

Transient Heat-Shock Disrupts Molecular Signals and Competence of Riverine *Bubalus Bubalis* Oocytes and Early Embryos in a Stage-Specific Manner

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Research

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Abstract

Background Thermal stress elicits detrimental effect in reproduction performance of *Bubalus bubalis*, buffalo. Riverine buffalo oocytes and early embryos possess differential ability to grow and survive under thermal stress condition. It is interesting to know how precisely the short and transient heat stress impacts growth and development of buffalo oocytes and early embryos in a stage specific manner. In this study, we aim to identify the most sensitive and vulnerable stage of oocytes and early embryos against transient heat stress as well as unravel the underlying molecular signals responsible for the stunted embryo development under thermal stress. It was assessed by utilizing six different groups of oocytes and embryos with an incorporation of 4 h transient heat shock at 40°C during different stage of in-vitro maturation (IVM) and in vitro culture (IVC).

Results The stressed oocytes of group (grp) 1, 2 and 3 exhibit comparable paces of attaining metaphase-II (M-II) phase at different time interval of IVM. The most detrimental effect of heat stress was observed in grp 2 with sharp reduction in morula to blastocysts transition rate ($p < 0.05$). Expression of mRNA transcripts of HSP8, MnSOD and Sirt-3 genes were significantly increased from mid-IVM to 4-cell embryo although subsequently, down-regulated in embryos of 8-16 cells, morulae and blastocysts. Expression of maternal to embryo transition (MET) genes viz. PAP, U2Af and eIF4A was significantly down regulated from 2-cell embryo to morula ($p < 0.05$). Maternal recognition of pregnancy (MRP) and morulae to blastocyst transition genes were poorly expressed in grp 2 than of the other stress groups ($p < 0.05$).

Conclusions Disruption of molecular signal has implicated in the poor formation of inner cell mass (ICM) and trophectoderm (TE) cells, this results in compromised development prospects of early buffalo embryos in a stage specific manner. Well-coordinated molecular signals associated with heat stress held responsible for reduced development of early embryo. We establish the most vulnerable stage of buffalo oocytes and presumptive zygotes against transient heat shock and observe a narrow window as the most critical stage for regulating the development potential of the prospective embryos under short heat stress environment.

Introduction

Development of oocytes and pre-implantation embryos are often considered as highly sensitive stages of embryogenesis toward abiotic stress including temperature. Environmental heat stress has negatively impacted the reproduction ability of female cattle [1]. In dairy animals, growing gametes and embryos are profoundly influenced by environmental changes that can induce developmental disorders and implantation failure in cattle [2]. Growing oocytes and embryos are reasonably susceptible to stress factors due to their inability to adapt stress factor and suboptimal culture conditions which exert stress to developing embryos [3, 4].

The consequences of heat shock on various developmental milestone of oocytes and developing cattle embryos have been reported in previous studies utilizing in vitro culture models [5]. Al-Katanani et al. [6]

demonstrated the reduced probability of successful embryos due to exposing oocytes to heat stress. Riverine *Bubalus bubalis* (buffalo) embryos exposed to increased culture temperature has shown their compromised development potential [7]. However, earlier investigations were largely focused on heat stress utilizing the varying temperature regime for relatively larger exposure period of in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC) for cattle [8]. It is understood that effect of heat stress on oocytes basically appears in a time dependent manner during the oocyte maturation and crucial stages of pre-implantation embryos. Suitable explanations have been offered in order to assess nuclear and cytoplasmic maturation as the basis of detrimental effect of stress in time dependent manners that prepare oocytes for fertilization and subsequent embryonic development [9]. Disruption of crucial mRNA transcripts deters the developmental ability of maturing oocytes. Cytoplasmic maturation of bovine oocytes at 42 °C for 4 h have altered spindle formation at the time of meiosis, reduced number of trophectoderm (TE) and inner cell mass (ICM) cells and a rapid fall in the production of blastocysts after in vitro fertilization [10]. Oocytes matured at elevated ambient temperature, had shown long-term effect on blastocyst development in cattle; but when heat stress was given to after 8–16 cell then there was less effect on the embryo development [11]. Exploring the molecular and cellular signal disruption during heat stress using in vitro culture model may enhance our understanding on buffaloes during pregnancy. To broaden the available information of effect of heat shock on buffalo oocytes and early embryos, the current study assessed the detrimental effect at a short exposure period along with specific time window for buffalo oocytes and early embryos utilizing an in-vitro culture model. In addition, the study deciphered disruptive molecular signals as the major implication of stress factor that hampers the developmental efficiency of oocytes and early embryo during early embryogenesis.

Material And Methods

All media and chemicals were procured from Sigma Aldrich, MO, USA unless otherwise specified. Disposable plastic wares used were procured from Falcon NJ, USA and Nunc, Denmark. Fetal bovine serum used was from Hyclone, Canada.

Development of in vitro experimental heat stress model

Overall experimental design of elevated temperature exposure model is as depicted in Fig. 1. The experiments consist of 6 groups i.e. grp1, 2 and 3 represent maturing oocytes exposed to elevated temperature respectively at 40°C for 4 h during 0–4, 8–12 and 16–20 h of IVM period. Whereas, remaining period these groups were maintained at 38.5°C. Similarly, experimental grp 4, 5 and 6 represent developing embryos exposed to elevated temperature of 40°C for short period of 4 h during 12–16, 84–88 and 104–108 hours post insemination (hpi), respectively and remaining period of culture the exposure groups were maintained at normal temperature of 38.5°C (Fig. 1). Concomitantly, a control group was maintained for assessment of IVM, IVF and IVC at 38.5°C. Development of embryos was recorded for their cleavage rate, 4-cell embryos, 8–16 cell embryos, morulae and blastocysts under individual stress groups. All experiments were performed at least three times in 2 replicates. Data were analyzed using

SYSTAT version 12 software packages and differences of means were calculated with one way ANOVA followed by Fisher's LSD. Level of significance of differences between means was at 5% ($P < 0.05$).

IVM of oocytes, IVF and IVC for production of early embryos

Buffalo ovaries were collected from slaughterhouse, Delhi, regardless of the estrous cycle and transported within 3–4 h to the culture laboratory in physiological saline (0.9%, w/v NaCl) containing strepto-penicillin (50 mg/l). Ovaries were washed 3–4 times in normal saline and cumulus oocyte complexes (COCs) were aspirated from visible ovarian follicles with the help of vacuum aspiration unit (K-MAR-5200 IN, USA) in hepes-buffered hamster embryo culture (HH) medium and placed the aspirated follicular fluid in a dry bath at 37.5⁰C for 35–40 min. Quality of cumulus oocyte complex (COCs) was observed and graded into grade A (> 5 layer cumulus layer) and B (3–5 cumulus layers) under stereo zoom microscope. Only COCs of grade A and B were selected for IVM and IVC. To perform IVM, selected COCs were briefly washed 3–4 times with maturation media (TCM199, HEPES) modified with 10% (v/v) fetal bovine serum (FBS), 0.005% (w/v) streptomycin, 0.01% (w/v) sodium pyruvate and 0.005% (w/v) glutamine supplemented with 5.0 µg/ml pFSH and 10 µg/ml LH, 1 µg/ml estradiol 17-β and 50 ng/ml epidermal growth factor (EGF), 64 µg/ml cysteamine and 50 µl ITS). After washing, 15 COCs were placed in drop of 100 µl maturation medium overlaid with mineral oil and cultured for 24 h at 38.5 °C in humidified atmosphere of 5% CO₂ incubator.

IVF was carried out in 100 µl droplets of BO medium supplemented with 1% (w/v) bovine serum albumin (BSA), 1.9 mg/ml caffeine sodium benzoate, 0.14 mg/ml sodium pyruvate and 0.01 mg/ml heparin. The matured COCs were washed thrice in BO medium and placed in BO medium droplets. The frozen thawed buffalo semen was processed for in vitro capacitation as per the procedure described earlier by Jain et al. [12] and 50 µl of the sperm suspension (at final concentration of 1×10^6 cells/ml) was added to each fertilization drops having 15 COCs and incubated at 38.5 °C with 5% CO₂ for 12 h. The presumptive zygotes were removed from the fertilization drops after 12 h of insemination, adhered cumulus cells were mechanically removed by vortexing and washed five times in modified Charles and Rosenkrans 2 amino acid (mCR2aa) medium. Following washing, 15 presumptive zygotes were co-cultured with monolayers of granulosa cells in 100 µl drops of IVC-I medium (mCR2aa supplemented with 0.8% (w/v) BSA, 1 mM glucose, 0.33 mM pyruvate, 1 mM glutamine, 1 x MEM essential amino acid, 1x non-essential amino acid and 50 µg/ml gentamycin). After 48 h of post insemination (hpi) zygotes were evaluated for evidence of cleavage. At 72 h of post insemination all cleaved embryos were transferred to IVC-II medium (same as IVC-I with 10% FBS) and maintained the embryos for 8 days post insemination at 38.5⁰C with 5% CO₂ and replaced culture medium after every interval of 48 h.

Assessment of metaphase-II (M-II) stage of oocytes by Hoechst 33342 staining

To determinate metaphase-II (M-II) stage of oocytes, denuded oocytes from grp1, 2, and 3 stained with Hoechst 33342 described briefly as follows. COCs were collected after maturation (24 h) followed by separation of cumulus cells using mechanical separation. Denuded oocytes were stained with Hoechst

33342 with slight modifications of protocol of Smith [13]. The denuded oocytes were fixed in 4% (w/v) paraformaldehyde solution (in PBS, pH 7.4) for 1 h at room temperature. Upon fixing and washing, groups of 40 oocytes were transferred to 150 μ l drop of 10 μ g/ml Hoechst 33342 stain prepared in PBS-PVP solution for 30 min under dark. The stained oocytes were washed three times in PBS-PVP solution and placed onto glass slides and mounted with Pro-Long mounting medium (Invitrogen, USA) and observed under the fluorescent microscope with UV filter (Olympus, Japan). Oocytes nuclei revealing both the oocyte nucleus along with its polar body counterpart were interpreted as matured M-II. Percentage of M-II was calculated for each IVM groups for at least 150 oocytes.

Estimation of Glutathione production during heat stress

In vitro matured oocytes (20 oocytes/pool) from four different stress pools, viz., P1, 2, 3, and 4 were collected respectively at 4, 8, 16 and 24 h of IVM, and utilized for estimation of glutathione using glutathione assay kit (Sigma- Aldrich, India) following the manufacturer's instructions. Briefly, groups of oocytes were suspended in 45 ml deionized water and pipetted into separate wells of a 96-well microtiter plate. In addition to the oocyte samples, 50 ml of standard (3.125–50 nmol glutathione per well) were pipetted into duplicate wells. This was immediately followed by the addition of 100 μ l reaction mixture (prepared by mixing 5.75 ml 100 mM NaPO₄ buffer with 1 mM EDTA, pH 7.5, 5 ml 1 mM DNTB, 5 ml 1 mM NADPH, and 0.1 ml 200 U/ml glutathione reductase dissolved in NaPO₄ – EDTA buffer) to the oocyte samples or standards. Absorbance was read at 405 nm after, 6–8 min incubation using a microtiter plate reader. The experiment was replicated on 3 different pools of oocytes (20 oocytes/pool) were assayed for each group.

Quantification of TE and ICM cells of blastocysts by differential staining

To determine TE and ICM cell number in blastocysts, we took hatch blastocysts from experimental grp 2 on day 7 for differential staining. Staining of hatched blastocysts was performed as per the protocol of Thouas et al. [14]. The blastocysts were stained with Hoechst 33342 (10 μ g/mL) for 30 min followed by permeabilization with 0.1% (v/v) triton-X for 30 s and propidium iodide (25 μ g/mL) staining for 20 s. Distinct TE and ICM nuclei, which stained pink and blue, respectively, were counted using epi-fluorescence microscope (Olympus, BX- 51, Japan).

Differential expression of cell stress, apoptotic, maternal to embryonic transition (MET), morula to blastocysts transition (MBT) and maternal recognition of pregnancy (MRP) genes of oocytes and embryos under stress

Extraction of total RNA was performed for different oocytes and embryonic stages, were used to prepare cDNA for relative quantification of transcripts of target gene at different embryonic cell stages by using qPCR. To isolate total RNA, 10 oocytes were collected at different maturation of time interval (0 h, 8, 16, 24) and subsequently different embryonic stages (2-cells, 4-cells, 8-16-cells, morulae and hatched blastocysts) from experimental grp2, processed using RNAqueous Micro Kit (Ambion, USA) as per manufacturer's instruction. Total RNA was eluted in 20 μ l of elution buffer and treated with RNase-free

DNase I (Ambion, USA). A constant amount of 50 ng total RNA from each samples were reverse-transcribed using Revert-Aid cDNA synthesis kit (Fermentas, USA) following the manufacturer's instructions. cDNA samples were diluted X3 with nuclease-free water and stored at -20⁰C till further use. Quantification of cDNA transcripts was performed by real-time PCR amplification using Sybr Green (Fermentas, USA) with RPS 18 as normalization control. The list of primers used for amplification of stress, apoptotic, MET, MRP and morula to blastocysts transition genes were designed by using primer3 software and primers are shown in Table 1. Primer optimization was carried out as per the protocol of Bettegowda et al. [15]. Real time PCR reaction mixtures were consisted of 5 ul of Sybr green qPCR mix, 2 ul of cDNA template, optimized primer quantities and nuclease free water to make the total reaction volume of 10 ul. Reactions were performed in duplicate for each samples using Mx3005P Real Time PCR System (Stratagene, USA). The universal PCR condition used was 95⁰C for 10 min followed by 40 cycles consisting of denaturation at 95⁰C for 30 s, annealing at 59⁰C for 20 s and extension at 72⁰C for 30 s. A dissociation curve analysis was run to confirm the specificity of the amplified product. Mean threshold cycle values (CT) for genes under study were calculated for duplicate samples and relative transcript abundance for target gene expression was calculated using the formula $2^{-(\Delta\Delta CT)}$ [16].

Table 1
Details of different gene primers developed and utilized in qRT-PCR assay

Gene	Forward sequence	Reverse sequence	Annealing Temperature (°C)
HSPA8	AACCACCCCAAGCTATGTCTG	TGGTGGGATTCATTGCGACT	59
Sirt3	TTGCTGGTGCCTCAAGTGAC	TCTGGCAGGCTCTGGTCTTA	59
MnSOD	GCTGGAAGCCATCAAACGTG	CCTGCTCCTTATTGAAGCCGA	59
Bcl2	GCAGGTATTGGTGAGTG	ATTGTTCCCGTAGAGTTCC	59
Bax	TTTCTGACGGCAACTTCAACTG	TCGAAGGAAGTCCAATGTCCAG	59
Caspase-9	ATCAAAGCCGAGCCAAGGAA	CCTGGGTACCTCTGGTCTGA	59
eIF1A	CTCCAAGTGGCTGAGAAAG	TCACTCTCCTCCTCGCTCTC	59
U2AF	GATGTCGAGATGCAGGAACA	TCTTCTTCACGGCGAAACTT	59
PAP	GGATGGTCATGGTTGAGGAG	GGCGTTGTTTTTCAGTTGGT	59
ZO1	AGCCAAAGAAGGCTTGGAG	GAGGTCAAGCAGGAAGAGGA	59
NaK ATPase β subunit	ATGCTGCTCACCATCAGTGAAT	ATAGCTCTTGGGATCGTTAGGAC	59
ETS2	CCATCTTGTTTACCTGTGGAGTC	CTTCTCTTTCCCATCTTGCTGA	59
Tead4	GGTGGAGAAAGTGGAGACCG	CATGTACTCACAGAGCGGGG	59
Cdx2	AAGGACGTGAGCATGTATCCC	CTGACGAAGTTCTGCGGGG	59
GATA3	CTCCAAGACGTCCATCCACC	CGGGAAGGTGAAGAGGTGTG	59
Ifn-τ	CGGTGACGGGAGAGAAAGAC	CTCTGACGATTTCCAGGCA	59
RPS18	GAAAATTGCCTTTGCCATCACTGC	GATCACACGTTCCACCTCATCCTC	59

Statistical analysis

Each experiment was repeated thrice in 2 replicates to determine development rate of oocytes and IVF embryos. Relative abundance values of mRNA was analyzed using SYSTAT version 12 software packages. Data was analyzed using one way ANOVA followed by Fisher's LSD. Significance of differences between means was calculated at 5% level of significance ($P < 0.05$).

Results

Effect of heat shock during IVM on development rate of oocytes and embryos

To determine the maturation state of oocytes to each metaphase-II (M-II) we assessed the M-II stage of IVM oocytes and found that on an average 80% of the oocytes reach metaphase II (M-II) stage irrespective of the stress given at early, mid and late-IVM under grp1, 2, and 3, respectively (Fig. 2). Contrary to nuclear maturation (M-II), cleavage and subsequent development rate of embryos revealed substantial effect of heat stress in grp 2 and 3 where thermal stress was employed during mid and late-in vitro maturation of oocytes, respectively. Our result clearly demonstrates that the reduced cleavage rate obtained in grp1, 2 and 3 (Fig. 3). Further analysis of data reveal that grp 2 did show normal growth rate at 4 and 8–16 cell stage of embryo, however, thereafter significant reduction in development rate of 16 cell embryos, morulae, and formation of blastocysts (Fig. 4). In addition, significant reduction in production of blastocysts was observed for grp 1, 3 and 4 suggesting that these groups are equally vulnerable to a short heat exposure. Our result convincingly demonstrates that grp 2 has shown the most striking and deleterious effect of heat stress with reduced blastocysts rate (5%) as compared to other exposure groups.

Effect of heat shock during IVC on production rate of early embryo

To confirm whether different embryonic stages have differential capacity to withstand heat shock, we determined thermal stress on embryos at 12–16 hours post insemination (hpi), 84–88 hpi and 104–108 hpi, under grp4, 5, and 6 respectively. Our results show that the cleavage rate was significantly reduced when presumptive zygotes were exposed to heat stress at the very beginning of IVC (12–16 hpi) under grp 4 there was significant reduction in development of 4-cell embryos, morulae and blastocysts except 8–16 cell embryos. Quite intriguingly, inhibitory effect of stress was not observed in case of grp 5 and 6 suggesting that early embryo (Fig. 4).

3.3 Production of Glutathione from oocytes under heat stress

The content of glutathione from different oocytes groups has shown increased pattern from 4 h to 16 of IVM (Fig. 5). However, the most significant glutathione content was observed from oocytes group to higher temperature during 12–16 h of IVM. Similarly, increased content of was obtained at 24 h of IVM. No difference in glutathione concentration was observed in grp 1 and 2 as compared to the control.

Effect of stress on TE and ICM of blastocysts

We assessed the TE and ICM ratio of blastocysts. Table 2 describes the TE and ICM cells derived from blastocysts under exposure grp 2. As it can be seen from data of Table 2, TE cells were substantially reduced under treatment group as compared to the control ($P < 0.05$). Similarly, ICM cells were sharply reduced by more than half when compared with the control. By accounting both TE and ICM cells, results have shown significant reduction in number in treatment group essentially indicating the implication of heat shock (Fig. 6).

Table 2
Quantitation of TE and ICM cells in grp 2 and control using differential staining method

Treatment	No. of Blastocyst examined	TE cells	ICM cells	Total cells
Control	10	178 ± 3.21 ^a	46 ± 5.41 ^a	207 ± 5.47 ^a
Grp 2 (40°C)	10	86 ± 4.15 ^b	22 ± 2.22 ^b	108 ± 6.25 ^b

Each treatment was repeated 3 times and each replicate consisted of 60 oocytes per group. (P < 0.05)

Expression of cell stress and apoptotic genes

Expression profile of grp 2 oocytes and embryo to evaluate the association of the expression of key cell stress related genes (HSPA8, Sirt3, MnSOD), and apoptotic cycle genes (pro-apoptotic: Bax, Caspase-9, and anti-apoptotic: Bcl-2) and their relationship with oocyte development ability. Expression level of heat stress genes HSPA8, Sirt3 and MnSOD was found to be significantly higher level in oocytes collected from 16 h of IVM (Fig. 7). The relative abundance of mRNAs for these genes was 15 folds higher upon exposure to heat stress (P < 0.05). Post IVF stage, pre-implantation embryos exhibited increased expression for HSPA8, MnSOD and Sirt3 continued until 4-cell embryos after which it dropped down to the basal level similar to that of the control. In similar trend with the stress response genes the expression of pro apoptotic genes Bax and Caspase 9 revealed a significant over expression at 16 h. Reflecting the overall apoptotic effect as a result of heat stress the anti-apoptotic gene (Bcl-2) was detected at significantly lower levels in heat stress group oocytes as compared to control (Fig. 8).

Status of MET genes in embryos generated from heat stressed oocytes

Figure 9 depicts the expression pattern of MET genes at 2, 4, 8-16-cell embryo and morulae produced from heat stressed oocytes. Expression level of eukaryotic translation initiation factor 1A (eIF1A) U2 auxiliary splicing factor (U2AF) and polyadenylate polymerase (PAP) were found to be upregulated in 8–16 cell embryos. The relative abundance of mRNAs for eIF1A and U2AF genes was nearly 2–3 fold significantly downregulated in the stressed group of 8–16 cell embryos (P < 0.05). Similarly, relative abundance of mRNAs for PAP gene was significantly lowered in 2-cell, 4-cell, 8–16 cell embryos (P < 0.05).

Expression of morulae to blastocysts transition genes

Expression of Zonula occludens 1 (ZO1), ETS Proto-Oncogene 2 (ETS2) and Na/K ATPase β subunit (NKB1) gene was analyzed with TE and ICM samples. Expression level was increased in trophectoderm as compared to ICM (Fig. 10). The relative abundance of mRNA transcripts of ZO1, ETS2 and Na/K ATPase β subunit (NKB1) genes were significantly 4–8 folds higher in trophectoderm that of ICM sample (P < 0.05). There was no effect on expression of ZO1, ET2 and NKB1 genes in morula, however, substantial inhibition in expression of ZO1 and ETS2 and NKB1 genes in blastocysts Relative abundance

of mRNAs for these genes was approximately 2–3 folds lower in stress blastocyst as compared to the control ($P < 0.05$).

Expression of maternal recognition of pregnancy genes

Expression profile of 8–16 cell, morula and blastocyst was generated in order to assess detrimental effect of heat stress on developmental related process. Figure 9 represents the expression pattern of Tead4, Cdx2, GATA3 and IFN- τ . Relative abundance of mRNAs for these genes was significantly down-regulated in heat stressed blastocyst ($P < 0.05$), however, transcript abundance of cdx2 and IFN-t in 8–16 cell embryo and morula was found to be non-significant as that of control (Fig. 11).

Discussion

Heat stress elicits severe detrimental effect on pre-implantation embryos in dairy animals and the damage due to stress is most pronounced in the tropical environment. The growing oocytes and embryos largely show varying levels of tolerance against heat stress. Still, largely it is unclear how precisely the transient heat shock affects the growth of buffalo embryos. In this study we explained the molecular and developmental capacity of buffalo oocytes and early embryos at elevated temperature utilizing buffalo IVF model. This study clearly suggests that a short period of heat stress to oocytes did not regulate the pace of nuclear maturation of oocytes in terms of their ability to enter M-II stage, however, there was noticeable imbalance in molecular signal and cytoplasmic maturation leading to poor generation of cleavage rate and subsequent embryonic development. To prove the cytoplasm maturation of buffalo oocytes we examined glutathione content of oocytes at different time interval of IVM. The increase in glutathione concentration was maximum during 12–16 h of IVM suggesting the degree of cytoplasmic maturation implicates in stronger glutathione response in order to counter heat stress at mid and late-stage of IVM of oocytes. Additionally, to understand the molecular signal associated with glutathione response we assessed the expression level of HSPA8 and Mn-SOD and Sirt-3 genes and found that expression of HSPA8 and Mn-SOD was intricately related with the increased production of glutathione. Our data comprehensively reveal that buffalo oocytes generate maximum glutathione signal due to the antioxidant responses from HSPA8, Mn-SOD and Sirt-3 primarily at mid-IVM under transient heat stress. Surprisingly, over expression of antioxidant genes owing to stress were conspicuously subsided during zygotic stage indicating the amelioration of transient heat stress in oocytes and this effect did not persist during zygotic stage.

Next we examined the effect of short heat stress on molecular signals responsible for oocyte maturation and achieving the developmental milestones of early embryogenesis. Transient heat stress implicates in increased expression of apoptotic genes (Bax/Bcl₂ & Caspase-9) specifically during mid-IVM stage as compared to any other stage of IVM and embryogenesis ($P < 0.05$). Our findings suggest that even a short exposure of nearly 4 h exerts an irreparable disruption in molecular signals with the substantial increase activity of apoptotic genes of mid-IVM oocytes; a stage homologous to post ovulatory oocytes of in vivo condition of buffaloes. Based on the circumstantial evidences of hyperactivity of apoptotic genes here

we enunciate that mid IVM stage of buffalo oocytes represents the most vulnerable stage against heat stress.

We assessed the effect of heat stress on oocytes maturation, oocyte to embryo conversion rate, and subsequent embryonic development capacity until formation of blastocysts. Our data on embryo development clearly reveal that heat stress on oocytes at mid-IVM elicited most significant deleterious effect specifically on morulae with the lowest morulae to blastocysts transition rate in grp 2 as compared to other exposure groups. This indicates that even a short and transient heat stress to oocytes at mid-IVM stage has potential to elicits deleterious effect to pre-implantation buffalo embryos. To relate the developmental milestones of oocytes such as nuclear and cytoplasmic reorganization process with the cellular and molecular signals in the form of essential mRNA transcripts during IVM, our findings support that significant reduction in mRNA transcripts of ZO1, EST2 and NKB1 genes, indicating the molecular mechanism for development process has weakened due to lack of vital factors viz. ZO1, EST2 and NKB1, deem to be essential for development of embryos. The observation of distinct and conspicuous effect of heat stress on morula to blastocysts transition rate of grp2 can be explained on the basis of poor transcripts abundance that implicated in subdued formation of TE and ICM cells. With the presence of lesser gene products, development of TE and ICM turns out weak, this thus, results in substantial reduction in morula to blastocysts transition rate (~ 71%). In concurrence with current results, study by Lawrence et al. [17] reported nearly 65% reduction in development rate of blastocysts by exposing oocytes to heat stress during maturation in cattle. Edwards et al. [18] explained the reduction in compaction and blastocyst production rates of heat stressed bovine oocytes bovine. Quite contrary to our results Ju et al. [19] reported that heat stress for 1 h at 40 °C or 42 °C to IVM oocytes did not impart detrimental effect on blastocysts formation. Taken together, our findings support that thermal stress given to buffalo oocytes during mid IVM (8–12 h) elicits the most deleterious effect on developmental ability of pre-implantation buffalo embryos.

Among the zygotic groups, we found the effect of thermal stress was relatively more pronounced on pre-morulae (grp 4) embryos as that of morulae (grp 5) and blastocysts (grp 6). This can be explained on the basis that early hours of post-fertilization phase of presumptive embryos undergo vital activity for activation of maternal to zygotic gene activation and this transition phase holds a critical stage in post-zygotic embryo development. Expression level of MET genes (PAP, U2Af and eIF4A), and their canonical signals are essential for zygotic genome activation. The reduced expression level polyadenylate, translation initiation and splicing factors have cascading effect on MET, which in turn has disturbed the development capacity of embryos. These results conforms our previous study where we explained PAP, U2Af and eIF4A as vital indicators of MET in buffalo embryos [20]. Our study reinforces previous findings of Rivera & Hansen [21] in which they observed the blastocysts production tends to decrease when early stage cattle embryos were exposed to heat stress. Blythe et al. [22] explained the pathways of thermal stress which triggers the checkpoints regulating MET.

Additionally, we assessed the molecular signals responsible for maternal recognition of pregnancy, an important milestone of embryogenesis. Effect of heat stress on expression of mRNA transcript of tead4,

cdx2, gata3 and ifn-t remained to be down-regulated suggesting that essential molecular support of tead4, cdx2, gata3 and ifn-t largely diminished owing to heat shock exposure. These molecular signals related to MRP undoubtedly supports that quality of pre-implantation embryos remained poor, hence, they will fail to achieve the developmental milestone. To dwell on molecular signal supporting MBT process, our data explicitly confirm the transcripts of ZO1, ETS2 and Na/K ATPase β subunit genes responsible for MBT discretely down-regulated under heat-stress conditions. This impairment in MBT signal due to heat shock to oocytes during IVM provides clear evidence of reduction of molecular signals essential for morula to blastocysts transition in early embryos. Given the fact that transient heat shock to oocytes elicits cascading effect through the molecular milieu which are essential for achieving developmental milestones of early embryos.

Conclusion

In this study, we answered the three pertinent questions; first, which is the most susceptible stage of buffalo oocytes and early embryos against heat stress, second, whether a short transient period of heat stress for four hours can affect the development ability of early embryos and, third, how will the dynamics of molecular signal be associated with heat stress at different stage of oocytes maturation and early development. Our findings clearly delineate a stage-specific effect of thermal stress to buffalo oocytes and early embryos, and proved mid-IVM period as the most critical and vulnerable stage under heat shock with a down-streaming effect with a severe reduction in morula to blastocysts progression rate, thus, overall, regulates the development ability of buffalo embryos. A well-coordinated molecular signal associated with heat stress emphasizing the basis for poor maturation of oocyte and early embryo development. This was further reflecting on generation of poor TE and ICM cell indices, thus provides an evidence for lopsided developmental ability of the embryos. Altogether, our findings provide a foundation from which further studies can continue to explore underpinning a suitable intervention preventing the pregnancy losses during heat stress. Additionally, necessary in-vivo validation study warrants in order strengthen the outcome of the current study.

List Of Abbreviations

IVM: In vitro maturation; IVF: In vitro fertilization; IVC: In vitro culture; grp: Group; M-II: Metaphase-II; MRP: Maternal recognition of pregnancy; ICM: Inner cell mass; TE: Trophectoderm; RT-qPCR: Reverse Transcription-quantitative Polymerase Chain Reaction; COCs: Cumulus oocyte complex; hpi: Hours post insemination; BSA: Bovine serum albumin; EGF: Epidermal growth factor; FBS: Fetal Bovine Serum; MET: Maternal to embryonic gene transition; MBT: Morula to blastocysts transition; MRP: Maternal recognition of pregnancy

Declarations

Ethics approval and consent to participate

This work has been carried out as per the approval of NDRI institutional animal ethics committee.

Consent for Publication

All the authors have given consent for publication.

Availability of data and material

Datasets of the current research are available with the corresponding author.

Competing interests

There is no competing interest exist.

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Authors' Contributions

DS and MK performed the experiments, data analysis and drafted the manuscript. TKD designed the experiments, involved in data analysis and revised the manuscript. RK helped in data analysis and writing of manuscript. All authors read and approved the final manuscript for publication.

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Nil

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Figures

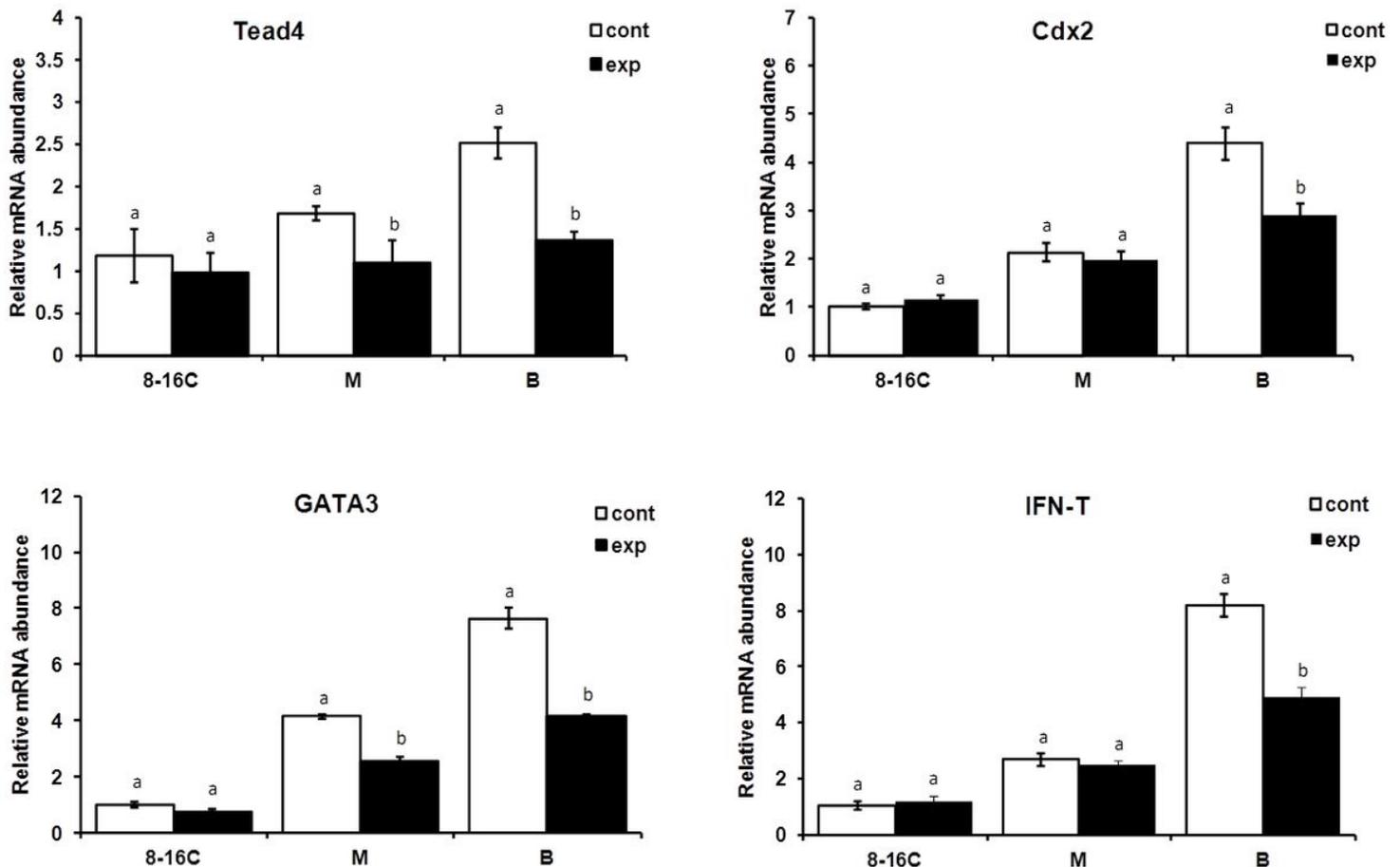


Figure 1

Relative mRNA expression of Tead4, Cdx2, GATA3 and IFN-T from exposure grp 2 (solid bar) and control (open bar) in 8-16 cell embryo (8-16C), morula (M) and blastocyst (B), $P < 0.05$.

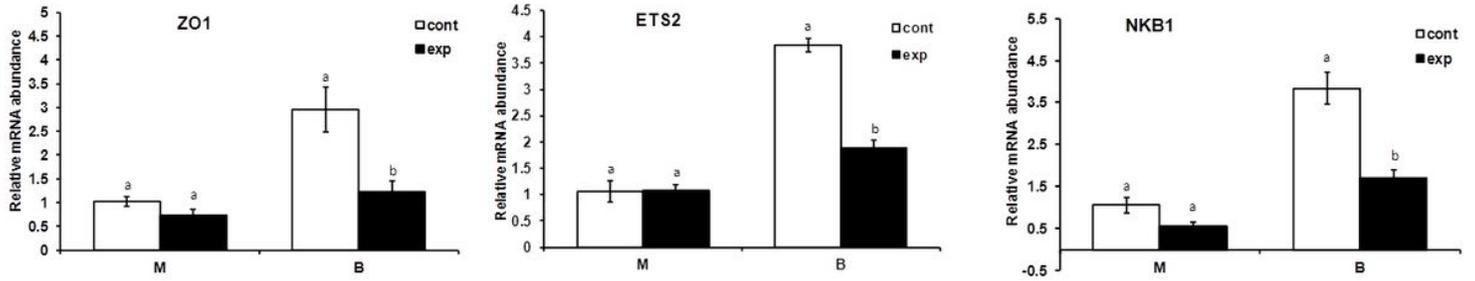


Figure 2

Relative mRNA expression of morula and blastocysts transition genes (ZO1, ETS2 and NKB1) from exposure grp 2 (solid bar) and control (open bar) in morula (M) and blastocyst (B), $P < 0.05$

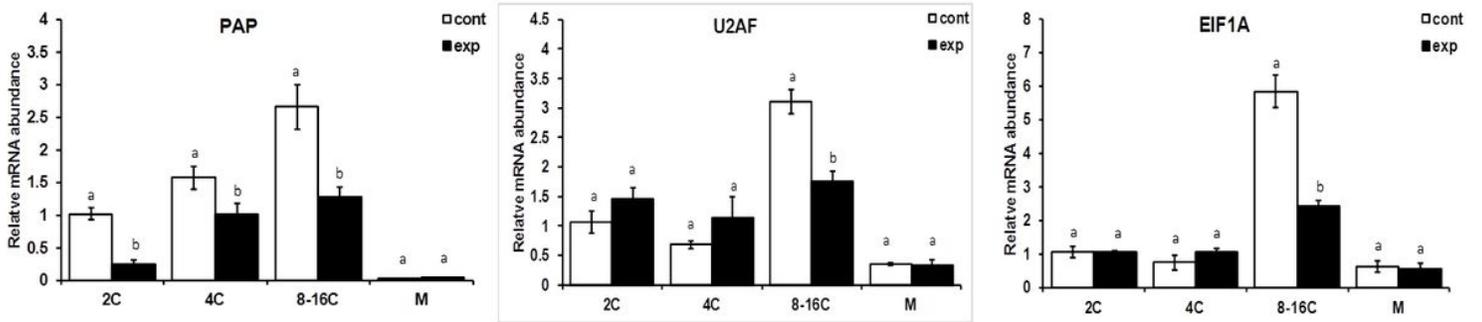


Figure 3

Relative mRNA expression of PAP, U2AF and EIF1A genes from exposure grp 2 (solid bar) and control (open bar) at early embryo stages (2-cell, 4 cell, 8-16 cell, morula and blastocysts shown as 2C, 4C, 8-16C, M & B, respectively), $P < 0.05$.

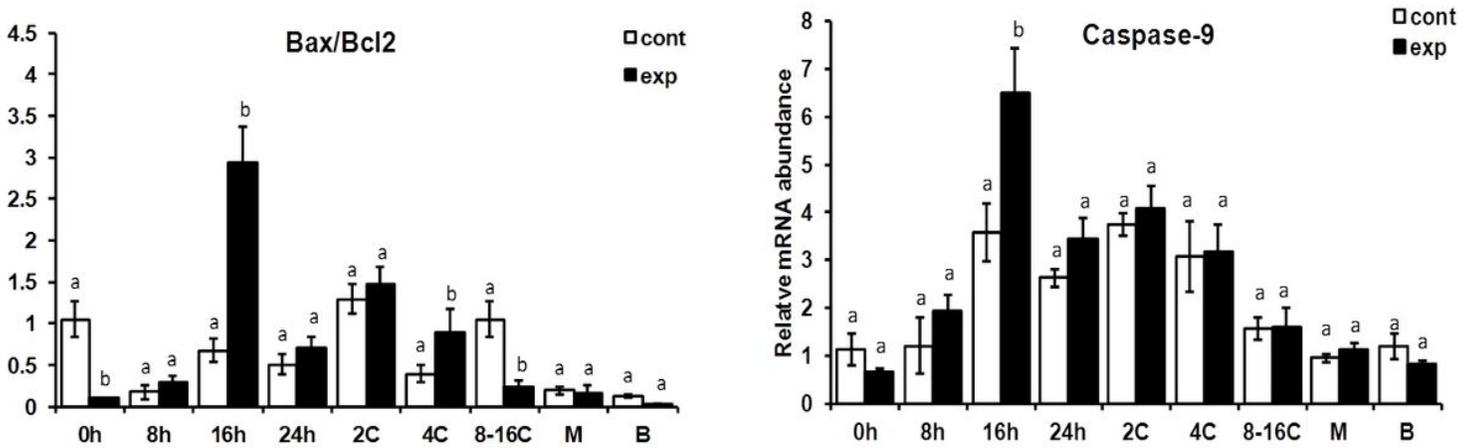


Figure 4

Relative mRNA expression of Bax/Bcl2 and Caspase-9 genes from exposure grp 2 (solid bar) and control (open bar) at different IVM (0,8,16, 24h) of oocytes and early embryo stages (2-cell, 4 cell, 8-16 cell, morula and blastocysts shown as 2C, 4C, 8-16C, M & B, respectively), $p < 0.05$.

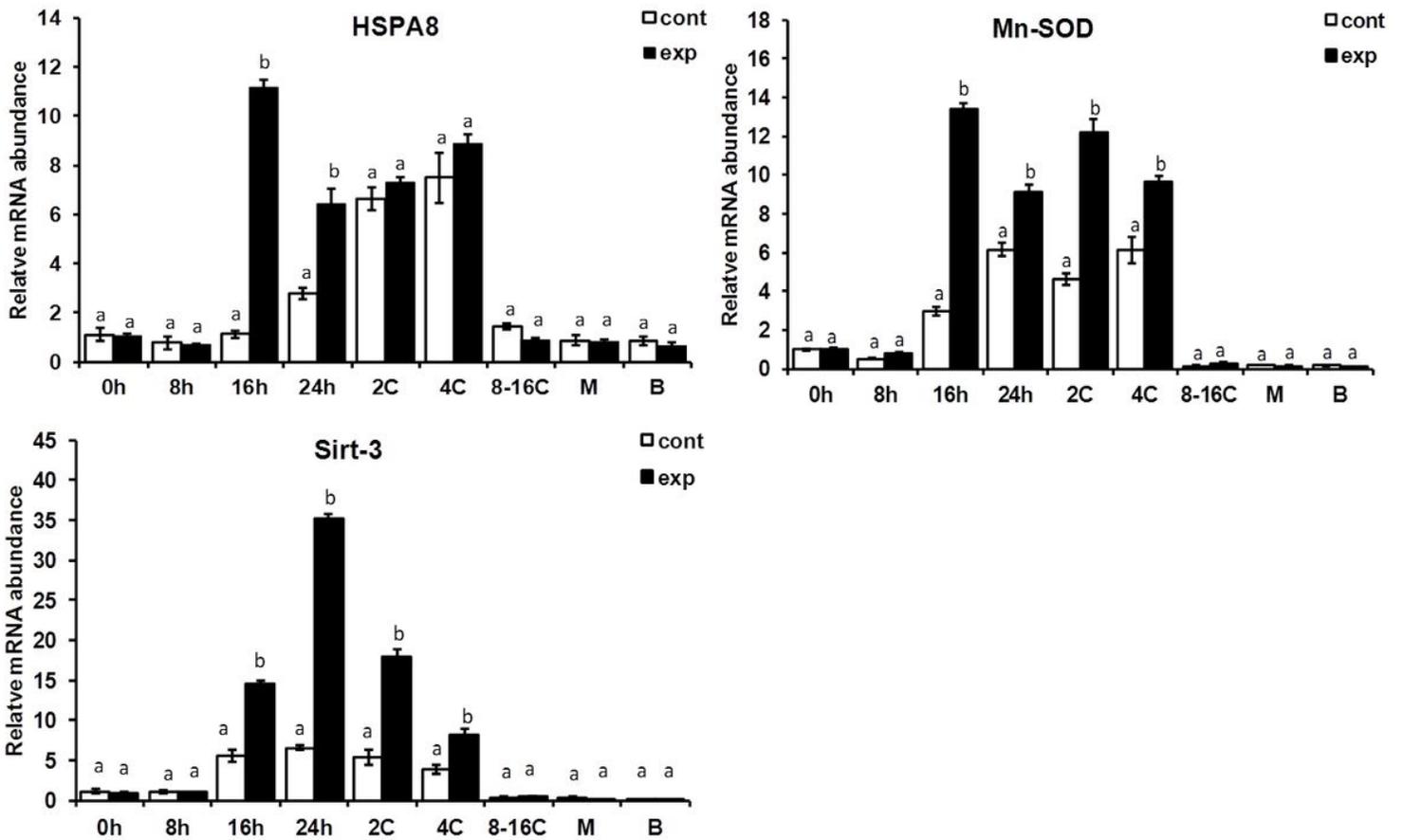


Figure 5

Relative mRNA expression of HSPA8, Sirt3 and MnSOD genes from exposure grp 2 (solid bar) and control (open bar) at different period of IVM (0,8,16, 24h) of oocytes and early embryo stages (2-cell, 4 cell, 8-16 cell, morula and blastocysts depicted as 2C, 4C, 8-16C, M & B, respectively), $P < 0.05$.

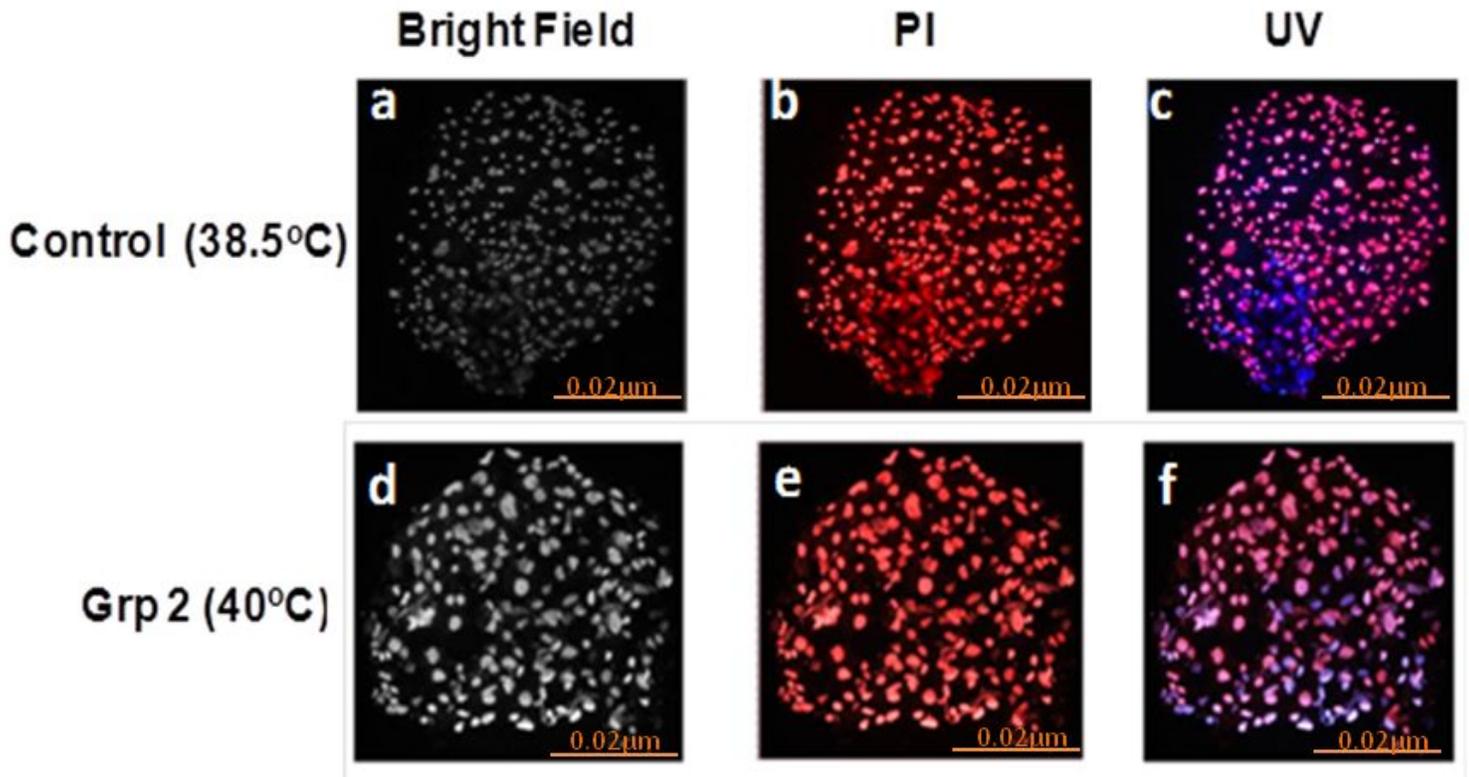


Figure 6

Effect of heat stress on quality of blastocysts generated from oocytes of exposure grp2. Blue and pink stains indicate ICM cells and TE cells, respectively. Magnification; X200

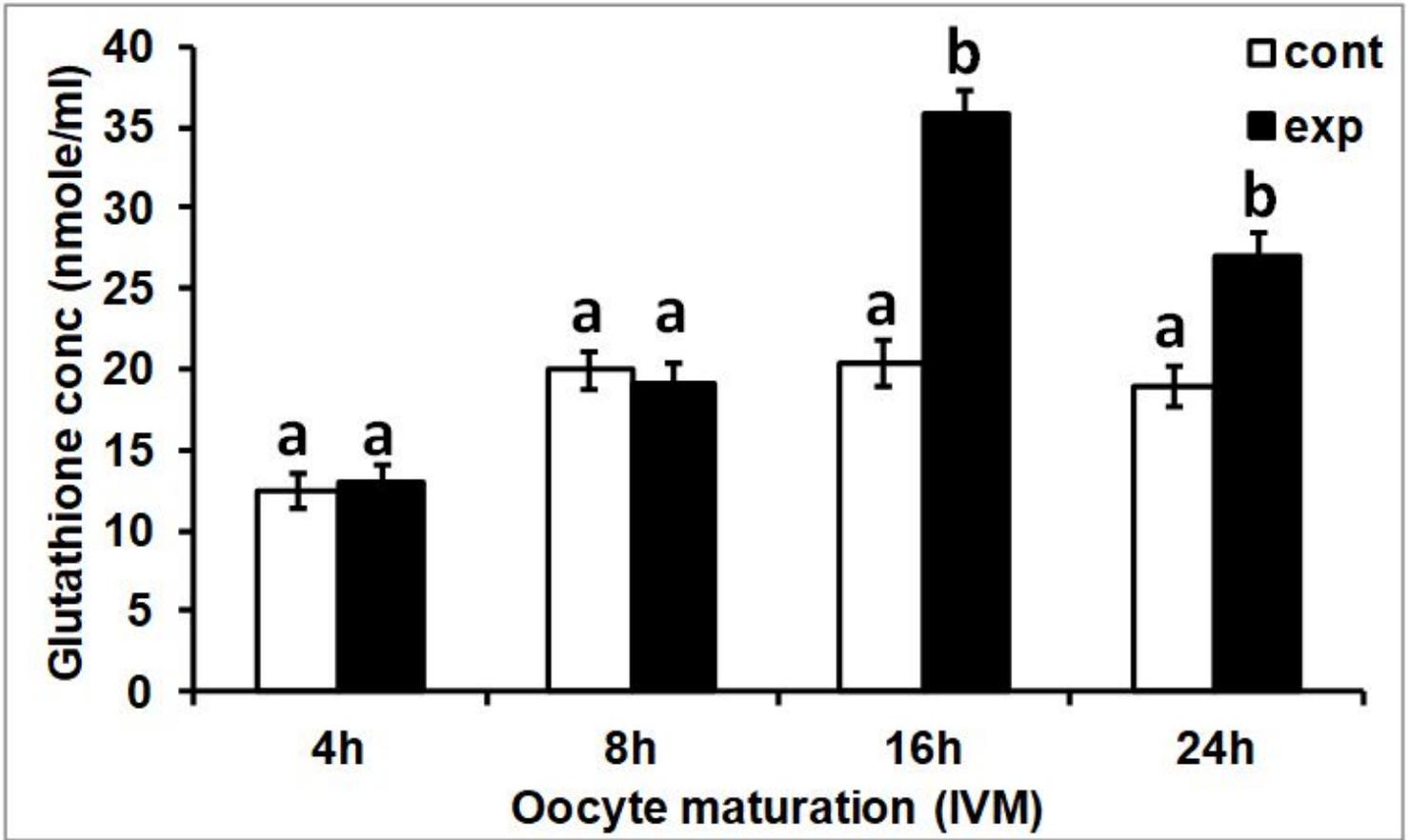


Figure 7

Glutathione content was determined for oocytes of 4 exposure groups during IVM. Each treatment was repeated thrice and each replicate consisted of 60 oocytes, ($P < 0.05$).

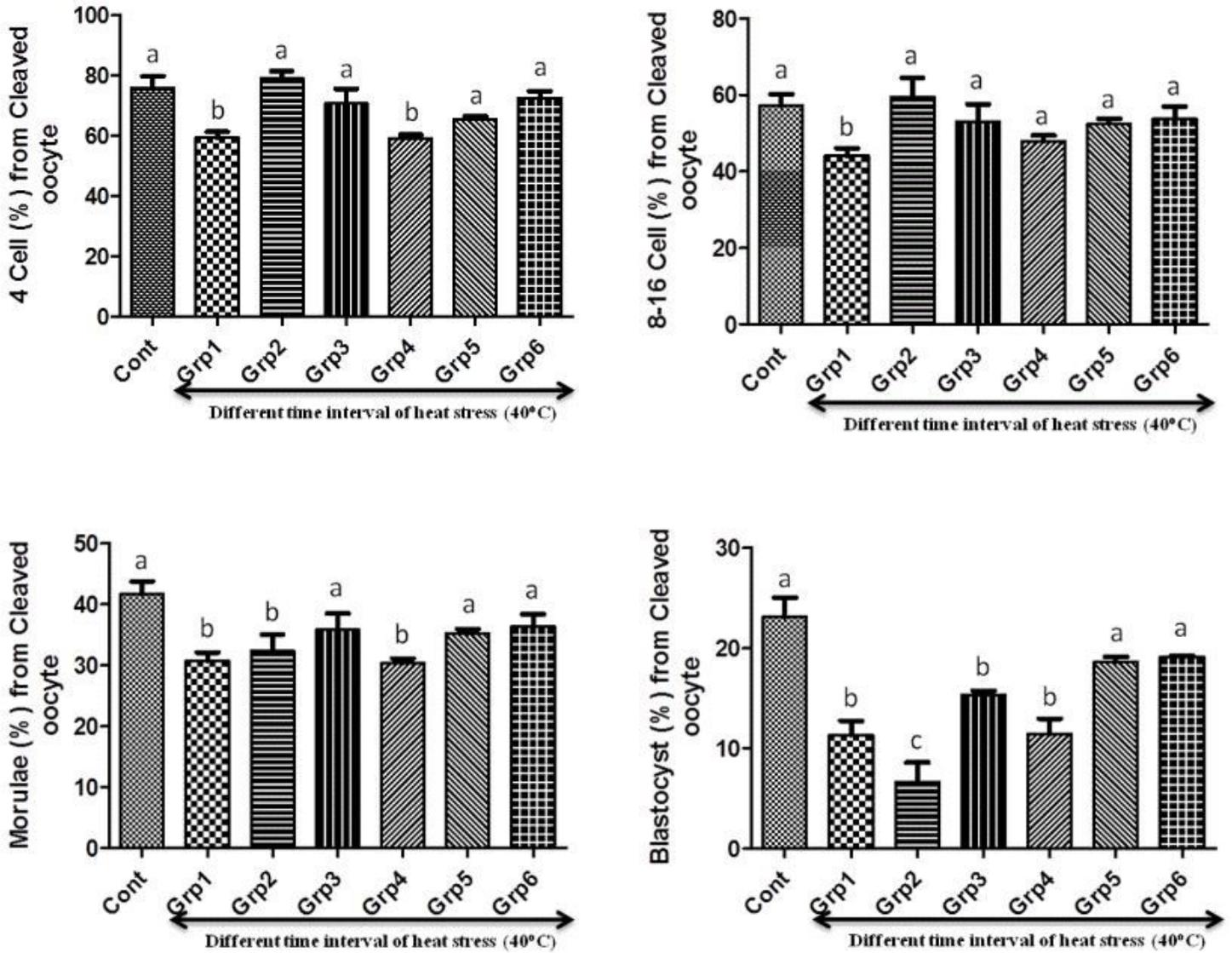


Figure 8

Development rate of embryos (a) 4 cell, (b) 8-16 cell, (c) morulae and (d) blastocyst under six different heat exposure groups. Enumeration of 4 cell embryo, 8-16 cell, morula and blastocysts were performed on 3rd day, 5th day and 7th day of hpi. Each treatment was repeated 3 times in 2 replicates and each replicate consisted of 60-70 oocytes. ($P < 0.05$).

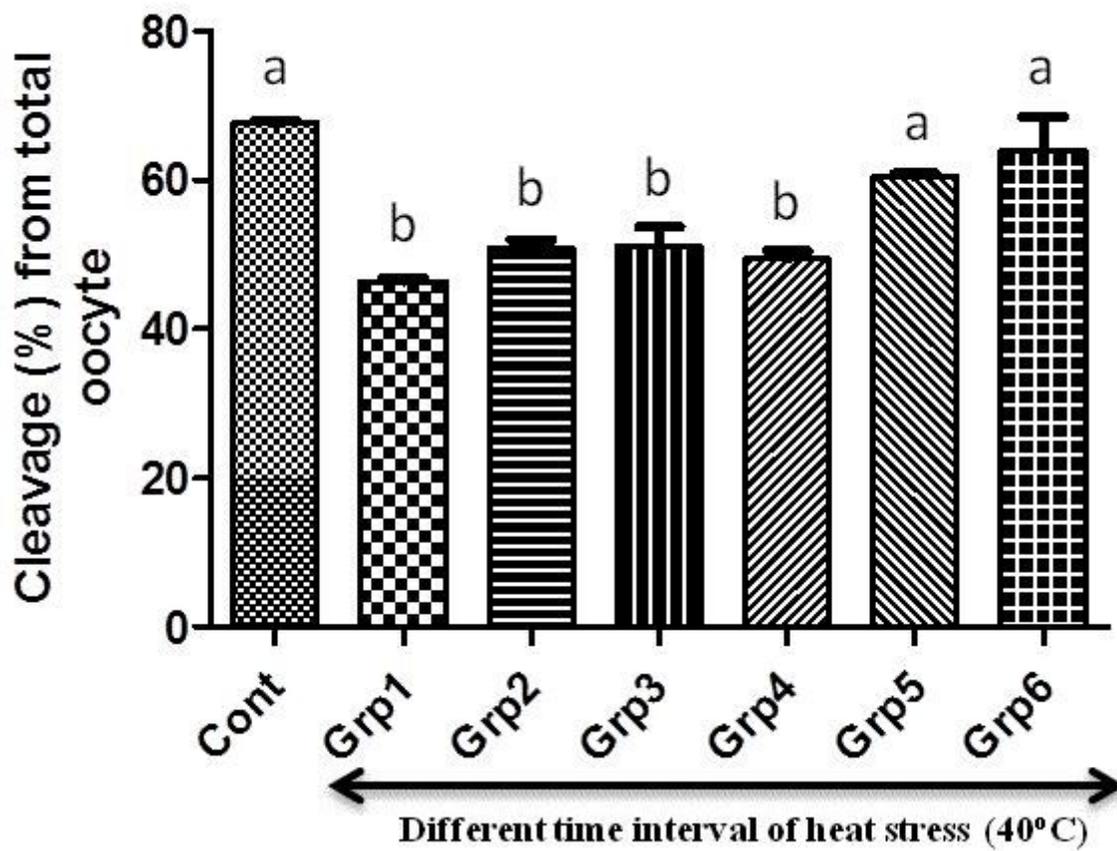


Figure 9

Cleavage rate of oocytes exposed to heat during IVM and IVC from different exposure groups. Cleavage rate was confirmed after 48 h post insemination (hpi). Each treatment was repeated 3 times in 2 replicates and each replicate consist of 60-70 oocytes, ($P < 0.05$).

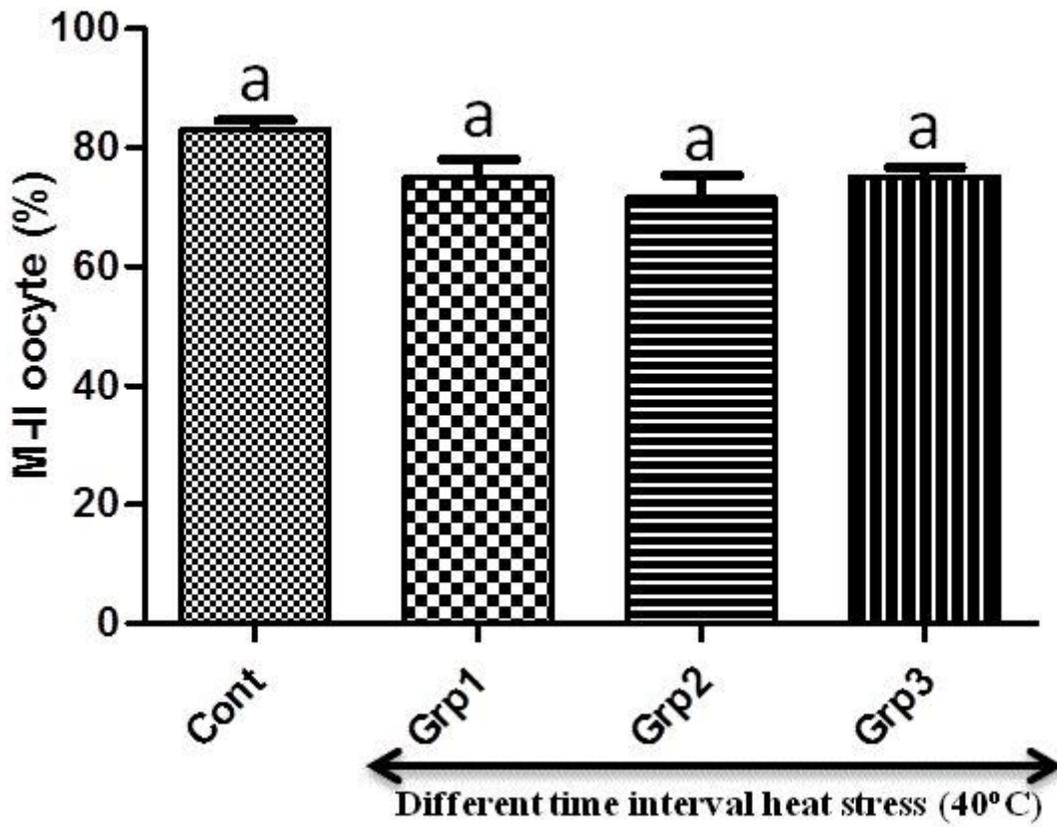


Figure 10

Effect of heat stress on oocytes maturation (M-II stage) at different time interval of IVM and maturation was assessed by Hoechst staining. Each treatment was repeated 3 times and each replicate consisted of 50 oocytes ($P < 0.05$).

In vitro oocyte stress Model

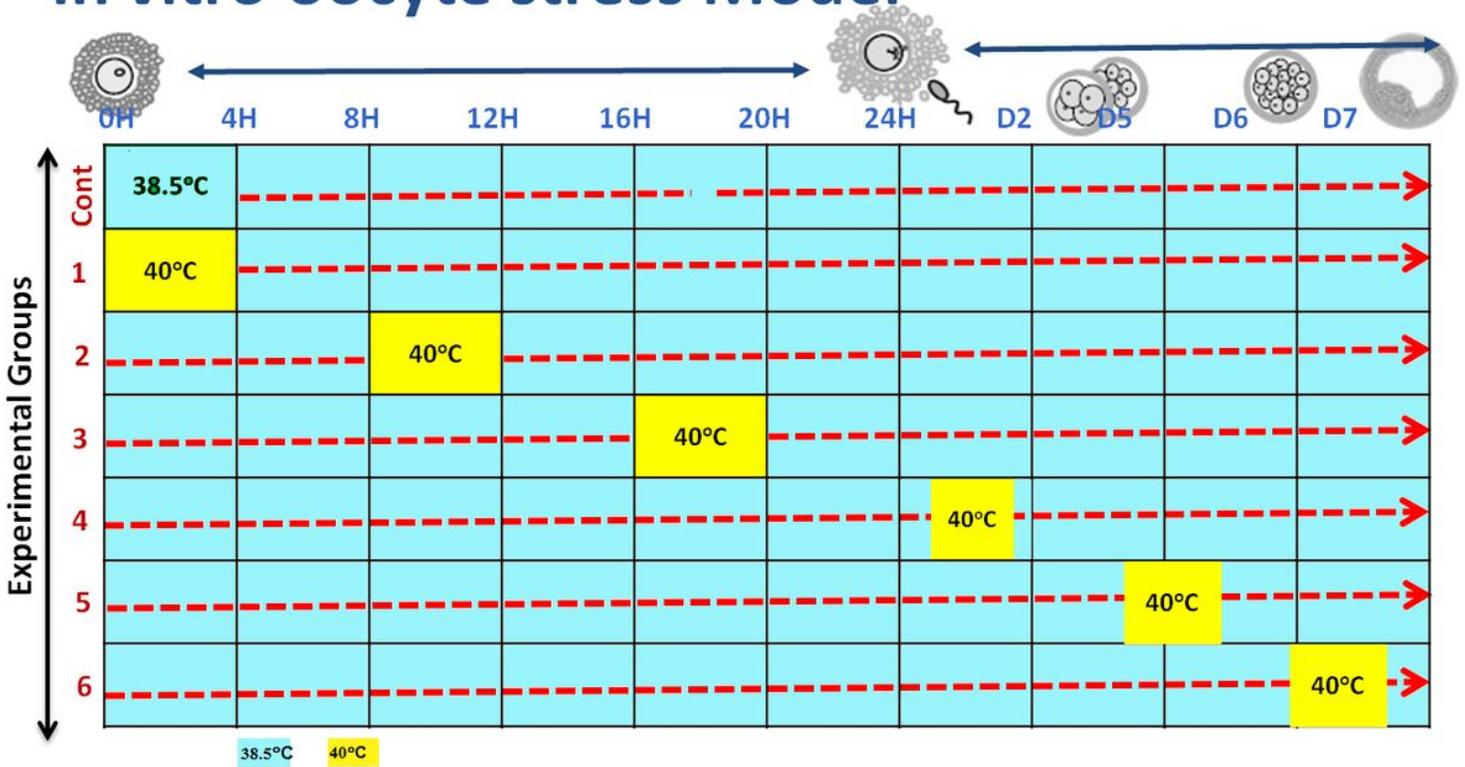


Figure 11

Schematic design of heat stress experimental groups (grp1, 2, and 3) of buffalo oocytes exposed at 40°C for 4 h during 0-4, 8-12 and 16-20 h of in vitro maturation (IVM) and experimental groups (grp3, 4, and 5) of embryo exposed at 40°C for 4 h during days; D2, D5-6 and D7 of IVC.