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Improved cryopreservability of stallion sperm using a sorbitol-based freezing extender

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Abstract

Cryopreservation of stallion semen is often associated with poor post-thaw sperm quality. Sugars are among the important components of a freezing extender and act as non-permeating cryoprotectants. This study aimed to compare the quality of stallion sperm frozen with glucose, fructose or sorbitol-containing freezing extenders. Semen was collected from six stallions of proven fertility and cryopreserved using a freezing extender containing different types of monosaccharide sugars (glucose, fructose or sorbitol). After thawing, the semen was examined for sperm motility, viability, acrosome integrity, plasma membrane functionality and sperm longevity. The fertility of semen frozen in the presence of sorbitol was also tested by artificial insemination. Sperm quality was significantly decreased following freezing and thawing ($P < 0.05$). Fructose was inferior for protecting sperm during cryopreservation when compared to sorbitol and glucose ($P < 0.05$). Although the viability, motility and acrosome integrity of sperm cryopreserved with a glucose-containing extender did not significantly differ from sperm frozen in the sorbitol-based extender when examined at 2 and 4 h post-thaw, all of these parameters plus plasma membrane functionality were improved for sperm frozen in the sorbitol extender than in the glucose extender when examined 10 min post-thaw. Two of four mares (50%) inseminated with semen frozen with a sorbitol-containing freezing extender became pregnant. It is concluded that different sugars have different abilities to protect against cryoinjury during freezing and thawing of stallion sperm. This study demonstrated that an extender containing sorbitol as primary sugar can be used to successfully cryopreserve equine sperm; moreover, the quality of frozen-thawed sperm appeared to be better than when glucose or fructose was the principle sugar in the freezing extender.

Keywords: Cryopreservation; Stallion; Sperm; Glucose; Fructose; Sorbitol; Extender

1. Introduction

During the last 3–4 decades, reproductive biotechnologies such as artificial insemination (AI) and embryo transfer (ET) have become increasingly important means of improving the reproductive potential of the most genetically desirable horses [1,2]. However, while fresh and cooled semen AI are useful ways of obtaining high pregnancy rates (65–70%: [3,4]), the pregnancy rates of mares inseminated with stallion sperm decreased during cold storage of more than 48–72 h [5]. For this reason, methods for cryopreserving stallion sperm without significant loss of viability and fertility are still sought by the equine breeding industry; suboptimal cryopreservation induces irreversible cellular

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injuries including disruption of the plasma membrane and premature capacitation or acrosome-like changes. In contrast, the acceptance of semen cryopreservation in stallions has, at least until relatively recently, been hindered by great variability in post-thaw sperm viability and pregnancy rates [1]. Several factors have been demonstrated to be involved in the poor freezability of equine sperm, such as freezing technique [6,7], the composition of freezing extender and the type of cryoprotectant used [8]. In addition, it has been estimated that about 20% of stallions produce semen that freezes well, 60% produce semen that freezes acceptably, and 20% consistently produce sperm that freezes poorly [7]. Per-cycle pregnancy rates of mares inseminated with frozen-thawed semen are variable (range 32–73%: [1]).

The composition of the freezing extender plays a central role in determining the viability of frozen-thawed sperm. Although several freezing extenders for equine semen have proven satisfactory for maintaining sperm viability and pregnancy rates in a high percentage of stallions [9], alternative freezing extenders or modifications of freezing techniques are still required [8,10]. Sugars, such as lactose, sucrose, glucose and trehalose are frequently supplemented into freezing extenders, where they act as non-penetrating cryoprotectants. As well as being energy substrates, these sugars contribute to sperm protection against osmotic damage and lethal intracellular ice formation, especially when used in a combination with penetrating cryoprotectants [11–13]. Among the monosaccharide sugars (glucose, fructose and sorbitol) naturally present in seminal plasma [14], glucose and fructose have been most frequently added to freezing extenders used for the semen of domestic animals [15–17]. The cryoprotective role of sorbitol during stallion sperm freezing and thawing has, however, been far less extensively studied. Sorbitol is a monosaccharide sugar alcohol that has molecules similar to glycerol [18,19] and has been demonstrated to osmo-stabilize plasma membranes [20]. Sorbitol has been used to improve cryotolerance in sperm from bulls [21] and men [22]. We hypothesized that addition of sorbitol to a freezing extender would reduce damage during freezing and thawing and thereby improve post-thaw stallion sperm quality. This study was therefore designed to examine the effect of sorbitol in a freezing extender on post-thaw sperm quality. For control purposes, the use of sorbitol in the freezing extender was compared with the two monosaccharide sugars most commonly included in freezing extenders, glucose and fructose.

2. Materials and methods

2.1. Animals

Six stallions of proven fertility but different breeds (Appaloosa, Arabian, Quarter horse, Standardbred, Thoroughbred and Warmblood) and aged between 5 and 12 years old were used for this study. The stallions were housed individually and fed with grass and concentrates. Water was provided *ad libitum*. The semen was routinely collected and evaluated for semen quality approximately 1–2 times a week. All semen to be used for cryopreservation contained ≥70% motile, viable and morphologically normal sperm.

2.2. Semen collection and handling

Four ejaculates were collected from each stallion, using an artificial vagina (Hannover model; Minitub, Germany). After semen collection, the semen was filtered through sterile gauze and then examined for volume, pH and concentration. The pH was measured using pH paper (Neutralit®, Merck, Darmstadt, Germany). The filtered semen was then diluted at a ratio of 1:3 to 1:5 with a non-fat dry milk glucose based semen extender (non-fat dry milk 2.4 g, glucose 4.9 g, 8.4% $NaHCO_3$ 2 mL, penicillin G sodium 100,000 IU, streptomycin 10 mg, double deionized water 98 mL; pH 7.35, 350 mOsm) and transported to the laboratory at 4–6 °C in a styrofoam box.

2.3. Freezing extenders and cryopreservation

The sperm-washing solutions (containing EDTA) and freezing extenders used in this study were classified into 3 groups based on the principal sugar included (glucose, fructose or sorbitol). The amount of sugar included in the extender was almost equal because the molecular weights of these three sugars are very similar (180.2 for both glucose and fructose and 182.2 for sorbitol). The formulation of the extenders is shown in Table 1. The fresh egg-yolk used in the freezing extenders was pooled to reduce the biological variation found in eggs. The pH and osmolality of these solutions were measured using a pH meter (EUTECH Instruments Pte Ltd., pH 510, Ayer Rajah Crescent, Singapore) and micro-osmometer (The FISKE® Micro-Osmometer model 210, Massachusetts, USA) and adjusted to 6.7 ± 0.1 and 400 ± 10 mOsm/kg for sperm washing, and 7.0 ± 0.1 and 300 ± 10 mOsm/kg for freezing extenders (before adding glycerol), respectively.

After transportation at 4–6 °C, the semen was divided into three parts and centrifuged at 400 g for 10