

# Cryopreservation of canine semen: the effect of two extender variants on the quality and antioxidant properties of spermatozoa

R. Strzeżek, M. Kozirowska-Gilun, M. Stawiszyńska

Department of Animal Biochemistry and Biotechnology, Faculty of Animal Bioengineering,  
University of Warmia and Mazury in Olsztyn, Oczapowskiego 5, 10-718 Olsztyn, Poland

## Abstract

The aim of this study was to determine the effect of two variants of Tris-citric acid-fructose (TCF) extender containing whole hen egg yolk (TCF-HEY) and lyophilized lipoprotein fractions extracted from ostrich egg yolk (TCF-LPF<sub>o</sub>) on selected biological properties of cryopreserved sperm cells. Post-thaw percentage of motile sperm (MOT) was significantly higher ( $P < 0.05$ ) for TCF-HEY extender ( $66.3 \pm 3.2\%$ ) than for TCF-LPF<sub>o</sub> extender ( $52.4 \pm 3.4\%$ ). Moreover, there was no significant difference in the percentage of sperm with progressive motility (PMOT). Both diluents effectively preserved sperm plasma membrane integrity and mitochondrial function. However, it was observed that cryopreservation impaired the functionality of antioxidant sperm enzymes. The above was manifested by reduced SOD activity, in particular in samples preserved in the TCF-HEY extender, as well as decreased GPx activity. Both diluents inhibited the rate of lipid peroxidation in sperm plasma membrane during freezing-thawing. Our results suggest that LPF<sub>o</sub> is a satisfactory alternative to hen egg yolk in the extender used for canine sperm cryopreservation.

**Key words:** dog, spermatozoa, freezing-thawing, extenders, antioxidant enzymes, lipid peroxidation

## Introduction

The cryopreservation process decreases the fertilizing ability of spermatozoa (Bilodeau et al. 2000, Cerolini et al. 2001). The above could result from enhanced peroxidation of sperm cell membrane lipids which is largely determined by excessive generation of reactive oxygen species (ROS) in semen (Alvarez and Storey 1992, Wang et al. 1997). The membrane of a sperm cell contains large amounts of polyunsatu-

rated fatty acids (PUFAs), which makes them highly susceptible to lipid peroxidation (LPO) in the presence of ROS, resulting in impaired sperm function (Aitken et al. 1996).

In contemporary techniques of canine semen cryopreservation seminal plasma is removed by centrifuging (Sirivaidyapong et al. 2001). The main disadvantage of this method is the elimination of natural antioxidants from seminal plasma (Strzeżek et al. 2009). In sperm cells, there are multiple protection

mechanisms designed to protect spermatozoa against oxidative injury (Mennella and Jones 1980). As a means of protection against the cytotoxic effects of internally generated ROS, canine spermatozoa contain antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Strzeżek et al. 2009). However, the sparse cytoplasmic volume in the sperm cell mid-piece limits its antioxidant capacity (Aitken et al. 1996).

Hen egg yolk (HEY) is the most commonly used compound in canine semen extenders for protecting spermatozoa against cold shock and disruption during the freezing-thawing procedure (Moussa et al. 2002). Recent studies investigating different animal species, including dogs, have demonstrated that low density protein (LDL) extracted from hen egg yolk could be largely responsible for protection against cold shock, improvement in sperm motility, acrosome and plasma membrane integrity and more effective protection against sperm DNA fragmentation (Demianowicz and Strzeżek 1996, Bencharif et al. 2008). Similar effects can be obtained by introducing lyophilized low molecular weight lipoprotein fractions extracted from the egg yolk of African ostriches (LPF<sub>o</sub>) to semen cryopreservation extenders (Fraser et al. 2007).

The objective of this study was to determine the effect of two TCF extender variants (TCF-HEY and TCF-LPF<sub>o</sub>) on selected biological properties of canine spermatozoa subjected to freezing-thawing treatments.

## Materials and Methods

### Animals and semen collections

Six sexually mature dogs of mixed breeds, aged from 2 to 10 years, were used in this study. The dogs were housed in individual pens and fed with commercial canine food. Water was available *ad libitum*. Animal experiments were carried out in accordance with the guidelines set out by the Local Ethics Committee.

The pre-sperm and sperm-rich fractions of ejaculate were collected by digital manipulation (Kutzler 2005) during eight sampling sessions held at weekly intervals. During each sampling, ejaculates collected from the same three dogs were pooled to obtain semen samples of sufficient volume and to rule out individual differences between ejaculates (Yu et al. 2002).

Sperm characteristics of the pooled semen (progressive motility, sperm concentrations, plasma membrane integrity and mitochondrial function, activity of antioxidant enzymes, lipid peroxidation) were evaluated in pre-freeze and post-thaw semen samples.

### Media and extenders

A Tris-citric acid-fructose extender (TCF) for semen dilution was prepared prior to equilibration according to the method proposed by Rota et al. (1995). Two variants of the TCF extender (with identical amounts of TCF) containing 20% hen egg yolk (TCF-HEY) or 10% lyophilized lipoprotein fraction extracted from the ostrich egg yolk (TCF-LPF<sub>o</sub>) were prepared for semen cooling and freezing treatments. The final concentrations of glycerol and Orvus Es Paste (OEP, Nova Chemical Sales, Inc., Scituate MA, USA) were 4% and 1%, respectively. A brief description of the LPF<sub>o</sub> preparation procedure can be found elsewhere (Strzeżek et al. 2005).

Semen dilution and the freezing-thawing procedure

Semen was processed using the method proposed by Rota et al. (1997) with some modifications (Kordan et al. 2010). The semen was centrifuged after collection ( $720 \times g$ , 6 min at room temperature). Seminal plasma was removed, the sperm pellet was divided into 2 aliquots and diluted in TCF at a concentration of  $200 \times 10^6$  spermatozoa/ml. The diluted semen was allowed to equilibrate for 30 min at room temperature, after which it was cooled to 5°C. One hour after the beginning the cooling procedure, TCF-HEY and TCF-LPF<sub>o</sub> variants of the extender, also at 5°C, were added. The final sperm concentration was  $100 \times 10^6$  spermatozoa/ml. At 75 min from the onset of cooling, semen samples were frozen in 0.25 ml plastic straws (Minitub GmbH, Tiefenbach, Germany) in a controlled programmable freezer (CryoCell 1205; SY-LAB, Austria) using an appropriate freezing protocol (cooled from +5°C to -20°C at 4.46°C/min and then further cooled from -20°C to -80°C at 5°C/min). The frozen straws were stored in liquid nitrogen (-196°C) until required for laboratory analysis. Thawing was performed for 5 sec at 70°C in a water bath (Nöthling and Shuttleworth 2005).

### Evaluation of sperm parameters

Sperm motility characteristics were evaluated using the Hamilton-Thorne Sperm Analyzer IVOS version 12.3 (Hamilton-Thorne Biosciences, Beverly, MA, USA). The motility parameters determined by the IVOS analyzer were: total motility (MOT, %), progressive motility (PMOT, %), average path velocity (VAP,  $\mu\text{m/s}$ ), straight line velocity (VSL,  $\mu\text{m/s}$ ), curvilinear line velocity (VCL,  $\mu\text{m/s}$ ), amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ), beat cross frequency (BCF, Hz) and linearity (LIN, %). A droplet of approximately 5  $\mu\text{l}$  was placed in a Makler counting

chamber (Sefi-Medical Instruments Ltd., Haifa, Israel) at 37°C, and six fields were examined per sample.

Sperm plasma membrane integrity was assessed with a dual fluorescent staining technique described by other authors (Garner and Johnson 1995), using SYBR-14 and PI (Live/Dead Sperm Viability Kit; Molecular Probes, OR, USA). Aliquots (10 µL) of sperm samples stained with SYBR-14/PI were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). For each aliquot, approximately 200 sperm cells were classified as spermatozoa with intact or damaged plasma membrane.

Mitochondrial function was assessed in sperm aliquots using a dual fluorescent staining technique with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (1 mg / ml DMSO) (Molecular Probes, Eugene, OR, USA) and PI (10 µl of PI solution in 0.5 mg/ml PBS), according to a previously described method (Thomas et al. 1998). Aliquots (10 µL) of samples stained with JC-1/PI were examined under an epifluorescence microscope (Olympus CH 30, Tokyo, Japan). For each aliquot, approximately 200 sperm cells were classified as spermatozoa with viable or non-viable mitochondria.

#### **Determination of antioxidant enzyme activity and lipid peroxidation**

Before and after freezing-thawing treatments, spermatozoa were washed by centrifugation (1000 × g, 5 minutes at room temperature). Sperm pellets were washed twice with 0.85% NaCl and later adjusted to a final concentration of 150 × 10<sup>6</sup> spermatozoa/ml. Sperm samples were homogenized at room temperature using an Ultra-turax T8 homogenizer (IKA-Werke, Germany). Homogenized samples were used to analyze the activity of antioxidant enzymes.

Total protein content was measured according to the method proposed by Lowry et al. (1951) using bovine serum albumin – BSA (Serum and Vaccine Production, Cracow, Poland) as a standard.

The activity of SOD and GPx was measured with the use of commercial kits according to the manufacturer's recommendations. All assays were run in duplicates.

SOD activity was determined using a Ransod kit (Randox Laboratories, U.K.) with xanthine and xanthine oxidase to generate superoxide radicals that react with 2- (4 – iodo-phenyl) – 3 – (4- nitrophenol)-5-phenyltetrazolium chloride (I.N.T) to produce red formazan dye. One unit of SOD was the amount that

caused a 50% inhibition in the rate of I.N.T. reduction. SOD activity was expressed in arbitrary units of absorbance normalized to mg of protein.

The activity of GPx was determined using the Ransel Glutathione Peroxidase kit (Randox Laboratories, UK). In this assay GPx catalyzes the oxidation of GSH with cumene hydroperoxide. In the presence of GR and NADPH GSSG is converted into GSH with concomitant oxidation of NADPH to NADP<sup>+</sup>. The decrease in absorbance was measured at 340 nm at 37°C (pH 7.2). GPx activity was normalized to mg of protein and expressed in mU/mg of protein.

Lipid peroxidation in spermatozoa was measured by determining malondialdehyde (MDA) production based on the thiobarbituric acid (TBA) reaction (Tappele 1973, Strzeżek et al. 2000). MDA content was determined spectrophotometrically at a wavelength of 530 nm. Lipid peroxidation was expressed in nM MDA/10<sup>8</sup> spermatozoa.

#### **Statistical analysis**

All values were expressed as the means ± standard deviation (SD). The data were analyzed by ANOVA and Duncan's multiple comparison test using the Statistica software package (StatSoft Incorporation, Tulsa OK., USA). Differences between means were considered significant at  $p \leq 0.05$ .

#### **Results**

In samples of cryopreserved semen a significant decrease in the percentage of motile sperm cells (MOT) and progressively motile sperm cells (PMOT) was observed in comparison with fresh semen samples (Table 1). Samples cryopreserved in TCF-HEY extender were characterized by a higher share of MOT ( $p < 0.05$ ). No significant differences in PMOT, VAP, VSL, BCF, STR, LIN parameters were observed between two extender variants after freezing-thawing. Similar values were also produced by evaluations of plasma membrane integrity (SYBR-14/PI staining) and analyses of the percentage of sperm cells with a high mitochondrial membrane potential (JC-1/PT staining) (Table 2).

Lower levels of SOD activity were reported in sperm cells cryopreserved in both extender variants in comparison with fresh semen. A dramatic drop in GPx activity was noted in spermatozoa cryopreserved in the extender containing TCF-HEY ( $p < 0.05$ ). Significant differences were not observed in the rate of induced membrane lipid peroxidation between sperm cells cryopreserved with both extender variants.

Table 1. Sperm motility characteristics of canine semen before and after the freezing-thawing procedure in two different extenders (n=16).

| Stage of cryopreservation | Motility parameters         |                             |                              |                              |                              |                              |                             |                             |                             |                             |
|---------------------------|-----------------------------|-----------------------------|------------------------------|------------------------------|------------------------------|------------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                           | MOT (%)                     | PMOT (%)                    | VAP ( $\mu\text{m/s}$ )      | VSL ( $\mu\text{m/s}$ )      | VCL ( $\mu\text{m/s}$ )      | ALH ( $\mu\text{m}$ )        | BCF (Hz)                    | STR (%)                     | LIN (%)                     |                             |
| Pre-freeze                | 92.3 $\pm$ 0.7 <sup>a</sup> | 52.9 $\pm$ 2.8 <sup>a</sup> | 140.5 $\pm$ 2.1 <sup>a</sup> | 110.8 $\pm$ 2.0              | 221.5 $\pm$ 5.7 <sup>a</sup> | 8.4 $\pm$ 0.2 <sup>a</sup>   | 18.9 $\pm$ 1.0 <sup>a</sup> | 78.0 $\pm$ 1.3 <sup>a</sup> | 51.8 $\pm$ 1.8 <sup>a</sup> |                             |
| Post-thaw                 | TCF-HEY                     | 66.3 $\pm$ 3.2 <sup>b</sup> | 40.3 $\pm$ 3.4 <sup>b</sup>  | 117.7 $\pm$ 3.7 <sup>b</sup> | 103.4 $\pm$ 3.9              | 159.7 $\pm$ 4.8 <sup>b</sup> | 6.2 $\pm$ 0.2 <sup>b</sup>  | 12.4 $\pm$ 0.9 <sup>b</sup> | 87.2 $\pm$ 0.8 <sup>b</sup> | 67.4 $\pm$ 1.1 <sup>b</sup> |
|                           | TCF-LPF <sub>o</sub>        | 52.4 $\pm$ 3.4 <sup>c</sup> | 32.1 $\pm$ 2.6 <sup>b</sup>  | 112.1 $\pm$ 2.7 <sup>b</sup> | 100.1 $\pm$ 2.5              | 144.2 $\pm$ 4.0 <sup>c</sup> | 5.6 $\pm$ 0.1 <sup>c</sup>  | 14.0 $\pm$ 1.3 <sup>b</sup> | 88.3 $\pm$ 0.5 <sup>b</sup> | 70.5 $\pm$ 1.0 <sup>b</sup> |

Values are expressed as the mean  $\pm$  SD.

The values with different superscripts (a-c) within the columns differ significantly ( $p \leq 0.05$ )

TCF-HEY – Tris- citrate-fructose extender containing whole hen egg yolk

TCF-LPF<sub>o</sub> – Tris-citrate-fructose extender containing lyophilized lipoprotein fractions of ostrich egg yolk

Table 2. Sperm viability and antioxidant properties of canine semen before and after the freezing-thawing procedure in two different extenders (n=16).

| Stage of cryopreservation | Sperm viability                            |                                     | Antioxidant properties      |                               |  |                |
|---------------------------|--|-------------------------------------|-----------------------------|-------------------------------|--|----------------|
|                           | Plasma membrane integrity (SYBR-14/PI) (%) | Mitochondrial function (JC1/PI) (%) | SOD activity (U/mg protein) | GPx activity (mU/mg protein)  | MDA production (nM/ 10 <sup>8</sup> spermatozoa) |                |
| Pre-freeze                | 90.1 $\pm$ 2.6 <sup>a</sup>                | 80.6 $\pm$ 5.0 <sup>a</sup>         | 1.91 $\pm$ 0.33             | 126.0 $\pm$ 19.2 <sup>a</sup> | 14.2 $\pm$ 1.5                                   |                |
| Post-thaw                 | TCF-HEY                                    | 51.7 $\pm$ 4.4 <sup>b</sup>         | 40.6 $\pm$ 2.6 <sup>b</sup> | 1.44 $\pm$ 0.13               | 12.5 $\pm$ 10.6 <sup>b</sup>                     | 10.7 $\pm$ 1.6 |
|                           | TCF-LPF <sub>o</sub>                       | 43.6 $\pm$ 2.4 <sup>b</sup>         | 36.6 $\pm$ 2.4 <sup>b</sup> | 1.34 $\pm$ 0.25               | 42.3 $\pm$ 17.1 <sup>c</sup>                     | 13.4 $\pm$ 1.3 |

Values are expressed as the mean  $\pm$  SD.

The values with different superscripts (a-c) within the columns differ significantly ( $p \leq 0.05$ )

TCF-HEY – Tris- citrate-fructose extender containing whole hen egg yolk

TCF-LPF<sub>o</sub> – Tris-citrate-fructose extender containing lyophilized lipoprotein fractions of ostrich egg yolk

## Discussion

The cryopreservation process is accompanied by excessive generation of reactive oxygen species which may induce changes in the structure and functions of the sperm plasma membrane (Wang Y. et al. 1997). The results of our study validate the above hypothesis. In thawed samples a significant decrease in the number of motile sperm cells with integral plasma membranes and active mitochondria was observed in comparison with fresh semen samples.

In the present study the percentage of motile spermatozoa (MOT) following freezing-thawing in extender containing whole hen egg yolk (TCF-HEY) was significantly higher than with extender containing LPF<sub>o</sub>. However, there was no significant difference for the percentage of sperm with progressive motility (PMOT), but the values tended to be better with the TCF-HEY extender. The values of sperm motility

parameters obtained in semen frozen in extender containing HEY are similar to those previously reported following the freezing of dog spermatozoa (Rota et al. 2005, Nizański 2006).

There are numerous reports on cryopreserving canine semen. However, because there is no available published information regarding the effect of extender containing LPF<sub>o</sub> on properties of canine sperm during freezing-thawing procedure, it is difficult to compare our results.

The results of this study indicate that cryopreservation induces changes in the antioxidant balance of canine spermatozoa. Thawed samples of semen treated with both extender variants were characterized by lower levels of activity of antioxidant enzymes. The range of changes in cryopreserved semen varies across different species. Frozen-thawed samples of bull semen showed more than a 50% drop in SOD activity (Bilodeau et al. 2000). In cryopreserved

human and ram spermatozoa a decrease in SOD activity was accompanied by stable levels of GPx activity (Alvarez and Storey 1992, Lasso et al. 1994, Marti et al. 2008). In our study an insignificant (ca. 30-35%) decrease in SOD activity levels was observed in both extender variants. A dramatic decline in GPx activity was noted in semen samples cryopreserved in TCF-HEY. The above could suggest that in canine semen SOD is less susceptible to cryogenic changes than GPx.

The mechanism responsible for the loss of antioxidant enzyme activity in cryopreserved semen remains unexplained. Lasso et al. (1994) suggested that organelle damage in sperm cells and enzyme leakage during cryopreservation may be responsible for a partial loss of SOD function. According to the above authors antioxidant enzymes are not denatured during cryopreservation.

Cryopreservation decreases semen quality (Oettle 1986, Wang et al. 1997). The degree to which the peroxidation of sperm membrane lipids is responsible for the above loss remains unknown. In our experiment lipid membrane peroxidation was not enhanced in canine semen samples subjected to freezing-thawing treatments with both extender variants. An analysis of human sperm cells cryopreserved with the use of the same method produced similar results (Wang et al. 1997). Negau et al. (2011) demonstrated a decrease in the peroxidation of membrane lipids in thawed samples of canine semen (fluorescent staining with Biodipy 581/591 stain). The above authors attributed their findings to the fact that cryopreservation impairs the function of mitochondria – the main source of reactive oxygen species which induce lipid peroxidation.

Natural antioxidants, including tocopherols and carotenoids in avian egg yolk, may be responsible for low levels of malondialdehyde (MDA) in frozen-thawed sperm cells (Surai 1999). Our findings indicate that antioxidants found in whole hen egg yolk decrease the rate of lipid peroxidation in cryopreserved semen.

The results of our study indicate that cryopreservation decreases the biological properties of canine spermatozoa. The quality of frozen-thawed semen extended with LPF<sub>0</sub> was similar to that of semen samples treated with a freezing extender supplemented with HEY.

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lipoprotein fractions extracted from the ostrich egg yolk as a protective component in extenders for preserving animal semen has been filed at the Patent Office of the Republic of Poland (P-388264).

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