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Cryopreservation of epididymal stallion sperm ☆

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ABSTRACT

Any event that makes semen collection or mating impossible, such as death, castration, or injury, may terminate a stallion's breeding career. Fortunately, stallion sperm which are capable of fertilization can be harvested from the epididymis, and frozen for future use. However, the fertility of frozen–thawed epididymal sperm has been found to be lower than that of ejaculated sperm. Therefore, this study aimed to optimize the fertility of frozen epididymal stallion sperm by investigating the effects of different cryoprotectants and freezing protocols on sperm quality. Dimethylformamide was tested alone or combination with pasteurized egg yolk as substitute of fresh egg yolk. In addition, the effect of the pre-freeze stabilization on sperm quality was analyzed. Heterospermic samples obtained from stallion epididymis were collected and cryopreserved in lactose–egg-yolk extender or in the same extender with varying content of cryoprotectant and content of egg yolk, stabilized and no-stabilized. Sperm motility, viability, hypoosmotic swelling test (HOST) and acrosome integrity were evaluated post-thawing. No improvement was observed on the replacement of fresh yolk by pasteurized egg yolk, whereas the results suggest that dimethylformamide is a cryoprotectant suitable for cryopreservation of equine epididymal semen, even better than glycerol. In addition, we found that the stabilization before freezing on epididymal stallion sperm, can improve sperm quality parameters.

Introduction

Cryopreserved semen is an important tool in assisted reproduction, in particular, for the equine industry sperm-freezing technology has become an area of increasing interest [31]. Although the first equine pregnancy using frozen semen was reported in 1957 [4], it has been estimated that only 30–40% of stallions produce semen that is suitable for cryopreservation, and a large inter-individual variation on sperm survival during the freezing and thawing procedures has been also reported [2,18]. These facts have limited the widespread application of frozen semen by horse industry, so that the improvement of freezing protocol and extender composition is of great importance.

Sudden death, catastrophic injury, castration or any other event that makes semen collection impossible may prematurely terminate a stallion's reproductive life [25]. Fortunately, stallion sperm capable of fertilization can be harvested from the cauda epididymis [4,15] and can be used for artificial insemination of either fresh or frozen semen [22]. Besides, a recent study demonstrated that the number of spermatozoa recovered from the cauda epididymis is higher than that recovered from artificial vagina on a single collection [23]. There are limited data regarding survival and fertility of frozen–thawed epididymal stallion sperm, although the first pregnancy using frozen–thawed stallion spermatozoa was reported in a mare inseminated with epididymal spermatozoa [4]. If sperm recovery from cauda epididymis is the last chance to obtain viable spermatozoa from a stallion, to test protocols to enhance the success rates of this biotechnology in stallions is a important point of study [25].

Glycerol has been the first cryoprotectant used [28], and it has been routinely used with success in freezing extenders for semen of many domestic or wild animals [7,38], including horses [3,19]. However, glycerol causes injury to spermatozoa during cryopreservation process (review by Fahy et al. [10]), therefore its use as a cryoprotectant could be a factor involved in poor post-thaw motility and fertility rates in frozen stallion doses [3]. Glycerol toxicity may result in protein denaturation, alteration of actin interactions and induction of protein-free membrane blisters, resulting in a detrimental effect on the fertility of fresh cooled and thawed equine semen [8,24]. Glycerol toxicity is partly due to osmotic stress, because glycerol permeates the cell membrane slower than other cryoprotectants [12]. Negative effects of glycerol have encouraged investigation on alternative cryoprotectants for equine sperm with similar properties but with less toxic effects. It was suggested that the ideal cryoprotectant must have low molecular weight, great water solubility and minimal toxicity. Most of the amides had a

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lower molecular weight compared with glycerol and these cryoprotectants may induce less osmotic damage [3,19]. Research on the effects of different cryoprotectants on ejaculated sperm shed some light on the potential beneficial effects of formamides on epididymal sperm.

Equine semen is generally cryopreserved using egg yolk-based freezing extender [27]. Egg yolk protects the sperm cells from the toxic effects of seminal plasma. The amount of egg yolk required in semen diluents to provide protection against seminal plasma toxins is proportional to the amount of seminal plasma in diluted semen [32]. Egg yolk also exerts a protective effect against cold shock, primarily due to its phospholipid components [36]. Jasko et al. [14] reported that 4% egg yolk in an extender was beneficial for maintenance of spermatozoa motion characteristics after cooling. However, recent arguments against the use of egg yolk have arisen, one of which is the wide variability of composition that makes it difficult to analyze the beneficial effects of a particular compound on sperm cryopreservation. Furthermore, egg yolk introduces a risk of microbial contamination, with the subsequent production of endotoxins capable of damaging the fertilizing capacity of spermatozoa [1,5,11,27].

Pasteurized egg yolk may be used as an alternative to prepare freezing extenders and reduces microbial contamination. van Wagtendonk-De Leeuw et al. [33] reported high percentages of motile sperm after thawing in bull sperm frozen in pasteurized egg yolk extender and 56-day non-return rates higher than 67%. Besides Botu-Crio® diluent, which contains pasteurized egg yolk, provided excellent fertility rates for stallion sperm in other studies [20,25].

Regarding freezing protocol, Cochran et al. [6] reported that exposure of lactose-egg yolk extended semen to prolonged pre-freezing cooling conditions are not required. This technique speeds up the process, and is currently being used for freezing stallion semen [20,26]. However, Woolley et al. [39] reported that a slow pre-freeze cooling rate could increase the cold injury resistance of the acrosomal membrane and the plasma membrane on human sperm. In addition, Watson et al. [37] reported that if stallion spermatozoa are cooled-rapidly, they undergo irreversible membrane changes, termed cold shock. Besides, Salazar et al. [30] found recently that sperm quality with slow pre-freeze cooling rate was higher than using fast pre-freeze cooling rate.

Therefore, the objective of this study was to optimize the fertility of frozen epididymal stallion sperm by investigating the effects of replacement of glycerol by dimethylformamide (DMF), totally or partially, and fresh egg yolk by pasteurized egg yolk for freezing equine epididymal sperm. In addition we analyzed stabilization before freezing as Cochran's protocol alternative.

Materials and methods

Unless otherwise indicated, all chemicals were from Sigma–Aldrich Co. (Alcohobendas, Madrid, Spain). Dimethylformamide and glycerol were from Panreac Quimica S.L.U (Barcelona, Spain), Equex Paste was from Minitub and pasteurized egg yolk was from Grupo Leche Pascual (Spain).

The medium used for washing and centrifugation was Citrate–EDTA, composed of 8.33 mM glucose, 88.23 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate. Lactose–egg-yolk extender containing 50% (v/v) of 290 mM L-lactose, 20% (v/v) of egg yolk, 25% (v/v) of Glucose–EDTA medium (322.20 mM glucose, 12.58 mM sodium citrate, 9.93 mM disodium EDTA, 14.28 mM sodium bicarbonate), 0.5% (v/v) of Equex Paste and 5%(v/v) of glycerol, was used as control freezing extender. The freezing extender for the different treatments was the same than for the control freezing extender varying the type of egg yolk and the concentration and type of cryoprotectant.

Experimental design

Six replicates were conducted, using on each replicate a heterospermic mixture from three stallion's epididymis collected at the slaughterhouse (Mercazaragoza, Zaragoza).

Five experimental freezing extenders were prepared varying the cryoprotectant concentration and egg yolk type: GP (5% glycerol and pasteurized egg yolk), G/DMFF (2.5% glycerol, 2.5% dimethylformamide and fresh egg yolk), G/DMFP (2.5% glycerol, 2.5% dimethylformamide and pasteurized egg yolk), DMFF (5% dimethylformamide and egg yolk fresh), and DMFP (5% dimethylformamide and pasteurized egg yolk). As control freezing extender we used GF (5% glycerol and fresh egg yolk).

In order to determine the stabilization effect in the cryopreservation procedure, samples were frozen by two different methods. In the first of them, extended semen is packaged in straws at 22 °C and immediately exposured to freezing conditions (as described by Cochran [6]). The second procedure consist of a stabilization step with a slow cooling rate, in which semen samples were cooled to 4 °C in 2 h, before packing at 4 °C.

Collection, dilution and freezing of stallion epididymis spermatozoa

Sperm was collected using a retrograde flushing. The cauda epididymides were dissected and cannulated with a 25 G needle connected to 10 mL syringe with 2 mL of Citrate–EDTA at 20 °C. Then manual pressure was applied by a syringe and spermatozoa were collected in a Petri dish. Semen samples were centrifuged at 1000*g* for 5 min at room temperature and the supernatant was discarded.

Remaining semen was slowly extended to achieve a concentration of 100×10^6 per mL with the medium (GF, GP, G/DMFF, G/DMFP, DMFF, DMFP, respectively).

Then, semen samples were cryopreserved using two different ways described above. After being packaged, straws were placed for 10 min in liquid nitrogen vapor, 4 cm above the level of liquid nitrogen. The straws were then plunged into liquid nitrogen and stored at −196 °C.

Semen quality

Frozen semen samples were thawed in circulating water at 38 °C for 30 s. Sperm motility, viability, acrosome integrity and hypoosmotic swelling test (HOST) at 0 and 2 h after incubation at 38 °C (thermoresistance test) were assessed. The percentage of total motile spermatozoa and percentage of progressively motile spermatozoa was analyzed by means of a computer assisted semen analysis (CASA) system (ISAS PROISER; Valencia; Spain). Sperm viability was evaluated using eosin–nigrosin stain [9]. A semen sample was diluted 1:1 (v/v) with stain solution (5% eosin, 10% nigrosin in a citrate solution) and smeared. Live spermatozoa remained unstained. In addition, the percentage of normal acrosome was evaluated under a phase contrast microscope. Samples were fixed in buffered 8% glutaraldehyde solution and the percentage of spermatozoa with intact acrosome was determined [29]. Membrane functional integrity was further assessed using HOST [17]. The technique consisted of incubating 10 μL of semen with 90 μl of lactose hypoosmotic solution (100 mOsm) at 37 °C for 30 min. The samples were then fixed in 8% glutaraldehyde buffered solution. The proportion of sperm with swollen or coiled tails was considered as HOST-positive.

Statistical analysis

Analysis was performed using SPSS 17.0 for Windows. Results were expressed as mean ± SD. Data concerning to effect of