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In vitro fertilization of in vitro matured canine oocytes using frozen–thawed dog semen

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Abstract

Experiments were conducted to evaluate in vitro fertilization (IVF) of in vitro matured (IVM) bitch oocytes using dog spermatozoa frozen in three different extenders. Sperm-rich fraction from eight ejaculates of five dogs was frozen in each one of three egg yolk Tris extenders with additional: (A) 1.4 g citric acid and 0.8 g glucose; (B) 0.7 g citric acid and 3.5 g glucose; or (C) 1.4 g citric acid and 0.8 g fructose (all with 5% glycerol in 100 mL milliQ water). Thawed sperm were co-incubated with IVM bitch oocytes for 6 h. Oocytes were fixed and evaluated under an epifluorescence microscope; penetrated oocytes were defined as those having sperm heads in the perivitelline space or in the oocyte cytoplasm. Higher penetration rates ($P < 0.05$) were obtained in oocytes cultured with spermatozoa frozen in extenders B and C than those frozen in extender A (33.1, 34.2 and 26.4%, respectively).

Keywords: Spermatozoa; In vitro fertilization; Cryopreservation; Canine

1. Introduction

Cryopreservation of semen results in reduced fertility compared with fresh semen, due to a combination of loss of sperm viability and reduced fertilizing ability of the surviving cells. Testing frozen–thawed sperm fertility by artificial insemination is expensive and necessitates several bitches to be used. However, interaction of canine gametes offers a useful approach to evaluate presumptive fertilizing ability of spermatozoa, because this interaction is a complex process that requires several sperm functions involving initial recognition, attachment followed by binding, acrosome reaction and penetration of zona matrix [1]. Homologous immature oocytes [2], salt storage of zona pellucida (ZP) or frozen ZP have been used to evaluate frozen–thawed sperm fertilizing ability in this species; nevertheless, the latter have a significantly negative effect on the sperm binding potential [3]. Matured oocytes could be more physiological. The aim of this study was to evaluate fertilizing capacity of dog spermatozoa frozen in three different extenders, through in vitro fertilization of in vitro matured bitch oocytes.

2. Materials and methods

Eight ejaculates were obtained (by manual stimulation) from five dogs (various breeds) with proven fertility. Aliquots of the sperm-rich fraction of each ejaculate were diluted in each one of the following three freezing media: (A) 2.4 g Tris, 1.4 g citric acid and 0.8 g glucose; (B) 2.4 g Tris, 0.7 g citric acid and 3.5 g glucose; or (C) 2.4 g Tris, 1.4 g citric acid and 0.8 g fructose. All media contained 5% glycerol, 20% egg yolk, 100 IU/mL penicillin G and 100 mL milliQ water. The semen was diluted to a final concentration of 100×10^6 sperm/mL, placed in 0.25 mL straws, and

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frozen at 30 °C/min to −196 °C. The straws were subsequently thawed at 60 °C for 8 s. Sperm fertilizing capacity was assessed by in vitro fertilization assays with in vitro matured bitch oocytes, previously cultured for 72 h in TCM-199 (Earle's salt, buffered with 25 mM Hepes; Invitrogen®, Grand Island, NY, USA), supplemented with 10% FCS, 11.2 mg/mL pyruvic acid, 10 IU/mL hCG (CG-5 Sigma®) and 5 μL/mL antibiotic solution (12.2 mg/mL penicillin and 20 mg/mL streptomycin) (Sigma®). In each replicate, spermatozoa were added to 100 μL Fert-Talp drops containing 10–15 in vitro matured oocytes, at a final sperm concentration of 5×10^6 spermatozoa/mL. Sperm and oocytes were co-cultured for 6 h at 38 °C under 5% CO_2 in air. At the end of the incubation period, the oocytes were fixed with 5% neutral formalin and stained with 200 μg/mL of propidium iodide and then examined with an epifluorescence microscope (Nikon Optiphot 2, Nikon, Kawasaki, Japan). Penetrated oocytes were defined as those having sperm heads in the perivitelline space or in the oocyte cytoplasm.

Each ejaculate frozen in the three extenders was considered an experimental replicate. Penetration percentages were arc-sine transformed, analysed with ANOVA, and differences were evaluated with a Tukey test (SAS Institute, Cary, NC, USA). Statistical significance was assumed at $P < 0.05$.

3. Results

In the present study, more than 350 oocytes were evaluated for sperm penetration throughout eight experimental replicates. The results of the sperm penetration assay with in vitro matured bitch oocytes are shown in Table 1. A low rate of polyspermia was observed throught the three treatments. The percentage of zona pellucida penetration (Fig. 1) was greater ($P < 0.05$) for sperm frozen in extenders B and C compared with extender A (no significant difference between extenders B and C).

Table 1
Penetration rates of in vitro matured canine oocytes by spermatozoa frozen in three different extenders after co-incubation for 6 h

Oocytes	Extender		
	A	B	C
Penetrated/total	29/110	40/121	41/120
Percentage	26.4[a]	33.1[b]	34.2[b]

Within a row, percentages without a common superscript letters (a and b) were different ($P < 0.05$).

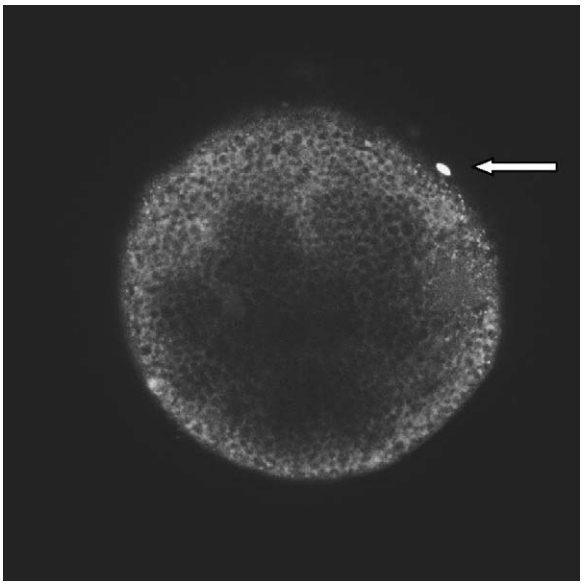

Fig. 1. Photomicrograph of a bitch oocyte penetrated by a single frozen–thawed dog sperm (arrow), stained with propidium iodide (×200).

4. Discussion

Different extenders have been described for cryopreservation of canine semen. Both glucose and fructose in Tris–citric acid extenders have been widely used for dog sperm [4]. These sugars provide energy substrate for sperm cells and can act as a cryoprotectant, giving a protective effect against the damage in spermatozoa exposed to fast cooling [5]. In the present study, higher rates of in vitro penetration of IVM bitch oocytes were achieved when spermatozoa were cryopreserved in media with a high glucose concentration or with fructose (extenders B and C) compared with those oocytes cultured with spermatozoa frozen in a medium with a low glucose concentration (extender A). Components in a frozen medium that can influence sperm post thaw activity include the energy substrates. Sperm (rather than the oocyte) need to metabolize sugars to achieve fertilization [6], and dog spermatozoa are able to use fructose and glucose. However, the formation of metabolic intermediaries is different for these sugars, thus the effects of the two hexoses on the canine sperm are also different [7]. This was reflected by the difference between A and C. Monosaccharides, especially fructose, improved post-thaw viability and intact acrosome percentages in dog sperm [5] and acrosome integrity was of paramount importance for the fertilizing ability of spermatozoa [1,3]. The

differences in penetration rates when using sperm frozen in media with different glucose concentration (extenders A and B) may be related to the dose-dependent response to glucose by dog sperm [7].

Although frozen–thawed canine spermatozoa can penetrate immature bitch oocytes, the use of IVM oocytes represents a more physiological evaluation of the fertilizing capacity. Therefore, evaluation of the post thaw fertilizing capacity of canine sperm through gamete interaction using in vitro matured bitch oocytes may provide an accurate estimation of potential fertility of frozen dog semen. However, further studies are needed regarding the influence of the sugar supplementation in the extender on fertilizing ability of frozen–thawed dog spermatozoa.

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