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Research Article

Superiority of a Non -Enzymatic Antioxidant Over Enzymatic Antioxidants in Improving the Post Thaw Function of Labrador Dog Semen

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Abstract

In the current study the effect of supplementation of non-enzymatic and enzymatic antioxidants to the Tris-citric acid-fructose egg yolk plasma extender on sperm attributes and antioxidant defence system in frozen-thaw semen was compared. Supplementation of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH) to the extender significantly ($p < 0.05$) improved motility, viability, PMI, AI and high IMMP compared to control. Malonaldehyde (LPO) concentration was significantly ($p < 0.05$) high in GSH supplemented extender followed by CAT/glutathione peroxidase (GPX), SOD and control. Significantly ($p < 0.05$) high H2O2 scavenging activity of spermatozoa was observed in GPX supplemented extender followed by SOD, CAT, control and GSH. NO2- concentration (μM/109 spermatozoa) was significantly ($p < 0.05$) less in GSH, CAT, GPX supplemented extenders compared to control. SO2- scavenging activity of spermatozoa was significantly high in GSH/GPX than SOD/CAT/control. SOD, CAT, GPX and GRE activity of spermatozoa was significantly ($p < 0.05$) high in GSH supplemented extender. Spermatozoa cryopreserved in SOD and CAT supplemented extenders indicated significantly ($p < 0.05$) higher SOD activity compared to control. GPX activity of spermatozoa was also significantly ($p < 0.05$) high in GPX/CAT supplemented extenders. Cryoinjury to the spermatozoa was protected to a maximum level in GSH supplemented extender followed by CAT, SOD, GPX and control. Study concluded that GSH is superior over antioxidant enzymes in protecting the spermatozoa from cryoinjury. Based on these observations, higher fertility rate is expected of canine semen cryopreserved in GSH supplemented TCFEYP extender compared to TCFEYP.

Keywords: Non-Enzymatic; Enzymatic; Antioxidants; Post Thaw Semen; Functional Analysis

Introduction

Spermatozoa are highly sensitive to oxidative stress and reactive oxygen species (ROS). Concentration of ROS in spermatozoa is controlled by their endogenous antioxidants. The process of semen cryopreservation boosts loss of sperm motility and decrease of sperm survival at post-thaw [1]. It occurs mainly through the generation of ROS [2]. ROS in small quantity promote physiological effects, such as sperm capacitation and fertilization. However, ROS in high quantity can be deleterious to sperm, causing oxidative stress [3]. There may be increase in injury in proteins, carbohydrates, plasma membrane, sperm motility, and in spermatic DNA integrity due to high concentration of ROS [4]. The effects of ROS during sperm cryopreservation may be reduced involving an addition of antioxidants to the extenders [5,6]. Antioxidants are produced physiologically by organisms, to minimize or prevent the oxidation by ROS effect in a determined substrate. Glutathione peroxidase (GPx), glutathione (GSH), Phospholipid hydroperoxide GPx combined with superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GRE) constituting a glutathione cycle, had an important effect in ROS fight, because these enzymes catalyse the chain reaction in which superoxide anion (O_2-), the first produced ROS, is converted into water [7]. In dogs, most of the

antioxidant defence system, present in semen is from the seminal plasma. Availability of antioxidant enzymes to the spermatozoa from seminal plasma is reduced during cryopreservation process as a result of extension in the extender. Moreover, in dog, seminal plasma antioxidants are totally removed during centrifugation before extension of the semen in extender. Therefore, protection against ROS generation during freezing-thawing process relies only on minute antioxidant defence system of spermatozoa, which may not be sufficient to protect the spermatozoa from deleterious effect of cryoinjury. Variable effects of supplementation of GSH, catalase, SOD to the extender have been evaluated in different species by various author [8-12,6,13-15]. Despite a number of studies on supplementation to the extender, there is no consensus about the optimum concentration of antioxidants that provides better results after thawing or even in chilled semen. More studies are necessary, searching the ideal concentration of antioxidants to be supplemented to the extender for beneficial effect on cryopreserved semen. We have standardized optimum concentrations of GSH, GPX, SOD and catalase for beneficial effect on dog semen preserved at 4ºC for 72 hrs [16]. Therefore, present study was aimed to compare the effect of enzymatic (GPX, SOD, catalase) and non-enzymatic (GSH) antioxidants on post-thaw sperm attributes, LPO, ROS scavenging activity and antioxidant capacity of post thaw spermatozoa in Labrador Retriever dog.

Materials and Methods

- **Maintenance of dogs and semen collection:** All the procedures were approved by the CPCSEA, New Delhi vide F. No 25-19-2018-CPCSEA, dated 22/11/2018. The experiments were conducted at Reproductive Biotechnology Lab, Department of Veterinary Gynecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141004, Punjab, India. Semen was collected from six Labrador retriever dogs. Dogs (2-3 years old) were maintained in individual pens in the university dog house and were daily fed cooked feed supplemented with vitamins and trace minerals twice daily and water provided ad libitum. Dogs were given regular exercise of walking/running for one hour daily in the morning and evening. Deworming of dogs was done and they were vaccinated for rabies, CDV, CAV2, CPV, CPI and CAV1.
- **Collection, Processing and Cryopreservation of semen:** Semen was collected from six dogs by message method at an interval of 3 days. Tris citric acid fructose egg yolk plasma glycerol (TCFEYP-G) extender was prepared as reported somewhere else [17]. Semen of dogs exhibiting >70% motility was pooled for each trial and divided into required aliquots. To each aliquot SOD (200 IU/ml), GPX (10 IU/ml), CAT (200 µg/ml) and GSH (7.5 mM/ml) was added and incubated at 37°C for 10 miN [16]. An aliquot without enzyme was also processed. To each aliquot TCFEYP-G extender was added and semen-extender mixture was centrifuged at 960g for 3 min. Loose pellet was re-suspended in extender containing respective concentration of the antioxidant to get a final sperm concentration between 150-200 spermatozoa/ml. Sperm suspension was again equilibrated at 37°C for 10 min and processed for cryopreservation [17].
- **Analysis of sperm attributes in frozen-thaw semen:** Motility was evaluated by wet mount and track method. Viability was assessed by staining the spermatozoa with eosin-nigrosin stain, a drop of semen was mixed with a drop of 0.5% aqueous eosin in normal saline, mixed for 60 sec followed by addition of a drop of 10% nigrosin on a slide. Semen-stain mixture was mixed thoroughly for 60 sec and a smear was prepared, dried and observed under a binocular microscope (Olympus) at 1000x. Hypo-osmotic swelling test (HOST) was performed to analyse the plasma membrane integrity of sperm (PMI) [18]. One drop of semen incubated in 60 mosm HOS solution for 30 min was placed on a slide, covered with cover slip and examined under bright field microscope (Olympus) at 400 X for coiled tailed spermatozoa. A control was also run in PBS, pH 7.4. The number of coiled tailed spermatozoa in PBS was deducted from the number in hypo-osmotic solution and the resultant figure was taken as the HOS-reactive spermatozoa. For acrosome integrity (AI), sperm smears prepared on clean glass slides were stained with Coomassie brilliant blue-R stain [19]. Spermatozoa with unstained (damaged) and stained (blue) acrosomes were observed under a binocular microscope (Olympus) at 1000x. About 200 spermatozoa were counted in different fields to calculate percentage motility, viability, PMI and AI. Fluorescent carbocyanine dye, JC-1 (JC-1 stain kit, Sigma –Aldrich) was used to measure IMMP of the spermatozoa. JC-1 dye and semen were mixed in the ratio of 1:10 and incubated at 37ºC for 20 min. A drop of incubated semen was placed on a glass slide and covered with coverslip. Slide was observed under a fluorescent microscope (Olympus CX-24) at 400X. About 200 spermatozoa with high (HIMMP, red/orange), medium (MIMMP, yellow) and low (LIMMP, green) IMMP were counted in different fields and percentage of spermatozoa with HIMMP and MIMMP was calculated.
- **Preparation of sperm extract for antioxidant enzymes and reactive oxygen species:** Neat semen was washed twice with phosphate buffered saline (PBS, pH, 7.4) and sperm pel-

let (1.0 x 10^9 sperm/2.0 ml) was suspended in Tris buffer (pH 7.5, 25 mM Tris, 2 mM Sodium EDTA, 2 mM dithiothretol and 250 mM NaCl containing 1% Triton X-100). The sperm suspension was agitated at 25ºC for 30 min and then centrifuged at 9615 g at 4ºC for 20 min. Supernatant (sperm extract) was stored in aliquots at -20ºC till analysis.

Reactive oxygen species

- **Superoxide anion radical assay (SO_2^-) [20]:** The reaction mixture was prepared by mixing 75 µl phosphate buffer (pH 7.4), 25 µl NBT (100 µM), 25 µl NADH (468 µM) and 25 µl sperm extract. The reaction was started by adding 25 µl phenazine methosulfate solution (60µM) in to the reaction mixture followed by incubation at 25ºC for 5 min. A control was also run without sample. The absorbance was measured at 560 nm against blank. Percentage of SO_2^- scavenging activity was calculated by using the following formula:
- (Abs sample -Abs control)/Abs sample x100
- **H_2O_2 radical assay [21]:** A solution of hydrogen peroxide (H_2O_2, 40 mmol/l) was prepared in phosphate buffer (pH 7.4). Reaction mixture was prepared by mixing 150 µl of 40 mmol H_2O_2 and 25 µl sperm extract and incubated at room temperature for 10 min. Absorbance of 40 mM H_2O_2 (control) and reaction mixture was measured at 230 nm against PBS, pH 7.4. H_2O_2 activity (%) was calculated by the following formula
- (Abs control - Abs sample)/Abs control x 100
- **Nitrite radical assay (NO_2^-) [22]:** Nitrite free radicals produced in sperm extract were measured by Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% 1- naphthyl ethylene diamine dihydrochloride in water). Griess reagent (50 µl) and sperm extract (25 µl) were mixed and incubated at room temperature for 10 mins. A blank consisting of 50 µl PBS, pH 7.4 and 25 µl sperm extract was also run along with the samples. The absorbance of the chromophore that formed during diazotization of the nitrite with sulfanilamide and subsequent coupling with 1-naphthyl ethylene diamine dihydrochloride was immediately read at 540 nm. NO_2^- concentration in the samples was calculated from standard curve plotted for 1mM-10 mM concentrations of sodium nitrite.
- **Lipid Peroxidation and Antioxidant enzymes:** Malondialdehyde (MDA) concentration (end product of LPO), SOD, GRE, GPx and CAT were estimated in sperm extracts as per modified and standardized methods in our earlier study [23]. MDA concentration and enzyme activity were calculated by using the following formulas:

$$\text{MDA content } (\mu M/10^9 \text{ spermatozoa}) = \frac{\text{OD x volume of assay mixture}}{\text{Volume of sample taken x coefficient extinction}}$$

$$\text{SOD } (IU/10^9 \text{ sperm/min}) = \frac{\Delta T \times 2}{\Delta C}$$

$$\text{GRE } (IU/10^9 \text{spermatozoa/min}) = \Delta T - \Delta C$$

$$\text{GPX } (IU/10^9 \text{spermatozoa/min}) = \Delta T - \Delta C$$

Where, ΔT - Change in OD $_{\text{Test}}$ at 60 sec interval and

ΔC - Change in OD $_{\text{Control}}$ at 60 sec intervals.

$$\text{CAT } (KU/10^9 \text{ spermatozoa}) = \frac{A \text{ (Sample)} - A \text{ (Blank 1)} \times 27.1}{A \text{ (Blank 2)} - A \text{ (Blank 3)}}$$

Statistical analysis

Significant differences (5% level) among the control and antioxidant supplemented samples were tested by one-way Anova using SPSS 16 program (Student version for windows, SPSS Inc. 233 South Wacker Drive, 11th floor Chicago, IL 60606-6412). Normality of the data was assessed using the Shapiro-Wilk test and homogeneity of variances was evaluated using the Levene test.

Results and Discussion

Effect of supplementation of enzymatic and non-enzymatic antioxidants to the extender on biological function of post thaw spermatozoa

Supplementation of SOD, CAT and GSH to the extender significantly ($p < 0.05$) improved motility, viability, PMI, AI and HIMMP compared to control in frozen thawed semen (Figure 1). There was a difference of 5.8-8.9%, 6.7-11.2 5 and 10.8-13.8% in motility, viability, PMI, AI and HIMMP between control and SOD, CAT, GSH supplemented samples.

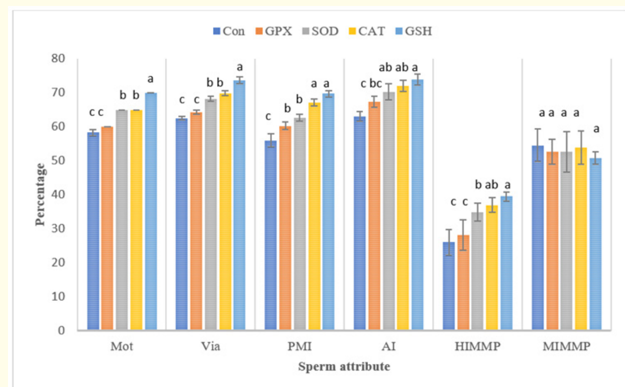

Figure 1: Effect of supplementation of enzymatic and non-enzymatic antioxidants on post thaw sperm attributes of Labrador dog. a, b, c indicates significant ($p < 0.05$) differences in sperm attributes among the extenders.

There are meagre studies on effect of supplementation of antioxidant enzymes to the extender on post-thaw canine semen. Supplementation of 5mM GSH to the extender improved quality of post-thaw semen in canine [5]. It was concluded in another study that addition of 10 mM GSH to the extender resulted in acrosome protection and preserved fertility of post thaw semen [6]. Supplementation of 5 mM GSH to the extender was also effective in improving motility, longevity and AI and inhibiting LPO levels in post thaw canine spermatozoa without any adverse impact on full term development after trans-cervical insemination [11]. Chilled and thawed samples supplemented with 7.5 and 10 mM GSH had lower percentage of spermatozoa with high and medium mitochondrial activity [14]. Contrary to these observations, addition of 7.5 mM/ml GSH to the semen significantly improved motility, viability, PMI, AI and HIMMP of spermatozoa at post thaw in our study. Beneficial effect of GSH is also reported on the survivability and fertility strength of spermatozoa in Turkey [23].

Catalase (300units/ml) had the most pronounced effect compared to vitamin C, taurine, N-acetyl cysteine, B-16 and tocopherol in improving post-thaw quality of canine spermatozoa [24]. But in the present study, addition of 200 µg/ml CAT significantly ($P < 0.05$) improved the sperm attributes at post-thaw. Contrary to these observations in canine, the addition of 100 IU/ml and 200 IU/ml CAT to citrate egg yolk extender did not improve motility and viability significantly in post thaw semen of cattle bull but values for both were higher compared to control [8]. Addition of CAT (200 IU/ml) and SOD (100IU/ml) alone to TEST-yolk buffer did not improve progressive motility of human post thaw semen compared to control [25]. However, addition of both CAT and SOD together showed a significant improvement in motility because of their combined and simultaneous activity on superoxide anions and H_2O_2. Addition of 400 µg/ml CAT to the extender resulted in the highest motility for chilled semen, but it resulted in significantly lowest motility after freezing thawing process in cattle bull [26]. In another study in ovine, the enzymatic antioxidants CAT, SOD and reduced GSH in extenders containing low density lipoproteins (LDL) showed no high positive effect on sperm viability during cryopreservation. However, CAT at concentrations of 100 and 200U/mL and SOD at a concentration of 200U/mL better preserved the kinetic parameters of VCL, VSL, and VAP. Câmara., *et al*. [27] evaluated the supplementation of very low concentrations of CAT (5, 10 and 20 U/mL), SOD (5, 10and 20 U/mL) and GSH (0.5, 1.0 and 2.0 mM) in Tris-yolk extenders during cooling and freezing of ram semen compared to concentrations reported in canine, human, ovine and cattle. They found no positive effect of supplementation on sperm motility, kinetics, PMI, AI and MMP. However, 200 IU of SOD in TCFEYP extender significantly improved sperm attributes in post thaw semen of Labrador dog.

GPx did not improve the sperm attributes compared to control at post-thaw in this study (Figure 1). There is not any report on effect of GPX on frozen-thaw semen, however, the addition of SOD, CAT and GPX in the extender allowed preservation of semen quality for up to 10 days of storage at 4ºC in both fertile and hypo fertile dogs [15]. Insignificant high values in post thaw semen supplemented with GPX may be due to fact that antioxidant defence system of mammalian spermatozoa is low in GPX and GRE activity [24].

Supplementation of SOD, CAT and GSH to the extender significantly improved motility as well as viability, PMI, AI and HIMMP in the post thaw canine semen. These observations are justified by the fact that

a motile sperm should be considered viable and viable sperm must have an intact plasma membrane, as later is essential for sperm interaction with the microenvironment and other cells [28]. There is also an association between PMI and HMMP [29]. Supplementation of antioxidants SOD, GSH, and CAT to Black Bengal buck semen also resulted in a beneficial effect during cryopreservation [30]. Sakhdary., *et al*. [31] were of the opinion that SOD alone and catalase in combination with vitamin C improved the quality of cryopreserved bull epididymal spermatozoa.

Effect of supplementation of enzymatic and non-enzymatic antioxidants to the extender on lipid peroxidation, reactive oxygen species and antioxidant enzymes of post thaw spermatozoa

MDA concentration (µM/10^9 spermatozoa) was significantly ($p < 0.05$) high in GSH supplemented extender (114.5 ± 7.9) followed by CAT/GPX (79.3 ± 5.2/79.7 ± 3.0), SOD (61.5 ± 2.3) and control (46.1 ± 1.9) (Figure 2,3). Many studies have shown that the key reason for abnormal sperm functioning is LPO and antioxidant imbalance, caused due to oxidative stress [32, 33]. Oxidative stress occurring in the sperm is a phenomenon associated with increased rate of oxidation of cellular components and excessive production of ROS [32]. It is mostly said that low intensity of oxidative stress may have beneficial effects on cells [34], while its high levels can cause destruction of nucleic acids, proteins, lipids and carbohydrates, ultimately leading to the cell death [35,36]. However, LPO was higher in frozen-thawed spermatozoa supplemented with SOD, CAT, GSH and GPX compared to control. It may be predicted here that significantly low values of sperm attributes in control compared to antioxidant supplemented spermatozoa were a re-

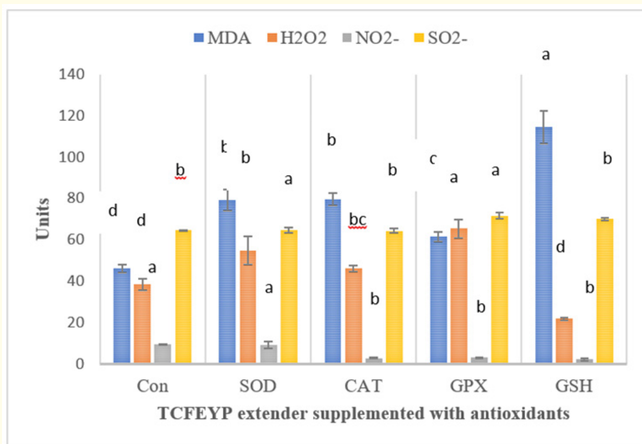

Figure 2: Effect of supplementation of enzymatic and non-enzymatic antioxidants on lipid peroxidation and reactive oxygen species of post thaw spermatozoa in Labrador dog. a, b, c indicates significant ($p < 0.05$) differences in sperm attributes among the extenders. H_2O_2/SO_2- (%), NO_2- (μM/10^9 spermatozoa).

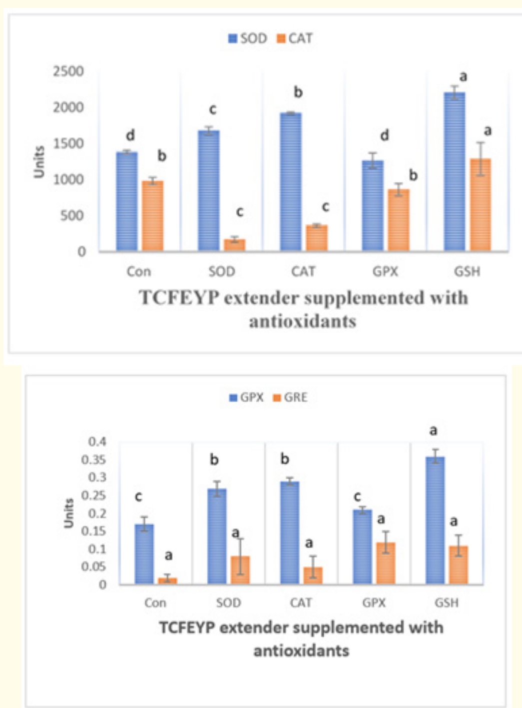

Figure 3: Effect of supplementation of enzymatic and non-enzymatic antioxidants on antioxidant enzymes of post thaw spermatozoa in Labrador dog. a, b, c indicates significant ($p < 0.05$) differences in sperm attributes among the extenders. TCFEYP-G, Tris citric acid fructose glycerol; superoxide dismutase (SOD, IU/109 spermatozoa); catalase (CAT, KU/10^9 spermatozoa); glutathione peroxidase (GPX, IU/10^9 spermatozoa; glutathione reductase (GRE, IU/10^9 spermatozoa); GSH, glutathione.

sult of imbalance between antioxidants and LPO. Therefore, antioxidant capacity of semen may be insufficient in preventing LPO during the freezing thawing process in control samples. The protective antioxidant systems in sperm are primarily of cytoplasmic origin and sperm discard most of their cytoplasm during terminal stages of differentiation [36] and main antioxidant defence system to spermatozoa is provided by seminal plasma, which is removed during cryopreservation process in canine. Hence, higher level of LPO in antioxidant supplemented spermatozoa may be a result of balance achieved between the LPO and antioxidant defence system of spermatozoa upon addition of external antioxidants. Balance of antioxidants and LPO in SOD, CAT, GSH and GPX supplemented spermatozoa generated higher motility, viability, PMI, AI and HIMMP compared to control.

Significantly ($p < 0.05$) high H_2O_2 scavenging activity of spermatozoa was observed in GPX supplemented (65.4 ± 4.5%) extender followed by SOD (55 ± 6.8%), CAT (46.2 ± 1.6%), control (38.4 ± 2.9%) and GSH (21.8 ± 0.8%). Whereas, NO_2^- concentration (μM/10^9 spermatozoa) was significantly ($p < 0.05$) less in GSH (2.2 ± 0.7), CAT (2.9 ± 1.0), GPX (3.1 ± 0.3) supplemented extenders compared to control (9.5 ± 0.2). NO_2^- concentration was even significantly ($p < 0.05$) less in GSH supplemented extender compared to CAT and GPX. SO_2^- scavenging activity of spermatozoa was significantly ($p < 0.05$) high in GSH/GPX (70.1 ± 0.7%/71.6 ± 1.5%) than SOD/CAT/control (64.7 ± 1.1%/64.4 ± 1.0%/64.7 ± 0.2%).

The generation of ROS is significantly increased in the presence of cryo-damaged spermatozoa [38]. H_20_2 appears to be the most important ROS for damage to sperm and is much more membrane permeable than the other free radicals [39]. The generation of H_20_2 is lower in semen cryopreserved in extenders containing some antioxidants, because these components may affect sperm quality by removing the H_20_2 and release into the extracellular environment [40]. NO_2^- has been shown to have detrimental effect on sperm function inhibiting motility and competence for zona binding [41]. Increased level of ROS has been correlated with decreased sperm motility [34], increased DNA sperm damage [35], sperm cellular membrane LPO [41] and decreased efficacy of oocyte-sperm fusion [43]. But physiological level of ROS is also necessary to maintain normal cell function. We observed that supplementation of SOD, CAT, GSH and GPX to the extender protected the spermatozoa from the damaging effect of SO_2^-, NO_2^- and H_20_2 during freezing-thawing process. It is demonstrated that ROS and LPO induced damage in living spermatozoa can be restricted or eliminated by the action of enzymatic and non-enzymatic antioxidants [10]. The antioxidant system comprising GSH, GPX, CAT and SOD have been described

as a defence mechanism against LPO in semen of men, dogs, stallion, ram and bulls [36, 44-46]. The impairment of antioxidant defence system of mammalian cryopreserved semen might be due to removal of high rate of dilution of seminal plasma during cryopreservation process [47], which is confirmed in stallion [44] and bull [48].

SOD, CAT, GPX and GRE activity of spermatozoa was significantly ($p < 0.05$) high in GSH supplemented extender than SOD, CAT, GPX supplemented extenders and control (Fig. 3). Spermatozoa cryopreserved in SOD and CAT supplemented extenders indicated significantly ($p < 0.05$) higher SOD activity at post-thaw compared to control. However, CAT activity in SOD/CAT supplemented extender was significantly ($p < 0.05$) less than control. GPX activity of spermatozoa was also significantly ($p < 0.05$) high in GPX/CAT supplemented extenders. It indicated that antioxidant gets absorbed to the sperm membrane and provided more protection to the spermatozoa compared to control during the freezing-thawing process. Low level of MDA concentration in control samples compared to antioxidant samples may be explained taking in account that cryopreservation impairs mitochondrial function [49]. Since the main source of ROS, are the sperm mitochondria. If the activity of mitochondria is reduced, the production of molecular oxygen also is reduced, and thus at the same time less SO_2^- anion is produced. Moreover, HIMMP was significantly high in antioxidant supplemented post-thaw semen. This factor may explain an increase in LPO/SO_2^- ions/H_2O_2 scavenging activity and low level of nitric oxide ions after thawing in antioxidant supplemented frozen thaw semen compared to control.

Comparison of antioxidants further revealed that motility, viability, PMI, AI and HIMMP were significantly high ($P < 0.05$) in the GSH supplemented extender than in SOD and CAT supplemented extenders. It is one of the major endogenous antioxidants produced by cells participating directly in the neutralization of free radicals and ROS, as well as maintaining exogenous antioxidants such as vitamins C and E in their reduced forms [50]. It protects the plasma membrane from LPO, scavenges SO_2^- and prevents oxygen formation. The glutathione/reductase system forms an excellent protection against the LPO of the sperm plasma membrane. GRE stimulates the reduction of glutathione disulphide, to reduced GSH, thereby recycling it. The protective action of GSH against ROS is facilitated by the interactions with its associated enzymes, such as GPx and GRE. In the body, the antioxidant defence capability consists of enzymatic and non-enzymatic systems, in which the latter is represented mainly by GSH [51]. Therefore, higher effect of GSH compared to enzymatic antioxidants on post thaw semen quality of canine semen justified it as a powerful non-enzymatic antioxidant.

Conclusion

These observations revealed that supplementation of antioxidants maintained an optimum level of ROS and antioxidant enzymes, which correspondingly resulted in less cryoinjury to the spermatozoa and higher values of sperm attributes were achieved at post thaw compared to control. However, cryoinjury to the spermatozoa was protected to a maximum level in GSH supplemented extender followed by CAT, SOD and GPX. Study concluded that GSH is superior over antioxidant enzymes in protecting the spermatozoa from cryoinjury. Based on these observations, higher fertility rate is expected of canine semen cryopreserved in GSH supplemented TCFEYP extender compared to TCFEYP.

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Conflict of Interest

None of the authors have any conflict of interest to declare.

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