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Effect of Heat Stress and Amelioration by Antioxidants on Expression Profile of Pro- and Anti-Apoptotic Genes in *in vitro* Matured Bovine Oocytes

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A B S T R A C T

Heat stress often leads to apoptosis of oocytes through generation of free radicals. The use of antioxidants has been found to mitigate the harmful effects of these free radicals and probably apoptosis itself. The present study was conducted to evaluate the effect of heat stress on expression profile of genes related to apoptosis (pro-apoptotic *Bad* and *Bax*; and anti-apoptotic *Bcl-2*) during oocyte maturation and the ameliorating effects of select antioxidants- *viz.* melatonin and zinc. In the experiment, bovine oocytes were divided into 4 groups and Group II, III, IV was matured under heat-stress at 41°C. Moreover, group III and IV were supplemented with antioxidant melatonin and zinc respectively, incorporated in the oocyte maturation medium (OMM), while Group II served as antioxidant control and was matured with OMM alone. Group I served as control and was matured without heat-stress (38.5°C) and antioxidant supplementation. After maturation, the total RNA was isolated for *Bcl-2*, *Bax* and *Bad* expression. It was found that there was up regulation of *Bad* and *Bcl-2* gene expression during induced heat-stress without any supplementation (Group-II). *Bax* was down regulated in all groups, while *Bad* was down-regulated in melatonin and zinc supplemented groups. It is speculated that supplementation with zinc probably induced early maturation changes in the oocyte and induced an early meiotic arrest, which was associated with a sharp decline in all apoptosis modulator transcripts. It sis concluded that by detoxifying ROS, antioxidants may therefore subsequently reverse the ROS-induced decline in *Bcl-2* and prevent apoptosis.

Introduction

The mechanism by which heat stress leads to a disruption in developmental competence of the oocyte remains unclear; however, one of the processes that may be involved is apoptosis, although there have been few studies on extrinsic or intrinsic control systems in reproduction for its activation (Roth and Hansen, 2004). Apoptosis is regulated by the interplay of the pro- and anti-apoptotic (pro-survival) factors, involving chiefly members of the B-cell lymphoma/ leukemia 2 (BCL-2, Bcl-2) family of proteins (Youle and Strasser, 2008). All pro-apoptotic and pro-survival (anti-apoptotic) proteins belong to the Bcl-2 family (Reed *et al.,* 1996). Bcl-2 protein counteracts Bax, and when Bax is in excess, cells execute a death command; but, when Bcl-2 dominates, the program is inhibited and cells survive. The pro or anti-apoptotic activities of the *Bcl-2* family members are regulated not only at the transcriptional level, but also at the post-translational level, including phosphorylation, cleavage, translocation, and dimerization (Gross *et al.,* 1999). Expression abundance of the Bax and Bcl-2 genes are good markers for oocyte apoptosis and subsequent embryo development (Li *et al.*, 2009).

Bax forms a heterodimer with Bcl-2, and functions as an apoptotic activator and have been reported to interact with, and increase the opening of, the mitochondrial voltage-dependent anion channel (VDAC), which leads to the loss in membrane potential and the release of cytochrome-c (Shi *et al.,* 2003).

Bad (Bcl-2-associated death promoter) is a member of the BH3-only subfamily of the Bcl-2 family. Bad is dephosphorylated and activated to form a heterodimer with anti-apoptotic proteins Bcl-2 and Bcl-xL and prevent them from avoiding apoptosis. Free radicals can initiate a chain of reactions involved in modulation of signal transduction pathways, including regulation of tissue growth and apoptosis. Studies have shown that the redox status of the cell, resulting from an accumulation of Reactive Oxygen Species (ROS) and a decrease of antioxidant levels, is involved in inducing apoptotic cell death (Hockenbery *et al.,* 1993) and GSH presumably plays a critical role in regulating apoptosis by influencing the redox status (Boggs *et al.,* 1998). Loven (1988) suspected that free radical production may be one mechanism by which heat shock alters cellular function.

Cellular exposure to heat stress increases the production of ROS, thereby promoting cellular oxidation events (Skibba *et al.,* 1991; Sikka *et al.,* 1995; Ikeda *et al.,* 1999; Kim *et al.,* 2005) and also associated cellular hyperthermia (Skibba and Stadnicka, 1986; Malayer *et al.,* 1990; Ando *et al.,* 1997). Incorporation of antioxidants has been reported to moderate the deleterious effects of heat-stress on oocytes (Hansen, 2009; Ahmed *et al.,* 2016) seemingly due to the generation of reactive oxygen species. This has also been amply documented in cattle with retinol *in-vitro* (Lawrence *et al.,* 2004) as well as in mice with epigallocatechingallate (EGCG) *in-vivo* during the preovulatory period (Roth *et al.,* 2008). Various studies suggest the role of antioxidants in mitigating the deleterious effects of ROS as an inducer of apoptosis.

The present study was undertaken to evaluate the expression of pro-apoptotic genes *Bad* and *Bax* and the anti-apoptotic *Bcl-2* gene by bovine oocytes during maturation under heat stress (41°C). Simultaneously, two candidate antioxidants *viz.* melatonin and zinc were added to the oocyte maturation medium (OMM) to evaluate if they had any amelioration effect, while influencing the expression of the apoptotic genes.

Materials and Methods

Collection of oocytes and *in vitro* Maturation (IVM)

Aspiration media and oocyte maturation media (OMM) were prepared according to Dutta *et al.*, 2013. Ovaries from cows were collected from local abattoirs immediately post-slaughter and transported to the laboratory in sterile pre-warmed normal saline containing antibiotic (Penicillin G @ 0.06g/1000 ml) at 37°C. The connective tissue covering the ovaries were removed, and washed thrice with normal saline containing antibiotic.

Cumulus oocyte complexes (COCs) were collected by aspiration of surface follicles with a sterile 18 gauge needle attached to a 10 ml syringe containing the aspiration medium. Only follicles of 2-8 mm diameter or greater were selected amongst those present on the surface. The COCs were separated from the debris and picked individually under a stereo-zoom microscope on to another petridish with washing medium and graded according to Hafez and Hafez, 2000, while only Grade 'A' and 'B' COCs were selected for in vitro maturation. OMM droplets were prepared by taking 50 µl of *in vitro* OMM in a 35 mm petridish and covered with sterile 0.2 µm filtered mineral oil and incubated for 1 hour in a CO_2 incubator at 38.5°C with 5% CO_2 and humidified air. Selected COCs (A and B grade) were washed six times in washing media and twice in OMM media. Approximately 10-12 washed COCs were then transferred into each OMM droplet for maturation and incubated for 24 hours in a CO_2 incubator at 38.5°C with 5% CO_2 and humidified air. For heat stress studies COCs were exposed to 41°C temperature during the first 12 hrs of *in vitro* maturation (IVM) as described by Roth and Hansen (Roth and Hansen, 2004).

Antioxidant supplementation

Oocyte Maturation Medium (OMM) was supplemented either with 1 nM melatonin (Sigma, India) modified from Jang *et al.*, 2005 and prepared according to Farahavar *et al.*, 2010; or 1.5 µg/ml (~11 mM) Zinc modified from Picco *et al.*, 2010 as zinc chloride (Sigma, India).

Experimental design

In the experiment, bovine oocytes were divided into 4 groups and Group II, III, IV was matured under heat-stress at 41°C. Moreover, group III and IV were supplemented with antioxidant melatonin and zinc respectively, while Group II served as antioxidant control and was matured with OMM alone. Group I served as control and was matured without heat-stress (38.5°C) and antioxidant supplementation.

Isolation of total RNA

Total RNA from oocytes was isolated using a commercially available kit (Promega, SV Total RNA Isolation System, #Z3100) according to manufacturer's instructions.

cDNA synthesis and quantitative real time PCR (qPCR)

The first strand cDNA was synthesized from the isolated total RNA. Reverse transcription of the RNA extracted from oocytes was performed using the following reagents-(a) RevertAid™ M-MuL Reverse Transcriptase (Thermo Scientific, #EP0441), (b) Ribolock (Ribonuclease inhibitor) (40 u/µL) (Thermo Scientific, #EO0381), (c) 10 mM dNTP mix (Thermo Scientific, #R0192) and (d) Random hexamer (0.2µg/µl) (Thermo Scientific, #SO142). Reverse transcription reaction was carried out with two-step PCR cycling condition at 70˚C for 5 min, 25°C for 10 min

(1st cycling condition) and 25˚C for 5 min,42°C for 60 min and 70˚C (2nd cycling condition) in a thermal cycler. Primers for *Bcl-2*, *Bad*, *Bax* and reference gene (*GAPDH*) were used (Table 1). The yield of total RNA and cDNA were routinely checked to be pure spectrophotometrically (Thermo, NanoDrop 1000). For total nucleic acid yield, sample concentration was expressed in nμ/μl as estimated at 260nm. The purity was estimated from the relative absorbance at 230, 260 and 280nm.The A260/A280 ratio of absorbance was used to assess the purity of DNA and RNA. A ratio of ~1.8 was generally accepted as "pure" for DNA; a ratio of ~2.0 was generally accepted as "pure" for RNA. The A260/A230 ratio of sample absorbance was also used as secondary measure of nucleic acid purity which were often higher (1.8 - 2.2) for "pure" nucleic acid than the respective 260/280 values.

The real time PCR reaction was carried out in Applied Biosystems, StepOnePlus™ Real-Time PCR System with 3.0 μl of c DNA template, 10.0 μl of Maxima SYBR green qPCR master mix and volume of *Bcl-2*, *Bad*, *Bax* and *GAPDH* sequence specific forward and reverse primers (5pmol/ μl) were used and final volume of 20 μl was made with nuclease free water (Table 2). The realtime PCR program (Table 3) consisted of initial heating at 95ºC for 10 min followed by 95ºC for 15 sec and samples were amplified for 40 cycles (60ºC for 45 sec and 95°C for 15 sec. The melt curve stage for one more cycle at 60ºC for 1 min and 95ºC for 15 sec.

The relative quantification of target genes expression was calculated using $2^{-\Delta\Delta Ct}$. The threshold cycle (Ct) values were based on triplicate measurements and each experiment was repeated twice. The quantification values obtained for target genes in control were used for calibration and were arbitrarily set to 1 and 0 for linear and log graph types respectively. The data analysis was carried out by StepOne® Plus software v2.2.2 using the Ct method employing GAPDH as reference gene for normalization [ΔCT = Ct of target gene (ΔCTT) - Ct of reference gene (ΔCTR)]. The threshold line was assigned to all PCR reactions and the cut-off C_T value was taken after 40 cycles. To confirm the specificity of each product, melt curve analysis was conducted.

The experimentation was cleared by Institutional Animal Ethics Committee (IAEC) under CPCSEA.

Results and Discussion

Verification of cDNA synthesis

After cDNA synthesis, PCR was performed for confirmation of product size of primers by electrophoresis on 2% agarose gel and also in 12% SDS PAGE (Figure 1).

Screening the transcription profile of *Bcl-2*, *Bad* and *Bax* genes by qPCR showed that they were expressed in oocytes matured in different antioxidant supplemented and non-supplemented OMM with heat stress as well as non-supplemented OMM without heat stress. The relative quantification (RQ) values of *Bcl-2*, *Bad* and *Bax* gene mRNA expression are presented in Figure 2. Melt curve analysis also gave a single peak in positive samples for each of the target products suggesting a single size product.

The relative quantification (RQ) values of *Bcl-2* indicated that the expression of *Bcl-2* gene was up-regulated in oocytes that were heat-stressed in non-supplemented OMM when compared with oocytes under normal temperature and non-supplemented control. RQ values for *Bad* expression was up-regulated only in non-supplemented heat-stressed OMM and down-regulated in

melatonin and zinc supplemented OMM. RQ values for *Bax* was down regulated in zinc and melatonin supplemented OMM. In heat stressed non-supplemented group there is down-regulation of *Bax* expression than reference control.

Heat stress and non-supplemented group

In the present study, it is speculated that the existence of pro-apoptotic signals due to heat stress would probably lead to the elevation of *Bad* mRNA to counter the pro-survival elevated expression of *Bcl-2*. This would result in increased translation and formation of heterodimers between dephosphorylated *Bad* and *Bcl-2*, thereby shifting the balance towards apoptosis by leaving the *Bax* pro-apoptotic protein free. Yang and Rajamahendran (2002) reported that the expression of *Bax* was found in all types of oocytes and embryos, with the highest expression in the denuded oocytes. Similarly, a high level of *Bax* has also been observed in degenerating oocytes (Felici *et al.,* 1999) indicating spontaneous apoptosis, and that good quality oocytes are resistant to apoptosis and are *Bax* deficient (Perez *et al.,* 1997). Reportedly, *Bax* is also significantly altered by the modification of culture conditions, or oocytes with different developmental competence (Nemcova *et al.,* 2006). Furthermore, expression of *Bax* is observed to be higher in blastocysts cultivated in a synthetic oviduct medium (SOF) than in those cultured in ovine oviduct or *in vivo* (Lonergan *et al.,* 2003). Similarly, expression of *Bax* mRNA was observed to be significantly higher ($p<0.05$) for the buffalo oocytes matured at higher temperatures (40.5 and 41.5°C) at both the incubations (12 and 24 h) compared to control, while mRNA expression of *Bcl-2* decreased significantly ($p<0.05$) in the treatment groups compared to control (Ashraf *et al.,* 2014). *Bax*/*Bcl-2* ratio has also been found to be almost six times higher in buffalo oocytes immediately after the heat stress that could lead to apoptosis (Singh, 2015). We further observed that elevated *Bad* profiles were associated only in non-supplemented control group and not in any of the oocytes supplemented with antioxidant melatonin and zinc.

Heat stress and melatonin

In the present study there was a down regulation of both *Bax* and *Bad* pro-apoptotic transcripts. *Bcl-2* decreased expression was also noticed by us and probably was due to the associated decrease of the pro-apoptotic transcripts. An alternative but not mutually exclusive hypothesis suggested by Hildeman *et al.,* (2003), is that ROS act to down-regulate endogenous *Bcl-2* levels within cells, and because levels of *Bcl-2* within cells are critical to anti-apoptotic activity, decreasing *Bcl-2* could be a mechanism to sensitize cells to apoptosis. By detoxifying ROS, antioxidants (i.e. melatonin, as in this case) may therefore subsequently reverse the ROS-induced decline in *Bcl-2* and prevent apoptosis. The entry of oocytes into a state of meiotic arrest may also be associated with reduced transcription and translational activities as observed in all three *Bcl-2*, *Bad* and *Bax* transcripts under investigation.

Heat stress and zinc

The supplementation of zinc in the media brought about a down-regulation of both pro-apoptotic transcripts *Bad* and *Bax* exceeding the levels induced by melatonin. However, the precise mechanism of zinc as an antioxidant is unclear. An alternate credible explanation is the importance of zinc in inducing meiotic arrest of the oocytes throughout the entire oocyte maturation process during the first (Kong *et al.,* 2012) and second (Kim *et al.,* 2010) meiotic arrest points.

Table.1 Primers used for expression and quantification studies of Bcl-2, Bax, Bad and GAPDH using SYBR® Green based qPCR

Gene primer sequence	Annealing temp.	Product size	Accession No.	Reference
Bcl-2				
TCGTGGCCTTCTTTGAGTTC	60	109	XM_586976.4	Fear and Hansen (2011)
CGGTTCAGGTACTCGGTCAT				
Bax				
CTCCCCGAGAGGTCTTTTTC	60	176	NM_173894.1	Fear and Hansen (2011)
TCGAAGGAAGTCCAATGTCC				
Bad				
CTTTTCTGCAGGCCTTATGC	59	151	NM_001035459.1	Fear and Hansen (2011)
GGTAAGGGCGGAAAAACTTC				
***GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase)**				
AAGGTCGGAGTGAACGGATTC	60	170	-	Hashem *et al.* (2013)
TTGACTGTGCCGTTGAACTTG				

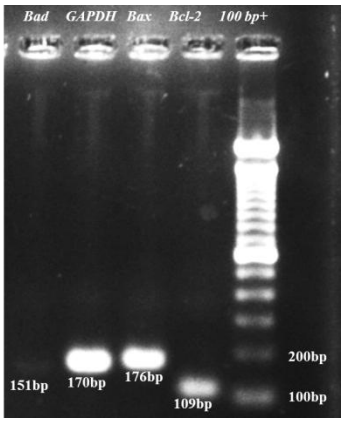
Table.2 Components of qPCR reaction mixture

Components	Reaction mixture	Non-Template Control (NTC)
Maxima SYBR green/ROX qPCR Master Mix (2X)	10.0 µl	10.0 µl
Forward primer (5pmol/µl)	0.50 µl	0.50 µl
Reverse primer (5pmol/µl)	0.50 µl	0.50 µl
cDNA template	3.0 µl	_
Nuclease free water	6.0 µl	9.0µl
Total reaction volume	**20.0 µl**	**20.0 µl**

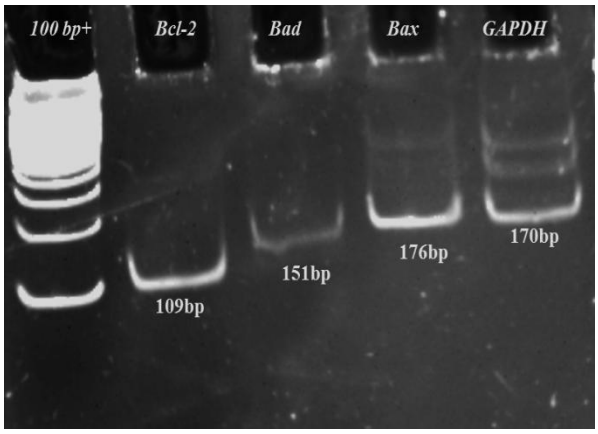
Table.3 Conditions for SYBR® Green based qPCR reaction

Step	Temperature (^{o}C)	Duration	Cycles
Initial Denaturation	95	10 min	HOLD
Denaturation	95	15 sec	40
Anneal/ Extend	60	45 sec	
Melt curve stage	95	15 sec	1
	60	1 min	
	95	15 sec	

Fig.1 Gel electrophoresis of amplicons generated by q-PCR showing specific bands for apoptotic genes Bcl-2, Bad, Bax and reference gene GAPDH

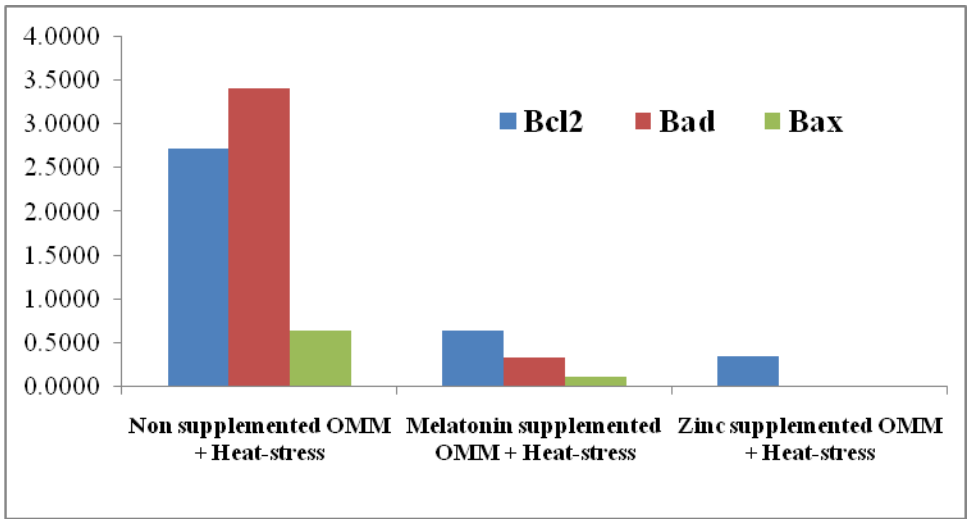


2% Agarose



12% Polyacrylamide gel electrophoresis (PAGE)

Fig.2 Relative quantification (RQ) by q-PCR of Bcl-2, Bad and Bax mRNA expression in bovine oocytes matured in non-supplemented, and antioxidant melatonin, and zinc supplement Oocyte Maturation Medium (OMM) matured under elevated temperatures (41°C).



It may be reasoned that the entry of the oocytes in a state of meiotic arrest could bring about a decrease in transcriptional activities. And since, the induction of meiotic arrest is profoundly modulated by zinc, it is reasonable that the transcriptional activities be more affected. Similarly, barely detectable *Bcl-2* has been described in oocytes entering into meiosis without changing its expression during the stage of meiotic prophase-I (Felici *et al.,* 1999). Jeon *et al.,* (2014) observed that treatment with adequate zinc concentrations during IVM improved the developmental potential of porcine embryos by regulating the intracellular GSH concentration, the ROS level and transcription factor expression, and transcript levels of *Bax* were decreased in zinc-treated cumulus cells and oocytes, whereas, *Bcl-2* transcript levels were significantly higher in zinc-treated IVF blastocysts. It is postulated that supplementation with zinc probably induced

early maturation changes in the oocyte and induced an early meiotic arrest, which was associated with a sharp decline in all apoptosis modulator transcripts. From the present study, it may be concluded that *Bax* expression may be lower in good quality oocytes, as only good quality eggs were selected for the experimentation. Elevated *Bad* profiles were associated only in non-supplemented control group and not in any of the oocytes supplemented with antioxidants melatonin or zinc. The ameliorating effects of antioxidants resulting in the decreased expression of pro-apoptotic genes, verifies an underlying oxidative stress mechanism for apoptosis, and that their incorporation in *in-vitro* medium is beneficial during heat stress. The meiotic arrest after maturation may be involved in an inhibition of transcription activity of the oocyte which was seen in expression profiles of apoptosis modulator genes. Supplementation with zinc probably induced early maturation changes in the oocyte and induced an early meiotic arrest, which was associated with a sharp decline in all apoptosis modulator transcripts.

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