



# Gene Expression Profile and Enzymatic Activities of Frozen Buck Sperm Supplemented with Melatonin in Cold and Hot Temperatures

Sherif Mohamed Dessouki<sup>1</sup>, Gamal Ashour<sup>1</sup>, Moataz El-Gayar<sup>2</sup>, Fakhri El-Hadi El-Azzazi<sup>2</sup>, Elias Kodi<sup>1,3</sup> and Nasser Ghanem<sup>1\*</sup>

<sup>1</sup>Department of Animal Production, Faculty of Agriculture, Cairo University, Giza, Egypt

<sup>2</sup>Department of Animal Production, Faculty of Agriculture, Suez Canal University, Ismailia, Egypt

<sup>3</sup>Department of Animal Production, College of Veterinary Science, Bahr El-Ghazal University, Wau, South Sudan

\*Corresponding author's E-mail: [nassergo@agr.cu.edu.eg](mailto:nassergo@agr.cu.edu.eg); ORCID: 0000-0002-0480-0959

## ABSTRACT

This study was carried out to improve the freezability of buck semen using two different types of cryoprotectants supplemented with melatonin as antioxidant in cold and hot temperature of breeding season. Ejaculates from four mature Egyptian baladi bucks were pooled after collection. Semen was extended with Tris-fructose-citric containing egg yolk using glycerol and dimethyl sulfoxide supplemented with two doses of melatonin ( $10^{-6}$ M and  $10^{-3}$ M) in addition to control group. Types of motility as well as velocity, enzymatic activity and expression profile of selected genes were measured. The results revealed that the progressive motility percentage was significantly higher in samples supplemented with low dose of melatonin ( $10^{-6}$  M) compared to high dose ( $10^{-3}$ M) in glycerol (74.4 versus 64.4) and Dimethyl Sulfoxide (DMSO) based extender (35.5 versus 32.9) in cold temperature. The same trend was found in samples cryopreserved with glycerol (75.1 versus 53.5) and DMSO (32.1 versus 22) in hot temperature. The results also demonstrated that CASA parameters (VAP and VCL) were significantly increased in low compared to high melatonin dose in glycerol based extender during cold and hot temperature. The activity of total antioxidant capacity (TAC) was significantly higher in samples supplemented with low (0.49 mM/L) than high melatonin dose (0.16 mM/L) in DMSO extender. CPT2, ATP5F1A and SOD2 genes were up regulated in glycerol based extender groups in cold temperature compared to other groups of this study. On the other hand, NFE2L2 gene was up-regulated in groups cryopreserved with DMSO in hot temperature compared with all other experimental groups. Therefore, it could be concluded that the glycerol based extender in cold season supplemented with low dose of melatonin improved semen quality, antioxidant defense capacity and transcriptional profile, which may maintain the post-thaw fertilizing ability of buck semen.

**Keywords:** Antioxidant enzymes, Bucks, Melatonin, Motility, Transcript abundance

## INTRODUCTION

Goat population contributes 3.37% of local meat production and approximately 0.3% of local milk production in Egypt (Khalifa et al., 2009). This contribution is considered low that is due to low genetic merit and performance of Egyptian goat (Khalifa et al., 2009). Therefore, genetic improvement of goats under Egyptian conditions is a necessary demand for improving the productivity of this species (Khalifa et al., 2009). Indeed, cryopreservation of semen is an important technique for breeding schemes focus on increasing livestock production, However, technical and biological factors limit the commercial application of this technique (Silva et al., 2000). The freezing technique, extender, as well as the type and concentration of cryoprotectant agent determine the quality of frozen semen. Glycerol and Dimethyl Sulfoxide (DMSO) have been extensively used as effective agents for preserving sperms (Fleming and Hubel, 2006).

Variation in annual environmental temperature and photoperiod represent key factors determining reproductive seasonality and defining the reproductive seasons of some farm animal species (Aguirre et al., 2007 and Abecia et al., 2012). Several breeds of sheep and goats are short-day seasonal breeders (Carolan et al., 1994; Hafez, 2000 and Abecia et al., 2012). In addition breed, season and type of ration are main factors affect sexual maturity of buck (Barkawi et al., 2006; Zarazaga et al., 2009; Elhammali and Elsheikh, 2014). Moreover, semen quality was improved during the breeding season in the late summer and early autumn, which coincides with decreasing photoperiod (Farshad et al., 2012). It is reported that semen quality and freezability were higher in breeding season than in non-breeding season (Wang et al., 2014). On the other hand, the decline in semen quality of bucks during hot months was due to increased alkalinity of semen that changed pH and subsequently compromised spermatozoa physical characteristics (Elsheikh et al., 2013).

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Oxidative stress is referred to an imbalance between the oxidant and antioxidant systems that is caused by the accumulation of free radicals such as Reactive Oxygen Species (ROS) and reactive nitrogen species (Deng et al., 2016). Free radicals have critical roles in sperm hyperactivation, capacitation, and the acrosome reaction (Zhang et al., 2016). Nevertheless, excessive level of ROS is produced during cryopreservation process that may cause lipid peroxidation and destroy the lipid bilayer structure of the spermatozoon membrane (Zhang et al., 2016).

Melatonin is a neuroendocrine hormone with a natural antioxidant activity. Melatonin stimulates the activity of antioxidant enzymes such as Superoxide Dismutase (SOD) and glutathione peroxidase (GSH-Px) (Jang et al., 2010). Melatonin improves semen characteristics in goat (Ramadan et al., 2009), rat (Sonmez et al., 2007), boar (Jang et al., 2010), ram (Ashrafi et al., 2011), and in human spermatozoa (Ortiz et al., 2011). Related enzymes includes catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase represent the most important enzymatic system involved in maintaining balance between ROS production and scavenging (Gadea et al., 2011 and Saraswat et al., 2014).

Transcriptional profile of specific candidate genes was linked with conventional semen physical and biochemical characteristics predicted male fertility in different farm animal species (Kadivar et al., 2016; Kim et al., 2019). The implication of sperm related genes in fertility was suggested by the involvement of sperm transcripts in different crucial reproductive processes like spermatogenesis, sperm motility (Bissonnette et al., 2009), fertilizing ability and subsequent embryonic development (Boerke et al., 2007). Therefore, identification of the possible role of genes regulating sperm quality, the transcripts related to mitochondrial activity (CPT2, ATP5F1A and SOD2) as well as oxidative stress (NFE2L2) for profiling all experimental groups was selected.

The objective of this study was to improve the freezability of buck semen through testing the effect of glycerol and DMSO as two different cryoprotectants in combination with melatonin supplementation of ( $10^{-6}$ M and  $10^{-3}$ M) in cold and hot temperature on motility characteristics of buck sperm post thawing. Moreover, to correlate between the activity of biomarkers and transcriptional profile of the selected genes and the antioxidant competence of melatonin in buck sperm.

## MATERIALS AND METHODS

### Experimental region

This study was carried out at the experimental farm station located on the Faculty of Agriculture and Faculty of Veterinary Medicine, Suez Canal University, Ismailia, Egypt. Semen samples were collected during breeding season in cold temperature (April and May, 2018) with average maximum and minimum of ambient temperatures 31.4°C and 17.4°C that corresponds to temperature-humidity index (THI) values of  $79.31 \pm 0.68$  and  $55.73 \pm 0.38$ , respectively. In addition, environmental data recorded during hot temperature (July and August 2018) indicated average maximum and minimum of ambient temperature 36.3°C and 22.5°C that correspond to THI values of  $98.32 \pm 0.37$  and  $73.97 \pm 0.22$ , respectively. The formula used to calculate THI was;

$$\text{THI} = T - 0.55 (1 - \text{RH})(T - 58),$$

T = air temperature in °F; RH = relative humidity percentage.

After cryopreservation, the analysis of semen samples was done in semen laboratory belongs to Animal Production Department, Faculty of Agriculture Cairo University.

### Experimental animals, feeding and management

Four healthy Egyptian native Baladi bucks were used in this experiment. The animals are sexually mature and their age ranged from 2.5 to 3 years. The bucks were nearly at the same body condition and with an average body weight of 45 kg. The bucks were kept under shaded yard and drinking water was provided *ad libitum*. Animals were fed concentrated feed (16 % total protein and 65 % total digestible nutrients), and roughage (rice straw).

### Semen collection and processing

The procedure of semen collection and physical analysis was done according to Hafez and Hafez (2000). Semen samples were collected using artificial vagina twice a week from four mature healthy bucks during cold (April and May) and hot temperature (July and August). The collection was done for eight successive weeks in each period. The volume, color and viscosity of all ejaculates (n=128) were recorded throughout the experiment. Progressive motility percentage was evaluated subjectively and only ejaculates of at least  $\geq 70\%$  motility with  $2 \times 10^9$  sperm/mL concentrations were pooled and cryopreserved according to experimental design (Evans and Maxwell 1987 and Leboeuf et al., 2000). Pooled samples were diluted at 1: 8 (v:v) with each extender. The concentration of sperm was adjusted to be 50 million/straw. The composition of 100 ml extender contained 3.605 g of Tris base (buffer), 1.490 g of fructose, 20 ml of egg yolk, 100,000 IU penicillin, 50,000 IU streptomycin, and 2.024 g of citric acid that all were mixed well and dissolved in distilled water. Two different cryoprotective agents, glycerol and DMSO were used for the composition of the two different types of extenders at 5% each. The melatonin (M5250, Sigma – Aldrich, 3050 Spruce Saint Louis, MO 63103,

USA) was diluted in each extender before extension at ( $10^{-6}$ M and  $10^{-3}$ M). Supplementation of melatonin was done by weighing 0.0023 g of melatonin powder, which was then dissolved in 10 ml of each extender to get  $10^{-3}$  M melatonin concentration, and further diluted to reach  $10^{-6}$  M as the second concentration. Fresh extender was prepared on every collection day and left in a fridge for maximum of 1 to 1.5 hours until semen was collected. The extender was pre-warm at  $37^{\circ}\text{C}$  in water bath prior to extension. Extended semen was packed in 0.25 ml French straws (IMV). The straws were cooled at  $5^{\circ}\text{C}$  for four hours as an equilibrium period. Thereafter, straws were collected and spread on a rack above liquid nitrogen for 15 minutes. Finally, the straws were submerged slowly into liquid nitrogen and kept at  $-196^{\circ}\text{C}$ .

#### **Post-thawing evaluation motion characteristics**

According to Ashrafi et al. (2013), three straws from each batch of semen were removed from liquid nitrogen tank and immersed immediately into a water bath at  $37^{\circ}\text{C}$  for 30 seconds. Thawed semen was evaluated immediately and the motion characteristics of spermatozoa were determined by using Computer Assisted Sperm Analysis (CASA; instrument SpermVision™ software Minitube Hauptstraße 41. 84184 Tiefenbach, Germany). The motion characteristics included the following measurements; distance curved line (DCL,  $\mu\text{m}$ ), distance average path (DAP,  $\mu\text{m}$ ), distance straight line (DSL,  $\mu\text{m}$ ), velocity curved line (VCL,  $\mu\text{m}/\text{sec}$ ), velocity average path (VAP,  $\mu\text{m}/\text{sec}$ ), velocity straight line (VSL,  $\mu\text{m}/\text{sec}$ ), linearity ( $\text{LIN}=\text{VSL}/\text{VCL}$ ), straightness ( $\text{STR}=\text{VSL}/\text{VAP}$ ), wobble ( $\text{WOB}=\text{VAP}/\text{VCL}$ ), beat cross frequency (BCF,  $\text{H}_2$ ) and amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ).

#### **Antioxidant biomarkers assays**

Two straws from each group were thawed for 30 seconds at  $37^{\circ}\text{C}$  and packed into 1.5 ml eppendorf tube. Then 1 ml of Dulbecco's phosphate buffer saline was added to each eppendorf tube and they were centrifuged for 10 minutes at 1030 rcf at  $4^{\circ}\text{C}$ . This procedure was done three times and every time the supernatant was discarded. Afterward, 1 ml of distilled water was added to each eppendorf and frozen in liquid nitrogen. Later on the contents were centrifuged at 1030 rcf at  $4^{\circ}\text{C}$  for 15 minutes. The pellets were discarded and supernatants were collected for enzymatic activity analyses. Three different biological replicates of each experimental group were used to profile all antioxidant enzymes; catalase, Total Antioxidant Capacity (TAC), Glutathione Peroxidase (GPX), and Malondialdehyde (MDA). The analyses of all enzymes were achieved by colorimetric assay using spectrophotometer (Sunostk SBA 733 plus, Bio diagnostic - Egypt) at 505 nm wavelength

#### **Catalase activity**

The activity of catalase was measured using standard procedure described by Fossati et al. (1980) and based on the company information (Cat No. CA 25 17, Bio Diagnostic, Giza, Egypt). The reaction was initiated by mixing the phosphate buffer, pH 7.0 ( $\text{R}_1$ ) with known quantity of  $\text{H}_2\text{O}_2$  ( $\text{R}_2$ ). Catalase inhibitor ( $\text{R}_3$ ) was added for one minute to inactivate the reaction. In the presence of peroxidase (HRP) ( $\text{R}_4$ ), remaining  $\text{H}_2\text{O}_2$  reacted with 3,5-Dichloro -2-hydroxybenzene sulfonic acid (DHBS) and 4-aminophenazone (AAP) to form a chromophore with a color intensity inversely proportional to the amount of catalase in the original sample. After incubation for 10 minutes at  $37^{\circ}\text{C}$ , samples were read at 510 nm (500 – 520 nm).

#### **Total antioxidant capacity (TAC)**

The TAC was determined using the same method according to Koracevic et al. (2001) by mixing antioxidants in the sample with a defined amount of exogenously provide hydrogen peroxide ( $\text{R}_1$ ) and incubated for 10 minutes at  $37^{\circ}\text{C}$ . The antioxidants in the sample eliminated a certain amount of the provided hydrogen peroxide. In the presence of chromogen ( $\text{R}_2$ ), the residual  $\text{H}_2\text{O}_2$  was measured by an enzymatic reaction which resulted in the conversion of 3,5-dichloro-2-hydroxy benzensulphonate ( $\text{R}_3$ ) to a colored product. The absorbance of blank (distilled water) and sample were read immediately at 505 (500 – 510 nm). All the chemicals were manufactured and the method was described in the manual of the company (Cat. No. TA 25 13, Bio Diagnostic, Giza, Egypt)

#### **Glutathione peroxidase activity**

The measurement of the GPx activity was performed as described by Paglia and Valentine (1967) and followed the company instruction (Cat. No. GP 25 24, Bio Diagnostic, Giza, Egypt). The chemical conversion of NADPH ( $\text{R}_2$ ) to  $\text{NADP}^+$  was followed by a reduction in measured absorbance by spectrophotometer at wavelength 340 nm ( $\text{A}_{340}$ ) which was a tool to measure the activity of GPx enzyme. Phosphate Buffer ( $\text{R}_1$ ) pH 7.0 was added to a solution containing glutathione, glutathione reductase, and NADPH ( $\text{R}_2$ ). The enzyme reaction was initiated by adding the substrate hydrogen peroxide ( $\text{R}_3$ ) and the decrease of absorbance at 340 nm/ min was recorded over a period of 3 minutes against deionized water.

### Malondialdehyde concentration

To measure the concentration of MDA, thiobarbituric acid (TBA) ( $R_2$ ) was reacted with MDA in the presence of chromogen in acidic medium at 95°C for 30 minute and formed thiobarbituric acid reactive product (Ohkawa et al., 1979). The absorbance of the resultant pink product was measured for sample against blank and standard ( $R_1$ ) against distilled water according to the procedure mentioned by (Cat No. MD 25 29, Bio Diagnostic, Giza, Egypt) at 534 nm.

### RNA isolation, cDNA synthesis and quantitative real-time PCR (qRT-PCR)

Three straws from each treatment were removed from the liquid nitrogen tank and thawed at room temperature. The contents were transferred to 1.5 ml eppendorf tube and centrifuged for 12 minutes on 680 rpm at 4 °C. The pellets of all samples were kept at -80°C till the extraction of RNA. The isolation of RNA was performed using GeneJET kit (ThermoFisher Sci., Vilnius, Lithuania), following the instructions of manufacturer. The digestion of genomic DNA was done by adding DNase I recommended by RNase-free kit (Thermo Scientific, California, USA). The Nano-drop 2000C instrument (Thermo Scientific, Wilmington, DE, USA) was used to measure the concentration of total RNA and purity was determined by the ratio reading at A260/280 nm. Reverse transcription Kit (Life Technologies Corporation, California, USA) was used to synthesize cDNA which includes MultiScribe™ Reverse Transcriptase and random primers. Gene-specific primers were designed from sequences available in GenBank database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) using Primer3 software (<http://primer3.wi.mit.edu/>) as shown in Table1. The real-time PCR reaction was done using using a StepOnePlus™ Real-Time PCR instrument (Applied Biosystems, California, USA). The reaction contained 0.2 µl reverse and forward primers, 10ul of SYBR Green Master Mix (ThermoFisher Scientific, California, USA), 7.6µl nuclease-free water and 2.0µl cDNA. The settings of PCR reactions were as following: 10 minutes at 95°C, 40 cycles of 15 seconds at 95 °C, 20 seconds at 60 °C, and 30 seconds at 72 °C and finally 1 min at 60°C. Delta Ct method was used for gene expression data analysis after normalization of the target transcript (CPT2, ATP5F1A, NFE2L2 and SOD2) to the housekeeping gene (GAPDH).

**Table1.** The primers used for quantitative real-time PCR analysis

Gene Name	Gene bank accession number	Primer sequence	Fragment size (bp)	Reference
ATP5F1A	NM_174684.2	F: 5'-CTCTTGAGTCGTGGTGTGCG-3' R: 5'-CCTGATGTTGGCTGATAACGTG-3'	184	(Ghanem et al., 2014)
CPT2	NM_001045889	F: 5'-CCGAGTATAATGACCAGCTC-3' R: 5'-GCGTATGAATCTCTTGAAGG-3'	152	(Ghanem et al., 2014)
NFE2L2	NM_001011678	F: 5'-TAAACAGCAGTGGCTACCT-3' R :5'-GAGACATTCCCCTTTGTAGA-3'	159	(Ghanem et al., 2014)
SOD2	NM_201527	F: 5'-GTGATCAACTGGGAGAATGT-3' R: 5'-AAGCCACACTCAGAAACACT-3'	163	(Ghanem et al., 2014)
GAPDH	NM_001034034.2	F: 5'- AGGTCGGAGTGAACGGATTC -3' R: 5'- GGAAGATGGTGATGGCCTTT -3'	219	(Ghanem et al., 2014)

PCR: polymerase chain reaction; bp: base pair.

### Experimental design

The impact of the two different levels of melatonin supplementation ( $10^{-6}$ M and  $10^{-3}$ M) in combination with the two different types of buck extenders (glycerol and DMSO based extenders) during cold and hot temperature were evaluated after cryopreservation in the present study. Motion characteristics and antioxidant enzymes activity were analyzed in all experimental groups. In addition, expression profile of SOD2, CPT2, NFE2L2, and ATP5F1A genes was quantified using Real-time PCR.

### Data analysis

The data of the current study was analyzed using GLM procedure (SAS, 2004) by applying the following statistical model:  $Y_{ijklm} = \mu + E_i + M_j + S_k + (E \times M \times S)_l + e_{ijklm}$ . The measured traits were as following:  $\mu$  = overall means;  $E_i$  = Effect of extender type;  $M_j$  = Effect of melatonin level;  $S_k$  = Effect of season;  $(E \times M \times S)_l$  = The interaction between extender type, melatonin level and season and  $e_{ijklm}$  = Experimental error. Duncan's multiple range tests was used to detect differences among means. The significance level was set at ( $P < 0.05$ ).

## RESULTS

### Motion characteristics

The percentage of total motility (Tables 2 and 3) was higher significantly ( $p \leq 0.05$ ) in samples extended with glycerol (control, low and high melatonin level) in cold and hot temperature than those supplemented with DMSO. The



progressive motility (Tables 2 and 3) was significantly higher in all groups of glycerol-based extender than in DMSO based extender in both cold and hot temperature. The percentages of total and progressive motility in samples supplemented with ( $10^{-6}$ M) melatonin was insignificantly higher than in samples supplemented with ( $10^{-3}$  M) melatonin in both two types of extenders in cold temperature however, these differences were significant in hot temperature. In addition, samples supplemented with ( $10^{-6}$ M) melatonin in glycerol extender recorded the highest percentages of total and progressive motility compared with other samples in hot temperature. All CASA parameters tended to be higher in semen samples extended with glycerol than those extended with DMSO during cold temperature (Table 2). However, there were no clear variations in CASA parameters between samples extended with either glycerol or DMSO during hot temperature (Table 3).

**Table 2.** Spermatic parameters of buck semen as affected by types of extender and levels of melatonin supplementation during cold temperature

Traits	Glycerol 5%			DMSO 5%		
	Control	Low melatonin	High melatonin	Control	Low melatonin	High melatonin
Total Motility (%)	83.5 <sup>a</sup> ±2.2	84.1 <sup>a</sup> ±2.3	80.8 <sup>a</sup> ±2.4	48.9 <sup>bc</sup> ±2.5	50.2 <sup>b</sup> ±2.3	43.5 <sup>c</sup> ±2.6
Progressive motility (%)	71.6 <sup>a</sup> ±2.3	74.4 <sup>a</sup> ±2.4	64.4 <sup>b</sup> ±2.5	32.9 <sup>cd</sup> ±2.5	35.5 <sup>c</sup> ±2.4	29.8 <sup>d</sup> ±2.6
DAP (um)	24.9 <sup>a</sup> ±0.5	24.6 <sup>a</sup> ±0.5	21.8 <sup>b</sup> ±0.5	18.9 <sup>c</sup> ±0.6	18.7 <sup>c</sup> ±0.5	18.8 <sup>c</sup> ±0.6
DCL (um)	45.5 <sup>a</sup> ±1.2	44.5 <sup>a</sup> ±1.3	39.2 <sup>b</sup> ±1.4	33.5 <sup>c</sup> ±1.4	33.4 <sup>c</sup> ±1.3	33.8 <sup>c</sup> ±1.4
DSL (um)	18.3 <sup>a</sup> ±0.3	18.2 <sup>a</sup> ±0.4	15.9 <sup>b</sup> ±0.4	14.5 <sup>b</sup> ±0.4	14.3 <sup>b</sup> ±0.4	14.2 <sup>b</sup> ±0.4
VAP (um/s)	58.6 <sup>a</sup> ±1.1	57.4 <sup>a</sup> ±1.1	51.3 <sup>b</sup> ±1.2	45.0 <sup>c</sup> ±1.2	44.0 <sup>c</sup> ±1.1	44.5 <sup>c</sup> ±1.2
VCL (um/s)	106.6 <sup>a</sup> ±2.8	103.5 <sup>a</sup> ±2.9	91.8 <sup>b</sup> ±3.0	78.8 <sup>c</sup> ±3.1	77.4 <sup>c</sup> ±2.9	78.7 <sup>c</sup> ±3.2
VSL (um/s)	43.0 <sup>a</sup> ±0.7	42.5 <sup>a</sup> ±0.8	37.7 <sup>b</sup> ±0.8	34.8 <sup>bc</sup> ±0.8	34.0 <sup>bc</sup> ±0.8	33.8 <sup>c</sup> ±0.9
STR (%)	72.9 <sup>b</sup> ±0.6	73.6 <sup>b</sup> ±0.6	73.2 <sup>b</sup> ±0.7	77.6 <sup>a</sup> ±0.7	76.8 <sup>a</sup> ±0.6	75.8 <sup>a</sup> ±0.7
LIN (%)	39.9 <sup>c</sup> ±0.8	40.7 <sup>bc</sup> ±0.8	40.9 <sup>c</sup> ±0.9	45.8 <sup>a</sup> ±0.9	44.7 <sup>a</sup> ±0.8	43.4 <sup>ab</sup> ±0.9
WOB (VAP/VCL)	0.54 <sup>d</sup> ±0.01	0.55 <sup>bcd</sup> ±0.01	0.55 <sup>cd</sup> ±0.01	0.58 <sup>a</sup> ±0.01	0.57 <sup>ab</sup> ±0.01	0.56 <sup>abc</sup> ±0.01
ALH (um)	5.0 <sup>a</sup> ±0.0	4.7 <sup>bc</sup> ±0.1	4.8 <sup>b</sup> ±0.1	4.4 <sup>cd</sup> ±0.1	4.2 <sup>d</sup> ±0.1	4.2 <sup>d</sup> ±0.1
BCF (H2)	23.8 <sup>a</sup> ±0.5	24.4 <sup>a</sup> ±0.5	23.5 <sup>a</sup> ±0.5	19.1 <sup>b</sup> ±0.5	19.1 <sup>b</sup> ±0.5	18.9 <sup>b</sup> ±0.6

Means having different superscripts within the same row differ significantly ( $P < 0.05$ ). DAP: Distance Average Path (microns); DCL: Distance Curved Line (microns); DSL: Distance Straight Line (microns); VAP: Velocity Average Path (microns/sec); VCL: Velocity Curved Line (microns/sec); VSL: Velocity Straight Line (microns/sec); STR: Straightness (VSL/VAP); LIN: Linearity (VSL/VCL); WOB: Wobble (VAP/VCL); ALH Amplitude of Lateral Head Displacement (microns); BCF: Beat Cross Frequency (Hz); DMSO: Dimethyl Sulfoxide.

**Table 3.** Spermatic parameters of buck semen as affected by types of extender and levels of melatonin supplementation during hot temperature

Traits	Glycerol 5%			DMSO 5%		
	Control	Low melatonin	High melatonin	Control	Low melatonin	High melatonin
Total Motility (%)	67.4 <sup>b</sup> ±2.1	82.0 <sup>a</sup> ±2.3	59.0 <sup>c</sup> ±2.3	24.1 <sup>f</sup> ±2.3	41.2 <sup>d</sup> ±2.0	31.1 <sup>e</sup> ±1.8
Progressive motility (%)	57.9 <sup>b</sup> ±2.0	75.1 <sup>a</sup> ±2.2	53.5 <sup>b</sup> ±2.2	19.7 <sup>d</sup> ±2.2	32.1 <sup>c</sup> ±1.9	22.0 <sup>d</sup> ±1.8
DAP (um)	20.9 <sup>d</sup> ±0.5	24.7 <sup>a</sup> ±0.5	23.1 <sup>bc</sup> ±0.5	23.3 <sup>ab</sup> ±0.5	21.6 <sup>cd</sup> ±0.4	20.5 <sup>d</sup> ±0.4
DCL (um)	35.6 <sup>bc</sup> ±0.8	42.3 <sup>a</sup> ±0.9	35.6 <sup>bc</sup> ±0.9	38.0 <sup>b</sup> ±0.9	35.6 <sup>bc</sup> ±0.7	34.3 <sup>c</sup> ±0.7
DSL (um)	16.4 <sup>c</sup> ±0.5	19.4 <sup>a</sup> ±0.5	19.5 <sup>a</sup> ±0.5	19.3 <sup>a</sup> ±0.5	18.2 <sup>ab</sup> ±0.5	16.7 <sup>bc</sup> ±0.4
VAP (um/s)	47.5 <sup>c</sup> ±1.0	56.2 <sup>a</sup> ±1.1	51.2 <sup>b</sup> ±1.1	51.5 <sup>b</sup> ±1.1	48.6 <sup>bc</sup> ±0.9	45.5 <sup>c</sup> ±0.8
VCL (um/s)	81.0 <sup>bc</sup> ±1.8	95.8 <sup>a</sup> ±2.0	79.0 <sup>bc</sup> ±2.0	83.3 <sup>b</sup> ±2.0	79.5 <sup>bc</sup> ±1.7	76.0 <sup>c</sup> ±1.6
VSL (um/s)	37.4 <sup>b</sup> ±1.0	44.1 <sup>a</sup> ±1.1	43.2 <sup>a</sup> ±1.1	42.6 <sup>a</sup> ±1.1	40.8 <sup>a</sup> ±0.9	36.9 <sup>b</sup> ±0.9
STR %	78.5 <sup>b</sup> ±0.0	78.0 <sup>b</sup> ±0.0	83.8 <sup>a</sup> ±0.0	82.2 <sup>a</sup> ±0.0	83.4 <sup>a</sup> ±0.0	80.5 <sup>ab</sup> ±0.0
LIN %	45.8 <sup>c</sup> ±0.0	45.4 <sup>c</sup> ±0.0	54.2 <sup>a</sup> ±0.0	50.6 <sup>ab</sup> ±0.0	50.8 <sup>ab</sup> ±0.0	48.2 <sup>bc</sup> ±0.0
WOB (VAP/VCL)	0.58 <sup>c</sup> ±0.0	0.58 <sup>c</sup> ±0.0	0.64 <sup>a</sup> ±0.0	0.61 <sup>b</sup> ±0.0	0.60 <sup>bc</sup> ±0.0	0.59 <sup>bc</sup> ±0.0
ALH (um)	3.4 <sup>ab</sup> ±0.1	3.9 <sup>a</sup> ±0.1	2.8 <sup>c</sup> ±0.1	3.1 <sup>bc</sup> ±0.1	2.9 <sup>c</sup> ±0.1	3.0 <sup>bc</sup> ±0.1
BCF (H2)	26.4 <sup>b</sup> ±0.9	28.3 <sup>ab</sup> ±1.0	29.6 <sup>a</sup> ±1.0	25.1 <sup>b</sup> ±1.0	26.5 <sup>b</sup> ±0.9	26.3 <sup>b</sup> ±0.8

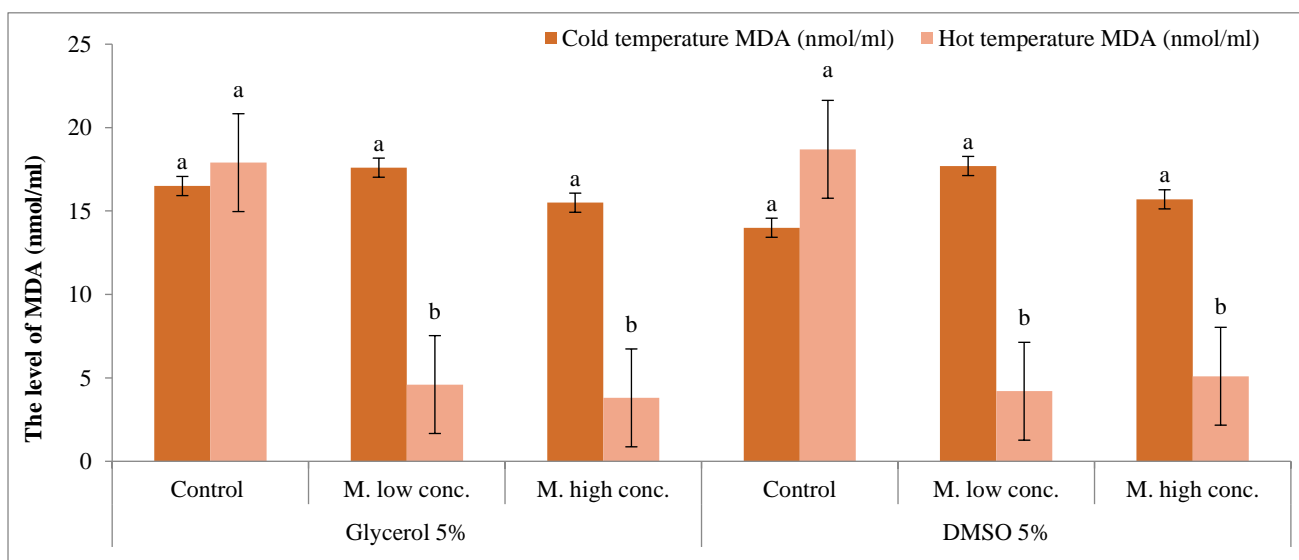
Means having different superscripts within the same row differ significantly ( $P < 0.05$ ). DAP: Distance Average Path (microns); DCL: Distance Curved Line (microns); DSL: Distance Straight Line (microns); VAP: Velocity Average Path (microns/sec); VCL: Velocity Curved Line (microns/sec); VSL: Velocity Straight Line (microns/sec); STR: Straightness (VSL/VAP); LIN: Linearity (VSL/VCL); WOB: Wobble (VAP/VCL); ALH Amplitude of Lateral Head Displacement (microns); BCF: Beat Cross Frequency (Hz); DMSO: Dimethyl Sulfoxide.

### Antioxidant biomarkers assays

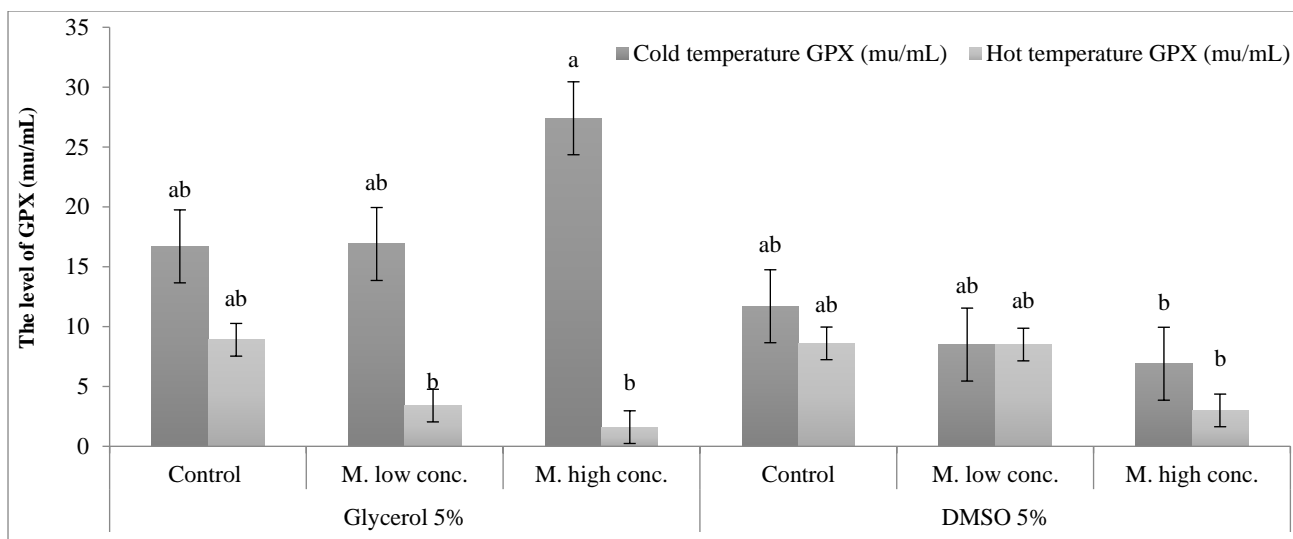
There were no significant differences between all samples cryopreserved with glycerol and DMSO on activity of MDA during cold temperature (Figure 1). However, the control group extended with glycerol and DMSO increased the level of MDA compared with samples supplemented with melatonin in hot temperature. The semen samples supplemented with melatonin in either glycerol or DMSO based extender had higher levels of MDA in cold than hot temperature. The activity of GPX (Figure 2) revealed insignificant difference between samples extended with glycerol and DMSO in cold season although samples cryopreserved with glycerol tended to show higher level of GPX than those cryopreserved with DMSO. While, there were no differences between all semen samples cryopreserved in hot temperature. Regarding the level of catalase (Figure 3), the activity of this enzyme was reduced in control samples with glycerol in comparison to all other experimental samples in cold temperature. However, the level of CAT was reduced in semen samples supplemented with low melatonin dose compared to all semen samples cryopreserved during hot temperature. The total antioxidant capacity values (Figure 4) increased in control groups cryopreserved with glycerol and DMSO compared to other experimental groups in hot temperature. Moreover, control and low level of melatonin-supplemented groups extended with either glycerol or DMSO revealed higher level of TAC than the groups supplemented with high melatonin level during cold temperature.

### Gene expression profile

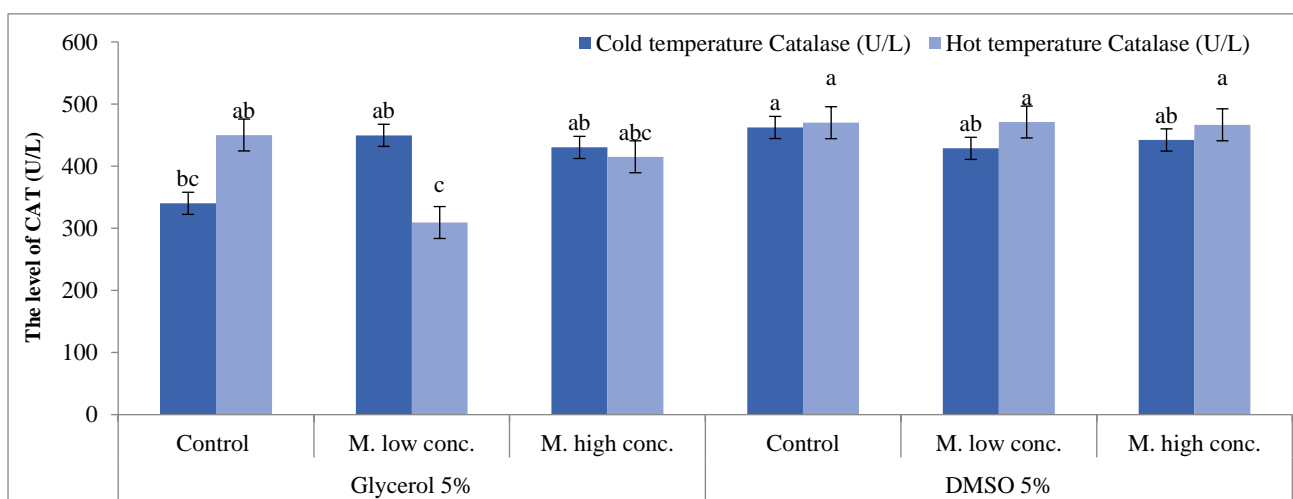
Transcript abundance of carnitine palmitoyl transferase 2 (CPT2) was up-regulated ( $P < 0.05$ ) in sperm samples cryopreserved in glycerol based extender compared with that cryopreserved with DMSO in cold temperature (Figure 5). Moreover, supplemented samples with low melatonin dose in DMSO based extender recorded higher ( $P < 0.05$ ) transcript abundance than all other sperm samples cryopreserved during hot temperature. The samples supplemented with low level of melatonin in both DMSO and glycerol based extender recorded the highest expression profile of this gene during cold and hot temperature. Expression profile of the ATP synthase F1 subunit Alpha (ATP5F1A) in sperm of glycerol based extender was increased significantly in comparison to those cryopreserved by DMSO during cold temperature (Figure 6). However, this gene had been down-regulated significantly in control group with DMSO compared to all semen samples cryopreserved during hot temperature. The expression of Nuclear Factor erythroid-derived 2-like 2 (NFE2L2, NRF2) was higher in samples cryopreserved in hot than cold temperature (Figure 7). In addition, the expression profile of this transcript was up-regulated in semen samples extended with DMSO than those extended with glycerol in hot temperature. There was no significant differences between all samples in the expression pattern of NFE2L2 during cold temperature although sperm samples cryopreserved with DMSO tended to show higher expression than those cryopreserved with glycerol. The transcript abundance of superoxide dismutase 2 (SOD2) gene was higher ( $P < 0.05$ ) in samples cryopreserved with glycerol than DMSO based extender during cold temperature (Figure 8). The expression of SOD2 was increased in control group extended with glycerol and low level of melatonin-supplemented group as well as with DMSO compared to all semen samples in hot temperature.



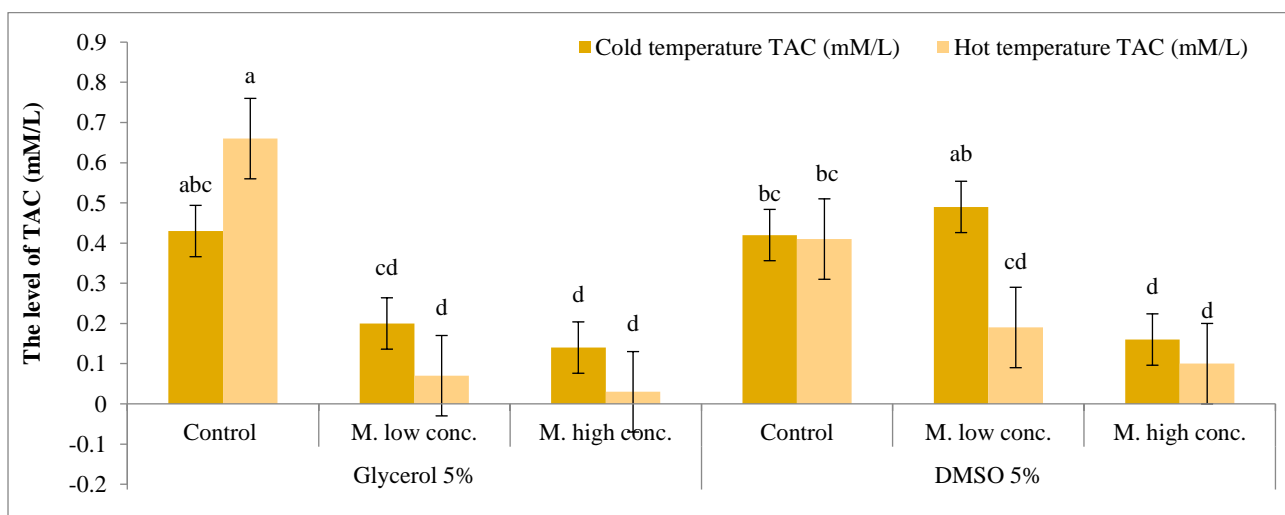
**Figure 1.** Enzymatic activity of Malondialdehyde of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. MDA: Malondialdehyde; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration



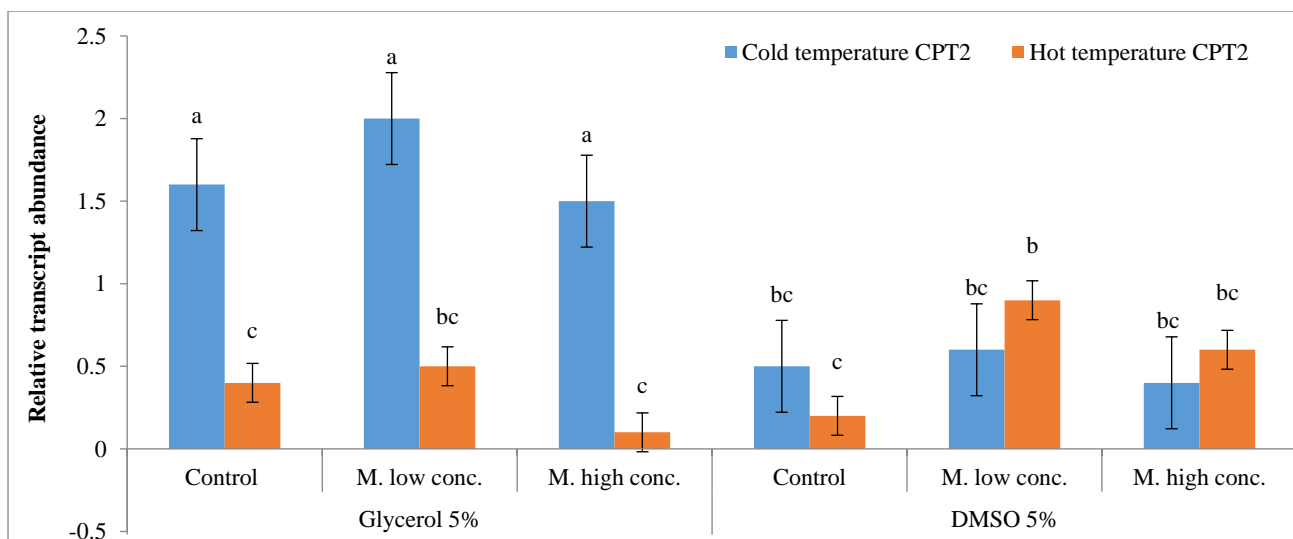
**Figure 2.** Enzymatic activity of glutathione peroxidase of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. GPx: glutathione peroxidase; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.



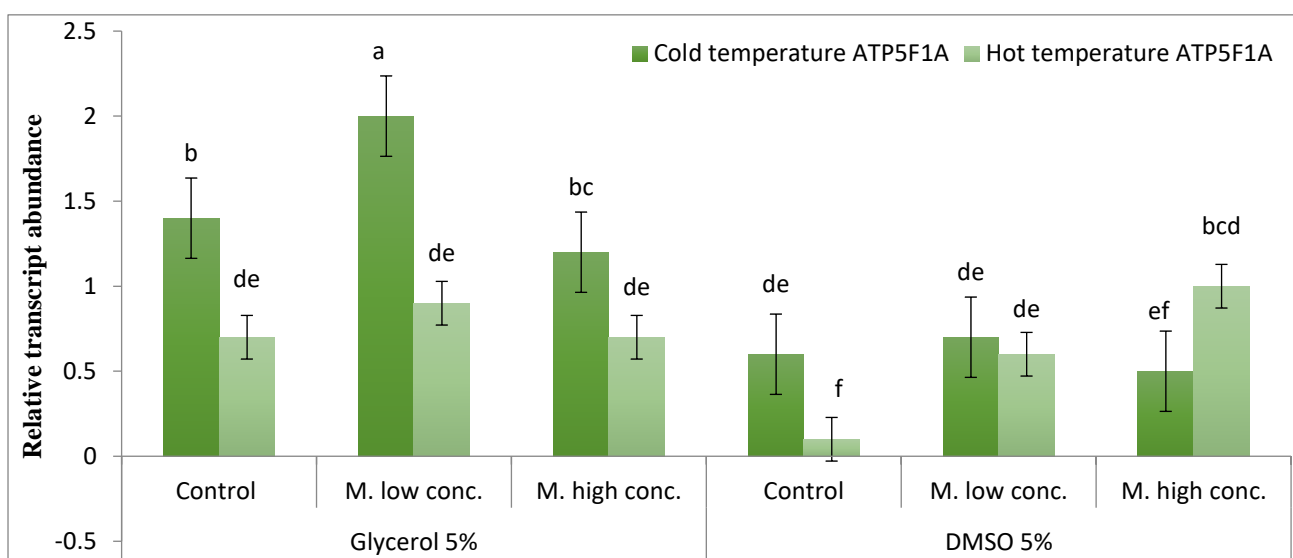
**Figure 3.** Enzymatic activity of Catalase of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. CAT: Catalase; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.



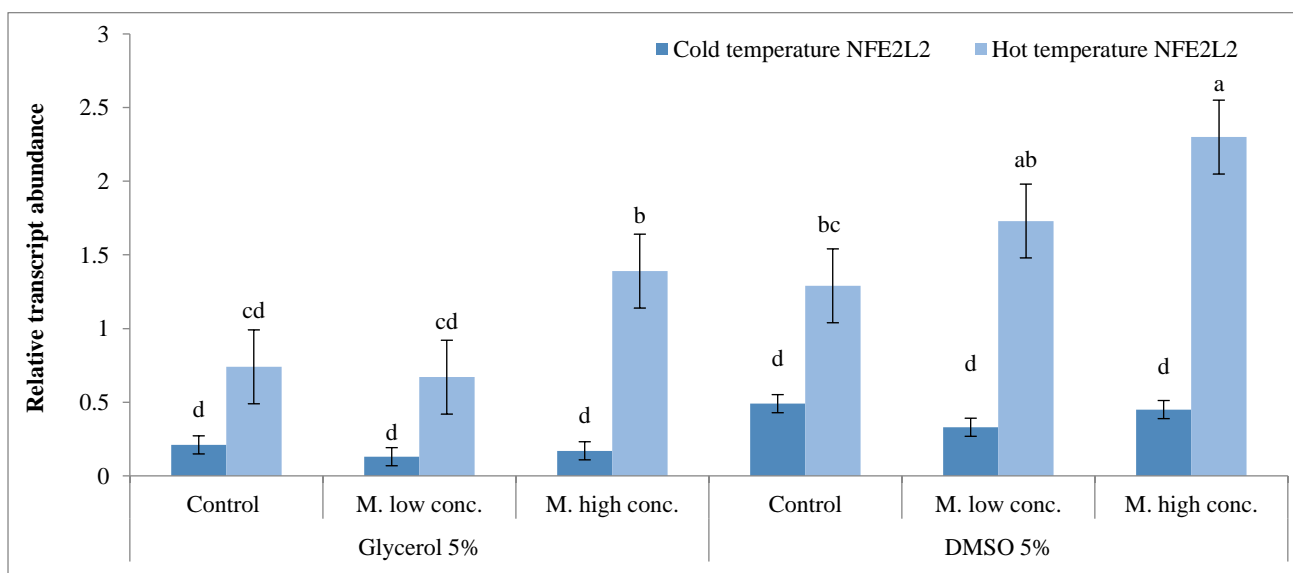
**Figure 4.** Total antioxidant capacity of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. TAC: Total Antioxidant Capacity; DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.



**Figure 5.** Relative transcript abundance of CPT2 gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

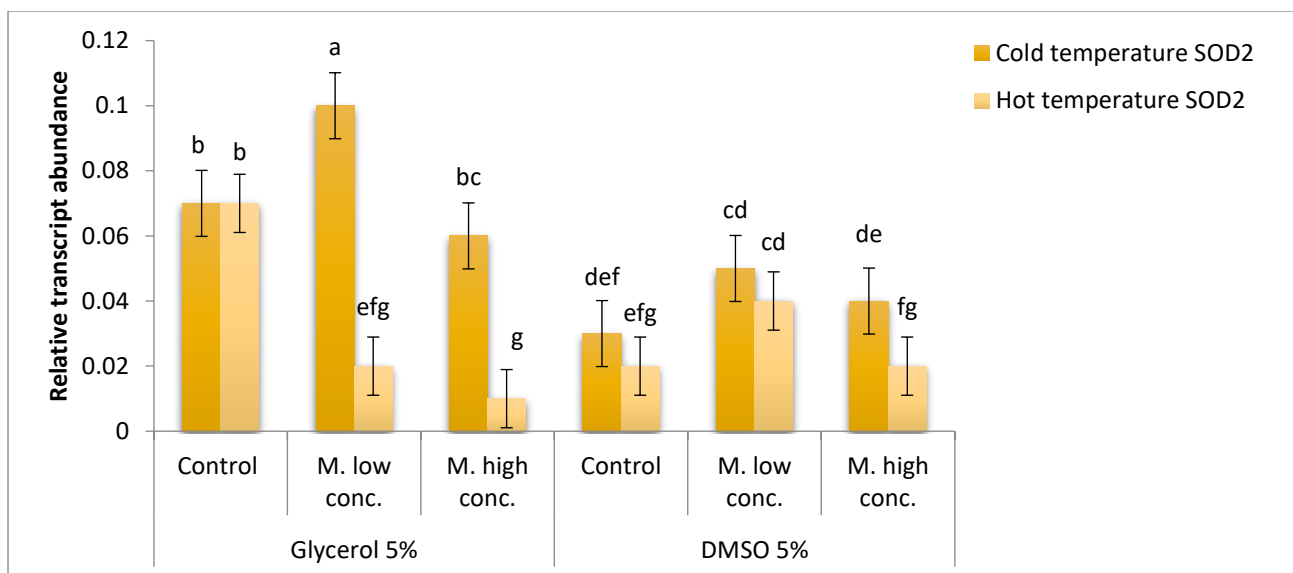


**Figure 6.** Relative transcript abundance of ATP5F1A gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.



**Figure 7.** Relative transcript abundance of NFE2L2 gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.





**Figure 8.** Relative transcript abundance of SOD2 gene of buck semen cryopreserved with two different extenders and supplemented with melatonin during cold and hot temperatures. DMSO: Dimethyl Sulfoxide; M. high Conc: melatonin high concentration; M. low conc: melatonin low concentration.

## DISCUSSION

Cryopreservation is an important method used in biotechnology of reproduction to preserve male semen and store it in a bank for future use (Sikarwar et al., 2015). It is an important method used in biotechnology of reproduction to preserve male semen and store it in a bank for future use. All metabolic activities are minimized during this process due to the freezing at low temperature (Sikarwar et al., 2015). However, freeze–thawing process could cause damage to sperm, mitochondria, acrosome and DNA that reduced its fertilizing ability (Watson, 2000 and Baumber et al., 2003). Moreover, cryopreservation could alter transcriptional profile of genes that considered biomarkers of spermatozoa quality (Valcarce et al., 2013; Hezavehei et al 2018).

The results of current study revealed that the percentages of total and progressive motility were improved in samples supplemented by low dose of melatonin ( $10^{-6}$  M) in comparison to control and high dose ( $10^{-3}$ M) in glycerol and in DMSO based extenders in hot season. This result confirmed the positive action of melatonin on sperm motility and viability (Ramadan et al., 2009; Du Plessis et al., 2010; Ashrafi et al., 2011 and 2013). Indeed, melatonin is a potent free radical scavenger through activation of antioxidant enzymes, which protect cells from oxidative stress (Ashrafi et al., 2013; Kilic et al., 2017). In addition, researchers have reported beneficial effects of melatonin on reducing the rate of lipid peroxidation in a dose dependent manner (Du Plessis et al., 2010; Succu et al., 2011 and Kilic et al., 2017). Succu, et al. (2011) have indicated that supplementing melatonin in a dose of 1.0 mM to ram extender maintained progressive motility, intracellular ATP concentrations, DNA integrity and fertilization rate in comparison to other doses (0.001, 0.01, 0.1, and 10 mM). In contrast, Souza et al. (2016) have stated that low concentration of melatonin (100 pM) increased the sperm motility, mitochondrial activity, integrity of plasma membrane and acrosome in ram semen in comparison to high concentrations (100 nM, and 100  $\mu$ M). Moreover, supplementation of melatonin at low dose (0.1 mM) to semen extender of buffalo bulls maintained the ultrastructure integrity of the sperm and increased pregnancy rate in compared to control and high doses groups (0.250, 0.500, 0.750 and 1 mM) (EL-Raey et al., 2015). The variation in melatonin concentrations supplemented to extender and their wide response on semen quality parameters could be due to animal species and breed used in these different experiments.

Present results (figures 1, 2 and 4) demonstrated that, melatonin supplementation has negatively correlated with some biochemical parameters of semen like MDA, GPX and TCA in glycerol and DMSO-based extenders during hot temperature. Indeed, the interaction among melatonin, season of collection and type of extender may be another factor as the quality and biochemical parameters changed with season of semen collection (Marti et al., 2007; Elsheikh et al., 2013 and Gabr et al., 2019). However, there are no available data describing the interaction among these three factors and downstream effect on semen cryopreservation. In general term, glycerol-based extender was better than DMSO as indicated from results presented in the current work which is in agreement with (Rasul et al., 2007). In accordance with our result, glycerol-based extender has enhanced post-thaw sperm motility, velocities (straight-line and average path), compared to other extenders that included DMSO in semen of buffalo bulls (Rasul et al., 2007). The post thaw sperm progressive motility rate was almost double in buck semen cryopreserved with 6% glycerol compared to that cryopreserved in 6% DMSO (Sikarwar et al., 2015). This could be due to rapid flow rate of DMSO due to its lower





