



Supplementation of Enzymatic and Non-enzymatic Antioxidants to the Extender improves Sperm Functionality during Storage at 4°C in Labrador Dog

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ABSTRACT

Several extenders to preserve fertilizing capacity of preserved canine semen have been successfully tested, but further studies are requisite to improve its quality. Effect of supplementation of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione (GSH) to Tris-citric acid-fructose-egg yolk extender on Labrador dog sperm survival during storage at 4° was evaluated. Different concentrations per antioxidant i.e. SOD (50-300 IU/ml), GPX (1.5-2.5 IU/ml), CAT (100-400 µg/ml) and GSH (2.5-10 µM/ml) were evaluated to look for an optimum dose. Semen was analyzed for motility, viability and plasma membrane integrity (PMI) after every 24 hrs till 72 hrs of preservation. Semen was also analyzed for acrosome integrity (AI) at 0, 72 hrs and lipid peroxidation at 72 hrs of storage. Values for motility, viability, PMI, AI were significantly ($p < 0.05$) higher in the presence of 200 IU/ml SOD, 2 IU/ml GPX, 200 µg/ml CAT and 7.5 µM/ml GSH compared to control and other tested doses. MDA concentration was non-significantly ($p > 0.05$) less in the presence of all doses of SOD and 2.0 IU GPX compared to control at 72 hrs of preservation. MDA concentration in the presence of 200 µg/ml catalase and 7.5 µM/ml GSH was non-significantly ($p > 0.05$) higher than control. It was concluded that SOD, catalase, GPX and GSH at a concentration of 200 IU, 2.0 IU, 200µg and 7.5 µM per ml were optimum concentrations to be supplemented to the extender for positive effect. Supplementation of antioxidants could improve sperm attributes by maintaining proportionate level of oxidative stress during preservation of Labrador dog semen at 4° for 72 hrs.

HIGHLIGHTS

- Effect of supplementation of different doses of antioxidants was observed on semen during preservation at 4°C.
- SOD, catalase, GPX and GSH at a concentration of 200 IU, 2.0 IU, 200µg and 7.5 µM per ml were optimum concentrations to be supplemented to the extender for positive effect

Keywords: Antioxidants, preservation, TEY extender, 4°C, Labrador dog semen

Artificial insemination (AI) with fresh semen in canines presents pregnancy rates almost similar to natural mating. The use of chilled or cryopreserved semen leads to the reduction in fertility rate (Yu *et al.*, 2002). It was verified that AI done with cryopreserved semen in dogs results in 30% reduced pregnancy rate and 31% lower litter size, compared to inseminations with fresh semen (Linde-Forsberg and Forsberg, 1993). The metabolic activities of spermatozoa during storage need to be reduced by using an appropriate medium and storage temperature. Several extenders to preserve fertilizing capacity of canine

semen have been successfully tested, but further studies are requisite to improve the quality of preserved semen (Kasimanickam *et al.*, 2012). There is little available information about the molecular mechanisms leading to death of spermatozoa during cooling, and apoptosis may be the reason (Tapia *et al.*, 2012). Sperm survival during

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storage at 4°C depends on oxygen consumption and metabolism, which increase the amount of reactive oxygen species (Agarwal *et al.*, 2003). Low ROS amounts are required for fertilizing capabilities, as they are involved in kinetic function, sperm capacitation, hyperactivation and fusion of the oocyte with the spermatozoa (Rota *et al.*, 1995). Excessive ROS production can damage sperm function, sperm– oocyte fusion, and impair fertilization.

ROS concentration is controlled by antioxidants of the seminal fluid and inside the sperm cell, thereby avoiding ROS overload and preserving semen quality (Michael *et al.*, 2003; Tapia *et al.*, 2012). Antioxidant systems consist of enzymatic antioxidants, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and non-enzymatic antioxidants [glutathione (GSH), amino acids, vitamin A, C, E, and uric acid. A study on dogs confirmed the presence of endogenous antioxidants in the seminal plasma of pre-spermiatic, spermiatic, and post-spermiatic fractions, with SOD representing the major enzymatic antioxidant in all dog ejaculate fractions, whereas GPX activity was present in the sperm-rich and post-spermiatic fractions, and CAT activity was deficient (Strzeżek *et al.*, 2009). CAT activity in dog ejaculates is demonstrated and the addition of SOD and CAT in the dilution extender of canine semen improved sperm quality (Kawakami *et al.*, 2007). In canine semen cryopreservation, seminal plasma is removed by centrifugation (Sirivaidyapong *et al.*, 2001) and hence there is elimination of natural antioxidants (Strzeżek *et al.*, 2009) and the sparse cytoplasmic volume in the sperm cell mid-piece limits its antioxidant capacity (Aitken *et al.*, 1996). Antioxidants supplementation to the extender allows the improvement of sperm quality after thawing and higher sperm longevity, which is considered an important indicator to the successful cryopreservation in dogs (Monteiro *et al.*, 2009). Lucio *et al.*, 2016) observed a higher acrosome protection and higher pregnant bitches with sperm cryopreserved with 10 mM of reduced glutathione (GSH). Hence, the aim of this study was to investigate the effect of supplementation of SOD, CAT, GPX and GSH to Tris-citric acid-fructose-egg yolk extender on Labrador dog sperm survival during storage at 4°C.

MATERIALS AND METHODS

Experimental design

Antioxidants were purchased from Sigma-Aldrich Company. Study was conducted in two phases. Effect of supplementation of SOD, GPx, CAT and GSH to TEY buffer was observed on Labrador dog semen stored at 4°C from 0-72 hrs. Different concentrations per antioxidant i.e. SOD (50-300 IU/ml), GPx (1.5-2.5 IU/ml), CAT (100-400 µg/ml) and GSH (2.5-10 µM/ml) were evaluated to look for an optimum dose.

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. Six Labrador dogs were maintained in individual pens. They were daily fed 500 g cooked feed twice daily and water provided ad libitum. Dogs were given regular exercise in the form of walking / running for one hour daily in the morning and evening. Dogs were dewormed and vaccinated for rabies, CDV, CAV2, CPV, CPI and CAV1. Semen was collected by message method twice a week.

Preparation of extenders for extension of semen

Tris - citric acid-fructose buffer (Tris, 3.08 g; citric acid, 1.78 g; fructose, 1.25 g /100 ml, pH 7.2, gentamycin, 5 mg) was prepared and autoclaved at 15 lb for 15 min. Buffer was filtered through 0.2-micron membrane filter. Egg yolk and gentamycin at a concentration of 15 % and 50µg/ml were added to the buffer on the day of experiment, again filtered through 0.2-micron membrane filter and kept at 37°C (TEY extender).

Processing of semen for storage at 4°C

Semen of dogs exhibiting >70 % motility was pooled for each trial and divided into required aliquots. To each aliquot SOD (50,100,200 and 300 IU/ml), GPX (1.5, 2.0, 2.5 µM/ml), CAT (100, 200, 300, 400 IU/ml) and GSH (2.5, 5.0, 7.5 and 10 µM/ml) were added and incubated at 37°C for 10 min. To each aliquot, extender was added and semen-extender mixture was centrifuged at 960 g 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 evaluated for motility, viability, membrane and acrosome integrity. Samples in tubes were shifted to a container containing warm water (37°C) and placed in a cooling cabinet at 4°C. Semen was analyzed for motility, viability and membrane integrity after every 24 hrs till 72 hrs of storage. Semen was also analyzed for acrosome integrity at 0, 72 hrs and lipid peroxidation at 72 hrs of storage.

Evaluation of semen

Motility was evaluated by wet mount and track method. Viability was assessed by staining the spermatozoa with syber green-propidium iodide stain kit (Sigma). Hypo-osmotic swelling test (HOST) was performed to analyze the integrity of sperm membrane (Jeyendran *et al.*, 1984). 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, sperm smears prepared on clean glass slides were stained with spermac stain kit (Minitube) as per manufacturer's instructions. About 200 spermatozoa were counted in different fields for all sperm attributes and percentage motility, viability, plasma membrane integrity and acrosome integrity were calculated.

Malondialdehyde concentration (end product of LPO) was estimated by the method of Buege *et al.*, 1978). Briefly, semen was washed twice with PBS, pH 7.4 by centrifugation at 865 g for 5 min. Sperm pellet was suspended in 0.2 ml PBS, mixed with 0.1 ml of 150 mM Tris HCl (pH 7.1) and incubated at 37 °C for 20 min. After incubation, 0.5 ml 10% TCA and 1.0 ml 0.375% TBA were added and kept for 20 min in the boiling water bath. Thereafter, mixture was centrifuged for 15 min at 2500 g and absorbance of supernatant was taken at 532 nm. The molar extinction coefficient for MDA was calculated as below:

MDA content (units) =

$$\frac{\text{OD} \times \text{volume of assay mixture}}{\text{Volume of sample taken} \times \text{coefficient extinction}}$$

Statistical Analysis

Significant differences (5% level) among the extenders 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 antioxidants on freshly extended semen

Treatment of fresh semen with different concentrations of SOD and GPX at 37° for 10 min did not show significant difference in sperm attributes of freshly extended semen. However, treatment with 200 µg/ml catalase and 7.5µM/ml GSH exhibited significantly higher values for sperm attributes in freshly extended semen compared to control and other tested doses (Table 1).

Effect of Superoxide dismutase on storage of semen at 4°C

Preservation of semen in TEY extender supplemented with 100 and 200 IU SOD revealed significantly higher values of individual motility, viability and PMI at 24 hrs, 48 hrs and 72 hrs compared to control and 300 IU (Table 1). Values for motility, viability and PMI were non-significantly ($p > 0.05$) higher in extender supplemented with 200 IU SOD than 100 IU during storage of semen at 4°C from 24-72 hrs. Acrosome integrity was non-significantly different among the freshly extended - control and SOD treated semen. But it was significantly ($p < 0.05$) higher in the presence of 200 IU SOD than control and other concentrations at 72 hrs of preservation. MDA production ($\mu\text{M}/10^9$ spermatozoa) was non-significantly ($p > 0.05$) higher in control than SOD treated semen samples at 72 hrs of preservation.

**Table 1:** Effect of supplementation of superoxide dismutase (100-300 IU/ml) to TEY extender on sperm attributes during storage of Labrador dog semen at 4°C for 72 hrs

Sperm parameters	0 Hr ^A				24 Hrs ^B				48 Hrs ^C				72 Hrs ^D			
	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300
MOT (%)	80.0 ^b ± 0.0	86.7 ^{ab} ± 1.7	85.5 ^a ± 2.9	80.4 ^b ± 2.9	68.3 ^b ± 1.7	75.0 ^b ± 0.0	78.7 ^a ± 1.3	71.7 ^b ± 63.3 ^b 3.3 ± 4.4	68.3 ^b ± 1.7	71.7 ^a ± 3.3	65.5 ^b ± 5.0	56.7 ^b ± 3.3	56.7 ^b ± 61.7 ^a 4.4 ± 6.0	56.7 ^b ± 61.7 ^a 4.4 ± 6.0	56.7 ^b ± 61.7 ^a 4.4 ± 6.0	56.7 ^b ± 61.7 ^a 4.4 ± 6.0
VIA (%)	86.0 ^{ab} ± 0.9	92.2 ^a ± 1.3 ± 5.5	90.0 ^{ab} ± 4.8	86.5 ^b ± 2.9	74.0 ^b ± 0.5	81.6 ^a ± 6.2	83.9 ^a ± 3.3	77.0 ^{ab} ± 4.0	69.2 ^b ± 1.3	75.4 ^a ± 3.9	77.5 ^a ± 5.2	71.6 ^b ± 2.2	63.2 ^b ± 2.2	66.9 ^{ab} ± 2.2	71.8 ^a ± 3.3	64.9 ^{ab} ± 1.9
PMI (%)	75.8 ^b ± 1.5	82.0 ^a ± 1.5 ± 3.3	82.4 ^a ± 3.0	79.7 ^a ± 3.0	70.0 ^b ± 2.2	76.1 ^{ab} ± 2.2	79.2 ^a ± 1.5	73.1 ^{ab} ± 2.8	63.5 ^b ± 3.1	71.3 ^a ± 1.3	74.1 ^a ± 3.8	65.7 ^b ± 5.1	58.4 ^b ± 1.2	61.4 ^a ± 65.3 ^a 0.1 ± 4.9	65.3 ^a ± 4.9	56.9 ^b ± 5.9 ± 3.3
AI (%)	76.3 ^a ± 1.5	78.7 ^a ± 2.0 ± 3.9	83.6 ^a ± 3.7	77.6 ^a ± 3.7									64.9 ^b ± 1.7	68.3 ^{ab} ± 2.2	74.7 ^a ± 3.4	67.1 ^{ab} ± 3.3
MDA (µM/10 ⁹ sperms)													46.6 ^a ± 33.6 ^a 21.9	35.2 ^a ± 34.6 ^a 9.3	34.6 ^a ± 34.6 ^a 12.1	34.6 ^a ± 34.6 ^a 11.2

Superscripts a, b indicates significance (p<0.05) differences among the treatments; Superscripts A, B, C, D indicate differences among the storage periods.

Table 2: Effect of supplementation of glutathione peroxidase (1.5-2.5 IU/ml) to TEY extender on sperm attributes during storage of Labrador dog semen at 4°C for 72 hrs

Sperm parameters	0 Hr ^A				24 Hrs ^B				48 Hrs ^C				72 Hrs ^D			
	0	1.5	2.0	2.5	0	1.5	2.0	2.5	0	1.5	2.0	2.5	0	1.5	2.0	2.5
MOT (%)	80.0 ^a ± 0.0	81.0 ^a ± 1.7	83.3 ^a ± 1.7	80.0 ^a ± 0.0	75.0 ^b ± 0.0	79.0 ± 1.0	78.3 ^a ± 1.7	75.0 ^b ± 1.9	70.0 ^b ± 0.0	73.3 ^{ab} ± 2.9	76.7 ^a ± 1.7	71.7 ^b ± 1.7	63.4 ^c ± 1.7	66.7 ^b ± 3.3	71.7 ^a ± 1.7	63.3 ^c ± 1.7
VIA (%)	86.1 ^a ± 0.6	86.8 ^a ± 0.6	88.7 ^a ± 0.6	87.3 ^a ± 0.3	80.6 ^b ± 1.5	83.9 ^{ab} ± 1.2	84.1 ^a ± 1.9	81.3 ^{ab} ± 2.5	75.5 ^c ± 2.2	79.4 ^b ± 1.5	82.4 ^a ± 1.6	77.5 ^b ± 2.5	68.7 ^c ± 1.2	72.9 ^b ± 2.0	76.6 ^a ± 2.2	68.8 ^c ± 2.6
PMI (%)	84.9 ^a ± 0.4	86.4 ^a ± 0.7	88.0 ^a ± 1.3	85.3 ^a ± 0.9	80.0 ^b ± 0.8	81.9 ^{ab} ± 0.7	83.4 ^a ± 2.7	80.8 ^b ± 3.1	76.0 ^b ± 2.5	79.5 ^a ± 0.1	81.6 ^a ± 1.3	73.2 ^b ± 2.0	69.9 ^c ± 2.8	72.4 ^b ± 2.9	76.4 ^a ± 2.3	70.4 ^c ± 2.5
AI (%)	77.0 ^b ± 1.2	82.1 ^a ± 0.9	85.3 ^a ± 0.9	81.8 ^a ± 1.0									68.0 ^c ± 1.2	74.1 ^{ab} ± 1.3	77.0 ^a ± 1.2	72.4 ^b ± 1.2
MDA (µM/10 ⁹ sperms)													23.8 ^a ± 3.6	24.1 ^a ± 4.3	18.1 ^b ± 3.1	22.0 ^a ± 2.8

Superscripts a, b indicate significance (p<0.05) differences among the treatments; Superscripts A, B, C, D indicate differences among the storage periods.

Effect of Glutathione peroxidase on storage of semen at 4°C

The values for motility, viability and plasma membrane integrity were significantly (p<0.05) high in TEY extender supplemented with 1.5 and 2.0 IU GPX / ml compared to control and other doses at 24 hrs of preservation (Table 2). However, these values remained significantly (p<0.05) high in TEY extender supplemented with 2.0 IU GPX / ml at 48 and 72 hrs of preservation. Acrosome integrity

was also significantly (p<0.05) high in 2.0 IU GPX / ml supplementation at 72 hrs of preservation. Corresponding to sperm attributes, MDA concentration was significantly (p<0.05) less in the presence of 2.0 IU GPX / ml compared to control and other doses.

Effect of Catalase on storage of semen at 4°C

Supplementation of TEY extender with 200 µg/ml catalase resulted in significantly (p<0.05) higher motility, viability

and plasma membrane integrity compared to control and other doses at 24, 48 and 72hrs of preservation (Table 3). Acrosome integrity also remained significantly ($p<0.05$) higher in 200 $\mu\text{g/ml}$ dose compared to control and other doses. Surprisingly, MDA concentration in the presence of 200 $\mu\text{g/ml}$ catalase was non-significantly ($p>0.05$) higher than control and 100 $\mu\text{g/ml}$ and lower than 300 $\mu\text{g/ml}$.

Effect of Glutathione on storage of semen at 4°C

Values for motility, viability and plasma membrane integrity were significantly ($p<0.05$) high in extender supplemented with 7.5 $\mu\text{M/ml}$ GSH compared to control and other doses from 24 – 72 hrs of preservation (Table 4). Acrosome integrity was significantly ($p<0.05$) higher in 7.5 $\mu\text{M/ml}$ GSH compared to control. But difference between different doses of GSH was non-significant ($p>0.05$). Similar to catalase supplementation, MDA concentration was non-significantly ($p<0.05$) high in the presence of 7.5 $\mu\text{M/ml}$ GSH compared to control and other doses.

A gradual decline in sperm attributes was noticed with increase in time of preservation irrespective of the antioxidant treatment. This observation supported previous studies on preservation of chilled dog spermatozoa in extenders (Iguer-ouada and Verstegen, 2001; Thiangtum *et al.*, 2012). Similar to our observations, higher viability

compared to motility was observed during preservation of semen at 5°C (Thiangtum *et al.*, 2012). Moreover, plasma membrane integrity and acrosome integrity were also higher than motility at 72 hrs of storage. Motility of Beagle dog spermatozoa was maintained up to 11 days by exchanging the old extender with fresh one (Verstegen *et al.*, 2005). It was also suggested that viable spermatozoa may be reactivated by medium supplementation *in vitro* or *in vivo* when interaction with the vagino-utero-oviductal secretion occurs. But semen was preserved in the same extender up to 72 hrs of preservation in the present study. Therefore, decrease in sperm attributes with increasing storage period may be due to utilization of energy by the highly active spermatozoa and change in pH and osmolarity of extender. Reduction in motility with increasing storage period was attributed to the consumption of glucose, decrease in pH and metabolite accumulation (Rota *et al.*, 1995; Verstegen *et al.*, 2005). Many other factors may also influence the deterioration of chilled semen quality especially the lipid peroxidation due to oxidative stress during sperm storage in cooling condition.

It may be inferred that SOD, GPX, catalase and GSH acted in a dose dependent manner and 200 IU SOD, 2.0 IU GPX, 200 μg catalase and 7.5 μM GSH per ml semen were optimum doses for preservation of Labrador dog semen at 4°C. Although motility, viability, plasma membrane integrity and acrosome integrity declined gradually in

Table 3: Effect of supplementation of Catalase ($\mu\text{g/ml}$) to TEY extender on sperm attributes during storage of Labrador dog semen at 4°C for 72 hrs.

Sperm Parameter	0 Hr ^A				24 Hrs ^B				48 Hrs ^C				72 Hrs ^D			
	0	100	200	300	0	100	200	300	0	100	200	300	0	100	200	300
MOT (%)	75.0 ^b ± 0.0	75.0 ^b ± 5.8	83.3 ^a ± 3.3	70.0 ^b ± 2.9	70.0 ^b ± 0.0	70.0 ^b ± 5.8	78.3 ^a ± 3.3	65.7 ^c ± 2.3	61.7 ^b ± 0.7	66.7 ^b ± 3.3	70.0 ^a ± 2.9	57.3 ^c ± 3.7	56.7 ^b ± 1.7	56.6 ^b ± 8.3	65.7 ^a ± 3.5	43.3 ^c ± 7.3
VIA (%)	83.9 ^b ± 2.2	81.2 ^b ± 2.9	88.3 ^a ± 4.6	78.5 ^c ± 1.5	74.1 ^b ± 2.3	72.5 ^b ± 2.5	79.6 ^a ± 4.1	70.4 ^b ± 0.9	68.8 ^b ± 2.2	70.0 ^{ab} ± 3.3	75.1 ^a ± 3.0	64.9 ^c ± 1.2	64.0 ^b ± 3.9	64.0 ^b ± 3.9	70.3 ^a ± 3.2	55.1 ^c ± 1.9
PMI (%)	85.1 ^b ± 2.1	80.0 ^b ± 3.0	89.7 ^a ± 0.6	80.4 ^b ± 1.2	75.0 ^b ± 1.2	70.5 ^b ± 3.7	81.6 ^a ± 1.1	70.2 ^b ± 1.4	70.6 ^b ± 2.9	69.0 ^b ± 7.1	76.5 ^a ± 3.7	58.3 ^c ± 5.1	64.9 ^b ± 4.4	61.7 ^b ± 10.7	74.1 ^a ± 3.0	47.7 ^d ± 8.9
AI (%)	69.5 ^a ± 6.6	74.9 ^a ± 11.4	84.8 ^a ± 6.8	68.7 ^a ± 2.8	—	—	—	—	—	—	—	—	59.8 ^a ± 6.6	65.4 ^a ± 12	73.9 ^a ± 12.0	58.7 ^a ± 2.4
MDA ($\mu\text{M}/10^9$ sperms)	—	—	—	—	—	—	—	—	—	—	—	—	35.4 ^a ± 10.1	46.2 ^a ± 12.7	66.6 ^a ± 25.6	77.4 ^a ± 31.6

Superscripts a, b indicate significance ($p<0.05$) differences among the treatments; Superscripts A, B, C, D indicate differences among the storage periods.

Table 4: Effect of supplementation of Glutathione (mM/ml) to TEY extender on sperm attributes during storage of Labrador dog semen at 4°C for 72 hrs

Sperm parameters	0 Hrs ^A				24 Hrs ^B				48 Hrs ^C				72 Hrs ^D			
	0	5.0	7.5	10.0	0	5.0	7.5	10.0	0	5.0	7.5	10.0	0	5.0	7.5	10.0
MOT (%)	73.3 ^{ab} ± 4.4	76.7 ^{ab±} 1.7 ± 0.0	80.0 ^a 3.3	71.7 ^{b ±} 0.0	65.0 ^{b ±} ± 2.9	60.0 ^b ± 1.7	73.3 ^a 2.9	60.0 ^{b ±} 1.7 ± 1.7	56.7 ^{b ±} ± 0.0	56.7 ^b ± 6.0	65.0 ^a ± 1.7	56.7 ^b ± 1.7	48.3 ^b 0.0	50.0 ^{b ±} ± 1.7	60.3 ^a ± 5.7	46.7 ^b ± 5.7
VIA (%)	80.0 ^{ab} ± 3.9	81.2 ^{ab} ± 1.9	84.6 ^a ± 0.9	78.5 ^{b ±} 5.1 ± 2.9	70.4 ^{ab} ± 1.7	65.9 ^b ± 0.9	78.3 ^a ± 6.2	67.8 ^{ab} ± 0.4	61.9 ^{ab} ± 2.4	60.4 ^b ± 1.0	70.8 ^a ± 7.2	62.5 ^{ab} ± 1.2	53.8 ^b ± 1.2	54.7 ^{b ±} 3.1 ± 2.2	62.8 ^a ± 4.5	53.5 ^b ± 4.5
PMI (%)	67.6 ^{b ±} 1.0	65.6 ^{b ±} 3.2 ± 3.0	76.0 ^a 3.8	52.4 ^{b ±} 1.9 ± 3.1	63.3 ^{b ±} ± 1.6	59.9 ^b 7.9	70.8 ^a 2.7	59.2 ^{b ±} ± 2.0	55.3 ^{b ±} ± 1.1	52.7 ^b ± 2.2	63.7 ^a ± 3.3	55.3 ^b ± 3.3	45.9 ^b 2.8	47.8 ^{b ±} ± 7.7	61.4 ^a ± 6.0	49.0 ^b ± 6.0
AI (%)	70.0 ^{b ±} 4.7	69.2 ^{b ±} 1.4	79.5 ^a ± 4.1	74.7 ^{b ±} 4.9									57.5 ^b ± 5.0	61.3 ^{ab} ± 2.3	68.9 ^a ± 5.1	59.2 ^{ab} ± 3.3
MDA (µM/10 ⁹ sperms)													52.7 ^a ± 26.7	55.9 ^a ± 29.3	63.5 ^a ± 23.4	50.5 ^a ± 33.6

Superscripts a, b indicate significance (p<0.05) differences among the treatments; Superscripts A, B, C, D indicate differences among the storage peri.

the presence of optimum dose of antioxidants, but still remained > 60% at 72 hrs of preservation. Contrary to our observations, extender containing 100, 400 and 1,600 U/ml of CAT or SOD or the combination did not enhance sperm motility, viability and acrosomal integrity during storage at 5°C (Thiangtum *et al.*, 2012). Comparable to our observations in dog, supplementing CAT and SOD and their combination to extender resulted in positive effect on chilled semen and during storage at 5°C in ram (Maxwell and Stojanov, 1996). Addition of CAT did not affect the maintenance of sperm motility during storage at 5°C in equine (Aurich *et al.*, 1997; Ball *et al.*, 2001). The addition of CAT to Tris-Lecithin extender did not improve even chilled dog semen (Beccaglia *et al.*, 2009), but moderate benefit was reported when catalase at 100 U/ml was added to Tris-glucose egg yolk (Michael *et al.*, 2009). The addition of CAT (100 and 200 U/mL) reduced the deleterious effects of cooling on total motility in ram sperm maintained at 5°C for 24 h, although it did not affect the functionality of the sperm membranes (Câmara *et al.*, 2011).

Addition of GSH at a concentration of 7.5 µM/ml was an optimum dose in improving sperm attributes compared to control during storage of Labrador dog semen at 4°C. Contrary to this observation, supplementation of the extender with 5 mM GSH improved the chilled and frozen-thaw semen quality (Angrimani *et al.*, 2018; Monteiro *et al.*, 2009). (Strezezek *et al.*, 2009) reported

that the physiologically normal GSH concentration in canine semen was closer to 5 mM, which is recommended for supplementation of semen extender/freezing solution. Variation in optimum dose of GSH had been reported in other species, as GSH @ 2.5 mM, 1 mM and 0.5 mM could preserve sperm quality of equine (de Oliveira *et al.*, 2013), boar (Zhang *et al.*, 2013) and bull (Tuncer *et al.*, 2010) during storage of semen at 4°C, respectively.

Among the three concentrations of GPX tested, only 2 IU/ml supplementation to the extender could improve the sperm attributes in Labrador dogs. But, Del Prete *et al.* (2018) supplemented 15 IU of GPX and observed an improvement in DNA integrity during storage of semen at 4°C in fertile and hypo-fertile dogs.

It may be inferred from these studies that the different species respond variably to the antioxidants. Moreover, influence of environment including temperature, extenders and endogenous antioxidants may also be possible reasons behind variation. Different concentration of antioxidants should be evaluated for a species under investigation as per the conditions of particular lab, as optimum dose for one species may not be effective or may be toxic for the other species due to the change in physiological condition of semen extender. We also observed higher doses of antioxidants as toxic to dog spermatozoa. Addition of 400 mM and 10 mM GSH caused negative effects on ram (Câmara *et al.*, 2011) and canine sperm (Ogata *et al.*, 2015). In ram, survival of spermatozoa will increase when

the dosage of SOD and CAT added to extender increases. However, CAT at dosage higher than 200 U/ml was toxic to ram spermatozoa (Maxwell and Stojanov, 1996).

There was no significant ($p < 0.05$) difference in MDA concentration in control and antioxidant treated samples except GPX. Rather MDA concentration was non-significantly high in the presence of optimum catalase and GSH concentration. The addition of catalase in semen extenders resulted in significantly ($p < 0.0005$) lower total ROS values compared with the controls in canine (Michael *et al.*, 2009). GSH supplementation at a concentration of 5mM and 1mM inhibited lipid peroxidation levels in post-thaw canine spermatozoa (Ogata *et al.*, 2015) and beetal buck semen storage at 4°C (Sarangi *et al.*, 2017). But, similar to our observations, MDA content of GSH treated groups was higher than that of the control group during storage at 4°C (Zhang *et al.*, 2016). In spite of higher MDA content viz a viz lipid peroxidation in semen samples supplemented with optimum antioxidants concentration, the sperm attributes were higher compared to control and other doses.

In the dog, seminal fluid has been found to be deleterious for the *in vitro* preservation of sperm quality (Rota *et al.*, 1995). It is also indicated that its presence before semen cryopreservation is also detrimental to sperm quality after thawing (Sirivaidyapong *et al.*, 2001; Nizański, 2006). Therefore, seminal plasma was removed for processing, extension and preservation of semen at 4°C and main disadvantage of this method is the elimination of natural antioxidants from seminal plasma (Strzeżek *et al.*, 2009). Semen has enzymatic (SOD, catalase and GPx) and non-enzymatic antioxidants (Michael *et al.*, 2009). Canine spermatozoa contain antioxidant enzymes, such as SOD and GPX for protection against the cytotoxic effects of internally generated ROS (Strzeżek *et al.*, 2009). However, the sparse cytoplasmic volume in the sperm cell mid-piece limits its antioxidant capacity (Aitken *et al.*, 1996). Moreover, intracellular antioxidant enzymes do not protect acrosomal and plasma membrane, so this must be protected by seminal plasma (Iwasaki and Gagnon 1992; Zini *et al.*, 2002). ROS are produced by sperm in physiological conditions and play an important role on mammal sperm function (Rota *et al.*, 1995; Beccaglia *et al.*, 2009). ROS in low concentrations are necessary to achieve sperm fertilizing capacity, as they are involved in sperm kinetic,

capacitation, hyper activation and fusion of the oocyte with the spermatozoa (Rota *et al.*, 1995). Free radical concentration is controlled by the antioxidants present in seminal plasma and spermatozoa. The contribution of each antioxidant system varies among species and it is believed that there is a synergistic action between them (Michael *et al.*, 2009). Antioxidants can act by inactivating free radicals (GSH, SOD, catalase, GPX and vitamin E) or repairing the oxidative stress lesions (Ferreira and Matsubara, 1997, ascorbate, glutathione reductase – GR and GPX). In the absence of seminal plasma, free radical concentration was maintained by supplemented antioxidants. Therefore, higher concentration of MDA and higher values of sperm attributes in the presence of optimum dose of antioxidants compared to control reveals that accomplished level of oxidative stress in preserved semen was not disproportionate and toxic to the spermatozoa. Otherwise, excessive production of free radical is responsible for alterations in semen quality, caused by decrease in sperm motility, as well as protein and DNA damage, resulting in cell apoptosis (Griveau and Le Lannou, 1997; Lucio *et al.*, 2016). It may be revealed that supplementation of optimum dose of antioxidants to the extender fulfilled the function of seminal plasma antioxidants and resulted in boosted sperm attributes in preserved semen.

CONCLUSION

It was concluded that 200 IU SOD, 2.0 IU catalase, 200µg GPX and 7.5 µM GSH per ml were optimum concentrations to be supplemented to the extender for positive effect. Supplementation of enzymatic and non-enzymatic antioxidants could improve sperm attributes by maintaining proportionate level of oxidative stress during preservation of Labrador dog semen at 4°C for 72 hrs. Artificial insemination using liquid semen supplemented with antioxidants and exhibiting enhanced sperm attributes will definitely results in achieving higher fertility rate.

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