Developmental competence and expression profile of genes in buffalo (*Bubalus bubalis*) oocytes and embryos collected under different environmental stress

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Abstract The study examined the effects of different environmental stress on developmental competence and the relative abundance (RA) of various gene transcripts in oocytes and embryos of buffalo. Oocytes collected during cold period (CP) and hot period (HP) were matured, fertilized and cultured in vitro to blastocyst hatching stage. The mRNA expression patterns of genes implicated in developmental competence (*OCT-4*, *IGF-2R* and *GDF-9*), heat shock (*HSP-70.1*), oxidative stress (*MnSOD*), metabolism (*GLUT-1*), pro-apoptosis (*BAX*) and anti-apoptosis (*BCL-2*) were evaluated in immature and matured oocytes as well as in pre-implantation stage embryos. Oocytes reaching MII stage, cleavage rates, blastocyst yield and hatching rates increased ($P < 0.05$) during the CP. In MII oocytes and 2-cell embryos, the RA of *OCT-4*, *IGF-2R*, *GDF-9*, *MnSOD* and *GLUT-1* decreased ($P < 0.05$) during the HP. In 4-cell embryos, the RA of *OCT-4*, *IGF-2R* and *BCL-2* decreased

($P < 0.05$) in the HP, whereas *GDF-9* increased ($P < 0.05$). In 8-to 16-cell embryos, the RA of *OCT-4* and *BCL-2* decreased ($P < 0.05$) in the HP, whereas *HSP-70.1* and *BAX* expression increased ($P < 0.05$). In morula and blastocyst, the RA of *OCT-4*, *IGF-2R* and *MnSOD* decreased ($P < 0.05$) during the HP, whereas *HSP-70.1* was increased ($P < 0.05$). In conclusion, deleterious seasonal effects induced at the GV-stage carry-over to subsequent embryonic developmental stages and compromise oocyte developmental competence and quality of developed blastocysts.

Keywords Seasonality · Oocyte quality · Gene expression · In vitro embryo production · Embryo development

Introduction

The goal of assisted reproductive technologies (ARTs) is maximizing the birth of healthy offspring. Among them, in vitro embryo production (IVP) offers unprecedented opportunities to understand the basic mechanisms involved in early development and to improve mammalian reproductive efficiency (Benjamin 2001). Despite rapid progress in the field of ARTs for humans and domestic animals, challenges remain. An example is highly fluctuating success rates of in vitro fertilization (IVF) and embryo transfer in the absence of explanation (Wunder et al. 2005). The

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influence of the seasons of the year on the characteristics of the oocytes, fertilization and pregnancy rates may be importantly related to this variability, as reported previously for bovine (Rutledge et al. 1999; Al-Katanani et al. 2002; Sartori and Mertens 2002; Vasconcelos et al. 2006; Paula-Lopes et al. 2008; Roth 2008; de Torres-Júnior et al. 2008; Ferreira et al. 2011; Gendelman and Roth 2012a, b, c; Vieira et al. 2014) and buffalo (Abdoon et al. 2014; Hozyen et al. 2014; Rossi et al. 2014; Ali 2015; Lal et al. 2015). Indeed, it is well-known that heat stress is a major concern for animal agriculture production in tropical and subtropical countries across the globe because of its negative impact on reproduction (Ealy et al. 1995). Bovine has become an increasingly popular animal model to study improvement in human IVF methodology due to similar biochemical and intrinsic paternal and maternal regulatory processes between bovine and human (Wrenzycki et al. 2001; Menezo and Herubel 2002). A question arises is whether different seasons have impact at molecular levels on oocyte and embryo in humans and domestic animals; still remains poorly understood. However, knowledge on the molecular mechanism of season induced altered oocyte competence and embryo viability, will improve success of IVF programs.

In buffalo (*Bubalus bubalis*) in vitro embryo production technology is considered most effective for improving genetic progress through the maternal lineage by increasing the number of embryos available per donor on a long-term basis (Francesco et al. 2011). Major restrictions of this technology in buffalo species are an innate low number of follicles, consequently a low number of cumulus–oocyte complexes that can be recovered per ovary (Gasparrini 2002) and seasonality (Gasparrini 2007). Studies in buffalo from different parts of the world have examined seasonal fluctuations revealing conflicting results (Nandi et al. 2001; Khairy et al. 2007). Oocytes recovered during summer exhibited lower quality (Rutledge et al. 1999; Al-Katanani et al. 2002), reduced developmental potential (Rutledge et al. 1999; Al-Katanani et al. 2002; Paula-Lopes et al. 2008) and pre-implantation embryonic development (Paula-Lopes and Hansen 2002). Differences are reported in transcript abundance among embryos produced by IVF of oocytes collected in summer versus winter in bovine (Gendelman and Roth 2012a). Seasonal effects on blastocyst formation generally result in decreased competence of cleaved

embryo development, rather than competence of the oocyte to cleave (Hansen 2015). Heat stress impairs augmentation of molecules essential for early embryonic development in the oocyte. It is possible that in summer oocytes are more reasonable to undergo aberrant chromosomal segregation so resultant embryos are chromosomally abnormal. Chromosomal abnormalities can influence the rate of embryonic development (Kawarsky et al. 1996).

Little is understood of the mechanisms underlying poor early embryonic development and reports investigating differences in gene expression correlated with aberrant early embryonic development or growth retardation of embryos during summer are scarce. The basic mechanisms behind the disturbance of oocyte maturational processes by summer are likely to be complex and possibly involve; decreased intracellular protein synthesis (Edwards and Hansen 1997), decreased glutathione content (Payton et al. 2003), disrupted cytoskeleton (Ju et al. 2005), microfilament and microtubule architecture, incompetent oocyte meiotic spindle (Drew 1999), distorted cortical granule types (Andreu-Vázquez et al. 2010) and initiation of oocyte death through apoptosis (Roth et al. 2001). Subsequently, the percentage of oocyte that completes nuclear maturation is reduced. The oocyte contributes the embryo one-half of its chromosomes and nearly all organelles, mRNA and other macromolecules required for embryonic development (Hansen 2014). During the maternal-embryonic transition (MET), the developmental program shifts from maternal to embryonic, i.e., there is degradation of maternal transcripts and expression of genes required for differentiation and successful implantation (Memili et al. 1998; Schultz 2002; Donnison and Pfeffer 2004; Schier 2007). In bovine embryos, MET occurs during the passage from the 8- to 16-cell stage (Memili et al. 1998). Therefore, one might expect that embryos developed from oocytes collected during summer would overcome previous maternal mRNA alterations upon activation of embryonic transcription. If this were the case, it may not be possible for deleterious carryover effects of summer on gene expression in developing embryos after MET stage. However, low expression of maternal *OCT-4* mRNA recorded before the 8- to 16-cell stage is also expressed at the blastocyst stage during summer (Gendelman and Roth 2012c). Transcription level of *OCT-4* has been shown to be higher in competent oocytes and embryos

(Schultz 2002; Amarnath et al. 2007). Thus, embryos that develop during summer which express low *OCT-4* levels may be considered of inferior quality.

The molecular and cellular mechanisms of season induced changes in Indian water buffalo oocyte competence and its effect on embryo viability remains poorly understood. Studies of the effects of seasonal temperature fluctuations on embryo gene expression are of importance in understanding the mechanism behind season influenced variation in developmental potential of embryos (Rutledge et al. 1999; Gendelman and Roth 2012b; Sakatani et al. 2012; Sharma et al. 2012). Therefore, the present study investigated the effect of season (winter and summer) in Indian water buffalo ovarian follicle—population, oocyte recovery, quality, in vitro developmental competence and expression of candidate genes related to developmental competence, heat stress, oxidative stress and apoptosis in cumulus oocyte complexes (COCs), in vitro matured oocytes and embryos.

Materials and methods

Chemicals and reagents

Chemicals and media were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise indicated. Most chemicals used in the present study were of embryo or cell culture tested grade. Fetal bovine serum (FBS) was sourced from Hyclone (Thermo Scientific, Wilmington, DE, USA) and same batch was used throughout the study. Disposable four-well multi-dishes and, six-well tissue culture plates were procured from Nunc (Roskilde, Denmark). Membrane filters (0.2 µm) were sourced from Pall Life Sciences (Pall Corporation, Ann Arbor, MI, USA). Primers were synthesized by Sigma (P) Ltd. (Delhi, India). Reagents for molecular biology work were of biotechnology grade and purchased from Invitrogen (Carlsbad, CA, USA) and Fermentas (Waltham, MA, USA) unless otherwise indicated.

Collection of oocytes

Ovaries were collected from reproductive organs of adult, apparently healthy female buffaloes of unknown reproductive status from an abattoir in New Delhi,

India, in April to October, 2013, the hot period (HP) of the year (n = 1460) and in November to March, 2013–2014, the cool period (CP) of year (n = 1476). The maximum temperature in HP was between 40 and 44 °C and the minimum temperature between 4 and 6 °C in CP. Within 30 min of slaughter ovaries were washed three times with warm isotonic saline (37 °C) containing 400 IU/mL penicillin and 500 µg/mL streptomycin and transported to the laboratory within 4 h. The number of antral follicles of 2–10 mm in diameter was recorded for each ovary. After counting the number of antral follicles for CP and HP collected ovaries, COCs from follicles (2–8 mm) were aspirated with an 18 gauge needle attached to 10 mL syringe (Sigma Chemical Co., # Z248029) containing aspiration medium (TCM-199 containing 0.3 % BSA, 0.1 mg/mL glutamine and 50 µg/mL gentamicin). Aspirated follicular fluid was transferred to 15 mL sterile tubes and kept in a water bath at 37 °C for 15 min. The resulting sediment was transferred to a culture dish and COCs were selected under a stereomicroscope (Nikon Inc., Tokyo, Japan) at a magnification of × 20. After collection, COCs were washed four to six times in washing medium which consisted of TCM-199 with 10 % FBS, 0.09 mg/mL sodium pyruvate, 0.1 mg/mL L- glutamine and 50 µg/mL gentamicin. The COCs were then classified into four grades according to Khurana and Niemann (2000) under an inverted microscope (Nikon Inc., Tokyo, Japan) at a magnification of x50. Grade A, B and C COCs were selected for further processing and grade D samples were discarded.

In vitro maturation (IVM)

The COCs from CP and HP were subject to maturation in IVM medium consisting of TCM-199 + sodium pyruvate (0.80 mM) + L-glutamine (2 mM) + 10 % FBS + 5 % follicular fluid (FF) + PMSG (20 IU/mL) + hCG (10 IU/mL) + gentamicin (50 µg/mL). The pH of the medium was adjusted to 7.4 and filtered through a 0.22 µm membrane filter immediately before use. The COCs were washed several times with IVM medium and groups of 15–20 COCs were placed independently in 100 µL droplets of IVM medium covered with sterilized mineral oil in 35 mm petri dishes and cultured for 24 h under 5 % CO₂ at 38.5 °C (Sadeesh et al. 2014b).

Cumulus expansion and polar body formation rate

Following the 24 h culture period, the degree of cumulus expansion of COCs was determined for CP and HP samples. Cumulus expansion was scored on a 0 to –4 scale as described by Fagbohun and Downs (1990). A representative number of expanded COCs ($n = 60$) from CP and HP were subject to examine polar body extrusion rate at 24 h of IVM as described by Sadeesh et al. (2014a) with some modifications. Cumulus oocyte complexes were transferred to 1.5 mL microcentrifuge tubes containing 500 μ L hyaluronidase in T2 and incubated for 1 min at 38.5 °C, followed by vortexing (2 min). Completely denuded oocytes with granular cytoplasm were selected and incubated in pronase (2 mg/mL in T10) for 10 min at 38.5 °C. Oocytes with completely digested zona pellucida were transferred to T20 and incubated for 10 min at 38.5 °C. Zona-free oocytes were observed and evaluated morphologically at x20 magnification under an inverted microscope for identification of oocytes with extrusion of the first polar body.

Sperm capacitation, in vitro fertilization (IVF) and in vitro culture (IVC)

In all experiments, frozen-thawed semen from the same bull was used. Spermatozoa were capacitated using Brackett and Oliphant (BO) (1975) medium and procedures for IVF and IVC as described earlier (Sadeesh et al. 2014b). Embryo production rate from each group was examined under inverted microscope (Nikon Inc., Tokyo, Japan) to record the number of embryos showing 2- to 4- cell stage at 48 h post-insemination (h.p.i), percentage of embryos with 8- to 16- cell at 94–96 h.p.i, compact morula at 120–144 h.p.i and blastocyst formation at 168–192 h.p.i. Hatching of blastocysts was recorded at 216–240 h.p.i. Randomly collected blastocysts from CP and HP ($n = 12$ per season) were stained with Hoechst 33342 for 1 h and number nuclei was counted under a fluorescent microscope.

Ribonucleic acid isolation, complementary DNA synthesis

Germinal vesicle (GV)- stage oocytes were collected immediately after aspiration and metaphase II (MII)-stage oocytes were collected after 24 h of maturation.

Both GV- and MII-stage oocytes ($n = 25$ oocytes per stage, per period; CP and HP) were denuded as described previously (Sadeesh et al. 2014b). Embryos at different developmental stages were collected at 2-cell, 4-cell and 8- to 16-cell, morula and blastocyst ($n = 10$ each) from CP and HP were used for preparation of cDNA using cDNA kit (Ambion Inc, The RNA Company, Austin, TX, USA) according to the manufacturers' protocol. Note that due to transcript level variations between early- and late-cleaved embryos (Roth and Hansen 2005), only early-cleaved embryos were collected for each season. For each stage, four replicates were taken from different in vitro production runs for each season. Oocytes/-embryos were washed with 200 μ L ice cold PBS after which 50 μ L of chilled cell lysis buffer was added and the mixture was incubated at 75 °C for 10 min in a thermal cycler. Genomic DNA was degraded by incubating cell lysates in DNase-I at 37 °C for 30 min and the remaining activity of DNase-I was inactivated by heating at 75 °C for 5 min. For cDNA synthesis, 10 μ L of cell lysates (RNA), 4 μ L of dNTP mix (2.5 mM of each dNTP) and 2 μ L of a random decamer were placed in a 200 μ L PCR tube. The reaction mixture was incubated at 70 °C for 1 min to denature RNA for easier primer binding in a thermal cycler. The tubes were cooled immediately on ice and remaining reverse transcription reagents; 2 μ L reverse transcription 10x buffer, 1 μ L MMLV reverse transcriptase (RT) and 1 μ L RNase inhibitor were added. The reaction mixture was again mixed and incubated in a thermal cycler at 42 °C for 60 min and 95 °C for 10 min to inactivate the reverse transcriptase. The synthesized cDNA was stored at –80 °C until used for quantification.

Reverse-transcriptase PCR

The candidate reference genes (*YWHAX* and *GAPDH*) were chosen based on a previous study using buffalo's oocytes during summer and winter (Macabelli et al. 2014). The target genes were selected taking into account their roles in developmental competence (*OCT-4*, *IGF-2R* and *GDF-9*), heat stress (*HSP-70.1*), oxidative stress (*MnSOD*), glucose metabolism (*GLUT-1*) and apoptosis (*BAX* and *BCL-2*). A polymerase chain reaction (PCR) was conducted in a 50 μ L final volume containing 45 μ L platinum PCR supermix (Invitrogen) and 5 μ L of primer (200 nM

each) and template DNA solution. The primer sequences are shown in Table 1. The PCR conditions were the same for all genes, except the annealing temperature, as 94 °C for 2 min (initial denaturation), denaturation at 94 °C for 30 s, elongation at 72 °C for 1 min (35 cycles). The amplified DNA fragments were resolved on 2 % agarose gel containing 0.5 µg/mL ethidium bromide against a 50-bp ladder and visualized under gel documentation system (Alpha Imager, Alpha Innotech, San Leandro, CA, USA). The RT-PCR analysis of gene amplifications in oocytes and pre-implantation embryos is shown in supplementary data.

Real-time PCR for relative quantification

Real-time PCR (Applied Biosystems 7500 Real-Time PCR system) was performed using SYBR Green qPCR supermix (Invitrogen SYBR Green qPCR supermix; Carlsbad, CA, USA) as a double-standard DNA specific fluorescent dye to assess gene expression of *OCT-4*, *IGF-2R*, *GDF-9*, *HSP-70.1*, *MnSOD*, *GLUT-1*, *BAX* and *BCL-2*. The primer sequences are

shown in Table 1. Validation studies were performed using *YWHAZ* as a reference gene and *YWHAZ* expression was consistent across various stages between embryos derived from different seasons, except for the 2-cell stage. For the latter, *GAPDH* was used as the reference gene as *YWHAZ* expression was diverse between seasons. All reactions were run in triplicates and three biological replicates were carried out. Amplification was carried out in 25 µL volume reaction mixture containing 12.5 µL SYBR Green qPCR supermix, 1 µL primer (10 pM each forward and reverse primer), 2 µL of cDNA template and 9.5 µL nuclease free water. Samples not exposed to RT were used as negative controls. For PCR, samples were activated at 95 °C for 10 min, followed by 40 cycles of denaturing at 95 °C for 45 s, then annealing at the specific primer annealing temperature for 30 s and extension at 72 °C for 30 s. The comparative CT method was used for relative quantification of target gene expression levels. Quantification was normalized to the internal control *YWHAZ* gene. Within the exponential phase of the amplification curve, each cycle doubled the amplified product. The ΔCT value

Table 1 Details of primer sequences

Gene name	Sequence (5'–3')	Fragment size (bp)	GenBank accession no.
<i>GAPDH</i>	Forward CCTGCCAAGTATGATGAGA	131	GU324291
	Reverse GAAGGTAGAAGAGTGAGTGT		
<i>YWHAZ</i>	Forward GCAGCTGGTGATGACAAGAA	124	NM_174814
	Reverse AGTTAAGGGCCAGACCCAGT		
<i>OCT-4</i>	Forward GACAAGGAGAAGCTGGAG	75	JF898834.1
	Reverse GCAAATGTTC AAGGTCTTTC		
<i>IGF-2R</i>	Forward GCAGATTTATTTCTTCTCCAC	186	J03527
	Reverse CACTCAAAC TCGTAGAAGCA		
<i>GDF-9</i>	Forward CCCTAAATCCAACAGAAGCC	148	NM_174681.2
	Reverse GTTCACAACAGTAACACGA		
<i>HSP 70.1</i>	Forward TCATCAACGACGGAGACAAGCCTA	104	GU-183097.1
	Reverse TTCATCTTGGTCAGCACCATCGAC		
<i>MNSOD</i>	Forward GGGCCATATCAATCACAG	193	JN687584
	Reverse GTCCCTGCTCCTTATTG		
<i>GLUT-1</i>	Forward CCGTCTCTTCCTATCCAA	194	HQ434959
	Reverse GGTCTTCTTGAATAGTGAGTT		
<i>BAX</i>	Forward ATCCACCAAGAAGCTGAG	82	HE661581
	Reverse CTGCGATCATCTCTGTGA		
<i>BCL-2</i>	Forward GGCCCCGTGTTGATTTCT	97	U92434
	Reverse CTTATGGCCCAGATAGGC		

was determined by subtracting the *YWHAZ* CT value for each sample from the target gene CT value. Calculation of $\Delta\Delta\text{CT}$ value involved using the highest sample method ΔCT as an arbitrary constant to subtract from all other ΔCT sample values. Fold changes in relative mRNA expression of target genes were determined using the formula $2^{-\Delta\Delta\text{CT}}$.

Statistical analysis

Data were analysed using GraphPad Prism (version 6.05). Experimental results were presented as mean $\pm$ SEM (standard error mean). Data were subject to analysis of variance (ANOVA) and the Tukey's test was used to compare means ($P < 0.05$) considered statistically significant.

Results

Seasonal effects on number of follicles, COC yield and morphology

The average number of ovarian follicles and COCs recovered per ovary were reduced ($P < 0.05$) in HP (2.38 ± 0.42 and 1.54 ± 1.14 follicles and COCs recovered, respectively) than CP (4.17 ± 1.38 and 2.64 ± 0.15 follicles and COCs recovered, respectively). In addition, oocytes collected during HP had heterogeneous dark ooplasm surrounded by few and sparse cumulus cells, while in the CP the ooplasm was homogenous and surrounded by several layers of compact cumulus cells, as showed in Fig. 1. A significantly reduced number of grade A, B, C and D COCs were recovered during HP than CP (Fig. 2).

Seasonal effects on maturation rate and developmental competence of COCs

Oocytes matured during CP showed healthy expanded cumulus cells and evenly granulated cytoplasm (Fig. 3a, b). In contrast, oocytes matured during HP showed degenerated cumulus cells and a higher rate of cytoplasmic degeneration, as indicated by the presence of large lipid vacuoles (Fig. 3a', b'). For COC maturation by cumulus expansion, no statistical ($P > 0.05$) differences were obtained between CP and HP ($82.64 \% \pm 6.62$ and $77.43 \% \pm 4.78$, respectively) (Fig. 4). A greater ($P < 0.05$) percentage of in vitro matured COCs collected during CP reached nuclear mature

MII stage than for HP (87.62 ± 3.61 and 57.94 ± 2.83 , respectively). In general, developmental competence of COCs collected during HP differed from that of COCs collected in CP. Cleavage rate was increased ($P < 0.05$) in COCs matured in CP compared with those harvested during HP (65.38 ± 12.24 vs 37.16 ± 6.73 , respectively). The same pattern was observed for embryonic development, where the percentage of cleaved embryos at the stages of 4- (53.53 ± 9.21 vs. 28.81 ± 5.25), 8- to 16- (35.17 ± 7.18 vs. 22.36 ± 4.16) cells, morula (26.51 ± 5.74 vs. 13.73 ± 3.82) and blastocyst (21.36 ± 4.81 vs. 8.85 ± 2.52) was greater ($P < 0.05$) during the CP compared to the HP (Fig. 5). Moreover, greater ($P < 0.01$) number of cells in the early-blastocysts (186.92 ± 4.24 vs. 162.48 ± 3.56) and greater ($P < 0.05$) percentage of hatched blastocysts (16.26 ± 2.23 vs. 5.21 ± 2.28) were registered in the CP compared to the HP.

Seasonal effect on gene expression in oocytes and pre-implantation embryos

The relative abundance (RA) of the gene transcripts in oocytes and pre-implantation stages of buffalo embryos during CP and HP is shown in Fig. 6. Transcription level of *GAPDH* was found to be equal between seasons in GV stage oocytes, morula and blastocysts, but not in MII-stage oocytes, 2-cell, 4-cell or 8- to 16- cell- stage embryos. Transcription level of *YWHAZ* was constant between seasons at all examined stages except the 2- cell stage (data not shown). Accordingly, *YWHAZ* served as the main reference gene for final analysis. It was found that mRNA expression of developmental competence related genes *OCT-4*, *IGF-2R* and *GDF-9* in GV stage oocytes did not show any difference ($P > 0.05$) between CP and HP. However, following maturation (i.e., putative MII stage oocytes) mRNA levels of *OCT-4*, *IGF-2R* and *GDF-9* were increased ($P < 0.05$) in oocytes collected in CP as compared to HP. In 2-cell- stage embryos, mRNA levels for *OCT-4*, *IGF-2R* and *GDF-9* were decreased ($P < 0.05$) in HP vs. CP. In 4-cell- stage embryos, expression of *GDF-9* was increased ($P < 0.05$) and *OCT-4* and *IGF-2R* were decreased ($P < 0.05$) in HP vs. CP. At 8- to 16- cells, morula and blastocyst stages, expression of *OCT-4* were decreased ($P < 0.05$) in HP vs. CP. No change ($P > 0.05$) in expression level of *IGF-2R* was found between HP and CP at the 8- to 16-cell stage. However, at morula and blastocyst stages, expression of *IGF-2R* was reduced

Fig. 1 Buffalo oocytes collected during CP and HP (200 μ m). **(a)** oocytes collected during CP showed homogenous cytoplasm and healthy cumulus cells; **(b)** oocytes collected during HP showed dark cytoplasm and degenerated cumulus cells (indicated by arrows)

Fig. 2 Seasonal effects on COCs grade. The aspirated COCs were graded based on morphological appearance of the cumulus cell investments and homogeneity of ooplasm. Proportion of COCs at grade A (oocytes with homogenous evenly granulated and many tight layers of cumulus cells), B (oocytes with homogenous ooplasm and two to three cumulus cell layers), C (oocytes with heterogeneous ooplasm surrounded by one cumulus cell layer) and D (denuded oocytes) are presented as mean $\pm$ SEM; $n = 1880$ – 1884 COCs from both season over eight independent experiments. * $P < 0.05$, calculated by one-way ANOVA with Tukey's test

($P < 0.05$) in HP vs. CP. Transcript abundance of *HSP-70.1* mRNA was increased ($P < 0.05$) in the pools of GV, MII, 8- to 16-cell, morula and blastocyst stages collected during HP than for those collected during CP. In HP the RA of *MnSOD* gene steadily increased from the 2-cell stage onward except its for down-regulation at morula and blastocyst stages.

MnSOD expression were reduced ($P < 0.05$) at MII, 2-cell, morula and blastocyst developmental stages during the HP vs. CP. No difference ($P > 0.05$) in RA of *MnSOD* was observed between HP and CP embryos at 4- and 8- to 16-cell stages. The RA of *GLUT-1* transcript was increased ($P < 0.05$) in embryos collected during CP vs. HP at 2-cell and blastocyst developmental stages. However, at GV, 4-, 8- to 16-cell and morula stages there was no difference ($P > 0.05$) between HP vs. CP. We observed increased ($P < 0.05$) mRNA abundance of *BAX* for 8- to 16-cell stage embryos from HP vs. CP. However, in immature or mature oocytes and embryos at the 2-, 4-, morula and blastocyst stages there was no difference ($P > 0.05$) between HP vs. CP. The RA of anti-apoptotic gene *BCL-2* was increased ($P < 0.05$) in CP vs. HP at the 4- and 8- to 16-cell stages, but not at GV, MII, 2-cell, morula and blastocyst stages ($P > 0.05$).

Discussion

Buffaloes are more predisposed to heat stress than cattle (Manjari et al. 2015). The cellular function in various tissues of the female reproductive tract, including the follicle, oocyte and embryo can be affected by hyperthermia (Hansen 2009). To the best of our knowledge, for the first time we report the developmental changes and molecular alterations in bubaline oocytes and embryos with respect to seasonality.

Fig. 3 In vitro matured buffalo oocytes during CP and HP (200 μ m). **a, b** Oocytes matured during CP showed healthy expanded cumulus cells and homogenous cytoplasm; **a', b'**

oocytes matured during HP showed degenerated cumulus cells and degenerated cytoplasm (arrows indicated lipid vacuoles)

The present study found that exposure of Indian water buffalo to HP resulted in significant decrease in antral follicle yield as compared to CP. Our findings are in agreement with the results of Wilson et al. (1998) who found a decrease in number of small ovarian follicles in bovine during summer. The number and quality of oocytes recovered per ovary were also affected by season in our studies. Heat stress altered endocrine patterns and reduced follicular development may be the reason behind the poor yield and low quality oocytes in HP. Our findings concur with earlier report by Francesco et al. (2011) in bovine. In addition, heat stress during HP induced a decrease in oocyte function due to a sequence of cellular changes disturbing nuclear and cytoplasmic compartments of the bovine oocyte which in turn destroys intracellular events associated with both nuclear and cytoplasmic maturation (Payton et al. 2003; Maya-Soriano et al. 2013). In the present work, it was also found that HP has deleterious effects on the nucleus

and cytoplasm of mature oocytes leading to impaired vitro maturation rates of buffalo oocytes. In earlier studies, summer season had a harmful effect on maturation rate of buffalo (Khairy et al. 2007) and bovine oocytes (Al-Katanani et al. 2002; Ferreira et al. 2011). The present study also revealed that IVM and IVF of buffalo oocytes during HP decreased ($P < 0.05$) cleavage rate and embryo development. Moreover, studies in bovine (Rivera and Hansen 2001; Zeron et al. 2001; Jousan and Hansen 2007) have shown similar results. The present findings, therefore, provide clear evidence of a carry-over effect of summer stressed oocytes, or maternal hyperthermia, up to blastocyst stage with a decrease in number at each stage of development. These findings provide fascinating evidence for a major effect of heat stress exposure during GV-stage on subsequent blastocyst development that may have a negative impact on Indian water buffalo reproduction. On the contrary, a series of in vivo (Rutledge et al. 1999) and in vitro

Fig. 4 Seasonal effects on cumulus cell expansion and nuclear maturation of in vitro matured buffalo oocytes. **(a)** cumulus expansion; **(b)** degree of cumulus expansion; **(c)** metaphase II stage of nuclear maturation. Values are presented as mean $\pm$ SEM; $n = 60$ in vitro matured oocytes per season over eight independent experiments. * $P < 0.05$, calculated by one-way ANOVA with Tukey's test

Fig. 5 Effect of season on cleavage rate and the development of in vitro produced buffalo embryos. Values presented as mean $\pm$ SEM; $n = 305$ – 308 embryos per season over eight independent experiments. * $P < 0.05$, calculated by one-way ANOVA with Tukey's test

studies (Barros et al. 2006) reported that elevated temperature did not alter oocyte cleavage rate, but blastocyst formation rate was lower than under normal temperature (Edwards et al. 2009). These inconsistencies among different studies may be due to genetic differences between species in their susceptibility to elevated temperature or severity of temperature or differences in length of exposure.

The present study also demonstrates that the detrimental effect of heat stress on embryonic development is related with seasonal variation in expression of genes involved in oocyte maturation and early embryonic development. Herein, we provide first evidence in Indian water buffalo that exposure of GV-stage oocytes to thermal stress is further expressed by alterations in maternal mRNA transcription before and after the MET stage.

The housekeeping gene *GAPDH* was differentially expressed in in vitro matured oocytes and embryos at different seasons, with a significant down regulation

Fig. 6 Relative expression profile of *GAPDH*, *OCT-4*, *IGF-2R*, *GDF-9*, *HSP-70.1*, *MnSOD*, *GLUT-1*, *BAX* and *BCL-2* in oocytes and pre-implantation stages of buffalo embryos during CP and HP. (a) *GAPDH*; (b) *OCT-4*; (c) *IGF-2R*; (d) *GDF-9*; (e) *HSP-70.1*; (f) *MnSOD*; (g) *GLUT-1*; (h) *BAX*; (i) *BCL-2*.

Values are presented as mean $\pm$ SEM; $n = 25$ oocytes/10 embryos from each stage per season over three independent experiments. * $P < 0.05$, ** $P < 0.01$, calculated by one-way ANOVA with Tukey's test

during HP. Transcription levels of *GAPDH* vary among cell types (Barber et al. 2005), developmental stages (Robert et al. 2002) and experimental conditions (Ito et al. 1996; Zhong and Simons 1999). These findings underline the priority of reference gene validation for each specific developmental stage and for each season. This report also raises concern as *GAPDH* has been generally used as an internal control gene in studies involving the effect of seasons on

oocyte and embryo gene expression in cattle and buffaloes (Sakatani et al. 2013; Yadav et al. 2013).

The most important seasonal variation in the present study was reduced expression of *OCT-4* mRNA for all embryonic stages in HP. *OCT-4* plays a role as an anti-apoptotic factor (Guo et al. 2007). Hence, a decrease in *OCT-4* expression may destroy the balance between pro- and anti-apoptotic factors which in turn could lead to elevated apoptotic cells

consequent to heat stress, as already reported for bovine oocytes and pre-implantation embryos (Soto and Smith 2009). Higher *OCT-4* expression has been reported for early- vs. late-cleaved 2- cell- stage embryos and has been associated with the proportion of embryos that further develop to blastocyst stage (Gendelman et al. 2010). As early-cleaved embryos were compared in the current study, reduced expression of *OCT-4* mRNA is involved in reduced oocyte developmental competence during HP.

The other differentially expressed gene was *IGF-2R*. Messenger RNA for *IGF-2R* was already reported in bovine and buffalo embryos (Yaseen et al. 2001; Pandey et al. 2009; Sadeesh et al. 2015, 2016). Cultured oocytes from in vitro showed fourfold fewer transcripts of *IGF-2R* in comparison with in vivo matured oocytes (Katz-Jaffe et al. 2009). The present results support the fact that lower *IGF-2R* mRNA expression in mature oocytes and embryos from HP results in the percentage of cleaved embryos arrested at the 2- and 4-cell stages and ability to develop to the morula and blastocyst stage was lower in HP than in CP.

It has been reported that *GDF-9* is critical for proliferation of granulosa cells throughout preantral and antral follicular development (Pennetier et al. 2004; McNatty et al. 2007). *GDF-9* transcript is greatly expressed in immature oocytes, but declines during early embryonic development with lower levels at the 8- cell- stage (McNatty et al. 2007). The reduced developmental competence reported for oocytes collected from HP in our studies suggested that seasonally induced changes in *GDF-9* expression are involved. Moreover, *GDF-9* expression in 4-cell-stage embryos developed in HP remained relatively high, suggesting a delayed decrease in *GDF-9* levels which is generally expected to occur at this embryonic developmental stage (McNatty et al. 2007). Such impairment indicates potential disruption of embryo developmental competence and might explain, in part, the reduced proportion of blastocysts developed during HP.

Transcription of heat shock proteins, *HSP-70.1* may be elevated by stressful conditions such as heat shock (Kiang and Tsokos 1998); its expression has been used as a signal of stress in bovine embryos (Balasubramanian et al. 2007). We found that relative mRNA abundance of stress-related *HSP-70.1* significantly increased in COCs, in vitro matured oocytes, 8- to 16- cell, morula and blastocyst stages in HP as

compared to CP. A study reported that exposure of COCs to elevated temperature increased *HSP-70.1* mRNA in cumulus cells and some adverse effects of heat stress on oocytes were intervened through cumulus cells (Payton et al. 2011). Besides, exposure of buffalo oocytes to elevated temperature (41.5 °C) for 1 h greatly disrupted oocyte maturation and resulted in upregulation of *HSP-70.1* mRNA expression at 8- to 16- cell and blastocyst (Pennarossa et al. 2012). It suggests that heat stress can promote *HSP-70.1* mRNA synthesis in buffalo and this could be used as marker of biological effect of heat stress in buffalo.

Subcellular allocation of mitochondria was distorted in oocytes in summer compared with oocytes collected in autumn and winter, and there was diminished expression of mitochondrial genes in summer (Gendelman and Roth 2012c). As *MnSOD* is a mitochondrial enzyme (Christianson 1997; Farin et al. 2001; Huo et al. 2005), the decrease in expression of *MnSOD* observed in buffalo oocytes and embryos collected in HP compared to CP may be related to altered mitochondrial activity after subjecting oocytes to heat stress, thereby negatively impacting developmental competence. However, the exact mechanism of down-regulation remains unclear and warrants further validation.

A study demonstrated the significance of glucose transporters (*GLUTs*) as regulatory molecules being crucial for early embryo development where down-regulation results in reduced developmental competence of embryos (Pantaleon et al. 2001). Relative mRNA abundance of *GLUT-1* at MII, 2- cell and blastocyst stages were low in HP vs. CP in our studies. Down- regulation of glucose transporters indicates that glucose uptake is severely compromised in response to increased ambient temperature. This indicates the importance of glucose transporters as regulatory factors in early embryo development and the heat-induced reduction in oocyte competence may be attributed to deficient expression of facilitative glucose transporters during subsequent development.

The *BAX* gene is the first pro-apoptotic member and when over-expressed the cellular apoptotic pathway is activated (Yang and Rajamahendran 2002). The temperatures inducing apoptosis (40 and 41 °C), revealed that apoptosis could be induced in pre-implantation embryos exposed to maternal hyperthermia. Jin et al. (2007) reported that relative mRNA abundance of *BAX* is increased in heat-shocked

porcine embryos. Our results revealed that exposure of oocytes to summer heat stress increased relative mRNA abundance of pro-apoptotic genes *BAX* at 8- to 16- cell stage. Absence of summer stress induced apoptosis in embryos at 2- and 4-cell stage may be correlated with an inadequacy of activation of group II caspases (Paula-Lopes and Hansen 2002). The anti-apoptotic (*BCL-2*) gene expression was elevated in the good quality oocytes and embryos (Elamaram et al. 2012). Surprisingly, the relative mRNA abundance of anti-apoptotic gene *BCL-2* was also decreased in HP at 4- cell and 8- to 16 –cell stages, whether this is a thermotolerance response of embryos exposed to summer stress needs to be examined.

Our findings indicate a deleterious carry-over effect of the hot season on oocyte developmental competence. Furthermore, the association between heat stress and hormones have an effect on reproductive process. It was shown that hormones FSH, Leptin and IGF-1 were able to prevent effect of heat stress on ovarian functions in porcine (Sirotkin 2010). This ability of hormones may be practically used in buffalo as an alternative option for elimination of negative effect of heat stress on ovarian functions as earlier reported in porcine.

In conclusion, exposing the ovarian pool of oocytes to environmental stress appears to reduce oocyte developmental competence mediated through maternal mRNA storage and/or the mechanism of transcription restoration, which in turn alters gene expression in the developing embryo. Further studies to examine seasonal differences in RNA stability, translation and degradation, as well as protein levels, are required to clarify the mechanism underlying the carry-over effect of summer heat stress. Confirmation of our findings will indicate that seasonal change should be taken into account in systematic breeding programs in buffalo.

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Authors' contributions EMS conceived of the study, carried out the experiments and drafted the manuscript. PS, AKB and

SB assisted with the sample analysis. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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