Reproductive Biology 17 (2017) 233–238

Contents lists available at ScienceDirect

Reproductive Biology

journal homepage: www.elsevier.com/locate/repbio

Cryopreservation of canine sperm using egg yolk and soy bean based extenders

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ARTICLE INFO

Article history:
Received 4 November 2016
Received in revised form 27 February 2017
Accepted 13 May 2017
Available online 31 May 2017

Keywords:
Canine spermatozoa
Cryopreservation
Liposome
Soy bean
Extender

ABSTRACT

Animal protein-based extenders are widely used despite being a potential source of bacterial or mycoplasma contamination. Its replacement with vegetal protein-based extenders could represent an interesting alternative for dog sperm cryopreservation. This technique could be further improved by the addition of Tris-Glucose-Citric acid (TGC) that could physically protect the spermatozoa and improve its homeostasis. The aim of this study was to evaluate a cryopreservation protocol for dog spermatozoa using a soybean-based extender (LP1) as well as the effects of the addition of (TGC) immediately after the semen collection. Eleven ejaculates from purebred adult dogs were collected, centrifuged in the absence or presence of TGC and processed as fresh or cryopreserved spermatozoa with: egg yolk-based extender (CaniPRO) or LP1. Freezing the spermatozoa in LP1 reduced the amplitude of the lateral head displacement, the percentage of spermatozoa that showed the intact acrosome and the mitochondrial function ($P < 0.05$). These samples also showed a trend towards increased percentage of apoptotic spermatozoa ($P < 0.05$). The addition of TGC before centrifugation did not improve the seminal parameters and adversely affected motility ($P < 0.05$) in the spermatozoa cryopreserved in CaniPRO. However, TGC did not affect motility and increased ($P < 0.05$) the percentage of intact acrosomes in the spermatozoa cryopreserved in LP1, reaching similar values than those cryopreserved in CaniPRO.

In conclusion, LP1® plus TGC provide the same level of protection to dog spermatozoa cryopreservation than the egg yolk based extender CaniPRO when comparing standard post-thaw sperm quality parameters.

1. Introduction

Sperm cryopreservation has enabled the development and optimization of assisted reproduction technologies (ARTs) allowing the conservation of endangered canine species and the long distance transportation of gametes [1–4]. A major concern of applying ART is the risk of bacterial or mycoplasma contamination, which can be a source of endotoxins able to reduce the fertilizing capacity of sperm and transmit diseases. A common source of contamination by components of animal origin are egg yolk or milk, included in most extenders [5].

Conventional freezing extenders are commonly supplemented with egg yolk [2–4]. Egg yolk is considered a non-penetrating cryoprotectant that lowers the freezing point of the medium and decreases the extracellular ice crystal formation [6]. However, egg yolk is a complex mixture of components and, although cryoprotection has been attributed to low density lipoproteins, phospholipids or triglycerides [7–10] the action mechanism is not clear yet. Regardless of their cryoprotection mechanism, egg yolk extenders are used extensively due to the reasonable results achieved. Despite its advantages, there have been increasing arguments against the use of egg yolk, mainly due to the variability in composition among batches and its proneness to contamination with animal pathogens [5]. These disadvantages, particularly the

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http://dx.doi.org/10.1016/j.repbio.2017.05.007
1642-431X/

sanitary risk, have boosted the development of freezing extenders free from components of animal origin.

The use of vegetal-based extenders can be an excellent alternative to egg yolk and, actually, there is a trend to evolve towards soy lecithin-based extenders [11]. Both egg yolk and soy lecithin contain phospholipids, from which the major component is phosphatidylcholine, together with phosphatidylethanolamine, and phosphatidylserine. Although lecithin is a synonym of phosphatidylcholine, the mixtures of soy phospholipids are commonly named as "lecithin", causing some terminology confusion. Phospholipids are molecules structured on a glycerol molecule. Phosphatidylcholine contains fatty acids in both glycerol positions 1 and 2, in addition to phosphocholine in position 3. Phosphatidylcholine is thus not a single molecule but a family of molecules that differ in the nature of the fatty acids. Due to its vegetal nature, soy phospholipids are richer in unsaturated fatty acids than egg yolk phospholipids, or than sperm membrane phospholipids. Therefore, soy phosphatidylcholine can increase membrane fluidity and resistance to cold shock [12].

Soy-based extenders have been applied to frozen dog spermatozoa with different results. Beccaglia et al. [13] have found that soy lecithin-based extenders were adequate to cryopreserve dog spermatozoa. More recently, Axner and Lagerson [14], who used higher concentrations of soy lecithin, found a significant decrease in the motility, acrosome and membrane integrity, indicators of lower sperm functionality. The effects of these type of extenders upon freezing dog spermatozoa require additional studies. The mitochondrial activity, frequently associated to motility, and cell necrosis/apoptosis, related to membrane damage, are parameters which have not been previously reported to study the influence of soy-based extenders on cryopreserved dog spermatozoa. Besides its composition, the efficiency of an extender depends on the freezing protocol. One of the factors worth to be considered is centrifugation. This first step is necessary to remove prostatic seminal fluids. However, it also exposes the spermatozoa to mechanical forces and deprivation of seminal plasma antioxidants, and leads to considerable loss of motility, as well as structural damage to the membrane and the acrosome [15,16]. A way to reduce the mechanical effects is to apply the cushioned centrifugation techniques, widely used in equine [17] and porcine [18], which consist in the centrifugation of semen in the presence of semen extender. TRIS-based extenders, such as Tris-Glucose-Citric acid (TGC) have been widely used for dog sperm preservation. The dilution with TGC before centrifugation modifies the volume and composition of the media. The increase in volume has shown to reduce the centrifugal force impact, whereas the carbohydrates present in TGC have shown cryoprotective effects on the acrosome and membrane integrity [19,20].

The aim of this study was to compare the efficiency of an egg yolk- based extender, CaniPRO, versus a soy lecithin-based extender, LP1, and investigate the effects of Tris-Glucose-Citric acid (TGC) addition immediately after semen collection, in the cryopreservation of dog spermatozoa.

2. Material and methods

2.1. Reagents

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich Química (Madrid, Spain).

2.2. Semen collection

A total of 11 ejaculates, one ejaculate per dog were collected from purebred adult male dogs aged between 2 and 8 years old. The three fractions of the ejaculate were collected separately into 15 mL falcon conical tubes (Becton Dickinson, Madrid, Spain) by digital manipulation using latex semen collection cones. Immediately after collection the first and third fractions were discarded and the second fractions (rich in spermatozoa) were maintained at 37 °C for analysis. The second fraction from each ejaculate was evaluated for color, volume, concentration (Makler Chamber, Sefi Medical Instruments, Haifa, Israel), pH (MicropH 2000, Crison Instruments, Barcelona, Spain), osmolarity (Advanced micro osmometer 3300, Advanced Instruments Inc., Norwood, MA, USA), sperm motility (Integrated Semen Analysis System, Projectes y Serveis R + D S.L., Valencia, Spain), viability (plasma membrane integrity), morphology and acrosome status.

2.3. Sperm cryopreservation

Only ejaculates with a concentration higher than 250×10^6 spermatozoa/mL with total motility higher than 70% and abnormal morphology values lower than 30% were used for cryopreservation (n = 11). Two different extenders were tested: an egg yolk-based extender widely used for dog sperm cryopreservation, CaniPRO freeze (Minitub Ibérica, Tarragona, Spain) and an animal protein free extender (LP1).

The LP1 was prepared as described by Pérez-Garnelo et al. [26] 10% soybean lipids rich in phosphatidylcholine diluted in Tris buffer stock solution (Tris-hydroxymethyl-aminomethane 30.28 g/L, mono-hydrated citric acid 17.8 g/L and D-fructose 12.5 g/L; 318 mOsm/L and pH 6.6) by stirring for 60 min at 50 °C. The hydrated suspension was homogenized by using a high pressure homogenizer (Nira Saovi, Parma, Italy) at a pressure of 900 bar to obtain lipid liposome in the size range of 15–50 nm.

Immediately after semen collection, each sperm sample was divided into six equal parts which were assigned to each experimental group: Fresh, fresh + TGC, LP1, LP1 + TGC, CaniPRO and CaniPRO + TGC. The buffer TGC (Tris 38 g/L, glucose 6 g/L and citric acid 22 g/L) was slowly added to groups fresh + TGC, LP1 + TGC and CaniPRO + TGC (1:1; v/v). Afterwards, all the samples, containing or not TGC, were centrifuged at $700 \times g$ for 6 min. The supernatants were discarded and the groups fresh and fresh + TGC were not resuspended. Whereas the groups LP1 and LP1 + TGC were resuspended in extender LP1 containing 3% glycerol and the groups CaniPRO, as well as CaniPRO + TGC were resuspended in CaniPRO fraction A, all to a final sperm concentration of 400×10^6 spermatozoa/mL. After 5 min at 37 °C the sperm solution was cooled to 5 °C (0.5 °C/min). After equilibration, samples were further diluted (1:1 v/v) with their respective extenders (LP1 containing 7% glycerol or CaniPRO fraction B). After 10 min at 4 °C, sperm samples were stored in 0.5 mL straws, placed at 13.5 cm above the liquid nitrogen for 10 min and frozen in liquid nitrogen until evaluation.

2.4. Sperm separation by swim-up

Samples from the experimental groups Canipro and LP1 were thawed at 37 °C for 50 s, and each seminal sample was diluted in 3 mL swim up medium (Tyrode's medium with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg mL^{-1} fatty-acid-free BSA and 10 mM HEPES). Then, each sample was centrifuged at $300 \times g$ for 10 min. After discarding the supernatant, the sperm pellet was gently overlaid with 1 mL of fertilisation medium [Tyrode's medium with 25 mM bicarbonate, 22 mM sodium lactate, 1 mM sodium pyruvate, 6 mg mL^{-1} fatty-acid-free BSA, 10 μg mL^{-1} heparin-sodium salt (184 U mg^{-1} heparin; Calbiochem, San Diego, CA), 1 μg mL^{-1} hypotaurine, 20 μL mL^{-1} basal medium Eagle's (BME) amino acids and 10 μL ml^{-1} minimum essential medium (MEM) aminoacids]. The tube was placed at 45° angle to allow the maximal number of motile spermatozoa to