stallions were previously submitted to frequent collections. During the experimental period, stallions were submitted to semen collection three times a week, in order to ensure stable semen characteristics thereafter. For statistical analysis, semen samples were collected at 15-day intervals throughout 60 days, for a total of five samples per stallion (n = 100 samples).

A Hannover model artificial vagina and an estrous mare were used for semen collections. The gel portion was filtered, and the semen was diluted with a commercial extender (BotuSêmen, Botucatu, São Paulo, Brazil) in the proportion of 1:1 and stored in plastic bags (12 × 5 cm). Average sperm concentrations were 7.2 ± 0.4 × 10^8 spermatozoa/mL. The cooled semen was kept at 5°C in plastic bags in a cooler, with ice and a thermometer, following a freezing rate of 0.5°C per minute [17].

In the freezing process, semen was placed in 15-mL tubes, centrifuged at 600g for 10 minutes for seminal plasma removal [18] and a cryoprotectant (BotuCrio, Botucatu, São Paulo, Brazil) was added to the remaining pellets. Semen was transferred into 0.5-mL straws, at 100 × 10^6 spermatozoa per pellet, sealed with polyvinyl powder (Minitub, Porto Alegre, Rio Grande do Sul, Brazil), and submitted to a cooling curve: 20 minutes at 5°C, 20 minutes at 7 cm over the liquid nitrogen, and then plunged into liquid nitrogen at −196°C [6]. For thawing, straws were plunged into water at 37°C for 30 seconds [5].

2.4. Evaluation of Semen Characteristics

The semen evaluations were performed in the fresh samples, cooled semen at 2, 6, 12, and 24 hours after cooling, and 24 hours after the freezing process in the frozen-thawed semen. The seminal characteristics analyzed were: sperm motility (MOT) and vigor (direct evaluation under contrast phase microscopy at × 100

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