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GAMETE BIOLOGY

Developmental competence and expression pattern of bubaline *(Bubalus bubalis)* oocytes subjected to elevated temperatures during meiotic maturation in vitro

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Abstract
Objective To determine the direct effect of physiologically relevant high temperatures (40.5 and 41.5 °C) for two time periods (12 and 24 h) on bubaline oocytes during in vitro maturation.
Method The control group oocytes were cultured at 38.5 °C for 24 h. The treatment 1 (T1) and 3 (T3) group oocytes were cultured at 40.5 and 41.5 °C respectively, for the first 12 h and at 38.5 °C for rest of the 12 h. However, treatment 2 (T2) and 4 (T4) group oocytes were cultured at 40.5 and 41.5 °C for complete 24 h.
Results Development of oocytes to blastocyst was severely compromised ($p<0.001$) when matured at 40.5 and 41.5 °C for both exposure periods (12 h and 24 h). It was found that the cleavage rates, blastocyst yield and mean cell number decreased remarkably ($p<0.001$) in the treatment groups compared to control. The relative mRNA expression of heat shock protein (*Hsp 70.1, 70.2, 70.8, 60, 10* and *HSF1*), pro-apoptotic (*caspases-3, −7, −8, Bid* and *Bax*) and oxidative stress (*iNOS*) related genes was significantly higher ($p<0.05$) in all the treatment groups compared to control. However, mRNA abundance of anti-apoptotic (*Bcl-2, Mcl-1, Bcl-xl*), glucose transport (*Glut1*, *Glut3* and *IGF1R)*, developmental competence (*ZAR1* and *BMP15*) and oxidative stress (*MnSOD*) related genes was significantly decreased ($p<0.05$) in the treatment groups compared to control.
Conclusion The present study clearly establishes that physiologically relevant elevated temperatures during in vitro meiotic maturation reduce developmental competence of bubaline oocytes.

Keywords Bubaline · Oocyte · Embryo · In vitro maturation · Heat stress

Capsule Oocyte maturation is the very first and critical stage in the overall embryo development. Therefore, exploiting the genetic determinants of oocyte might prove helpful in alleviating the embryonic losses in buffalo due to heat stress.

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Introduction

Elevated ambient temperature results in heat stress which is a major concern for production agriculture in tropical and subtropical countries across the globe due to its negative impact on reproduction [1]. The physiological state of animals is conducive in a certain comfort zone, immediately crossing this zone, animals experience either hypothermy or hyperthermy. The animals adjust at lower temperatures with a variation of about 15–25 °C, however, a rise of temperature by only 3–6 °C over the comfort zone is experienced as heat stress, which explains the higher concern for heat stress compared to cold stress [2]. Buffalo, the principal dairy animal of India, contribute over half of the milk production in the country. The core body temperature of buffalo ranges between 38.5 and 39 °C and may reach up to 41.5 °C on exposure to solar radiation during summer [3–5]. Although, buffalo are very well adapted to hot and humid climatic conditions but selection for this tolerance has traditionally resulted in their impaired productive and reproductive performance [6].

Buffalo are more prone to heat stress due to scarcely distributed sweat glands, dark body colour and sparse hair

on body surface [4] and therefore, get easily distressed reducing their reproductive capacity. Studies in cattle suggest that oocytes are directly susceptible to heat stress in addition to the alterations in follicular gonadotropic environment. However, such studies still need to be carried out in buffalo. In buffalo, reduced fertility during summer months is mostly because of hyperthermia. There are several possible mechanisms by which heat stress may affect the reproduction in buffalo. Heat stress may indirectly affect the follicular surge of gonadotropins [7] or may directly affect the quality of oocyte [8]. A close correlation was observed between seasons of the year and percentage of good quality oocytes. Buffalo in spring season had higher good quality oocytes, recovery and better maturation than the oocytes recovered in summer season [8, 9]. In cattle, the oocytes harvested from cows during the summer season were of lower quality, had reduced ability to develop into blastocysts in vitro and reduced potential of undergoing fertilization and subsequent embryo development [10, 11]. Moreover, through in vivo experiments, increased proportion of abnormal and retarded embryos following exposure of heifers to heat stress between the onset of estrus and insemination, further explains the susceptibility of oocyte maturation to heat stress [12]. However, the molecular and cellular mechanisms of heat induced reduced buffalo oocyte competence and its effect on embryo viability still remains poorly understood. Moreover, the direct effect of physiologically relevant elevated temperature on the maturation of bubaline oocytes in vitro has not been evaluated. Therefore, this study was designed to ascertain the direct effects of physiologically relevant elevated temperature on the maturation of buffalo oocytes and its effect on the expression of candidate genes related to heat stress, developmental competence, apoptosis, oxidative stress and metabolism.

Materials and methods

In the present investigation, the direct effect of high temperatures (40.5 and 41.5 °C) on bubaline oocytes during in vitro meiotic maturation for 12 h and 24 h was evaluated. Control group oocytes were cultured at 38.5 °C for 24 h. The treatment 1 (T1) and 3 (T3) group oocytes were cultured at 40.5 and 41.5 °C respectively, for the first 12 h and at 38.5 °C for rest of the 12 h. However, treatment 2 (T2) and 4 (T4) group oocytes were cultured at 40.5 and 41.5 °C respectively for entire 24 h.

A total of 1,036 in vitro matured oocytes in each group (ten replicates) were assessed for cleavage rate, percent of four-cell, eight-sixteen cell, morulae, blastocyst production and blastocyst quality on days 2, 3, 4, 6 and 8th day post insemination. The blastocyst quality (total cell number TCN) was determined on day 8 post insemination in both control and experimental groups (T1, T2, T3 & T4) respectively. The number of blastocysts used for total cell number (TCN) was 27 for all the groups. The mRNA expression of heat shock genes (*Hsp70.1, 70.2, 70.8, 60, 10 and HSF1*), transport and metabolism of glucose (*Glut1, Glut3 and IGF1R*), oxidative stress and mitochondrial activity (*MnSOD, iNOS and Dna J*), pro-apoptotic (*caspases-3, −7, −8, Bid and Bax*), anti-apoptotic (*Mcl-1, Bcl-2 and Bcl-xl*) and developmental competence (*GDF9, BMP15 and ZAR1*) related genes was determined with qRT-PCR in bubaline oocytes after 24 h of in vitro maturation.

Chemicals and reagents

Chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Disposable cell culture grade plastic ware was purchased from Becton, Dickinson and Co., Lincoln Park, NJ, USA, or Nunc, Roskilde, Denmark. Syringes of assorted sizes were purchased from Henke Saas Wolf GmBH, Tuttlingen, Germany, whereas the 0.22 μm filters were taken from Millipore Corp., Bedford, MA, USA. Foetal bovine serum (FBS) was taken from Hyclone (Logan, UT, USA).

In vitro embryo production

In vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC)

Buffalo ovaries were collected from a nearby abattoir and washed thrice with warm (37 °C) isotonic saline (penicillin 400 IU/ml and streptomycin 500 μg/ml). Cumulus Oocyte Complexes (COCs) were aspirated from 2 to 8 mm diameter follicles in aspiration medium (TCM-199, 0.3 % BSA and 50 μg/ml gentamicin sulphate) with a 19-gauge needle. The COCs, which had a compact mass (≥2 layers of cumulus cells) and homogenous granular ooplasm were used for IVM. The COCs were then washed (4–6 times) with the washing medium (TCM–199, 10 % FBS, 0.81 mM sodium pyruvate and 50 μg/ml gentamicin sulphate) followed by washing (3 times) with IVM medium (TCM-199, 10 % FBS, 5 μg/ml pFSH, 1 μg/ml estradiol-17b, 0.81 mM sodium pyruvate and 50 μg/ml gentamicin sulphate). Groups of COCs (15–20) were cultured in droplets (100 μl) of the IVM medium and subsequently overlaid with sterile mineral oil and finally incubated for 24 h at 38.5 °C and respective treatments in a humidified incubator (CO_2 5 %; RH 95 %).

The expanded COCs were washed three times with IVF medium (Bracket and Oliphant, BO medium containing 10 μg/ml heparin, 137.0 μg/ml sodium pyruvate, 1.942 mg/ml caffeine sodium benzoate and 10 mg/ml fatty acid-free BSA) and transferred into IVF drops (15–20 oocytes/50 μL drops). Simultaneously, the spermatozoa were processed by the method provided by Chauhan et al. [13] and were taken from the same donor tested for IVF earlier. Briefly, two straws of frozen-thawed buffalo semen were washed twice with BO medium and the pellet obtained was resuspended in 0.5 ml

of the capacitation-fertilization medium (BO). For IVF, 50 μl of the spermatozoa suspension (2–4 million spermatozoa/ml) was added to each droplet containing oocytes followed by addition of sterile mineral oil and finally incubated for 18 h at 38.5 °C with 5 % CO_2.

The presumptive zygotes obtained after IVF were denuded by gentle repetitive pipetting, washed 5–6 times with IVC medium (Modified Charles Rosenkrans Medium, mCR2aa, 0.8 % BSA, 10 % FBS) and subsequently cultured in the same medium for 48 h post-insemination. The processed zygotes suspended in 100 μl droplets of the IVC medium were cultured on the beds of granulosa cells (IVM droplets) for 9 days post-insemination at 38.5 °C in a humidified CO_2 incubator. The medium was replaced with 50 % of fresh IVC medium every 48 h. The cleavage rate was checked on day 2 post-insemination, and the percentage of oocytes that developed to 4, 8, 16 cell-stage, morulae and blastocyst stage were recorded on the day 3, 4, 6 and 8 post-insemination.

Bis-benzimide staining (Hoechst 33342) for total cell number assessment

The blastocysts were washed (3 times) in phosphate buffered saline with 0.3 mg/ml polyvinylpyrrolidone (PBS-PVA) and then immediately suspended in 0.5 % triton-X followed by incubation in Hoechst 33342 (bis-benzimide,1 μg/mL) for 45 min in the dark. The stained blastocysts were placed on the microscopic slides followed by the addition of a few drops of glycerol and antifade gold solution. Finally, the cover slip was mounted and the blastocysts were visualized on an inverted fluorescent microscope at a magnification of 200X (Nikon fluorescence microscope, Japan).

RNA extraction and cDNA preparation

RNA extraction was carried out in matured oocytes after stripping the surrounding cumulus cells by treating with 500 μl of 0.5 mg/ml hyaluronidase and gentle repetitive pipetting. The denuded oocytes from control and experimental groups were washed (3 times) in calcium and magnesium free phosphate buffered saline (PBS). Total RNA was isolated from these denuded oocytes with RNAqueous-microkit (Ambion Inc. TX, USA) according to the manufacturer's instructions. RNA integrity was checked on 1.4 % denaturation agarose gel electrophoresis. Total isolated RNA was treated with DNase I (Ambion Inc. TX, USA) and converted to cDNA using oligo $(dT)_{20}$ primers and fermentas reverse transcriptase (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's guidelines. The prepared cDNA was analyzed using PCR and stored at −80 °C until further use for qRT-PCR. The primers were designed using primer 3 software from NCBI database and the Ensembl (EMBL-EBL Wellcome Trust Sanger Institute, Cambridegeshire, UK).

Quantitative-real time PCR (qRT-PCR)

PCR was performed to amplify target and reference genes on Real Time Thermocycler (Biorad CFX96™, C1000™) with FastStart DNA Master SYBR Green I mix (Bio-Rad, Hercules, CA,USA) containing $MgCl_2$, dNTP, and FastStart Taq DNA polymerase by following manufacturer's instructions. The sequence of primers, annealing temperatures and length of gene fragments amplified is shown in Table 1. The PCR amplification was carried out using one cycle of initial denaturation at 94 °C for 5 min, 35 cycles of denaturation (94 °C for 20 s), primer specific annealing temperature for 15 s and extension (72 °C for 15 s). The qRT-PCR specificity was confirmed by the analysis of the melting curve after each run. Also, agarose gel electrophoresis analysis (1.5 %) was carried out to determine the length of the amplified PCR product. After amplification, threshold (Ct) values of both control and experimental groups with reference genes were taken for calculating fold change in target–gene expression. Expression of *Gapdh* was taken as an endogenous reference. In negative controls, nuclease free water was substituted for template.

Statistical analysis

Data were analysed using the GraphPad Prism (version 5.01). Experimental results were presented as mean ± SEM (standard error mean). Data were subjected to analysis of variance (ANOVA), and the Tukey test was used to separate the means ($p<0.05$) that were considered statistically significant.

Results

Developmental competence and quality of blastocysts

Developmental competence (cleavage rate, percentage of oocytes that developed to 2-cell, 4-cell, 8–16 cell, morulae and blastocysts) and quality of blastocysts produced in experimental groups was compared with control. The cleavage rate on day-2 post insemination, percentage of oocytes that developed to 4-cell, 8–16 cell, morulae and blastocysts were considerably lower ($p<0.001$) for oocytes of all the treatment groups (T1, T2, T3 and T4) compared to control as depicted in Fig. 1. However, non-significant changes were observed amongst the experimental group oocytes matured at 40.5 and 41.5 °C for both time durations (12 and 24 h).

Analysis of mRNA expression

Developmental competence and heat shock related genes

In the present investigation, it was found that mRNA expression of developmental competence related genes (*BMP15* and

Table 1 Primer sequence for quantitative real time PCR

Gene	Sequence	Annealing temperature	Base pairs	Accession no.
Hsp 70.1	F-5′TCATCAACGACGGAGACAAGCCTA 3′ R-5′TTCATCTTGGTCAGCACCATCGAC3′	60	103	GU-183097.1
Hsp 70.2	F-5′AAGCACAAGAGACATTGCACCC3′ R-5′AAGTGTAGAAATCCACGCCCTCGT'3′	58.5	130	NM_174344.1
Hsp 70.8	F-5′ CGGTGATGCAGCAAAGAACCAAGT3′ R- 5′CACCACCATGAAGGGCCAATGTTT3′	58	133	NM_174345.3
Hsp 60	F-5′ACTGGCTCCTCATCTCACTC3′ R-5′TGTTCAATAATCACTGTCCTTCCC3′	56	146	NM_001166610.1
Hsp 10	F-5′GAGTATTAGTTGAAAGAAGTGCGG3′ R-5′TGACTTTGGTGCCATATTC3′	58	198	NM_174346.2
HSF-1	F-5′ACATAAAGATTCGCCAGGAC3′ R-5′GAGATGAGGAACTGGATGAG3′	56	198	GQ396661.1
Glut-1	F-5′ACAGGCAGCTGGATGAGACT3′ R- 5′TGTGGGTGAAGGAGACTCTG3′	60	230	AJ812564
Glut-3	F-5′CATCCCTGTGGTCCTTGTCT3′ R-5′CAGCATTTCAACCGACTCTG 3′	60	182	NM_174603.3
IGF1 R	F-5′ GAACTGTCATCTCCAACCTC3′ R-5′GAATGTCATCTGCTCCTTCTG3′	58	145	NM_001244612.1
MnSOD	F-5′TCCACGTCCATCAGTTTGGAGACA3′ R-5′TTGTCATGCTGTCACATTGCCCAG3′	60	140	NM_174615.2
iNOS	F-5′TGTCCACGGCATGTGAGGATCAAA3′ R-5′TCATGATGGATGCCAGGCAAGACT3′	60	121	DQ_676956.1
Dna J	F- ATCTTCATGCTTGTCGTGTC R- CAGTGGTAGTGTGGTAAGGA	60	123	NM_174068.2
Bcl-2	F-5′GCAGGTATTGGTGAGTG3′ R-5′ATTGTTCCCGTAGAGTTCC3′	58	100	HM_630302.1
Bcl-xl	F-5′TTGTGGCCTTTTTCTCCTTC3′ R-5′GATCCAAGGCTCTAGGTGGT3′	58.5	128	ENSBTAT00000008572
Mcl-1	F-5′TCGGAAACTGGACATCAAAA3′ R-5′CCACAAAGGCACCAAAAGAA3′	58	128	ENSBTAT00000020159
Bax	F-5′CCTTTTGCTTCAGGGTTTCA3′ R-5′CGCTTCAGACACTCGCTCA3′	60	130	AJ812564
Bid	F-5′CTGTCGGAGGAGGACAGGAG3′ F-5′GTGGTCGGCTATCTTTTTGG3′	60	135	NM_001075446.1
Caspase 3	F-5′GACCATAGCAAAAGGAGCAG 3′ R-5′CCTCAGCACCACTGTCTGTC3′	60	220	NM_001077840.1
Caspase 7	F-5′AAACCCTGTTAGAGAAACC3′ R-5′GAATAGGCAAAGAGAAAGTCGG3′	60.5	156	XM_002698509.1
Caspase 8	F- GACATCTGACACCAGTTTACCGA R- CATCAAAGTCTGTTCCAAGTCCT	60	163	NM_001045970.2
BMP15	F-5′ CATCCCTTACGGTATATGCTG3′ R-5′ GTTTGGTCTCAGAGGAAAGTC3′	56	179	DQ463368.1
GDF9	F- 5′CCCTAAATCCAACAGAAGCC3′ R-5′ GTTCCACAACAGTAACACGA3′	60.5	148	NM_174681.2
ZAR1	F-5′ GCTTCCAGTTCTTAGAGCAG3′ R-5′TGTAGTAAACCTTGTTAGTGCC3′	56	110	NM_001076203.1
Gapdh	F-5′TCAAGAAGGTGGTGAAGCAG 3′ R-5′CCCAGCATCGAAGGTAGAAG 3′	57	122	GU324291.1

ZAR1) decreased significantly ($p < 0.01$) in all treatment groups (T1, T2, T3, T4) compared to control as shown in Fig. 2. However, results depicted that the oocytes matured at high temperature conditions (40.5 and 41.5 °C) and incubations (12 and 24 h) showed significantly higher ($p < 0.01$) mRNA expression of heat shock protein related genes (*Hsp 70.1, 70.2, 70.8, 60, 10 and HSF1*) compared to control as depicted in Fig. 3.

Oxidative stress and glucose transport related genes

The effect of elevated temperatures (40.5 and 41.5 °C) during maturation on mRNA expression of oxidative stress and mitochondrial activity related genes (*MnSOD, iNOS* and *Dna J*) is depicted in Fig. 4. Results depicted an increase ($p<0.01$) in mRNA expression of *iNOS* gene and a decrease ($p<0.01$) in mRNA expression of *MnSOD* gene in all the treatment groups (T1, T2, T3, T4) compared to control. However, it was observed that mRNA expression of *Dna J* gene did not alter ($p>0.05$) at high temperature conditions (40.5 and 41.5 °C) at both the incubations (12 and 24 h). Further, current study revealed a significant decrease ($p<0.05$) in the mRNA expression of glucose transport related genes (*Glut1, Glut3* and *IGF1R*) in all the treatment groups (T1, T2, T3 and T4) compared to control (Fig. 5).

Pro-apoptosis and anti-apoptosis related genes

In the current study, we observed a significantly higher ($p<0.05$) mRNA abundance of pro-apoptosis related genes (*caspase-3, −7 −8*, *Bid* and *Bax*) for the oocytes matured at higher temperatures (40.5 and 41.5 °C) at both the incubations (12 and 24 h) compared to control as shown in Fig. 6. However, it was found that mRNA expression of anti-apoptosis related genes (*Mcl-1, Bcl-2* and *Bcl-xl*) decreased significantly ($p<0.05$) in the treatment groups compared to control as depicted in Fig. 7.

Discussion

Several stages of embryo development are susceptible to elevated ambient temperature such as oocyte germinal vesicle-stage [10], the stage near or after fertilization and the early cleavage-stage embryos [14]. The majority of early embryonic losses in dairy cattle with environmental heat stress occur before day 20 of gestation [15]. The losses are more pronounced during temperature induced stressful conditions and may continue up to day 40 to 50 of gestation [16]. Effects of elevated temperature manifested as heat stress are particularly prominent during estrus (day-1) when the oocyte is undergoing meiotic maturation in preparation for fertilization [17, 18]. It has already been established that maternal hyperthermia affected the meiotic maturation of an oocyte [19, 20] in addition to a reduction in embryonic development with direct or in vitro exposure of bovine oocytes to elevated temperature [21, 22] to a similar degree as seen in vivo [12].

To the best of our knowledge, for the first time we report the developmental changes and molecular alterations in bubaline oocytes with physiologically relevant elevated temperatures that manifest in the form of heat stress. It has already been reported that exposure of buffaloes to direct solar radiation increased the rectal temperature to 40.5 °C after 2 h exposure and a further increase up to 41.5 °C after 4 h of exposure [3]. The present investigation was further validated by the study conducted by Das et al. [4] who have established a direct relationship between the solar radiation and rectal temperature of buffalo which increased up to 40.8 °C when exposed for a longer duration of time. Similarly, Sethi et al. [5] reported that the rectal temperature increased to 41.2 °C when the buffaloes were exposed to direct sunlight in the summer months (June and July) when the ambient temperature was 45 °C. Keeping in view the foregoing discussion, it is evident that the temperatures (40.5 and 41.5 °C) used in the present investigation are quite relevant in order to ascertain the effect of physiologically relevant elevated temperature on the developmental competence of bubaline oocytes. Moreover, it is likely that the exposure to elevated temperature or heat stress may coincide with the day of estrus when the oocyte is undergoing maturation in vivo.

In the present study, it was found that exposure of buffalo oocytes to higher temperatures (40.5 and 41.5 °C) during in vitro maturation (first 12 h only or entire 24 h) resulted in a significant decrease ($p< 0.05$) in the cleavage rate and therefore blastocyst production. Our results are in agreement with the findings of Mishra et al. [23] who have also found a decreased cleavage rate and subsequent embryo development in bubaline oocytes which were collected from buffaloes slaughtered at high ambient temperature (>40 °C) compared to low ambient temperature (<40 °C). Similarly, Torres-Junior et al. [24] while studying the in vivo effect of heat stress on the *Bos indicus* breed (Gir) found a reduced ability of oocytes to become blastocysts after fertilization compared to control group. Therefore, it is suggested that high ambient temperature may affect the process of maturation, fertilization and subsequent embryo development through oocyte competence as reflected by decreased cleavage rate and blastocyst production.

Recently, Yadav et al. [25] have found that physiologically relevant heat shock (40.5 °C) to buffalo oocytes or embryos for 2 h once every day throughout IVM, IVF and IVC resulted in a decreased ($p< 0.05$) percentage of buffalo oocytes that developed to 8–16 cell or blastocyst stage. The effect of 2 h heat shock throughout IVM, IVF and IVC was so profound that not a single blastocyst was formed at 40.5 °C. These findings supported our present data where a similar pattern, i.e., a remarkably decreased ($p< 0.001$) percentage of buffalo oocytes that developed to 2, 4, 8–16 cell, morulae or blastocyst stage was observed. Moreover, studies carried out in bovines have also shown the similar results, for example, it has been reported that exposure of bovine oocytes to increased temperature (40–41 °C for 12 h) reduced the development of oocytes to blastocysts, the number of embryos developing to the blastocyst stage, 4–8 cell embryos, percentage of cleaved embryos and blastocysts [19, 26, 27]. In a similar attempt, the

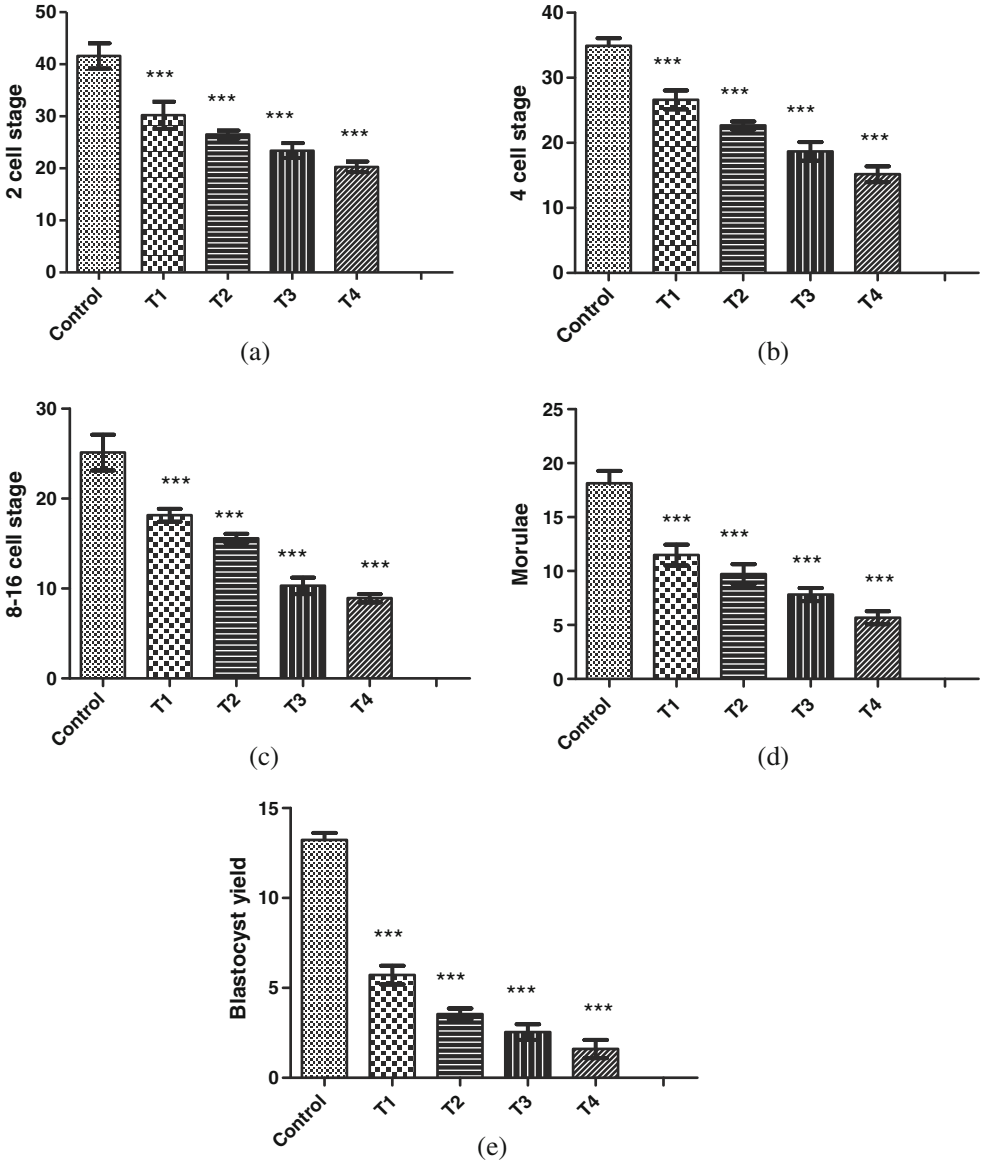

Fig. 1 Proportion (percentage) of 2–cell, 4–cell, 8–16 cell, morulae and blastocyst embryos formed in control group and treatment groups. Control group represents the optimal culture conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C, 12 h; 41.5 °C, 12 h, 40.5 °C, 24 h; & 41.5 °C, 24 h respectively. The values are expressed as mean ± SEM. *Asterisk* indicates the significant difference, ***$p<0.001$ as compared to the control group

reduced quality of buffalo oocytes was reported in summer and autumn compared to winter and spring [28]. Therefore, these results point towards the higher degeneration of buffalo oocytes during hot periods that may partly reflect for their lower developmental competence at higher temperatures. Our results were further in accordance with findings of Tseng et al. [14] who have found a decreased developmental competence of porcine embryos at 2 and 4 h time periods respectively at 41.5 °C compared to the control group (39 °C). Likewise, Payton et al. [29] have reported that the physiologically relevant elevated temperature compromised the continued development of antral follicle and germinal vesicle- stage oocytes in a direct manner. It has also been reported that oocytes experiencing heat stress during first 12 h of in vitro maturation have a lower ability for blastocyst development after fertilization than non-heat-stressed oocytes [30].

The present findings, therefore, provide the clear evidence of carryover effect of heat stressed oocytes or the maternal hyperthermia up to the blastocyst stage with a decrease in cell number at each stage of development. Hence, it is put forward that high ambient temperature may affect the process of maturation and subsequent embryo development at various cell stages that may ultimately cause negative impact on buffalo reproduction. Collectively, these findings along with previous results in bovines [31–34] provide compelling evidence for a major effect of heat stress exposure during meiotic maturation on subsequent blastocyst development.

In the present investigation, an increased ($p<0.05$) mRNA expression of pro-apoptotic genes (*caspases-3, −7, −8, Bid and Bax*) and a decreased ($p<0.05$) mRNA expression of anti-apoptotic genes (*Bcl-2, Bcl-xl and Mcl-1*) was observed in buffalo oocytes matured in vitro at 40.5 and 41.5 °C for both

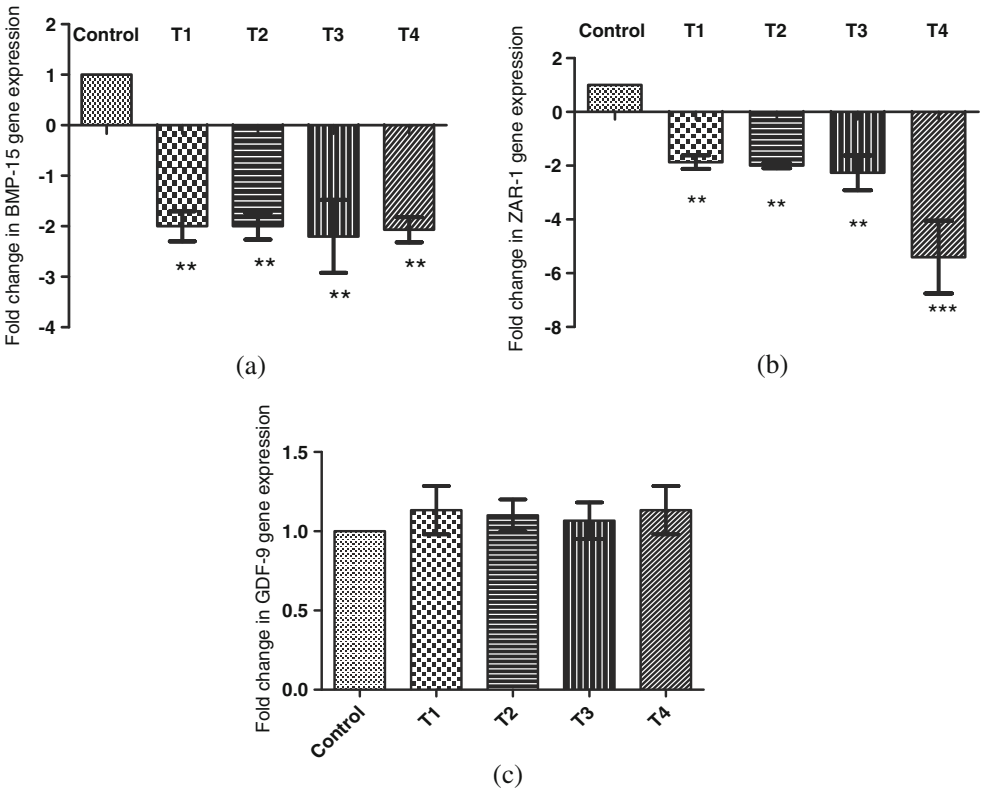

Fig. 2 Relative abundance of developmental competence gene transcripts in bubaline oocytes. **A** *BMP–15* **B** *Zar–1* and **C** *GDF–9*. Control group represents the optimal maturation conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C, 12 h; 41.5 °C, 12 h, 40.5 °C, 24 h; & 41.5 °C, 24 h respectively. *Gapdh* was used as a housekeeping gene for normalization. The values are expressed as mean ± SEM. *Asterisk* indicates the significant difference, $^{**}P<0.01$ and $^{***}P<0.001$as compared with the control group

time durations (only first 12 h and entire 24 h) compared to control group (38.5 °C for entire 24 h).

These results are in agreement with the Yadav et al. [25] who have also observed an increased ($p< 0.05$) mRNA expression of pro-apoptotic genes in 8–16 cell stage embryos following a heat shock of 2 h every day once in IVM, IVF and IVC. However, the inconsistency in the mRNA expression of anti-apoptotic genes in their study may be either the difference

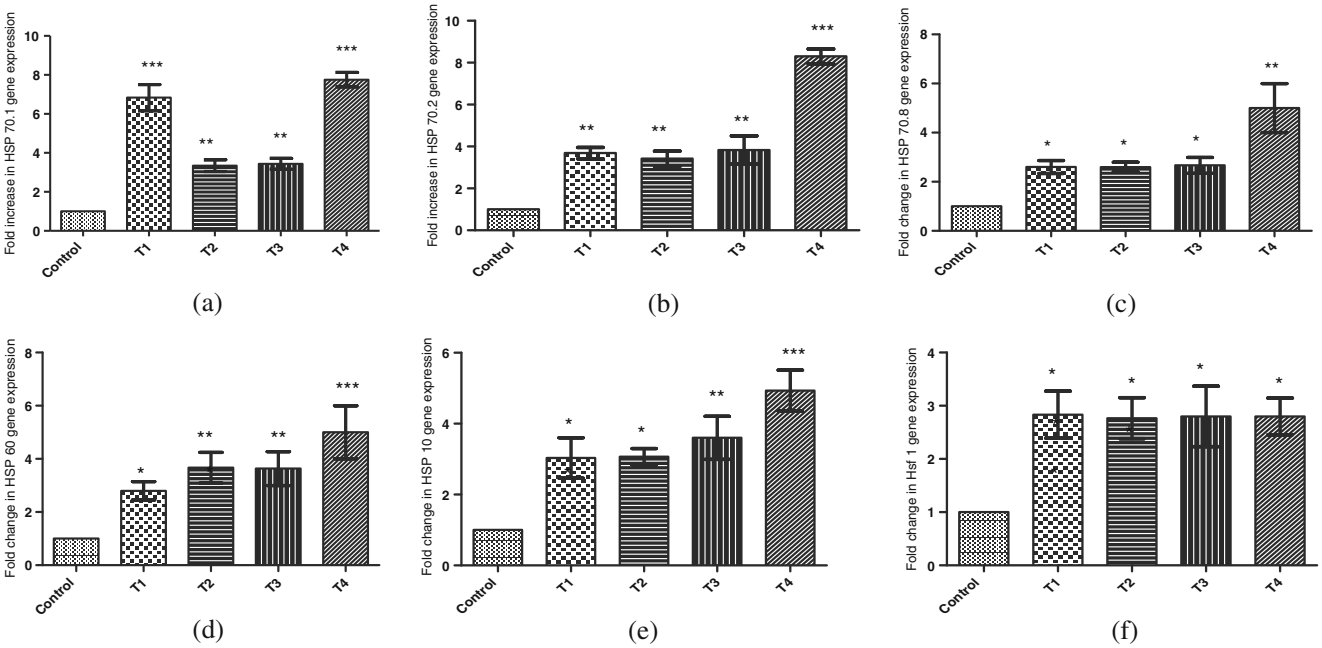

Fig. 3 Relative abundance of heat shock protein genes in bubaline oocytes. **A** *Hsp 70.1* **B** *Hsp 70.2* **C** *Hsp 70.8* **D** *Hsp 60* **E** *Hsp 10* and **F** *HSF–1*. Control group represents the optimal maturation conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C, 12 h; 41.5 °C, 12 h, 40.5 °C, 24 h; & 41.5 °C, 24 h respectively. *Gapdh* was used as a housekeeping gene for normalization. The values are expressed as mean ± SEM. *Asterisk* indicates the significant difference, $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ as compared to the control group

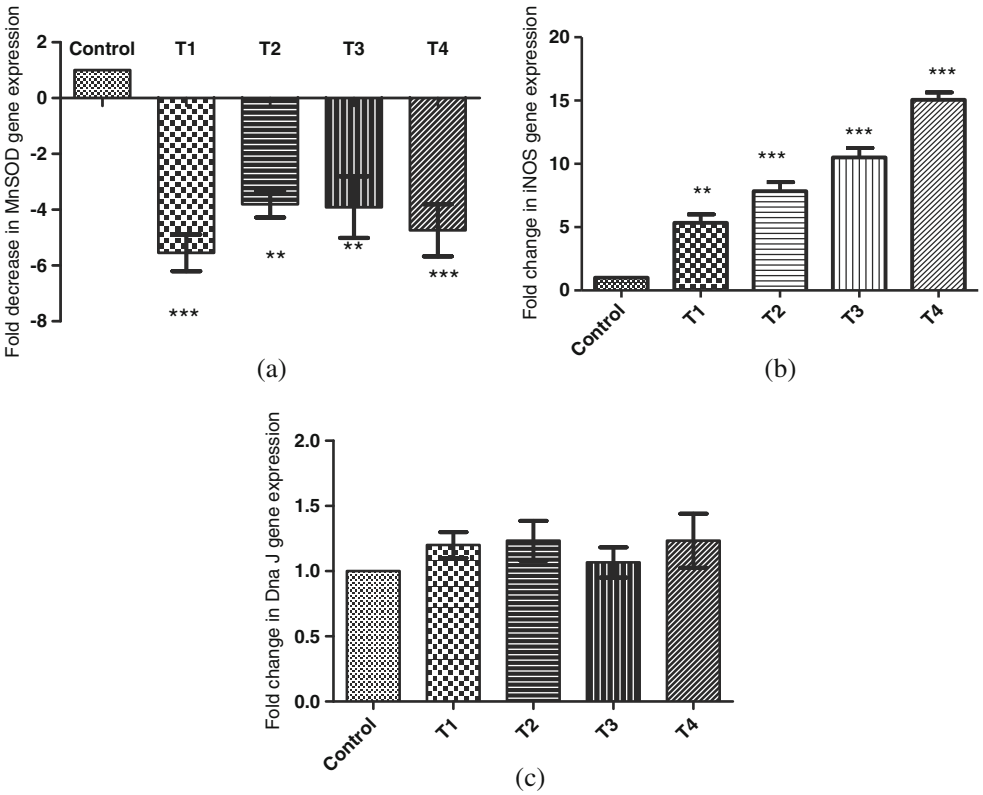

Fig. 4 Relative abundance of oxidative stress related gene transcripts in bubaline oocytes. **A** *MnSOD* **B** *iNOS* and **C** *Dna J*. Control group represents the controlled culture conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C,12 h; 41.5 °C, 12 h, 40.5 °C, 24 h; & 41.5 °C, 24 h respectively. *Gapdh* was used as a housekeeping gene for normalization. The values are expressed as mean ± SEM. *Asterisk* indicates the significant difference, **$p<0.01$ and ***$p<0.001$as compared with the control group

in the stage of embryo development or the duration of heat exposure. Likewise, Jin et al. [35] also reported the higher mRNA expression of pro-apoptotic genes (*BAK* and *caspase-3*) in porcine embryos exposed to 41 °C for more than 12 h compared to the non-treated control. Similarly, in bovines, Roth et al. [19] observed a higher activity of group II caspases (*caspases-3 & -7*) in heat shocked oocytes cultured at 40 and 41 °C (first 12 h of maturation) compared to control group

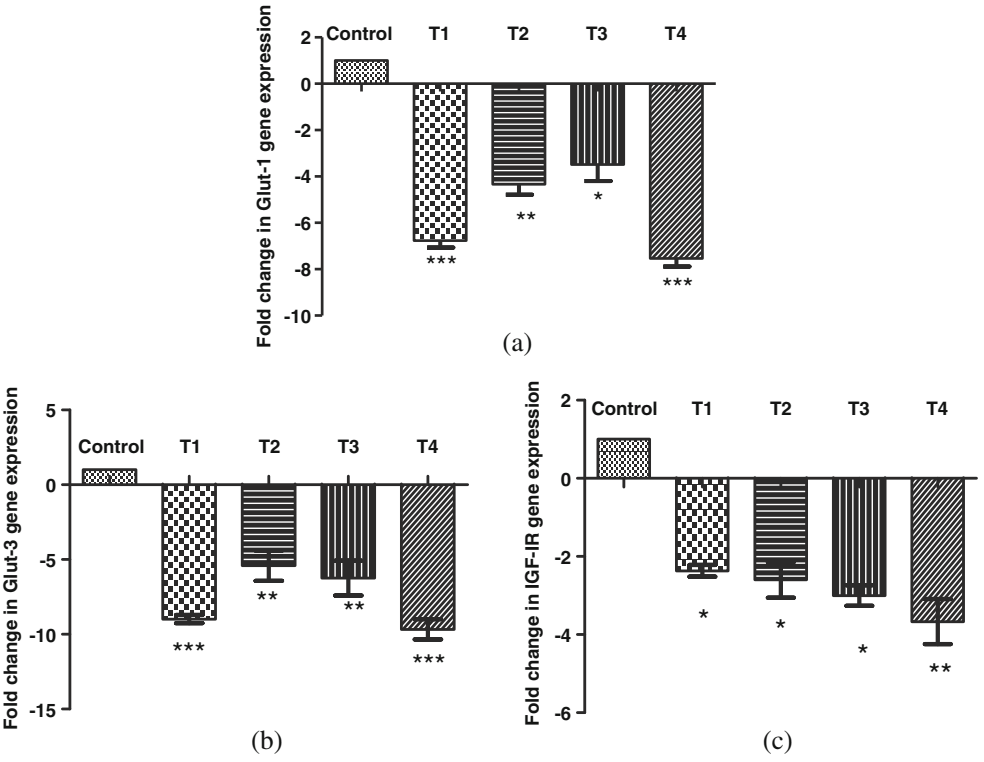

Fig. 5 Relative abundance of metabolism related gene transcripts in bubaline oocytes. **A** *Glut–1* **B** *Glut–3* and **C** *IGF-1R*. Control group represents the controlled culture conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C, 12 h; 41.5 °C, 12 h, 40.5 °C, 24 h; & 41.5 °C, 24 h respectively. *GAPDH* was used as a housekeeping gene for normalization. The values are expressed as mean ± SEM. *Asterisk* indicates the significant difference, *$P<0.05$, **$P<0.01$ and ***$P<0.001$ as compared to the control group

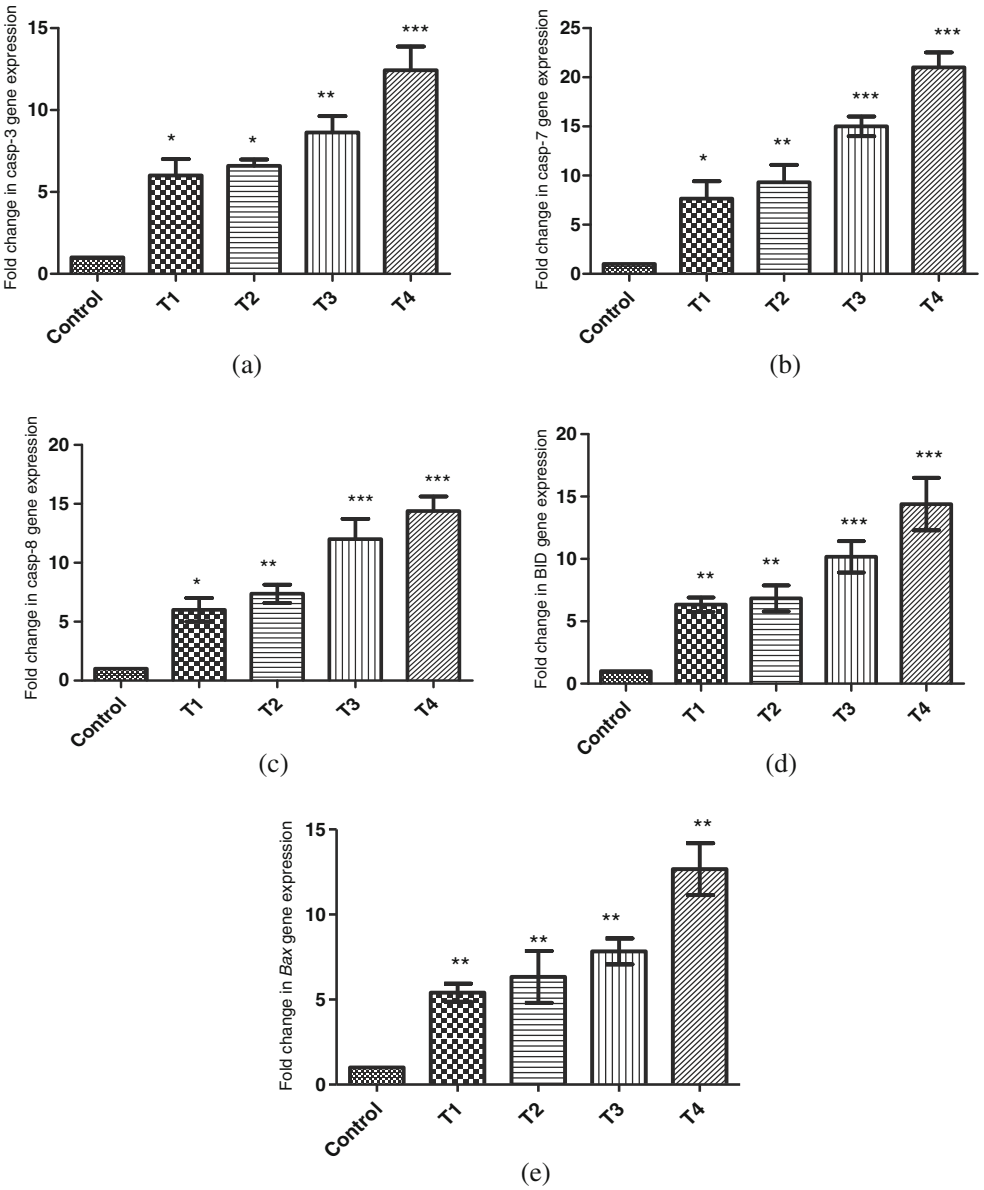

Fig. 6 Relative abundance of pro apoptotic gene transcripts in bubaline oocytes. **A** *Caspase–3* **B** *Caspase–7* **C** *Caspase–8* **d** *Bid* and **e** *Bax*. Control group represents the optimal maturation conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C, 12 h; 41.5 °C, 12 h, 40.5 °C, 24 h respectively. *Gapdh* was used as a housekeeping gene for normalization. The values are expressed as mean ± SEM. *Asterisk* indicates the significant difference, *$p<0.05$, **$p<0.01$ and ***$p<0.001$ as compared to the control group

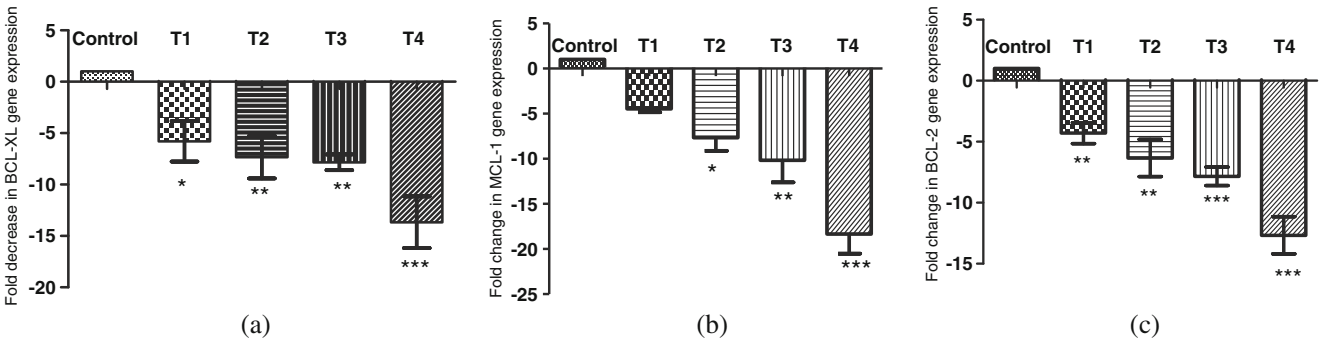

Fig. 7 Relative abundance of anti apoptotic gene transcripts in bubaline oocytes. **a** *Bcl–xl* **b** *Mcl–1* and **c** *Bcl–2*. Control group represents the optimal maturation conditions (38.5 °C for 24 h), T1, T2, T3 & T4 represent oocytes matured at 40.5 °C, 12 h; 41.5 °C, 12 h, 40.5 °C, 24 h; & 41.5 °C, 24 h respectively. *Gapdh* was used as a housekeeping gene for normalization. The values are expressed as mean±SEM. *Asterisk* indicates the significant difference, *$p<0.05$, **$p<0.01$ and *** $p<0.001$ as compared with the control group

oocytes (cultured at 38.5 °C for 24 h). These studies, therefore, support the current investigation where a similar effect (i.e., increase) on the mRNA expression in the pro-apoptotic genes (*caspases-3 & -7*) was observed. The increased expression of pro-apoptotic genes in the present study is further evidenced with the fact that programmed cell death is the mechanism affecting the oocyte negatively during early stages of follicular growth [36]. It is thought that *caspases-3* and *7* (executioners) may activate *caspases-8* and *9* (initiators) [37] to induce downstream signalling to cause apoptosis in buffalo oocytes during in vitro maturation at higher ambient temperatures. It is quite possible that heat stress could lead to apoptosis not only during the early follicular development but also during the maturation and oocyte competence may not be restored even after the fertilization. However, the exact mechanism through which pro/anti-apoptotic proteins generate downstream signalling in buffalo oocytes during heat stress is not known and warrants further exploration of signal cascade mechanisms.

Hsp 70 family (*70.1, 70.2, 70.8*) and other small *Hsps* (*10, 60*) form the most important group of stress responsive elements in embryonic cells [38, 39]. The relative importance of these proteins in oocyte/embryo growth and development could be potentially used as biomarkers for in vitro as well as in vivo studies concerning stressful conditions [40]. The rapid induction of *Hsp70* and other small *Hsps* represents a unique feature of the physiological function of these molecules. It is well known that *Hsp* expression is accomplished by mechanisms of transcriptional activation and translation involving heat shock transcription factors (*HSFs*). The current study revealed that maturation of bubaline oocytes at 40.5 and 41.5 °C for both time durations (only first 12 h and entire 24 h) increased ($p<0.05$) the mRNA expression of *Hsp* family genes *Hsp 70* family (*70.1, 70.2, 70.8*), small *Hsps* (*10, 60*) and *HSF1*. The results obtained are in accordance with Yadav et al. [25] where a similar increase ($p<0.05$) in the mRNA expression of these genes (*Hsp 70.1* and *70.2*) was observed in 8–16 cell stages and blastocyst stage. These results are also in accordance with the previous findings carried out in cattle, pig, horse and mouse. Jin et al. [35] reported the higher expression of *Hsp 70.2* in porcine embryos exposed to 41 °C for a prolonged duration of 12 h. In a similar study by Mortensen et al. [41] in equines, the expression of *Hsp 70.1* was higher in the blastocysts developed from oocytes exposed to 42 °C (2 h to 4 h) during the late stages of in vitro maturation. Also, Fiorenza et al. [42] have observed the higher transcriptional activity of *Hsp 70.1* in mouse embryos/oocytes and the initial nuclear localization of *HSF1* in mouse oocytes/embryos in response to osmotic shock. Members of the *HSF* family (*HSF1, HSF2, HSF3 and HSF4*) bind to heat shock elements in the promoters of *Hsp* genes and regulate their transcription [43, 44]. *HSF1* is ubiquitously expressed and is the most effective transactivator of stress induced expression of *Hsp70* genes. Therefore, in the present study, the expression of *HSF1* also increased in the oocytes matured at 40.5 and 41.5 °C for both time durations (only first 12 h and entire 24 h). However, Yadav et al. [25] did not observe any change in mRNA expression of *HSF1* during the IVM phase of in vitro development. This may be the result of short exposure duration of only 2 h compared to the exposure duration of 12 h and 24 h in the present study.

In the current investigation, a decreased mRNA expression ($p<0.05$) of *MnSOD* and increased *iNOS* mRNA expression was observed in buffalo oocytes matured in vitro at higher temperatures (40.5 and 41.5 °C) for both time durations (only first 12 h and entire 24 h) compared to control group (38.5 °C for entire 24 h). However, no changes ($p> 0.05$) were observed in mRNA expression of *Dna J* in all treatments groups compared to control. *MnSOD* protects cells against oxidative damage [45] and *iNOS* has a role in germinal vesicle breakdown and the anaphase-telophase transition of oocytes [46], therefore, its upregulation may negatively impact buffalo oocytes during maturation and subsequent embryo development. Our results are in accordance with the study by Balasubramanium et al. [47] where the expression of *MnSOD* decreased when embryos were cultured under high oxygen concentration (20 %) compared to the 5 % oxygen concentration normally occurring in vivo. Since *MnSOD* is a mitochondrial enzyme [45–47], the decrease in its expression may be related to the altered mitochondrial activity after subjecting the oocytes to heat stress thereby, negatively impacting the developmental competence. However, the exact mechanism of down regulation remains unclear and warrants further validation. The results obtained are in agreement with the previous studies [47, 48] where in the presence of all the isoforms of *NOS* in buffalo oocytes and embryos were reported. Tesfaye et al. [49] observed a higher mRNA expression of *eNOS* and *iNOS* in immature oocytes and in 2 and 4–cell stage bovine embryos than in 8-cell stage embryos, in morulae and blastocysts.

In bovine oocytes and embryos, glucose is the energy source and its uptake occurs through glucose transporters *Glut1* (preimplantation periods) and *Glut3* (post-compaction) [50–52]. In the present study, the relative mRNA abundance of *Glut1*, *Glut3* and *IGF1R* decreased ($p<0.05$) in the buffalo oocytes matured at 40.5 and 41.5 °C for two time periods (first 12 h or complete 24 h) compared to the oocytes matured at 38.5 °C for 24 h (control). On the contrary, Yadav et al. [25] didn't observe any change (p 0.05) in the mRNA expression of *Glut1* when buffalo oocytes/embryos were given a heat shock of 40.5 °C for 2 h once every day throughout IVM, IVF and IVC. The unaltered changes ($p> 0.05$) in mRNA expression of *Glut1* may be the short durations of heat exposure (2 h once every day) compared to 12 h and 24 h in the present study. The down regulation of glucose transporters and *IGF1R* in heat stressed oocytes indicates that the glucose uptake is severely compromised in response to increased maturation temperature for prolonged duration. This indicates the importance of glucose transporters as regulatory factors in early

embryo development and the heat-induced compromised oocyte competence may be attributed to a deficient expression of facilitative glucose transporters.

In this study, the mRNA expression of developmentally important genes *(ZAR1* and *BMP15)* was reduced in the buffalo oocytes without affecting *GDF9* expression during in vitro maturation at higher temperatures (40.5 and 41.5 °C) for both time durations (first 12 h and entire 24 h) compared to control group (38.5 °C for entire 24 h). The present results were in agreement with the most recent study in buffalo embryos where no change was observed in mRNA expression of *GDF9* genes when oocytes/embryos were exposed to physiologically relevant heat shock of 40.5 °C for 2 h once every day throughout IVM, IVF and IVC. Also, Payton et al. [52] did not find any significant difference in the relative abundance of *BMP15* and *GDF9* in germinal vesicle stage and matured oocytes on exposure to 41 °C for first 12 h of in vitro maturation. However, the heat-induced perturbations occurring at molecular level during maturation are inherited by later stage embryos even after fertilization [53].

In conclusion, the data presented in this paper clearly depicts that physiologically relevant elevated temperature (40.5 and 41.5 °C) for two time periods (first 12 or complete 24 h) during in vitro maturation reduced the developmental competence of bubaline oocytes and subsequent embryo development, most likely through apoptotic and developmentally regulated mechanisms. Oocyte damage caused by heat shock in vitro is likely to be relevant to the understanding of early embryonic losses in buffalo following heat stress conditions. Therefore, it is concluded that physiologically relevant elevated temperatures have a negative impact on buffalo reproduction.

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Conflict of interest None of the authors have any conflict of interest to declare.

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