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Comparing ethylene glycol with glycerol for cryopreservation of canine semen in egg-yolk TRIS extenders

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Abstract

The objective of this work was to evaluate the possibility of substituting glycerol (G) for ethylene glycol (EG) when cryopreserving dog semen. A total of 15 ejaculates from 13 dogs was pooled into five samples and frozen in egg-yolk Tris extenders with variable ethylene glycol and glycerol concentrations, with or without Equex® STM Paste. Two widely used glycerol extenders (Uppsala Equex II and Norwegian) were utilized as controls. Semen quality parameters assessed after thawing were total subjective motility (TSM), computer assisted sperm analysis (CASA), eosin-nigrosin staining, and flow cytometry (FC) after staining with the PI/Fitc-PSA (fluorescein isotiocianate conjugated with the agglutinin of *Pisum sativum*, PSA) fluorochromes. No advantages were seen in using EG to replace G when freezing dog semen or combining EG and G in the freezing medium. The Uppsala Equex II provided the best overall post-thaw parameters, followed by the egg-yolk Tris experimental extender with 5% EG and Equex® STM Paste. The extender with 4% EG produced similar results to the Norwegian extender. High correlations ($r > 0.98$) were obtained between eosin-nigrosin staining and FC, as well as between subjective and computerized motility assessment ($r > 0.90$).

Keywords: Dog; Frozen semen; Glycerol; Ethylene glycol; Equex® STM Paste

1. Introduction

Glycerol (G) is the most commonly used cryoprotectant for dog sperm. After the first artificial insemination of dogs with semen frozen using a Tris extender with 8% glycerol [1], several other extenders with variable glycerol concentrations have been tested. The results of these studies [2–6] varied by extender and utilized a wide range (2–8%) of glycerol concentrations. Discrepancies between studies as to the ideal glycerol concentration could be due to several factors such as using different extenders, using different cryopreservation protocols, using other cryoprotectants (such as Equex STM Paste) and using different criteria/methods to evaluate post-thaw sperm quality. Conversely, glycerol, besides its cryoprotective properties, can induce alterations in the organization and viscosity of the sperm cytoplasm and in the permeability and stability of the plasma membrane through disruption of phospholipid and protein structural organization [7–9]. These phenomena can cause potential deleterious effects on the sperm fertilizing ability. Recently, the incorporation of Equex STM Paste in freezing extenders for dog sperm was shown to have a beneficial effect in post-thaw motility and integrity of the membrane [10–12], which could enhance the attachment

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of the sperm cell to the zona pellucida [13]. This paste is composed of sodium-dodecyl-sulphate, a biological detergent that exerts a protective action. This action seems to be related to the interaction between the egg-yolk and the sperm membrane, thereby increasing sperm permeability and reducing osmotic stress during the freezing and thawing processes [14].

Despite the success of freezing canine sperm using glycerol as a cryoprotectant, other cryoprotectants such as dimethylsulfoxide (DMSO) and ethylene glycol (EG) have been tried. The incorporation of DMSO, an intracellular cryoprotectant, did not improve post-thaw motility and survival rates of frozen-thawed dog semen [2] or ram semen [15]. EG has successfully been used to freeze mouse and chinchilla sperm [16,17]. Results of previous work in dogs comparing EG and G as a sperm cryoprotectant are still few and the results contradictory. Cavalcanti et al. [18] reported lower post-thaw motility with EG than with G when the same concentration was used, whereas Soares et al. [19] observed similar post-thaw motility with EG at 0.25, 0.5 and 1 M as with the glycerol at 0.8 M. Santos et al. [20] obtained similar results for motility, vigor and morphology preservation of thawed semen, using either EG or G at 5%. More recently, Rota et al. [21] using 5% EG or 5% G with Equex STM Paste saw a positive effect of EG at thawing in both motility and membrane integrity, as determined by the hypoosmotic swelling test (HOS).

The chemical structures of EG and G are quite similar, both having the same ratio of carbon atoms and C/OH hydroxyl groups, an indicator of the molecule lipophilia/ hydrophilia [22]. However, EG has a smaller (62.07 versus 92.10) molecular weight, a characteristic that may result in lower toxicity and higher permeability to cells [23]. Consequently, EG is widely used for embryo freezing in various mammalian species [23–25], as well as freezing of ovarian tissue [26,27]. The effects of using EG in frozen/thawed semen varied among species. With bull semen, EG resulted in higher post-thaw motility when compared with G or DMSO. This may be because of a reduction of the ''osmotic lesions'' [28]. The possibility that EG could cause less ''osmotic lesions'' had already been suggested for stallion spermatozoa [29]. When used to cryopreserve stallion semen, EG had results similar to those of glycerol, and successfully replaced it when used in the same or lower concentrations [30,31].

This experiment was designed to determine the feasibility of using different concentrations of EG as a cryoprotectant for canine semen, alternatively or combined with G or with Equex STM Paste®. Our hypothesis was that EG would improve post-thaw motility, viability and integrity of the acrosome and plasma membrane. The experimental extenders were compared with the Norwegian [1] and the Uppsala Equex II [32] extenders that are widely used for clinical and research purposes.

2. Materials and methods

2.1. Animals

Semen from thirteen dogs, two to five years old, of different breeds (seven German Shepherd Dogs, two Rottweilers, three Labrador Retrievers and one Cocker Spaniel) kept in the kennel of the Policia Nacional of Madrid was collected. Two dogs were collected twice. The dogs were clinically healthy and had proven fertility after natural mating.

2.2. Extenders

Extenders composed of TRIS-citric acid-egg-yolk and variable ethylene glycol concentrations with or without Equex STM Paste, as well as a glycerol-EG combination were used. Percentages of EG in the TRIS extender were: 4% EG (extender EG4), 8% EG (extender EG8), 4%EG and 4% G (extender EG4-G4), and 5% EG with 0.5% Equex STM Paste (extender EG5Eq). The Norwegian (NWG: 8% glycerol) [1] and Uppsala Equex II (UEqII: 5% glycerol with 0.5% Equex STM Paste) [32] extenders were used as controls. The composition of the control extenders was: UEqII component A: 3.025 g TRIS[1], 1.7 g citric acid monohydrate[2], 1.25 g fructose[3], distilled water to 77 mL, 3 mL glycerol[4], 0.06 g benzyl-penicillin[5], 0.1 g streptomycin[6], 20 mL egg-yolk, pH 6.74, 988 mOsm; UEqII component B: 3.025 g TRIS, 1.7 g citric acid monohydrate, 1.25 g fructose, distilled water to 72 mL, 7 mL glycerol, 1 mL Equex STM Paste[7]; 0.06 g benzyl-penicillin, 0.1 g streptomycin, 20 mL egg-yolk, pH 6.74, 2905 mOsm [32]; NWG: 6.05 g TRIS, 3.4 g citric acid monohydrate, 2.5 g fructose, 16 mL glycerol, 0.12 g benzylpenicillin, 0.2 g streptomycin, 40 mL egg-yolk, distilled water to 200 mL, pH 6.62, 331 mOsm [1]. The composition of the experimental extenders was: EG4: 6.05 g TRIS, 3.4 g citric acid monohydrate,

[1] Panreac 141940, Madrid, Spain.
[2] Panreac 141018.
[3] Panreac 142728.
[4] Panreac 141339.
[5] Sigma P-3032, St. Louis, MO, USA.
[6] Sigma S-9137.
[7] Nova Chemical Sales Inc., Scituate, MA, USA.