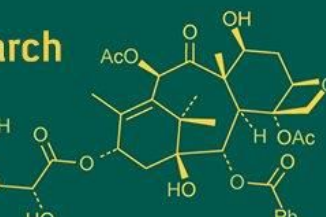
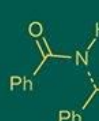


International Journal of Advanced Biochemistry Research



ISSN Print: 2617-4693
ISSN Online: 2617-4707
NAAS Rating (2025): 5.29
IJABR 2025; 9(12): 1237-1241
www.biochemjournal.com
Received: 05-10-2025
Accepted: 11-11-2025

MV Gopala Krishna
Assistant Professor, Veterinary
Gynaecology & Obstetrics,
College of Veterinary Science,
Korutla, PVNR Telangana
Veterinary University,
Telangana, India

K Venkata Ramana
Professor, Veterinary
Gynaecology & Obstetrics,
College of Veterinary Science,
Mamnoon, PVNR Telangana
Veterinary University,
Telangana, India

E Sunil Anand Kumar
Professor, Veterinary
Gynaecology & Obstetrics,
College of Veterinary Science,
Korutla, PVNR Telangana
Veterinary University,
Telangana, India

K Padmaja
Professor and HOD, Dept. of
Veterinary Medicine, College of
Veterinary Science,
Rajendranagar, PVNR
Telangana Veterinary
University, Telangana, India

CH Priyadarshini
Principal, Animal Husbandry
Polytechnic, Mahabubnagar,
PVNR Telangana Veterinary
University, Telangana, India

Corresponding Author:
MV Gopala Krishna
Assistant Professor, Veterinary
Gynaecology & Obstetrics,
College of Veterinary Science,
Korutla, PVNR Telangana
Veterinary University,
Telangana, India

Effect of antioxidant supplementation on biochemical parameters of Deccani Ram semen preserved at refrigeration temperature

MV Gopala Krishna, K Venkata Ramana, E Sunil Anand Kumar, K Padmaja and CH Priyadarshini

DOI: <https://www.doi.org/10.33545/26174693.2025.v9.i12o.7055>

Abstract

Liquid preservation of ram semen at refrigeration temperature (5 °C) is commonly practiced in artificial insemination programs; however, oxidative stress during storage leads to deterioration of sperm quality. The present study was undertaken to evaluate the effect of antioxidant supplementation on lipid peroxidation and antioxidant defense mechanisms in Deccani ram semen stored at 5 °C for up to 72 h. A total of 60 ejaculates collected from six healthy Deccani rams were pooled, diluted with egg yolk citrate extender, and divided into four groups: catalase (3 mM/mL), melatonin (3 mM/mL), methionine (4 mM/mL), and a control without antioxidant. Semen samples were evaluated at 0, 24, 48, and 72 h of storage for malondialdehyde (MDA) levels, glutathione peroxidase (GSH) activity, and total antioxidant capacity (TAC).

Results revealed a significant ($P < 0.05$) increase in MDA levels and a concomitant decline in GSH activity and TAC with increasing storage duration across all groups. Antioxidant supplementation significantly mitigated oxidative stress compared to the control. Among the antioxidants tested, methionine supplementation resulted in the lowest MDA levels and the highest GSH activity and TAC at all storage intervals. Overall mean MDA was lowest in the methionine group (30.82 ± 1.03 nmol/mL), while GSH activity (73.20 ± 0.90 IU/mL) and TAC (146.81 ± 0.80 mmol/g) were significantly higher than those in catalase, melatonin, and control groups. The control group consistently exhibited the highest lipid peroxidation and lowest antioxidant status.

In conclusion, liquid storage of Deccani ram semen at 5 °C induces oxidative stress, adversely affecting semen quality. Antioxidant supplementation, particularly methionine, effectively reduces lipid peroxidation and enhances antioxidant defenses, thereby improving the biochemical quality of semen during refrigerated storage.

Keywords: Ram semen, oxidative stress, antioxidants, methionine, malondialdehyde

Introduction

Liquid preservation of ram semen at refrigeration temperature (5 °C) is widely employed in artificial insemination programs due to its simplicity, cost-effectiveness, and suitability for short-term semen transport and use. This technique plays a vital role in genetic improvement and conservation of indigenous sheep breeds such as the Deccani ram. However, despite its practical advantages, prolonged liquid storage is associated with a progressive decline in semen quality, primarily attributed to oxidative stress-induced cellular damage.

During low-temperature storage, spermatozoa are exposed to an imbalance between the production of reactive oxygen species (ROS) and the antioxidant defense mechanisms of seminal plasma. Excessive ROS generation initiates lipid peroxidation of polyunsaturated fatty acids presents in the sperm plasma membrane, leading to loss of membrane integrity, reduced motility, compromised viability, and impaired fertilizing capacity. Owing to the high lipid content and limited cytoplasmic antioxidant enzymes, sperm cells are particularly vulnerable to oxidative injury during refrigeration.

Malondialdehyde (MDA), a stable end product of lipid peroxidation, is widely recognized as a reliable biochemical marker for assessing oxidative damage in semen. Elevated MDA levels reflect increased membrane lipid oxidation and are strongly associated with deterioration of sperm function. Although seminal plasma contains endogenous antioxidant

systems, including enzymatic antioxidants such as glutathione peroxidase, catalase, and non-enzymatic antioxidants, these protective mechanisms are gradually depleted during extended storage, rendering them insufficient to counteract oxidative stress.

In recent years, supplementation of semen extenders with exogenous antioxidants has emerged as an effective strategy to enhance oxidative balance and preserve sperm quality during liquid storage. Antioxidants such as catalase, melatonin, and methionine have been shown to scavenge free radicals, improve antioxidant enzyme activity, and stabilize sperm membranes. Methionine, in particular, plays a crucial role in glutathione synthesis and cellular redox regulation, while melatonin and catalase contribute through direct and enzymatic antioxidant actions, respectively.

Therefore, the present investigation was undertaken to evaluate the effect of catalase, melatonin, and methionine supplementation on lipid peroxidation, glutathione activity, and total antioxidant capacity of Deccani ram semen stored at 5 °C for up to 72 hours.

Materials and Methods

The study was conducted on 60 number of ejaculations collected from 6 Deccani rams at the rate of 10 number of ejaculations from each which were, aged between 2-5 years maintained at Livestock Farm Complex, College of Veterinary Science, Rajendranagar, Hyderabad under PVNR Telangana Veterinary University, Telangana State.

Selection and Management of Rams

Six healthy Deccani rams, aged between 2-5 years with an average body weight around 45 kg were selected for this study. All the selected rams were maintained under intensive feeding and management in loose cage, raised flat form housing system and were fed daily with 300 g of concentrate feed per ram along with adlibitum quantity of green fodder and fresh drinking water facility round the clock.

Semen Collection and Experimental Design

A total of sixty number of semen ejaculates were collected from six rams with the frequency of once in a week from each ram. The study was conducted during the period from June, 2022 to January, 2023. On the day of semen collection 3-4 rams were used for semen collection. Soon after semen collection, recorded the volume, colour, and consistency of individual ejaculate and kept in water bath at 37 °C, if any abnormality in colour and consistency that sample was not used for further process. All the collected ejaculates were pooled, transferred to andrology laboratory and diluted with equal quantity of diluent Egg Yolk Citrate (EYC) in Dept. of Veterinary Gynaecology and Obstetrics, College of Veterinary Science, Rajendranagar, Hyderabad. Andrology laboratory was maintained at room temperature 25-30 °C and water bath was maintained at 37 °C. the pooled sample was used for determining the semen concentration, later diluted with EYC extender at a final concentration of 500 X 10⁶ / mL of diluted semen. Pooled and diluted semen sample was randomly divided in to 4 equal parts and each part of sample was named as group I (added with Catalase @ 3 mM/mL), group II (added with Melatonin @ 3 mM/mL), group III (added with Methionine @ 4 mM/mL), group IV (Control - without antioxidant).

- **Group I:** Catalase

- **Group II:** Melatonin
- **Group III:** Methionine
- **Group IV:** Control (without antioxidant)

Samples were stored at 5 °C and evaluated at 0, 24, 48, and 72 h.

Estimation of Lipid Peroxidation (Malondialdehyde)

Semen lipid peroxidation was determined using the method previously described by Ohkawa *et al.* for the determination of lipid peroxidation products, mainly MDA (Ohkawa *et al.* 1979) [8]. The method was based on the reaction of two molecules of thiobarbituric acid with one molecule of malondialdehyde, at high temperatures and low pH, resulting in a pink chromogen that can be quantified with a spectrophotometer.

Procedure

1. 500 µL of seminal plasma was added to 1000 µL of 10% (w/v in water) trichloroacetic acid (TCA).
2. Centrifuge at 16,000 rpm for 15 min at 15°C for protein removal.
3. 500 µL of the supernatant was add to 500 µL of 1% (v/v in 0.05 M sodium hydroxide) TBA in another tube, which was maintained for 10 min in boiling water (90–100 °C). The sample was cooled on ice to stop the chemical reaction.
4. The TBARS molecules were quantified by a spectrophotometer (Ultrospec 3300pro, Healthcare® GE) at a wavelength of 532 nm.
5. Results were compared to a standard curve previously prepared with a standard solution of malondialdehyde.
6. The TBARS concentration was determined using the value of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ as the MDA extinction coefficient.
7. Lipid peroxidation in the seminal plasma was described as TBARS in nanograms/milliliter of seminal plasma.
8. The TBARS values were also normalized by the sperm concentration, because the sperm was the major source of MDA production in semen.
9. Therefore, the TBARS (ng/ml) was divided by the concentration of sperm (10⁶/ml) to obtain the TBARS/10⁶ sperm values (Lacerda *et al.* 2011, Sposito *et al.* 2017).

Lipid peroxidation was assessed by measuring malondialdehyde (MDA) levels using the thiobarbituric acid reactive substances (TBARS) method described by Ohkawa *et al.* (1979) [8]. Seminal plasma was separated by centrifugation and stored at –20 °C until analysis.

The absorbance of the pink chromogen formed was measured at 532-535 nm using a spectrophotometer. MDA concentration was calculated using the molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol/mL seminal plasma.

Glutathione Peroxidase (GSH) Activity

GPx enzyme activity was assessed indirectly by measuring the NAD phosphate (NADPH) consumption, using the Beutler protocol modified by Bittencourt (de Bittencourt *et al.* 1998).

1. This method was based on measuring the consumption of NADPH.

- The reaction between a hydroperoxide and reduced glutathione (GSH) that was catalyzed by the GSPH-Px together with the enzyme glutathione reductase (GSSGr) was induced.
- This reaction causes the conversion of glutathione disulfide (GSSH - glutathione oxidized) to GSH, which in turn consumes NADPH (measured with a spectrophotometer).

Procedure

- 100 μL of seminal plasma were mixed to 20 μL of 0.25 mM sodium azide, 20 ml of 0.25 UI GSSGr, 100 ml of 0.12 mM GSH, 660 μL of a solution containing 143 mM sodium phosphate
- 6.3 mM EDTA, and 1000 μL of 0.12 mM NADPH.
- The cuvette was stirred in vortex and maintained in the spectrophotometer (Ultrospec 3300pro, Healthcare® GE) for 300 s to stabilize the reaction.
- 100 ml of tert-butyl hydroperoxide in 1.2 mM hydroperoxide (TBHP) were added.
- The consumption curve of NADPH was measured in a wavelength of 340 nm for 10 min at 37°C (measured at every 5 s) and compared to a blank.
- Calculations considered the value of 0.0062/M cm as the molar extinction coefficient of NADPH.
- Glutathione peroxidase activity was calculated based on the formula:

$$\text{GPx activity} = (\text{final absorbance} - \text{initial}) / 0.0062 \times \text{dilution. IU/mL}$$

Glutathione peroxidase activity was estimated indirectly by measuring NADPH consumption following the method of Beutler as modified by de Bittencourt *et al.* (1998). The decrease in absorbance was recorded at 340 nm, and enzyme activity was expressed as IU/mL using a molar extinction coefficient of $0.0062 \text{ M}^{-1} \text{ cm}^{-1}$.

Total Antioxidant Capacity (TAC)

Total AOA values were measured using the methods of Koracevic *et al.* (2001) [5] with a slight modification. In brief, 490 ml of PBS solution were added to 10 mL of the sample.

- Additionally, sodium benzoate, acetic acid, Fe-EDTA and H_2O_2 were added to the tubes, respectively.
- The tubes were incubated at 37 °C for 60 min. Acetic acid and thiobarbituric solution were subsequently added to the tubes.
- The tubes were incubated in 100 °C water bath for 10 min. The absorbance of the samples was measured at 532 nm wave length.

- Total AOA values were expressed as mmol/g protein in the spermatozoa and seminal plasma samples.

Total antioxidant capacity was measured according to the method described by Koracevic *et al.* (2001) [5]. The reaction mixture was incubated at 37 °C followed by heating in a boiling water bath, and absorbance was measured at 532 nm. TAC was expressed as mmol/g protein.

Statistical Analysis

Data were analyzed using SPSS version 16.0. Analysis of variance (ANOVA) was employed to compare treatments, and Duncan's multiple range test was used for post hoc analysis. Differences were considered statistically significant at $P < 0.05$, and results were expressed as Mean $\pm$ SE.

Results

Lipid Peroxidation (MDA)

MDA levels increased significantly ($P < 0.05$) with the duration of storage in all groups. At all storage intervals (0, 24, 48, and 72 h), semen supplemented with methionine exhibited significantly lower MDA levels compared to catalase, melatonin, and control groups. The control group consistently showed the highest MDA values, indicating elevated lipid peroxidation.

Overall mean MDA levels were lowest in the methionine group ($30.82 \pm 1.03 \text{ nmol/mL}$) and highest in the control group ($42.98 \pm 0.86 \text{ nmol/mL}$).

Glutathione Reductase (GSH)

GSH activity declined progressively with increased storage duration across all groups. However, antioxidant-supplemented groups maintained significantly higher ($P < 0.05$) GSH activity compared to the control. Methionine supplementation resulted in the highest GSH levels at all storage intervals.

The overall mean GSH activity was highest in the methionine group ($73.20 \pm 0.90 \text{ IU/mL}$) and lowest in the control group ($52.19 \pm 0.92 \text{ IU/mL}$).

Total Antioxidant Capacity (TAC)

Total antioxidant capacity showed a significant decrease ($P < 0.05$) with increasing storage time. Antioxidant supplementation significantly enhanced TAC values compared to the control group at all storage periods. Methionine-supplemented semen consistently recorded the highest TAC values, followed by melatonin and catalase.

The overall mean TAC values were highest in the methionine group ($146.81 \pm 0.80 \text{ mmol/g}$) and lowest in the control group ($113.38 \pm 0.83 \text{ mmol/g}$).

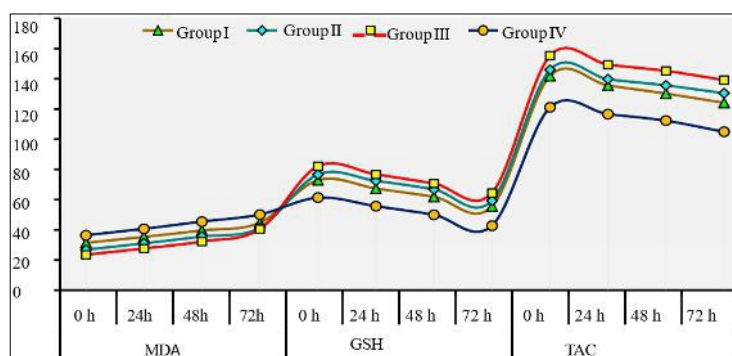


Fig 1: Bio chemical Parameters MDA, GSH and TAC levels in Deccani ram liquid semen diluted with egg yolk citrate and supplemented with and without antioxidants stored at 5°C refrigeration temperature during 0 h to 72 h.

Table 1: Mean ($\pm$ S.E) of Bio-Chemical Parameters MDA, GSH and TAC of Deccani ram liquid semen diluted with egg yolk citrate and supplemented with and without antioxidants stored at 5 °C of refrigeration temperature during 0 h to 72 h.

S. No.	Parameter	Period	Group I (Catalase)	Group II (Melatonin)	Group III (Methionine)	Group IV (Control)	TOTAL
1	MDA	0 h	31.20 $\pm$ 1.00 ^{ay}	26.67 $\pm$ 0.88 ^{ax}	23.33 $\pm$ 1.33 ^{aw}	36.27 $\pm$ 1.14 ^{az}	29.37 $\pm$ 0.83 ^A
2		24 h	35.13 $\pm$ 1.02 ^{by}	30.87 $\pm$ 0.98 ^{bx}	27.47 $\pm$ 1.20 ^{bw}	40.47 $\pm$ 1.03 ^{bz}	33.48 $\pm$ 0.82 ^B
3		48 h	39.40 $\pm$ 1.00 ^{cy}	35.40 $\pm$ 0.93 ^{cx}	32.13 $\pm$ 1.29 ^{cw}	45.27 $\pm$ 1.16 ^{cz}	38.05 $\pm$ 0.83 ^C
4		72 h	44.53 $\pm$ 0.91 ^{dy}	41.13 $\pm$ 0.92 ^{dx}	40.33 $\pm$ 1.26 ^{dx}	49.93 $\pm$ 1.06 ^{dz}	43.98 $\pm$ 0.71 ^D
5		Total	37.57 $\pm$ 0.80 ^Y	33.52 $\pm$ 0.83 ^X	30.82 $\pm$ 1.03 ^W	42.98 $\pm$ 0.86 ^Z	36.22 $\pm$ 0.53
6	GSH	0 h	72.63 $\pm$ 0.44 ^{dx}	76.37 $\pm$ 0.40 ^{dy}	81.83 $\pm$ 0.61 ^{dz}	61.07 $\pm$ 0.49 ^{dw}	72.98 $\pm$ 1.02 ^A
7		24 h	67.06 $\pm$ 0.34 ^{cx}	72.12 $\pm$ 1.18 ^{cy}	76.42 $\pm$ 0.35 ^{cz}	55.43 $\pm$ 0.43 ^{cw}	67.76 $\pm$ 1.07 ^B
8		48 h	61.64 $\pm$ 0.50 ^{bx}	66.49 $\pm$ 0.56 ^{by}	70.44 $\pm$ 0.52 ^{bz}	49.75 $\pm$ 0.40 ^{bw}	62.08 $\pm$ 1.04 ^C
9		72 h	55.32 $\pm$ 0.35 ^{ax}	59.06 $\pm$ 0.49 ^{ay}	64.12 $\pm$ 0.54 ^{az}	42.51 $\pm$ 0.48 ^{aw}	55.25 $\pm$ 1.07 ^D
10		Total	64.16 $\pm$ 0.86 ^X	68.51 $\pm$ 0.92 ^Y	73.20 $\pm$ 0.90 ^Z	52.19 $\pm$ 0.92 ^W	64.52 $\pm$ 0.67
11	TAC	0 h	141.41 $\pm$ 0.35 ^{dx}	145.51 $\pm$ 0.39 ^{dy}	154.77 $\pm$ 0.41 ^{dz}	120.72 $\pm$ 0.72 ^{dw}	140.60 $\pm$ 1.64 ^D
12		24 h	135.15 $\pm$ 0.45 ^{cx}	139.28 $\pm$ 0.88 ^{cy}	148.95 $\pm$ 0.33 ^{cz}	116.29 $\pm$ 0.85 ^{cw}	134.92 $\pm$ 1.58 ^C
13		48 h	129.73 $\pm$ 0.76 ^{bx}	135.21 $\pm$ 0.42 ^{by}	144.74 $\pm$ 0.41 ^{bz}	111.92 $\pm$ 0.52 ^{bw}	130.40 $\pm$ 1.58 ^B
14		72 h	123.68 $\pm$ 0.49 ^{ax}	129.99 $\pm$ 0.68 ^{ay}	138.76 $\pm$ 0.72 ^{az}	104.60 $\pm$ 0.36 ^{aw}	124.26 $\pm$ 1.66 ^A
15		Total	132.49 $\pm$ 0.89 ^X	137.50 $\pm$ 0.80 ^Y	146.81 $\pm$ 0.80 ^Z	113.38 $\pm$ 0.83 ^W	132.55 $\pm$ 0.89

A, B, C, D. Total means of different period values with different superscripts in columns differ significantly ($P \leq 0.05$) W, X, Y, Z. Total means of groups values with different superscripts in rows are differ significantly ($P \leq 0.05$)

a, b, c, d...period wise values with different superscripts in columns are differ significantly ($P \leq 0.05$) w, x, y, z group wise values with different superscripts in rows are differ significantly ($P \leq 0.05$)

MDA - Malondialdehyde, GSH - Glutathione reductase and TAC - Total antioxidant capacity

Discussion

The significant increase in MDA levels with prolonged storage indicates enhanced lipid peroxidation due to oxidative stress. Methionine supplementation effectively reduced lipid peroxidation, likely due to its role as a precursor of glutathione and its ability to scavenge free radicals. Similar observations were reported by Çoyan *et al.* (2010), Dai *et al.* (2019)^[3], and Kumar *et al.* (2022)^[6].

Methionine plays a critical role in cellular redox regulation as it serves as a precursor for glutathione synthesis, a major intracellular antioxidant. Additionally, methionine residues can undergo reversible oxidation, acting as scavengers of reactive oxygen species and protecting essential cellular components from oxidative injury. This dual antioxidant function may explain the superior performance of methionine in maintaining lower MDA levels and higher antioxidant capacity during semen storage.

Melatonin exerts potent antioxidant effects through both direct free radical scavenging and indirect upregulation of antioxidant enzymes. Its amphiphilic nature allows it to readily penetrate sperm membranes and protect mitochondrial integrity, thereby reducing oxidative damage during liquid storage.

Higher GSH activity in antioxidant-treated groups suggests improved antioxidant defense mechanisms, particularly in methionine-supplemented semen. Catalase supplementation also improved oxidative stability during prolonged storage, supporting findings by Michael *et al.* (2009)^[7].

Catalase contributes to oxidative stability by decomposing hydrogen peroxide into water and oxygen, thereby preventing the formation of highly reactive hydroxyl radicals. Its beneficial effects were more evident during prolonged storage periods when hydrogen peroxide accumulation becomes more pronounced.

Enhanced TAC levels in antioxidant-treated groups further confirm the protective role of antioxidants against oxidative stress during semen storage. These results are consistent with earlier findings reported by Dai *et al.* (2019)^[3], Bhalothia *et al.* (2022), and Kumar *et al.* (2022)^[6].

Conclusion

Supplementation of Deccani ram semen with antioxidants significantly improved biochemical parameters during liquid storage at 5 °C. Among the antioxidants tested, methionine was most effective in reducing lipid peroxidation, maintaining glutathione activity, and enhancing total antioxidant capacity. Prolonged refrigeration resulted in oxidative stress; however, antioxidant supplementation, particularly methionine, effectively mitigated these adverse effects and improved semen storage quality.

Acknowledgments

The authors are grateful to the hon'ble Vice Chancellor, PVNR Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana and the Director of Research, Registrar and Dean of faculties PVNR Telangana Veterinary University, Rajendranagar, Hyderabad, Telangana for providing necessary facilities to carry out research work. The authors are also thankful to the HOD, Department of Veterinary Gynaecology, and Associate Dean C.V. Sc, Rajendranagar for timely analysis of the semen samples.

References

- Beutler E. Red cell metabolism: a manual of biochemical methods. New York: Grune & Stratton; 1984.
- Coyan K, Başpınar N, Bucak MN, Akalin PP, Ataman MB, Ömür AD, *et al.* Influence of methionine and dithioerythritol on sperm motility, lipid peroxidation and antioxidant capacities during liquid storage of ram semen. *Res Vet Sci.* 2010;89:426-431.
- Dai G, Meng Y, Zhang L, Du Y, Wen F, Feng T, *et al.* Effect of addition of melatonin on liquid storage of ram semen at 4 °C. *Andrologia.* 2019;51:e13236.
- de Bittencourt PH Jr, Miyasaka CK, Curi R, Williams JF. Effects of the antiproliferative cyclopentenone prostaglandin A1 on glutathione metabolism in human cancer cells in culture. *IUBMB Life.* 1998;45(6):1255-1264.

5. Koracevic D, Koracevic G, Djordjevic V, Andrejevic S, Cosic V. Method for the measurement of antioxidant activity in human fluids. *J Clin Pathol.* 2001;54(5):356-361.
6. Kumar T, Kumar P, Saini N, Bhalothia SK, Prakash C, Mahla AS, Kumar A. Shielding effect of melatonin improves seminal quality and oxidative stress indices during chilled storage of ram semen. *Trop Anim Health Prod.* 2022;54(3):197.
7. Michael AJ, Alexopoulos C, Pontiki EA, Hadjipavlou-Litina DJ, Saratsis P, Ververidis HN, *et al.* Effect of antioxidant supplementation in semen extenders on semen quality and reactive oxygen species of chilled canine spermatozoa. *Anim Reprod Sci.* 2009;112:119-135.
8. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem.* 1979;95(2):351-358.
9. Snedecor GW, Cochran WG. Statistical methods. 8th ed. Calcutta: Oxford and IBH Publishing House; 1994. p. 1-491.