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Effects of α-tocopherol and freezing rates on the quality and heterologous *in vitro* fertilization capacity of stallion sperm after cryopreservation

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The effects of supplementation of α-tocopherol and different freezing rates (FRs) on the ability of stallion sperm to fertilize bovine oocytes with intact zona pellucida were investigated, in an attempt to develop a model to assess cryopreserved sperm function. Semen was obtained from four purebred Lusitano stallions ($n = 4$). Each ejaculate was subjected to cryopreservation with a commercial extender (Ghent, Minitub Iberia, Spain), without any supplementation (control) or supplemented with 2-mM α-tocopherol. The semen was exposed to two different FRs between 5 °C and −15 °C: slow (5 °C/min) and moderate (10 °C/min). After thawing, the viability (SYBR®-14 and propidium iodide [PI]), mitochondrial membrane potential (JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′tetraethylbenzimidazolyl carbocyanine iodine) and membrane lipid peroxidation (C_{11}-BODIPY$^{581/591}$) of each sample were determined by flow cytometry. Moreover, the heterologous IVF rate was measured to evaluate the fertilization capacity of postthaw semen in the four different treatments. For both extenders, the viability was higher for spermatozoa cooled slowly (39.40 ± 2.17 vs. 17.59 ± 2.25—control; 31.96 ± 2.19 vs. 11.46 ± 1.34—Tocopherol; $P < 0.05$). The α-tocopherol extender improved ($P < 0.05$) postthaw lipid peroxidation (10.28 ± 0.70 vs. 15.40 ± 0.95—slow FR; 10.14 ± 0.40 vs. 13.48 ± 0.34—moderate FR); however, it did not improve viability and mitochondrial membrane potential. Regarding the IVF rate, in the moderate FR, α-tocopherol supplementation reported a higher percentage of IVF (20.50 ± 2.11; $P < 0.05$), comparing with the control (14.00 ± 1.84). Regarding the slow FR, no significance differences were observed for percentage of IVF between the two extenders and the FRs. However, it seems that the α-tocopherol supplementation improved the IVF rate. In conclusion, this research reported that bovine oocytes intact zona pellucida can be used to evaluate the quality of postthaw stallion semen and α-tocopherol supplementation in the stallion freezing extender might exert a protective effect against oxidative damage during heterologous IVF.

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1. Introduction

Cryopreservation induces cell death or sublethal cryodamage in most sperm cells of the surviving population, leading to reduce their life span either in the female reproductive tract or *in vitro* [1,2]. The peroxidation of plasma membrane lipids (lipid peroxidation, LPO) and other cellular components have been defined as important aspects of oxidative stress of the spermatozoon [3,4]. The susceptibility of stallion spermatozoa to oxidative damage is attributed to the individual differences in fatty acid composition of their membranes, limited antioxidant

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capacity, and ability to generate reactive oxygen species (ROS) [5]. The effects of oxidative stress are particularly important during sperm cryopreservation, because much of the antioxidant capacity in semen resides with seminal plasma, and this is removed during the freezing process [6]. Many attempts using antioxidants as sperm additives in the freezing extender have been used in recent years to improve the quality of this process; specifically, vitamin E (a-tocopherol), which is considered the major membrane protectant against ROS and membrane LPO [7–13]. This low-molecular weight antioxidant can inhibit LPO reaction in the membrane by eliminating peroxyl, alkoxyl, and other lipid-derived radicals. Furthermore, vitamin E can be recycled to function again, even when its concentration is low [13]. This small-molecule antioxidant is a chain breaking antioxidant and not a scavenging antioxidant found in the sperm's cell membrane [7] and acts by neutralizing H_2O_2 and quenching free radicals, hence halting chain reactions that produce lipid peroxides and protecting the membrane from the damage induced by ROS [14]. The ability of α-tocopherol to maintain a steady state rate of peroxyl radical reduction in the plasma membrane depends on the recycling of α-tocopherol by external reducing agents such as ascorbate or thiols [15]. Furthermore, it improves the activity of other scavenging oxidants [16], helping to preserve both sperm motility and morphology [17].

For a successful freezing/thawing protocol, the optimal freezing rate (FR) is extremely important to maintain cellular integrity, because this determines whether the spermatozoa will remain in equilibrium with their extracellular environment or become supercooled with the increasing possibility of intracellular ice formation or osmotic shock [18,19]. During slow cooling, the dehydration of the spermatozoa can proceed to the point of osmotic equilibrium between intracellular and extracellular space with maximal, often detrimental, cellular dehydration. On the other hand, raising the cooling rate too much will not prevent the formation of intracellular ice because of the slow dehydration [20,21]. However, up to date, the cryopreservation of stallion semen is complex and still in a suboptimal level of development [2,5].

Developing new cryopreservation technologies for stallion spermatozoa is hampered by the inability to efficiently evaluate the fertility of treated spermatozoa, because fertility trials are expensive and time consuming. Moreover, it is becoming ever more apparent that the standard variables of sperm motility, morphology, concentration, and fertility on both fresh and frozen-thawed stallion semen are insufficient for predicting fertility [22]. The sperm-oocytes binding and IVF assays are easier and less-expensive methods to evaluate the fertilizing capacity of the spermatozoa [23]. This type of procedure allows the evaluation of sperm-oocyte interactions that occur during IVF and permits monitoring different endpoints in the early stages of embryo development. Homologous IVF assays have been used to predict male fertility using oocytes with intact zona pellucida (ZP) [24–26]. Noteworthy, the availability of equine oocytes for research is still limited because of the high cost and difficulty of collecting equine oocytes when compared with the sperm donor; therefore, heterologous oocyte binding assays have been developed [27–29].

The main goal of this study was to test the hypothesis that supplementation of α-tocopherol on the freezing extender and in two different FRs (between 5 °C and −15 °C) of frozen-thawed stallion spermatozoa may increase the heterologous IVF rate, by preventing oxidative damage to the plasma membrane. The study also aimed to prove that the heterologous IVF can predict male fertility, validating a new heterologous IVF assay using bovine oocytes with ZP-intact and equine sperm.

2. Material and methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich Chemical (St. Louis, MO, USA).

2.1. Semen collection and processing

Semen (four ejaculates per stallion) was obtained from four purebred Lusitano stallions (n = 4) of proven fertility individually housed in the Quinta do Malhinha, Angra do Heroísmo, Terceira. They were maintained according to institutional and European regulations. Semen was collected on a regular basis (two collections per week) during the breeding season. Ejaculates were collected by routine procedure with a Missouri model artificial vagina (Minitube, Tiefenbach, Germany), with an in-line filter to separate the gel fraction, lubricated, and prewarmed to 45 °C–50 °C. Immediately after collection, the filtered ejaculate was diluted 1:1 (v/v) in Kenney extender preheated at 37 °C [30], transported to the laboratory in a Styrofoam box for evaluation and processed in approximately 30 minutes.

2.1.1. Estimation of motility

Aliquots (5 ìL) of the extended ejaculates were examined by phase contrast light microscopy (× 40) equipped with a heated microscope stage (37 °C). A total of approximately 200 spermatozoa were evaluated in several fields on the slide. Only ejaculates with at least 60% progressive motility were included in the study.

2.1.2. Freezing and thawing procedure

Extended semen was centrifuged at 600 × g for 10 minutes and the sperm pellet reextended, to a final concentration of 100×10^6 sperm/mL, in a commercial freezing medium (Ghent, Minitube Iberia, Spain; Composition: skim milk, egg yolk, sugar, buffer, gentamycin, and glycerol [cryoprotector]; pH: 6.6–6.8; osmolarity: 330–360) and further divided into two parts: without any supplementation (control, extender A) and supplemented with 2-mM α-tocopherol (extender B; pH: 6.6–6.8). Afterward, semen was packed in 0.5-mL French straws and frozen in two different FRs between 5 °C and −15 °C, in a controlled rate freezer (IceCube 14S, SyLab, Purkersdorf, Austria), as follows:

- Slow FR: −0.5 °C/min from 20 °C to 5 °C, −5 °C/min from 5 °C to −15 °C, and −25 °C/min from −15 °C to −150 °C;
- Moderate FR: −0.5 °C/min from 20 °C to 5 °C, −10 °C/min from 5 °C to −15 °C, and −25 °C/min from

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