SUMMARY AND CONCLUSIONS

The present study was aimed at evaluating the effect of physiologically relevant elevated temperature of 40.5° and 41.5 °C for both 12 h and 24 h on bubaline COCs during meiotic maturation in vitro. For evaluating the developmental competence, gene expression and apoptotic cell ratio, the control group COCs were matured at 38.5 °C for 24 h. The treatment 1 (T1) and 3 (T3) group oocytes were matured at 40.5° and 41.5 °C respectively, for the first 12 h and at 38.5 °C for next 12 h and treatment 2 (T2) and 4 (T4) group oocytes were matured at 40.5° and 41.5 °C for complete 24 h. The developmental competence of bubaline COCs was evaluated through in vitro maturation (IVM), in vitro fertilization (IVF) and in vitro culture (IVC). The cumulus oocyte complexes (COCs) after isolation from follicles measuring 2-8 mm in diameter were matured at 38.5 °C for complete 24 h of in vitro maturation for control group and 40.5° and 41.5 °C for either only first 12 h or complete 24 h of maturation depending upon the treatment respectively. For evaluating the developmental competence, COCs were fertilized after 24 hours of in vitro maturation with frozen buffalo semen and co-incubated for about 16-18 h. Subsequently, the putative zygotes were cultured for 9 days and the number of embryos reaching the 2-cell, 4-cell, 8-16 cell, morulae and blastocyst stage was recorded on days 2, 3, 4, 5 and 8 post insemination.

The hypothesis underlying the research described herein was that exposure to a physiologically-relevant elevated temperature may alter RNA to account for some of the observed reductions in embryo development after fertilization. To test this hypothesis, separate experiment was performed to determine the effect of heat stress on maternal pools of RNA within maturing bubaline oocytes and their surrounding cumulus cells. After maturing the COCs at 38.5°, 40.5° and 41.5 °C for respective time periods of 12 h and 24 h respectively as carried out for the first objective, total RNA was isolated and reverse transcribed using reverse transcriptase polymerase chain reaction (RT PCR). The prepared cDNA was validated by the expression of constitutive mRNA transcript and further used for amplification of desired candidate genes through semi-quantitative PCR. Further, Real Time PCR analysis was done to look for differences in relative expression levels of heat shock proteins genes viz. Hsp70.1, 70.2, 70.8, 60, 10 and Hsf1, metabolism related genes viz. Glut1, Glut3 and IGF1R, mitochondrial and oxidative stress related genes viz. MnSOD, iNOS and DnaJ, apoptosis related genes viz. caspase-3, -7, -8, Bid, Bax, Bcl-2, Bcl-xl and
Mcl-1, developmentally important genes viz. ZAR1, BMP15 and GDF9 and endolysosomal proteases viz. cathepsin - B, K, S, Z in control group (38.5 °C for entire 24 h) and treatment groups (40.5° and 41.5 °C for first 12h and complete 24 h of maturation).

A detailed investigation into apoptosis in COCs matured in control group and treatment groups and blastocysts produced from in vitro fertilized embryos was carried out by evaluating the percentage of apoptotic to normal nuclei through TUNEL assay. Further, in order to investigate the possible mechanisms through which heat stress exerts its impact on developmental competence of bubaline oocytes, total ROS pool and antioxidant enzyme activity in COCs, oocytes and cumulus cell mass was separately determined by carrying out enzymatic assays. In order to fulfill this objective, the COCs matured at 38.5 °C was designated as control group. The heat stress was applied separately to two groups of COCs at 40.5° and 41.5 °C for 0-12 h of maturation and subsequently matured at 38.5 °C for remaining12 h (i.e. up to 24 h). After maturation, the COCs of the respective groups were denuded and the resulting denuded oocytes and cumulus cell mass was used for enzymatic assays. Following results were obtained:

- The cleavage rate observed was significantly (p<0.001) higher in embryos matured from control group oocytes (41.5 ± 0.91%) when compared with T1 (30.2±0.98%), T2 (26.5±0.29%), T3 (23.4±0.54%) and T4 (20.2±0.38%) respectively.
- The percentage of oocytes that developed to 4-cell and 8-16 cell stage was significantly (p<0.001) higher in control group (34.9±1.0 and 25.1±1.72%) compared to T1 (26.6±0.47 and 18.1±0.23%), T2 (22.2±0.19 and 15.5±0.16), T3 (18.6±0.46 and 10.3±0.29%) and T4 (15.1±0.39 and 8.9±0.15%) respectively.
- The percentage of oocytes that developed to morulae stage was significantly lower (p<0.001) for the oocytes of T1 (11.4±0.31%), T2 (9.7±0.29%), T3 (7.8±0.19%) and T4 (5.6±0.19%) respectively compared to the control group (18.1± 0.99%).
- The blastocyst rate declined significantly (p<0.001) in the treatment groups with the lowest yield in T4 group (1.6±0.16%) followed by T3 (2.5±0.14%), T2 (3.5±0.10%) and T1 (5.7±0.16%) groups respectively in comparison to control group (13.2±0.33%). In general, developmental competence of bubaline COCs decreased with increase in maturation temperature.
- Quality assessment of embryos done using bis-benzimide staining (Hoechst 33342) revealed that total cell number was significantly higher in control group embryos
(237.8±20.9) when compared with T1 (165.4 ± 7.71), T2 (134 ± 6.36), T3 (108.8 ± 2.8) and T4 (77 ± 2.81) respectively.

- Real Time PCR analysis revealed significantly higher expression of Hsp genes in oocytes matured at 40.5° and 41.5 °C for both the time durations (12 h and 24 h), as compared to control group. The mRNA expression of Hsp70.1 increased (p< 0.01) in T1 (6.81 folds), T2 (3.34 folds), T3 (3.44 folds) and T4 (7.75 folds) as compared to control.
- mRNA expression of Hsp70.2 also increased significantly (p<0.01) in T1 (3.10 folds), T2 (3.02 folds), T3 (3.50 folds) and T4 (7.97 folds) as compared to control.
- The relative mRNA abundance of Hsp70.8 increased (p<0.05) by almost similar proportion of 1.61 folds in the oocytes of T1, T2, T3 and T4 groups respectively in comparison to control.
- Similar pattern of expression was observed in Hsp60 and Hsp10 with a significant increase (p<0.05) in abundance at 41.5 °C for entire 24 h of maturation; T1 (2.63 and 2.92 folds), T2 (3.25 and 2.75 folds), T3 (3.47 and 3.45 folds) and T4 (5.08 and 4.95 folds) respectively.
- The relative abundance of Hsf-1 increased significantly (p<0.05) in all the treatment groups T1 (2.83 folds), T2 (2.76 folds), T3 (2.80 folds) and T4 (2.80 folds) as compared to control group.
- The mRNA expression of glucose transporter Glut1 decreased (p<0.05) in T1 (6.77 folds), T2 (4.33 folds), T3 (3.48 folds) and T4 (7.50 folds) as compared to control group.
- The results indicated the same pattern of decreased (p<0.01) Glut3 expression in treatment groups; T1 (8.90 folds), T2 (5.41 folds), T3 (6.24 folds) and T4 (9.66 folds) as compared to control. However, remarkable decrease (p<0.001) in mRNA expression of Glut1 and Glut3 was observed in T4 group oocytes as compared to control.
- A decreased mRNA expression of IGF1R (p<0.05) was observed in T1 (2.30 folds), T2 (2.61 folds), T3 (3.07 folds) and T4 (3.66 folds) as compared to control.
- No difference was observed (p<0.05) in mRNA expression of DnaJ gene in different treatment groups compared to control but a significant decrease (p<0.01) in mRNA expression of MnSOD gene was observed in T1 (5.75 folds), T2 (4.03 folds), T3 (4.34 folds) and T4 (4.92 folds) groups as compared to control.
**Summary and Conclusions**

- Results also demonstrated a considerable increase \( (p<0.01) \) in mRNA expression of **iNOS** gene in T1 (5.33 folds), T2 (7.83 folds), T3 (10.5 folds) and T4 (15 folds) groups as compared to control.

- Furthermore, for investigating the additional possible mechanisms that compromised oocyte developmental competence during heat stress, the relative mRNA abundance of pro-and anti-apoptotic genes was evaluated in the present study. The expression of proapoptotic genes (**caspases-3,-7 and -8, Bid and Bax**) was found to be significantly higher \( (p<0.05) \) for the oocytes matured at higher temperatures in comparison to control.

- Significant increase \( (p<0.05) \) was observed in mRNA expression of **Bid** and **Bax**: T1 (6.33 and 5.44 folds), T2 (6.83 and 6.33), T3 (10.1 and 7.83 folds) and T4 (14.4 and 12.6 folds) respectively as compared to control group oocytes.

- The relative abundance of executioner **caspases-3, -7 and -8** increased significantly \( (p<0.05) \) with increase in temperature (40.5° and 41.5 °C) and time interval of exposure (12 and 24 h) during *in vitro* maturation respectively. The relative abundance of **caspases-3, -7 and -8** was significantly higher \( (p<0.05) \) in T1 (6.04, 7.66 and 6.06 folds), T2 (6.61, 9.30 and 7.31 folds), T3 (8.66, 15.1 and 12.2 folds) and T4 (12.4, 21.2 and 14.4 folds) respectively as compared to the corresponding control group.

- Real time PCR expression analysis of anti apoptotic genes revealed the opposite trend as observed for pro apoptotic genes.

- A significant decrease \( (p< 0.05) \) was observed in mRNA expression of **Mcl-1** in oocytes matured at 40.5° and 41.5 °C for 12 and 24 h as compared to control. The mRNA expression of **Mcl-1** decreased in T1 (4.46 folds), T2 (7.66 folds), T3 (10.1 folds) and T4 (18.3 folds) as compared to control group.

- mRNA expression of **Bcl-2** and **Bcl-xl** genes decreased in T1 (4.30 and 5.80 folds), T2 (6.33 and 7.33 folds), T3 (7.83 and 7.83 folds) and T4 (12.6 and 13.6 folds) respectively as compared to control group.

- A significant decrease \( (p<0.01) \) was observed in mRNA expression of **BMP15** and **ZAR1** genes in oocytes matured at slightly elevated temperature in T1 (2.20 and 1.80 folds), T2 (2.20 and 2.10 folds), T3 (2.20 and 2.26 folds) and T4 (2.20 and 5.40 folds) respectively as compared to control with non-significant change in **GDF9** expression.
• The expression pattern of cathepsins revealed a significantly higher expression of *cathepsins*-B, K, S and Z in the oocytes matured at 40.5˚ and 41.5 °C for both 12 and 24h compared to control group.

• Significant increase (*p*<0.01) in mRNA abundance of *cathepsin B* and *K* was observed in T1 (5.03 and 2.81 folds), T2 (5.21 and 2.93 folds), T3 (6.23 and 3.04 folds) and T4 (8.17 and 3.12 folds) respectively as compared to control group.

• The mRNA abundance of *cathepsin S* and *Z* was significantly higher (*p*<0.01) in T1 (3.51 and 2.41 folds), T2 (4.06 and 2.43 folds), T3 (4.12 and 2.61 folds) and T4 (4.30 and 2.90 folds) respectively as compared to control group.

• TUNEL staining revealed that the percentage of TUNEL-positive cells was significantly higher (*p*<0.05) in the COCs in T1 (8.33%), T2 (10.0%), T3 (19.3%) and T4 (25.0%) respectively as compared to control group (4.0%). The apoptotic cell ratio in COCs increased to a considerable level with increase in the maturation temperature.

• TUNEL staining of blastocysts also revealed that blastocysts developed from COCs matured at higher maturation temperatures of 40.5˚ and 41.5 °C for both 12 and 24 h had significantly higher (*p*<0.05) TUNEL-positive blastomeres than those developed from control group COCs (38.5 °C for entire 24 h).

• The apoptotic cell ratio (ACR) in the blastocysts of T1, T2, T3 and T4 group was 5.26, 5.80, 8.36 and 12.0%, respectively compared to the control group (1.53%).

• Significantly higher production of ROS was observed in treatment groups as compared to control. Significant increase (*p*<0.01) in production of ROS by about 33.5, 35.5, 43.7 and 53.5% respectively in COCs and cumulus cell mass that were subjected to heat stress during meiotic maturation compared to the corresponding control group.

• The increase was remarkably (*p*<0.001) higher in denuded oocytes matured at 40.5˚ and 41.5 °C for 12 h compared to the control group by about 336.6 and 358.8 as compared to control group.

• The effect of heat stress on production of NO was remarkably higher (*p*<0.05) in DOs compared COCs and cumulus cell mass. Significant increase in the production of NO by about 23.8 and 29.2% was observed in heat stress treatment groups compared to the control group.

• NO production increased significantly (*p*<0.001) in denuded oocytes matured at 40.5˚ and 41.5 °C for 12 h up to 214.6 and 261.1% as compared to control group.
Summary and Conclusions

- Same trend was observed in cumulus cell mass in which the production of NO was significantly ($p<0.01$) higher by about 53.3 and 64% in treatment groups as compared to the control group.

- The effect of elevated temperature (40.5° and 41.5 °C) during maturation on the level of lipid peroxidation increased significantly ($p<0.001$) by about 18 and 21.2% in COCs as compared to control group.

- A remarkable ($p<0.001$) increase in lipid peroxidation level by about 242.1 and 289.5% was observed in denuded oocytes when matured at 40.5° and 41.5 °C for 12 h compared to control group.

- The present study also indicated that level of lipid peroxidation was significantly higher ($p<0.05$) in cumulus cell mass of the treatment groups by about 21.2 and 20% as compared to corresponding control group.

- The activity of SOD significantly increased ($p<0.01$) by 34.8 and 41.2% respectively in the COCs matured at 40.5° and 41.5 °C for first 12 h of maturation compared to control group.

- Significant increase ($p<0.01$) in SOD activity was observed in denuded oocytes (DOs) by about 156.6 and 166 % in the COCs matured at 40.5° and 41.5 °C for first 12 h of maturation as compared to control group.

- The activity of SOD increased significantly ($p < 0.01$) by 57% in cumulus cell mass cultured at 40.5° and 41.5 °C for first 12 h of maturation as compared to control group. Overall, the magnitude of increase in SOD activity in DOs was remarkably higher as compared to COCs and cumulus cell mass when subjected to heat stress during maturation.

- The activity of catalase was significantly ($p< 0.05$) higher in COCs matured at 40.5° and 41.5 °C by 13.2 and 22.0% compared to control group.

- Significantly higher ($p<0.01$) activity of catalase (129 and 168.2%) was observed in denuded oocytes matured at 40.5° and 41.5 °C for first 12 h of maturation compared to control group.

- Likewise, the activity of catalase was significantly ($p<0.05$) higher by about 23.6 and 36.5% in the above mentioned treatment groups compared to control group.

- The activity of GSH-Px was significantly higher ($p<0.05$) in COCs matured at 40.5° and 41.5 °C by about 12.7 and 16% compared to control group.
Summary and Conclusions

- Significant induction ($p<0.001$) of GSH-Px by about 151.1 and 165.1% was observed in denuded oocytes matured at 40.5° and 41.5 °C compared to control group.
- Following the same trend, significantly higher ($p<0.01$) enzymatic activity of GSH-Px by about 36.4 and 40.7% was observed in cumulus cell mass cultured at 40.5° and 41.5 °C as compared to control group.
- The effect of heat stress (40.5 and 41.5 °C for 12 h) on the GSR activity in COCs, indicated significantly higher ($p <0.05$) activity by about 10.4 and 20.1% as compared to control group.
- Significant induction ($p<0.001$) in the activity of GSR by 160.5 and 184.9% was observed in denuded oocytes when matured at 40.5° and 41.5 °C for 12 h as compared to control group.
- The activity of GSR was about 17.3 and 30.5% higher ($p<0.001$) in the cumulus cell mass cultured at 40.5° and 41.5 °C for 12 h as compared to control group.
- The concentration of GSH was assessed in the COCs matured at 38.5 °C (control group) and 40.5° and 41.5 °C (heat stress temperatures). Significant reduction ($p<0.05$) in the concentration of GSH by about 20.5 and 25.2% was observed in COCs matured at 40.5° and 41.5 °C for 12 h compared to the control group.
- The concentration of GSH significantly ($p<0.01$) decreased by 63.5 and 82.0 % in denuded oocytes matured at 40.5° and 41.5 °C for 12 h as compared to corresponding control group.
- The same pattern of significant ($p<0.05$) decrease in GSH levels was observed for cumulus cell mass which declined by 23.1 and 29.1% compared to the control group.

**Following conclusions were drawn from the present study:**

- Our findings suggest a deleterious carryover effect of heat stress on bubaline oocyte developmental competence as evidenced by significant reduction in cleavage rate and blastocyst yield. Embryos developed from heat stressed oocytes have lower ability to develop to blastocyst stage probably due to their less cell number.
- The present study suggests that exposing the oocytes to physiologically relevant elevated temperatures of 40.5° and 41.5 °C appears to impair maternal mRNA storage and the mechanism of transcription renewal, which in turn affects gene expression in developing embryo.
Summary and Conclusions

- Mechanisms through which the effects of heat stress are mediated in bubaline oocytes and embryos function at molecular and biochemical level in the cytoplasm and nucleus, and are based on alterations in the mRNA, pool of heat shock proteins, metabolism related genes, oxidative stress, developmental competence and most importantly apoptosis.

- Significant increase in expression levels of heat shock protein genes may in some way be responsible for inducing thermotolerance in oocytes and embryos.

- Significant increase in expression pattern of pro apoptotic genes (caspases-3, -7 and -8, Bid, Bax) with concomitant decrease in expression of anti apoptotic genes viz. Bcl-2, Bcl-xl and Mcl-1 suggests that apoptotic mechanisms play a pivotal role in compromising the developmental competence of bubaline oocyte.

- The present study suggests that apoptotic processes are mediated by group II caspases, as defined by both TUNEL assay and caspase gene expression analysis and that apoptosis is a critical mechanism responsible for the disruption of oocyte capacity for cleavage and subsequent development.

- Controlling the activity of group II caspases or intracellular events upstream from activation of execution caspases might be a useful strategy for blocking heat-shock-induced apoptosis in oocytes and thereby improving fertility of heat-stressed females.

- The present results also implicate cathepsins- B, -K, -S and -Z as one of the possible pathways that are responsible for heat stress-induced apoptosis in bubaline COCs and these endolysosomal proteases can be conveniently used as biomarkers for depicting the quality of oocytes in heat stress conditions.

- Oocyte damage caused by heat stress in vitro is likely to be relevant to the understanding of early embryonic losses in buffalo following heat stress conditions. Therefore, it is suggested that physiologically relevant elevated temperatures have a negative impact on buffalo reproduction.