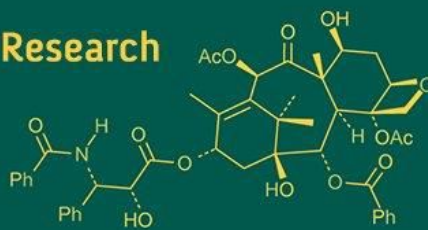


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Antioxidant modulation of apoptotic gene expression under thermal stress: Insights into mitigating summer-induced cellular damage

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Abstract

The present research work was undertaken to investigate the revitalizing potential of antioxidants such as Vitamin E, Alpha-lipoic acid and Melatonin against seasonal thermal stressed granulosa cells steroidogenic activity and survivability in *in vitro* cell culture model in buffalo. Different doses of antioxidants were prepared to assess the protective role of the respective antioxidants. Ovaries were collected from the local abattoir in different seasons i.e. summer season and winter season and were processed for the granulosa cells. The granulosa cells were cultured and further treatment was given with different antioxidants with different doses. We found that the mRNA expression of BCL-XL was significantly ($p < 0.05$) upregulated whereas, CASPASE 3 was significantly ($p < 0.05$) downregulated at a dose rate of Vitamin E (50 μ M), Alpha-lipoic acid (100 μ M) and Melatonin (30 μ M) during the winter season respectively. The mRNA expression of BCL-XL was significantly ($p < 0.05$) upregulated whereas, CASPASE 3 was significantly ($p < 0.05$) downregulated at a dose rate of Vitamin E (100 μ M), Alpha-lipoic acid (250 μ M) and Melatonin (50 μ M) during the summer season respectively.

Keywords: Revitalizing potential, vitamin E, alpha-lipoic acid, melatonin, mRNA expression

Introduction

The reproductive performance of buffaloes (*Bubalus bubalis*) is highly sensitive to environmental stressors, particularly thermal stress caused by seasonal temperature variations (Dash *et al.*, 2016) [3]. Heat stress disrupts the physiological homeostasis of animals, impairing ovarian function and granulosa cell activity, which are crucial for follicular development and oocyte maturation (Sammad *et al.*, 2022) [10]. Granulosa cells play a pivotal role in folliculogenesis by producing steroid hormones and providing support to the developing oocyte. However, thermal stress induces oxidative stress and apoptosis in granulosa cells, compromising fertility and reproductive efficiency (Liu *et al.*, 2023) [6].

Two key regulators of granulosa cell survival and apoptosis under stress are BCL-XL, an anti-apoptotic protein, and Caspase-3, an executioner enzyme in the apoptosis pathway (Valdez *et al.*, 2005) [12]. The balance between these proteins determines cell fate, and any disruption can have severe consequences for ovarian function (Regan *et al.*, 2018) [6].

The use of antioxidants such as vitamin E, alpha-lipoic acid, and melatonin has emerged as a promising strategy to mitigate the adverse effects of thermal stress. Vitamin E is a lipid-soluble antioxidant that protects cell membranes from oxidative damage by scavenging free radicals (Böhm, 2018) [2]. Alpha-lipoic acid is a potent antioxidant that functions both in aqueous and lipid environments, regenerates other antioxidants, and reduces oxidative stress-induced apoptosis (Xia *et al.*, 2019) [15]. Melatonin, a hormone with strong antioxidant and anti-apoptotic properties, regulates oxidative balance and supports mitochondrial function, thereby protecting granulosa cells from stress-induced damage (Xu *et al.*, 2023) [16]. Understanding their interaction could provide insights into strategies for enhancing buffalo reproductive performance and mitigating the detrimental effects of heat stress on fertility (Kandil *et al.*, 2024) [5].

Materials and Methods

Collection of Ovaries and isolation of Granulosa Cells

Bovine granulosa cells were processed as per Batalha *et al.* (2023) ^[1] with some modifications. Ovaries from healthy, cyclic female buffaloes were procured from a local abattoir following their slaughter process (40-50 ovaries per collection) and transported in a thermos containing normal saline (0.9% NaCl) at 37 °C, arriving at the laboratory within 1-2 h of post-slaughter. Ovaries underwent three washing procedures with pre-warmed normal saline solution, followed by wiping with 70% ethanol. The follicles housing the granulosa cells were aspirated with an 18-gauge needle using a 10 ml syringe containing a minimal volume of collection media under sterile conditions. The follicular aspirate was placed in a 15 mL sterilized Falcon tube and subjected to centrifugation at 277 g for 10 minutes. The residual supernatant was discarded, and the cell pellet was washed twice with 1X PBS by centrifugation at 277g for 10 minutes, followed by washing with complete media at 277g for 5 minutes. Following the removal of the supernatant, 1 mL of pre-warmed complete culture medium (DMEM supplemented with 10% FBS and 1.5% antibiotics) was added to resuspend the granulosa cells pellet. The viability of granulosa cells was assessed using the 0.4% Trypan blue exclusion method as described by Somal *et al.* (2017) ^[11].

Total RNA extraction and cDNA synthesis from granulosa cell culture

The total RNA was extracted from cultured bubaline granulosa cells using the QIAzol® method according to the manufacturer's protocol. Briefly, total RNA was extracted from the cell pellet using one mL of QIAzol®, followed by RNA pellet was resuspended in 20 µL of nuclease-free water. The concentration of extracted RNA was quantified using a spectrophotometer and samples were stored at -80 °C for further analysis. Consistent quantities of 1µg of total RNA from bubaline granulosa cells were reverse transcribed into complementary DNA (cDNA) utilizing the iScript™ as per the manufacturer's protocol.

mRNA expression analysis by qRT-PCR

The resultant cDNA served as a template in the qRT-PCR analysis for the relative mRNA expression of anti-apoptotic gene BCL-XL and pro-apoptotic gene CASPASE 3 with GAPDH as a reference gene. The qRT-PCR was done in duplicate according to the manufacturer's instructions. The specifications of the primer are delineated. Quantitative real-time polymerase chain reaction (qPCR) was employed to evaluate mRNA expression. Relative quantification of gene expression to the housekeeping gene GAPDH was determined using threshold (CT) values and PCR reaction efficiencies as per Pfaffl. (2001) ^[8].

Statistical Analysis

All experimental data are shown as mean±SEM. The statistical significance of differences, in mRNA expression of all target genes across different groups of antioxidant treatment with all the factors were assessed using two-way ANOVA followed by Tukey's multiple comparison test (GraphPad PRISM v 8.0, GraphPad software, Inc., USA). Significant differences were considered at the level when $p < 0.05$.

Results and Discussion

1. Effect of antioxidants on mRNA expression of CASPASE 3 in the cultured bGCs exposed to seasonal thermal stress

Vitamin E at the dose rate of 50 µM significantly ($p < 0.05$) downregulated CASPASE 3 (Fig. 1 A) expression in cultured bGCs of winter season but at 100 µM significantly ($p < 0.05$) downregulated CASPASE 3 (Fig. 1 A) expression in cultured bGCs in summer season as compared to other doses. Similarly, Alpha lipoic acid at the dose rate of 100 µM significantly ($p < 0.05$) downregulated CASPASE 3 (Fig. 1 B) expression in cultured bGCs in the winter season and at the dose rate of 250 µM significantly ($p < 0.05$) downregulated CASPASE 3 (Fig. 1 B) expression in cultured bGCs in the summer season. Melatonin at the dose rate of 30 µM and 50 µM dose significantly ($p < 0.05$) downregulated CASPASE 3 (Fig. 1 C) expression in the cultured bGCs of winter and summer season respectively. Significant ($p < 0.05$) difference in CASPASE 3 expression was observed between the cultured bGCs of winter and summer season in Vitamin E, Alpha lipoic acid and Melatonin treated groups. The capacity of ALA to neutralize free radicals either directly or indirectly through the preservation of antioxidants, including the recycling of GSH. CASPASE 3 activation is proposed to function as a downstream event of oxidative stress and is implicated in apoptosis. A reduction in CASPASE 3 mRNA in the testes of arsenic-treated mice may indicate the anti-apoptotic properties of ALA (Zoheir *et al.*, 2016) ^[17]. According to Wang *et al.* (2021) ^[13] pretreatment of granulosa cells with Vitamin E in H₂O₂ induced oxidative stress significantly decreased the mRNA expression level CASP3 levels. Similarly, in a study conducted by Wu *et al.* 2024 ^[14] demonstrated that the rat granulosa cells treated with melatonin and H₂O₂ showed downregulation of CASPASE 3 gene.

2. Effect of antioxidants on mRNA expression of BCL-XL in the cultured bGCs exposed to seasonal thermal stress

Vitamin E at the dose rate of 50 µM and 100 µM significantly ($p < 0.05$) upregulated BCL-XL (Fig. 2 A) expression in the cultured bGCs as compared to other doses in winter and summer season respectively. Similarly, Alpha lipoic acid at the dose rate of 100 µM and 250 µM significantly ($p < 0.05$) upregulated BCL-XL (Fig. 2 B) expression in the cultured bGCs in winter and summer season respectively. Melatonin at the dose rate of 30 µM and 50 µM dose significantly ($p < 0.05$) upregulated BCL-XL (Fig. 2 C) expression in cultured bGCs of winter and summer season respectively. Significant ($p < 0.05$) difference in BCL-XL expression was observed in between the cultured bGCs in winter and summer season in Vitamin E, Alpha lipoic acid and Melatonin treated groups. Antioxidant treatments, such as N-acetylcysteine (NAC), have demonstrated protective effects against hydrogen peroxide (H₂O₂)-induced apoptosis by modulating BCL-XL expression (Liu *et al.*, 2019) ^[7]. NAC treatment mitigates these effects by reducing reactive oxygen species (ROS) levels, preserving mitochondrial integrity, and enhancing BCL-XL expression, thereby promoting granulosa cell survival (Gottlieb *et al.*, 2000) ^[4].

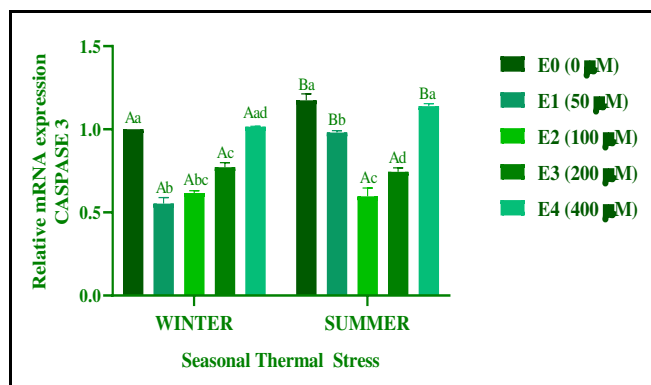


Fig 1A: Relative mRNA expression of CASPASE 3 in Vitamin E treatment. All values are shown as mean $\pm$ SEM. Different superscripts denote statistically different values ($p < 0.05$), a-e shows within group and A-B represents in between the group.

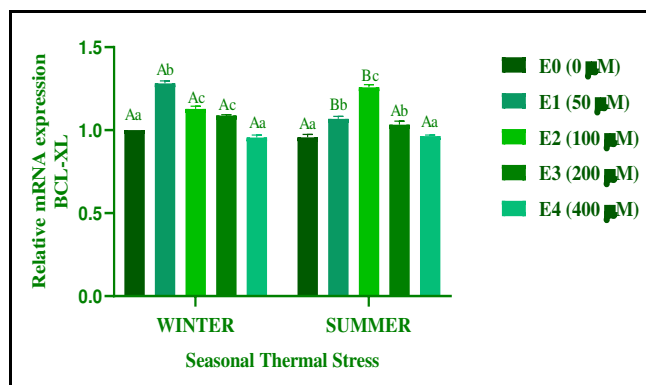


Fig 2A: Relative mRNA expression of BCL-XL in Vitamin E treatment. All values are shown as mean $\pm$ SEM. Different superscripts denote statistically different values ($p < 0.05$), a-e shows within group and A-B represents in between the group.

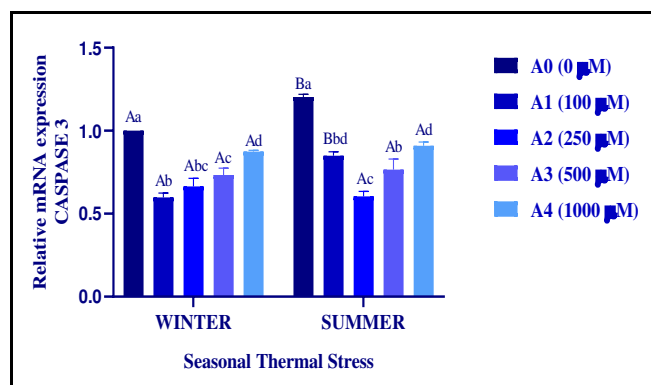


Fig 1B: Relative mRNA expression of CASPASE 3 in Alpha-Lipoic Acid treatment. All values are shown as mean $\pm$ SEM. Different superscripts denote statistically different values ($p < 0.05$), a-e shows within group and A-B represents in between the group.

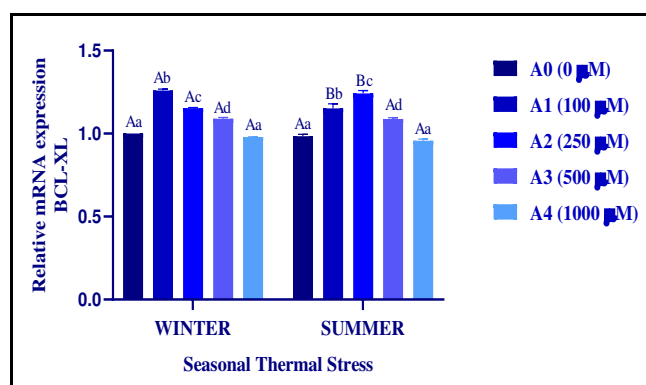


Fig 2B: Relative mRNA expression of BCL-XL in Alpha-Lipoic Acid treatment. All values are shown as mean $\pm$ SEM. Different superscripts denote statistically different values ($p < 0.05$), a-e shows within group and A-B represents in between the group.

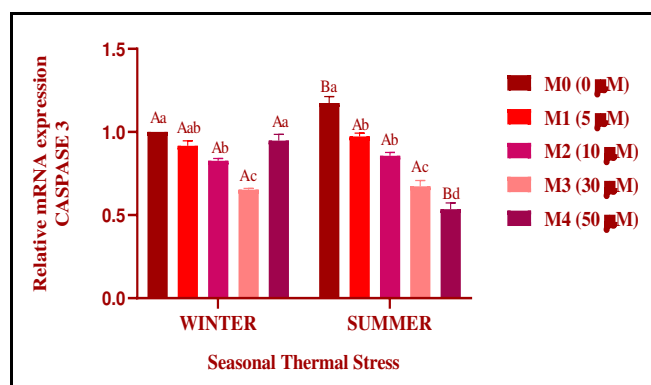


Fig 1C: Relative mRNA expression of CASPASE 3 in Melatonin treatment. All values are shown as mean $\pm$ SEM. Different superscripts denote statistically different values ($p < 0.05$), a-e shows within group and A-B represents in between the group.

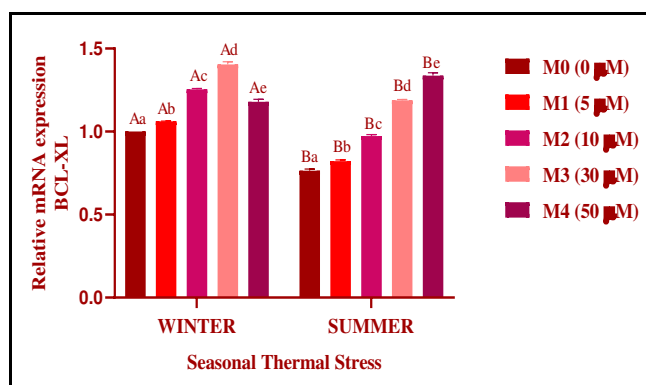


Fig 2C: Relative mRNA expression of BCL-XL in Melatonin treatment. All values are shown as mean $\pm$ SEM. Different superscripts denote statistically different values ($p < 0.05$), a-e shows within group and A-B represents in between the group.

Table 1A: Mean $\pm$ SEM of Vitamin E treatment group

Vitamin E/CASPASE 3	Winter	Summer
E0 (0 μ M)	1 ^{Aa} $\pm$ 0	1.17 ^{Ba} $\pm$ 0.03
E1 (50 μ M)	0.55 ^{Ab} $\pm$ 0.03	0.98 ^{Bb} $\pm$ 0.01
E2 (100 μ M)	0.61 ^{Abc} $\pm$ 0.01	0.59 ^{Ac} $\pm$ 0.04
E3 (200 μ M)	0.77 ^{Ac} $\pm$ 0.02	0.74 ^{Ad} $\pm$ 0.02
E4 (400 μ M)	1.01 ^{Aad} $\pm$ 0.00	1.13 ^{Ba} $\pm$ 0.01

Table 1B: Mean ± SEM of Alpha-Lipoic Acid treatment group

Alpha-Lipoic Acid/CASPASE 3	Winter	Summer
A0 (0 µM)	1 ^{Aa} ±0	1.20 ^{Ba} ±0.01
A1 (100 µM)	0.59 ^{Ab} ±0.02	0.84 ^{Bb} ±0.02
A2 (250 µM)	0.66 ^{Abc} ±0.04	0.60 ^{Ac} ±0.03
A3 (500 µM)	0.73 ^{Ac} ±0.04	0.76 ^{Ad} ±0.06
A4 (1000 µM)	0.87 ^{Ad} ±0.00	0.90 ^{Ba} ±0.02

Table 1C: Mean ± SEM of Melatonin treatment group

Melatonin/ CASPASE 3	Winter	Summer
M0 (0 µM)	1 ^{Aa} ±0	1.17 ^{Ba} ±0.03
M1 (05 µM)	0.91 ^{Ab} ±0.03	0.97 ^{Bbd} ±0.02
M2 (10 µM)	0.82 ^{Abc} ±0.01	0.85 ^{Ac} ±0.01
M3 (30 µM)	0.65 ^{Ac} ±0.00	0.67 ^{Ab} ±0.03
M4 (50 µM)	0.94 ^{Ad} ±0.03	0.53 ^{Ad} ±0.03

Table 2A: Mean ± SEM of Vitamin E treatment group

Vitamin E/ BCL-XL	Winter	Summer
E0 (0 µM)	1 ^{Aa} ±0	0.95 ^{Aa} ±0.02
E1 (50 µM)	1.28 ^{Ab} ±0.02	1.06 ^{Bb} ±0.02
E2 (100 µM)	1.12 ^{Ac} ±0.02	1.25 ^{Bc} ±0.02
E3 (200 µM)	1.08 ^{Ac} ±0.00	1.03 ^{Ab} ±0.02
E4 (400 µM)	0.95 ^{Aa} ±0.02	0.96 ^{Aa} ±0.00

Table 2B: Mean ± SEM of Alpha-Lipoic Acid treatment group

Alpha-Lipoic Acid/ BCL-XL	Winter	Summer
A0 (0 µM)	1 ^{Aa} ±0	0.98 ^{Aa} ±0.01
A1 (100 µM)	1.25 ^{Ab} ±0.01	1.15 ^{Bb} ±0.03
A2 (250 µM)	1.15 ^{Ac} ±0.00	1.24 ^{Bc} ±0.02
A3 (500 µM)	1.08 ^{Ad} ±0.01	1.08 ^{Ad} ±0.00
A4 (1000 µM)	0.97 ^{Aa} ±0.00	0.95 ^{Aa} ±0.01

Table 2C: Mean ± SEM of Melatonin treatment group

Melatonin/BCL-XL	Winter	Summer
M0 (0 µM)	1 ^{Aa} ±0	0.76 ^{Ba} ±0.01
M1 (05 µM)	1.06 ^{Ab} ±0.00	0.82 ^{Bb} ±0.01
M2 (10 µM)	1.25 ^{Ac} ±0.00	0.97 ^{Bc} ±0.01
M3 (30 µM)	1.40 ^{Ad} ±0.02	1.18 ^{Bd} ±0.00
M4 (50 µM)	1.17 ^{Ac} ±0.02	1.33 ^{Bc} ±0.02

Conclusion

Our findings suggest that Vitamin E, Alpha lipoic acid and Melatonin as antioxidants hold significant revitalizing potential in mitigating the adverse effects of thermal stress on granulosa cells by reducing oxidative stress, scavenging reactive oxygen species (ROS), and stabilizing mitochondrial function under challenging environmental conditions.

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