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Effects of Skim Milk and Tris Extender on Frozen-Thawed Canine Sperm Morphology

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Abstract: This study was aimed to compare the effects of freezing dog semen in straws in 0.1% fat bearing Skim Milk-Glucose (SMG) extender with the routinely used TRIS-Fructose-Citric acid (TFC) extender regarding the post-thaw motility, acrosome and total morphologic defects rate. Four German shepherd male dogs of 2-3 years under the same managemental conditions on a private kennel were used. Six ejaculates per each dog (total 24 ejaculates) were collected digital manipulation. Two extenders used were 20% egg yolk containing TFC and 10% egg yolk containing SMG extenders. Semen was divided into two split parts and one was extended with TFC and the other with SMG extenders at 26°C at a rate 1:1 and cooled to 5°C in an hour. Cooled semen samples were further extended with 10% (v/v) glycerol containing extender (for an hour) at equal rates (final glycerol rate 5%). Following glycerolisation semen samples were filled into 0.5 mL straws and equilibrated for an hour at 5°C. Straws were frozen at −110°C liquid nitrogen vapour for 7 min. Labeled straws were stored in separate canisters in liquid nitrogen at −196°C. Six straws for TFC extender and six straws for SMG extender were stored to be examined. Straws were thawed at 45°C for 60 sec. Post-thaw motility rate of TFC and SMG extended semen samples were, respectively 48.54±8.27 and 51.97±7.51%. Acrosome and total morphologic defect rates were 41.04±9.44 and 51.17±9.05% for TFC; 38.04±13.60 and 46.67±12.68% for SMG extender, respectively. It has been founded that when the post-thaw sperm traits of straws frozen dog semen was evaluated, SMG extender have been successful at least as much as TFC extender.

Key words: Canine, semen, freezing, extender, post-thaw, abnormal spermatozoa

INTRODUCTION

The first successful conception using frozen dog semen involved semen pellets and was achieved by Seager (1969). Various extenders have been described for cryopreservation of dog semen (Baran *et al*., 2000a; Ivanova-Kicheva *et al*., 1997). For this purpose, different cryoprotective agents, freezing and thawing protocols and various extenders containing PIPES, TES and TRIS, for example have been used; the latter is the most frequently used diluents for canine semen preservation (England, 1993; Silva *et al*., 2002; 2003). Therefore, an applicable method for freezing dog semen has not yet been established. It is recognized that dog sperm have poor freezing resistance and progressive sperm motility cannot be maintained for a prolonged time after post thawing (England and Ponzio, 1996; Ivanova-Kicheva *et al*., 1995).

TRIS is one of most used extenders for cryopreservation of canine semen and has been tested by several researchers (Alamo *et al*., 2005; Baran *et al*., 2000a, b; Rota *et al*., 1997; Pena *et al*., 2003). Strom *et al*. (1997) compared the most used extenders in Scandinavia, TRIS and CLONE and obtained approximately 70% sperm motility. Egg yolk is the most commonly used compound in canine semen extenders for protection of spermatozoa from cold shock and disruption during the freezing and thawing process (England, 1993). Cryoprotective substances like as glucose, lactose and raffinose are generally more beneficial as cryoprotectants when rapid cooling and freezing rates are required. There are a limited number of sugars that can be metabolized by sperm, namely glucose, fructose and mannose but the protection afforded to sperm by sugars during freezing is not apparently dependent on this as there are many types of sugar that enhance post-thaw sperm quality. Glucose appeared to result in recovery of more viable sperm post-thaw than other sugars when freezing ram sperm (Salamon and Visser, 1972). Milk is used as a medium for sperm preservation. Caseins, the major proteins of milk, appear to be responsible for the protective effect of milk on sperm. Skim milk extender is the most commonly used extender for mouse (Nakagata, 2000) and goat (Dorado *et al*., 2007) sperm. However, limited studies have

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been conducted to examine the influence of egg yolk containing skim milk-glucose extender for canine (Baran *et al*., 2000a).

This study was aimed to compare the effects of freezing dog semen in straws in egg yolk containing skim milk-glucose extender with the routinely used TRIS-fructose-citric acid extender regarding the post-thaw motility, acrosome and total morphological defects rate.

MATERIALS AND METHODS

Four German shepherd male dogs of 2-3 years under the same managemental conditions on a private kennel were used. The experiment was repeated six times. The male dogs were clinically healthy and had proven fertility after natural mating.

TRIS-citric acid-egg-yolk and skim milk-glucose diluents were used in this study. All chemicals were from Sigma Aldrich and were of the highest available grade. The Skim Milk based extender (SMG) comprised 10 mL of skim milk and 1.0 g of glucose, formulated on the basis of 100 mL. Next, 10% (v/v) egg yolk was added to the extender. Egg Yolk-Tris-citrate-glucose (EY) extender composed of 20% (v/v) egg yolk, 3.02 g Tris (hydroxymethyl) aminomethane, 1.78 g citric acid monohydrate, 1.25 g glucose, 100.000 U mL^{-1} penicillin G potassium and 0.10 g mL^{-1} streptomycin sulphate.

Sperm concentration was measured by counting sperm cells in five large squares of a Neubauer haemocytometer chamber (light microscope, 400x). Sperm Progressive Motility (SPM) was examined under a phase-contrast microscope (400x) equipped with a thermal stage at 37°C.

Semen samples from all dogs were collected twice (once a week for 2 weeks) before starting the study, to check the sperm quality of the ejaculates. Semen was collected weekly by digital manipulation during 6 consecutive weeks. The first and third fractions (seminal plasma) of the ejaculate were discarded. Only the sperm-rich fraction of the ejaculates was collected; this was evaluated to determine motility, concentration and proportions of abnormal spermatozoa. The percentage of motile spermatozoa was estimated by subjective microscopic examination at magnification 200x using phase contrast microscope and the sperm concentration was determined using a hemocytometer. Spermac® stain has been used to detect sperm morphology and acrosome abnormality.

A drop of each sperm specimen was removed at the end of incubation and an air-dried smear was made for the Spermac acrosome procedure as earlier reported Chan *et al*. (1996). Briefly, each dried smear was fixed (5 min) in formalin solution (fixative I) provided in the Spermac kit (Stain Enterprises, Onderstepoort, South Africa). Each slide was washed through stain solutions A-C (1 min). The stained slides were air-dried and analyzed under oil immersion (1000x) for the percentage of acrosome intact sperm. A total of 100 sperm cells were counted on each slide. Sperm morphology was classified accordingly to Christiansen (1984).

Two extenders used were 20% Egg Yolk containing TRIS-Fructose-Citric acid (EY-TFC) and 10% (v/v) Egg Yolk containing Skim Milk-Glucose (EY-SMG) extenders Table 1). Semen with a motility of ≥90% and a proportion of abnormal sperm of ≤20% was regarded suitable for freezing. Semen was divided into two split parts and one was extended with TFC and the other SMG extenders at 26°C at a rate 1:1 and cooled to 5°C in an hour. Cooled semen samples were further extended with 10% (v/v) glycerol containing extender (for an hour) at equal rates (final glycerol rate 5%). Following glycerolisation semen samples were filled into 0.5 mL medium PVC straws (Minitub, Germany) with approximately 1.0×10^8 spermatozoa/mL and equilibrated for an hour at 5°C. The straws were placed in an atmosphere of liquid nitrogen vapor, placed horizontally 6 cm above the surface of LN_2 in a closed styrene foam box (30×20×20 cm) retained there for 10 min and then plunged into the LN_2. All straws belong to the groups were stored in separate in containers that are included liquid nitrogen. Out of the six ejaculates from each dog, six straws for EY-TFC extender and six straws for EY-SMG extender were stored to be examined.

Table 1: Composition of extenders

	EY-TFC		EY-SMG	
Ingredients	1st extender	2nd extender	1st extender	2nd extender
Tris-(Hydroxymethyl) aminomethane	3.02 g	3.02 g	-	-
Glucose	-	-	1.00 g	1.00 g
Fructose	1.25 g	1.25 g	-	-
Citric acid	1.78 g	1.78 g	-	-
Egg yolk	20 mL	20 mL	10 mL	10 mL
Glycerol	-	10 mL	-	10 mL
Skim-milk to final volume	-	-	100 mL	100 mL
Distilled water to final volume	100 mL	100 mL	-	-
Penicillin potassium	100.000 U	100.000 U	100.000 U	100.000 U
Streptomycin sulfate	0.10 g	0.10 g	0.10 g	0.10 g

Thawing was achieved by placing the straws in a water bath at 45°C for 60 sec. The content of each straw was expelled into a 1.5 mL microtube. A small drop of semen was placed on a slide and evaluated for motility. Progressive motility was evaluated by hot plate phase-contrast microscope at 200x magnification. Post-thaw and after equilibration acrosome and total morphologic defect rates were examined by staining the semen samples with Spermac® stain.

Statistical evaluations of spermatological traits were done SPSS 16.0 ANOVA variance analysis student t-test. The results are expressed as means±standard deviations.

RESULTS AND DISCUSSION

Fresh canine semen has a white milky appearance. The volume of the sperm-rich fraction was 2.2±0.5 mL. Mean (±SD) fresh semen parameters were 85.83±3.76% motility, 19.10±3.5% of cells with abnormal morphology and a concentration of 379.27±168.11 $\times 10^6$ sperm cells/mL. Post-equilibration progressive motility rate in EY-TFC and EY-SMG extended semen samples were respectively, 78.25±4.00 and 72.85±5.35%. Acrosome and total morphologic defect rates were 12.74±3.02 and 24.55±3.25% for EY-TFC and 10.50±2.20 and 25.25±4.50% for EY-SMG extender, respectively. There was no statistical significance among these values (Table 2) ($p>0.05$).

Post-thaw progressive motility rate of EY-TFC and EY-SMG extended semen samples were respectively, 48.54±8.27 and 51.97±7.51%. Acrosome and total morphologic defect rates were 41.04±9.44 and 51.17±9.05% for EY-TFC; 38.04±13.60 and 46.67±12.68% for EY-SMG diluent, respectively. No statistical significance was observed between the two extenders regarding progressive motility, acrosome and total morphological defect rate (Table 3) ($p>0.05$).

Cryopreservation of dog semen is used to facilitate long-term storage and international transportation. This is of special importance for use in intra-uterine artificial insemination of bitches. When semen is frozen, cells are exposed to cold shock, ice crystal formation and cellular dehydration which all can cause irreversible damage (Hammerstedt *et al*., 1990). Various extenders have been described for cryopreservation of dog semen (Hay *et al*., 1997; Ivanova-Kicheva *et al*., 1997; Olar *et al*., 1989) and researchers are always trying to develop more practical and less-expensive methods. TRIS-citric acid-fructose extenders have been preferred for freezing semen packaged in straws. The skim milk-glucose extender (Baran *et al*., 2000b) was proven to be effective for freezing dog semen. Frozen dog spermatozoa are known to have a reduced thermo tolerance post-thaw (Rota *et al*., 1997). Some types of detergents in the cryopreservation extender such as Orvus Es Paste (Thomas *et al*., 1992; Tsutsui *et al*., 2000) and Equex STM Paste (Pena and Linde-Forsberg, 2000; Rota *et al*., 1997) was found to improve significantly the post-thaw survival and longevity of dog spermatozoa during *in vitro* incubation.

The spermatological chracteristics of twenty four fresh ejaculate collected from four German shepherd dogs were investigated and similar results with some researchers were obtained (Baran *et al*., 2000a; Theret *et al*., 1987).

In agreement with the findings, Oettle (1986) also pointed out that the percentage of dog sperm cells with acrosomal damage increased significantly during equilibration. In this study, motility and morphological defects rates of the SMG extender resemble to those values of lactose-egg yolk extender in some studies (Lees and Castleberry, 1997; Thomas *et al*., 1993; Yubi *et al*., 1987). The motility rates of the TFC in the present study were similar reported by Baran *et al*. (2000a). There were no difference between EY-TFC and EY-SMG extenders in motility and morphologic defects after equilibration ($p>0.05$).

In the present study, the post-thaw average progressive motility rates of semen samples extended with EY-TFC extender was 48.54±8.27%. This value was close to Olar *et al*. (1989). However, Pena and Linde-Forsberg (2000) and Pena *et al*. (2003) obtained the greatest sperm motility using TRIS plus Equex STM paste.

Mean post-thaw progressive motility rates of EY-SMG diluents was 51.97±7.51%. These means values for the EY-SMG extender have been found superior to the EY-TFC diluents. But comparison was not possible since no research dealing with this extender could be found. This superiority could be attributed to do cryoprotective agents like milk proteins, albumin, casein, globulin, calcium ions and synergic effect of milk and yolk. In the bull it has been reported that egg yolk protects sperm function by preventing the binding of sperm to the major

Table 2: Spermatological characteristics after equilibration

		Morphologic defects (%)	
Extender	Motility (%)	Acrosome	Total
EY-SMG	72.85±5.35	10.50±2.20	25.25±4.50
EY-TFC	78.25±4.00	12.74±3.02	24.55±3.25

Table 3: Spermatological characteristics after thawed

		Morphologic defects (%)	
Extender	Motility (%)	Acrosome	Total
EY-SMG	51.97±7.51	38.04±13.6	46.67±12.68
EY-TFC	48.54±8.27	41.04±9.44	51.17±9.050

($p>0.05$)

seminal plasma proteins, thereby preventing seminal plasma protein-mediated stimulation of lipid loss from the plasma membrane (Bergeron and Manjunath, 2006). As in the case of egg yolk, skim milk prevents the binding of seminal plasma protein to sperm and reduces sperm lipid loss while also maintaining sperm motility and viability during storage at 4°C (Bergeron *et al*., 2007).

Motility is one of the many important attributes of a fertile spermatozoon (Pena-Martinez, 2004). However, motile sperm are not necessarily fertile because of acrosomal and membrane changes that may occur after cryopreservation and thawing that affect fertility but not motility (Eilts, 2005). Overall, both semen-freezing extenders successfully cryopreserved canine semen.

CONCLUSION

The results presented here clearly demonstrate that an effective, simple extender composed of Egg Yolk Skim Milk Glucose (EY-SMG) is available for the cryopreservation of canine spermatozoa as an alternative to Egg Yolk Tris Citric acid Fructose (EY-TFC) extender and this may contribute to both efficient exchange of genetic materials and production of guide dogs for the blind. Many researchers can be use skim milk diluents because of simplified freezing techniques and benefit effects of frozen canine semen.

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