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Comparison of two different extenders for cryopreservation of epididymal dog spermatozoa

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Abstract

Post-mortem recovery and cryopreservation of viable sperm from the epididymis of genetically valuable animals or endangered species is an important technique for preserving male gametes and thus for maintaining germ-plasm banks. The extender type used to freeze sperm is important to avoid spermatozoal membrane damage and to preserve semen quality after cryopreservation. The objective of this study was to determine the effects of a commercial bovine extender (BullXcell ®; IMV, L'Aigle. France) and TRIS-citric acid -fructose-egg yolk-7% glycerol extender (TCF) on cryopreservation of canine epididymal sperm. The testes of ten adult Egyptian domestic dogs were kept at 5 °C in saline solution, and transported to laboratory within 24 h. Diluted samples were cooled slowly to 5°C over 2 h and equilibrated at that temperature for 2 h. Aliquots of samples were loaded into 0.25 ml straws and frozen in liquid nitrogen vapor for 10 min and stored in liquid nitrogen. The semen parameters; Post-thaw progressive motility, viability index, membrane integrity (HOST) and acrosomal integrity (sliver nitrate stain) were evaluated. Results indicated that membrane integrity (HOST) and intact acrosome were statistically higher in BullXcell (®) than TCF extender. We concluded that BullXcell (®) is a suitable alternative for the freezing of canine epididymal sperm.

Key words: Dog, Epididymal sperm, Cryopreservation, BullXcell

Introduction

The post-mortem recovery and cryopreservation of viable sperm from the epididymis of dead animals from genetically valuable animals or endangered species was an important technique for preserving male gametes and thus for maintaining germ-plasm banks. Sperm stored in the cauda epididymis had usually good quality and a high level of maturation, being able to fertilize oocytes **(Matas et al., 2010; Pamungkas et al., 2012).** After an animal dies its germ cells remain alive after certain period of time and it may be possible to produce progeny of an animal after its death **(Hishinuma et al., 2003).** However, in order to get good quality samples, sperm collection and processing should be carried out immediately after the death of the animal since the conditions(time and temperature) under which epididymis is handled could cause important changes in the viability of spermatozoa **(Martinez-Pastora et al., 2005).**

Thus, the present study aimed to evaluate the effect of the commercial bovine extender (BullXcell ®; IMV, L'Aigle. France) and Tris–citrate–fructose buffer extender (TCF) on the morphology and viability of cryopreserved canine epididymalspermatoza.

Materials and Methods

Experimental animals

Harvesting epididymal sperm

For this purpose, testes were obtained from ten, 2-5 yrs-old healthy dogs of various breeds after routine castration at the dog shelter of the Egyptian Society of Mercy to Animals (ESMA).

Each testis with the epididymis and surrounding tunics intact was put in a thermos flask containing 0.9 % NaCl at 30 °C and

transported to the laboratory at a maximum period of 24 hours. For processing, the tunics were removed from each testicle with special care taken not to damage the epididymis at its attachment to the testis. The blood vessels leading to the testis and the vas deferens were trimmed away along the natural curve of the testicle using a curved scissors. The cauda epididymis was dissected out of each testis and placed in a clean, dry Petri dish. Sperm samples were collected by repeated slicing of the cauda epididymis **(Hewitt, Leahy, Sheldon and England, 2001)**.

After 5- min incubation at 39 °C, Sperm samples were assessed for the percent forward motility to the nearest 5% by placing a small droplet of the sample to be examined on a pre-warmed glass slide **(Taha, 1980)**.

Preparation of extenders

Tris-Fructose (TCF) extender: Tris buffer was prepared as described by **Rota et al. (1995)**.The extender composed of Tris (3.025 g), monohydrated citric acid (1.7 g) and D-fructose (1.25g) in 100 ml of ultrapure distilled water.The pH of the solution was adjusted to 6.74. Egg yolk (20%), glycerol (7%). Antibiotics (Na-benzyl-penicillin (100,000 IU) and dihydrostreptomycin sulphate (100 mg) were added both to the so composed extenderand the commercial bovine extender (BullXcell ®; IMV, L'Aigle, France).

Cryopreservation procedure

Each epididymal sample was diluted either with BullXcell® and Tris–citric acid fructose (TCF) extenders (10 replicates).

The extended samples of epididymal sperm was cooled to 5°C gradually over 2 h equilibrated at 5°C for 2 hours gradually **(Songsasen et al., 2002)**. Diluted samples were then packaged in 0.25 ml French straws and frozen horizontally for 10 min in liquid nitrogen vapour in a foam box filled with liquid nitrogen according to **Silva et al. (2005)**. Straws were then rapidly plunged in the liquid nitrogen, transferred to a storage tank and left there for at least one week before thawing. Thawing was performed at 37 °C for 30 sec.

Evaluation Procedures

Sperm motility Estimation:

The percentages of progressively motile spermatozoa were estimated after cold equilibration (immediately before freezing)and after thawing.

Viability assay:

Post-thaw samples were incubated in a water bath at 37 °C for three hours during which the percentage of progressive motile sperm was recorded at 0, 1, 2, and 3 hours of incubation. The post-thaw viability index was calculated according to **Milovanov (1962)**.

Morphological Assays:

Sperm acrosomal integrity:

Acrosome integrity was assessed using silver nitrate stain in a procedure slightly modified from the method described by **Chinoy et al. (1992).**

Samples of thawed semen were spread on microscope slides and left to dry at room temperature. The preparations were fixed firstly in 70% ethyl alcohol for 2 minutes followed by 95% ethyl alcohol for a similar period. The slides were stained with silver nitrate solution for 2 hours in an incubator at 65°C in a completely humid atmosphere. After the preparations turned gold in colour, the chemical reaction was interrupted and the preparation rinsed several times with distilled water and dried at room temperature. The stained preparations were examined for acrosomal integrity using the Olympus BX50 light microscope under a 100X objective. The percentage of spermatozoa with deteriorated or lost acrosomes was counted in at least 300 sperm cells per each slide.

Plasma membrane integrity:

Plasma membrane integrity (PMI) of canine spermatozoa was assessed by hyposmotic swelling (HOS) assay **(Jeyendran et al.,1984)**. The solution of HOS consisted of sodium citrate 0.73 g and fructose 1.35 g, dissolved in 100 ml distilled water. The assay was performed by mixing 50 µl of frozen-thawed semen sample to

Table (1) Effect of BullXcell and TCF extenders on the cryopreserved epididymal sperm (Mean± SEM).

Type of extender	Motility before freezing (%)	Post-thaw motility (%)	Viability Index	Acrosomal abnormalities (%)	HOST (%)
BullXcell (n=10)	68.00 ± 2.14^{a}	30.00 ± 3.60^{a}	39.00 ± 6.21^{a}	15.60 ± 0.42^{b}	55.40 ± 1.56^{a}
TCF (n=10)	63.00 ± 2.57^{a}	25.00 ± 3.16^{a}	34.50 ± 5.88^{a}	20.40 ± 0.83^{a}	47.40 ± 1.73^{b}
Overall mean (n=20)	65.50 ± 1.81	27.50 ± 2.53	36.75 ± 4.42	18.00 ± 0.73	51.40 ± 1.50

. Values with different superscripts in the same column are significantly different at ($P < 0.05$).
. Data were obtained from 10 samples (one /dog).

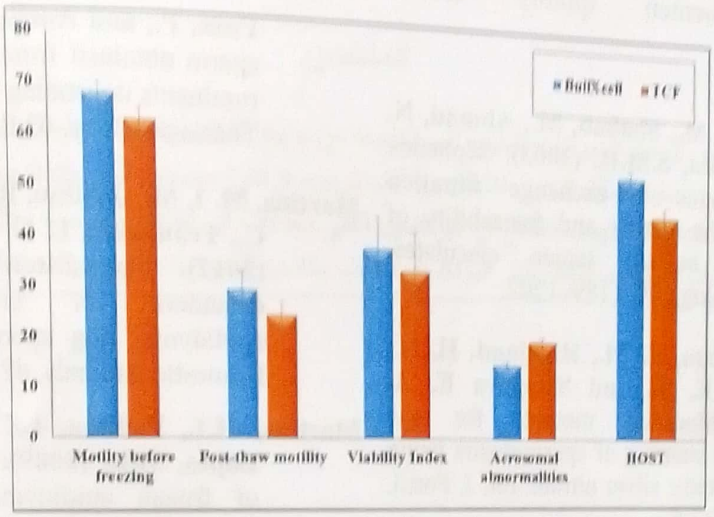

Fig.1: Effect of BullXcell and TCF extenders on the studied canine epididymal sperm parameters.

500µl of HOS solution and incubated at 37 °C for 40 min. After incubation, drop of semen sample was examined under microscope. Two hundred spermatozoa were counted for their swelling characterized by coiled tail indicating intact plasma membrane **(Ahmad et al., 2003; Pinto and Kozink, 2008).**

Statistical analysis

All experiments were repeated ten times. All statistical analysis were calculated with commercial software (SPSS, (ver. 15.0; SPSS Inc., Chicago, IL). Appropriate statistical analysis was carried out according to **Snedecore and Cochran (1976).** Analysis of variance (ANOVA) was used to check the statistical significance at $p < 0.05$ level.

Results

The effect of BullXcell and TCF extenders on the different canine semen characteristics are shown in Table 1, Fig 1.

Upon dilution, cooling and equilibration of epididymal sperm, no significant differences between the two used extenders were noted and so was the case for post-thaw motility and viability index.

On the other hand, the percentage of abnormal acrosomes was significantly lower

($P< 0.05$), but the per cent HOST reactive cells was higher in BullXcell than in TCF extender, respectively

-Discussion

Domestic dogs are not only companions but also excellent experimental models because of the similarity of the reproductive physiology with the wild species and humans. Obtaining semen directly from the epididymal tail and vas deferens is a technique that has been frequently used for the purposes of assisted reproduction (**Martinset al., 2009**).

The extender used to freeze sperm cells is important to avoid membrane damage and to preserve semen quality after cryopreservation. As expected, cryopreservation induced great changes in the values of morphologic and other sperm parameters. The results of motility sperm parameters obtained in this study, frozen with both extenders, were similar to those noted by **Martins et al. (2009)and Martins et al. (2012)**. There were statistical differences between the two extenders examined in this study. The commercial extender (BullXcell®) appeared to give better results regarding membrane integrity and the percentage of intact acrosome than TCF. We can conclude that the BullXcell®, a product used to freeze bovine semen, can successfully be used to freeze canine epididymal sperm.

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الملخص

مقارنه مخففين مختلفين لتجميد الحيوانات المنويه المستخلصه من بربخ الكلاب. تمت الدراسه باستخدام تاثير مخفف البولكسيل المستخدم في تجميد السائل المنوي للابقار ومخفف ترس- فركتوز-حمض الستريك لتجميد الحيوانات المنويه المستخلصه من بربخ الكلاب واظهرت النتائج ان البولكسيل اعلي معدلات في اختبار الهوست واقل معدل للتشوهات في الاكروسوم للحيوان المنوي للكلاب لذلك يعتبر البولكسيل مخفف مناسب للحيوانات المنويه المستخلصه من بربخ الكلاب