Present study was conducted to evaluate the effect of heat stress on the buffalo COCs during \textit{in vitro} meiotic maturation. The main aim was to assess the developmental competence of COCs when subjected to physiologically relevant elevated temperatures of 40.5\degree and 41.5 \degree C for 12h and 24 of \textit{in vitro} meiotic maturation and to examine the effect of heat stress on the molecular and enzymatic characteristics. Developmental competence of COCs was investigated by \textit{in vitro} maturation, \textit{in vitro} fertilization and subsequent \textit{in vitro} culture for 9 days at control and respective treatment groups in a humidified CO\textsubscript{2} incubator. The effect of heat stress on the quantitative expression of candidate genes related to heat stress (\textit{Hsp70.1, Hsp70.2, Hsp70.8, Hsp10, Hsp60, Hsf-1}) mitochondrial and oxidative stress (\textit{DnaJ, MnSOD and iNOS}) and apoptosis (\textit{Bcl-2, Bax, Bcl-xl, Bid, Mcl-1, Caspase -3, -7, -8}), developmental competence genes (\textit{BMP-15, GDF-9, ZAR1}),metabolism (\textit{Glut1, Glut3 and IGF1R}) and endolysosomal proteases (\textit{Cathepsins B, S, K and Z}) were investigated by quantitative real-time PCR (qRT-PCR). To determine the heat stress induced apoptosis, terminal deoxynucleotidyl transferase (TdT)-mediated fluorescein-deoxyuridine triphosphate (fluorescein-dUTP) nick end labeling (TUNEL) assay were performed in COCs and blastocysts subjected to maturation at physiologically relevant elevated temperatures and normal temperature. Biochemical assays were performed in COCs, denuded oocytes (DOs) and cumulus cell mass in order to evaluate the effect of heat stress on enzymatic antioxidant status and to establish the relationship between heat stress and generation of reactive oxygen species (ROS) in COCs, DOs and cumulus cell mass. Furthermore, the level of important non-enzymatic antioxidant viz. glutathione was determined in COCs, denuded oocytes (DOs) and cumulus cell mass in order to investigate the effect of heat stress on the intracellular pool of glutathione. Details of protocols, materials and methods used and the experimental design to carry out specific objectives have been presented here.

3.1. MATERIALS

3.1.1. Glassware and plasticware

The glassware used in the present study was made of high-grade pyrex glass. The glassware, wherever used, was thoroughly cleaned and rinsed with triple distilled water and then heat sterilized at 250 \degree C for 4 h. The pasture pipettes were purchased from Labco, Ambala, India.
Materials and Methods

The plasticware, which included disposable 35 mm x 10 mm and 60 mm x 15 mm cell culture Petri dishes, 100 mm x 100 mm square Petri dishes with 13 mm grid, 4 and 6 well plates, 15 and 50 ml Falcon tubes and tissue culture flasks were purchased either from Becton, Dickinson and Co., Lincoln Park, NJ, USA or from Nunc, Roskilde, Denmark. The 0.45 and 0.22 μm filters were from Millipore Corp., Bedford, MA, USA. Disposable, nontoxic and non-pyrogenic plastic syringes were from Norm-Ject, Henke-Sass Wolf GmbH, Tuttingen, Germany. Microblades™ (Model MTB-05) were purchased from Micromanipulator Microscope, Company Inc. Sterile disposable 19 gauge hypodermic needles of Dispovan make were purchased from Hindustan Syringes & Medical Devices Ltd., Faridabad, India whereas autoclavable disposable tips for micropipettes were from Tarsons Products Pvt. Ltd, Kolkata, India.

3.1.2. Chemicals, cell culture media and supplements

The different media used in the present study for culture of oocytes/embryos, which included tissue culture medium-199 (TCM-199) and Dulbecco’s phosphate buffered saline (DPBS) were purchased from Sigma Chemical Co., St. Louis, MO, USA. The media were in the ready-to-use liquid form. Various supplements, which included bovine serum albumin (BSA), antibiotics (gentamicin, penicillin and streptomycin), porcine follicle stimulating hormone (FSH-p), sodium pyruvate, fatty acid-free BSA, heparin, caffeine and all other chemicals used for in vitro embryo production in various experiments were also purchased from Sigma Chemical Co., St. Louis, MO, USA, unless otherwise indicated. Most of the chemicals used in present study were of embryo or cell culture tested grade. Fetal bovine serum (FBS, Hyclone, Logan, UT, US, cat no. CH30160.02) of same batch was used for in vitro embryo production.

3.1.3. Equipments

3.1.3.1. Microscopes

a) Zoom stereomicroscope

Low magnification zoom stereo microscopes (Nikon, Japan, Model SMZ-745T or Olympus SZX 7) were used for searching the aspirated oocytes, for evaluating the quality of oocytes and for collection of various embryonic stages.

b) Inverted microscope

An inverted microscope (Nikon, Japan, Model TMD) was used for examination of embryos for monitoring health, morphological characteristics and growth of various
embryonic stages. The microscope with light source at the top and a long working distance allowed embryos in culture dishes to be viewed and photographed whenever needed. The microscope was equipped with an incubator attachment to enable maintenance of optimum temperature during working. The inverted microscope was also equipped with UV fluorescence and differential interference contrast (DIC) attachment, which helped in capturing the images of in vitro produced embryos. The microscope was equipped with programmable still photography and video recording facilities.

3.1.3.2. Laminar flow hood

Experiments including searching, grading and in vitro embryo production (IVM, IVF, IVC) procedures were carried out in Laminar flow cabinet (CLEANAIR Laminar Flow Systems, India), which served the purpose of minimizing the incidences of microbial contamination and ensuring safety of the operator. UV irradiation and thorough cleaning of working places with ethanol (70% v/v) was used to maintain hygienic and sterile environment throughout experiments.

3.1.3.3. CO\textsubscript{2} incubator

For studying the effects of heat stress, the oocytes and embryos were cultured in a Thermo Fisher Scientific (Marietta, Ohio, USA, Model 3131) CO\textsubscript{2} incubator, which provided a 5% CO\textsubscript{2} in air (90-95% relative humidity) environment. Three CO\textsubscript{2} incubators were used at specific temperatures 38.5 °C, 40.5 °C and 41.5 °C for conducting the experiment.

3.1.3.4. Centrifuge

Refrigerated centrifuge (Sigma 3K30, Germany) with facilities to adjust centrifugation speed, time and temperature was used for centrifugation of chemicals and washing of oocytes and embryonic stages etc. as and when needed.

3.1.3.5. Thermal cycler

A thermal cycler (My Cycler, BIO-RAD, Hercules, CA, USA) was used for synthesizing cDNA from mRNA of buffalo oocytes through reverse transcription in the presence of reverse transcriptase enzyme for amplification of genes of interest with gene specific primers and heat stable Taq polymerase. It gave \(2^n\) number of DNA strands, where \(n\) = number of cycles.
3.1.3.6. **Real time PCR**

Real time PCR (CFX96 Real time system, BIO-RAD, Hercules, USA) was used for quantitative expression of genes related to heat shock, embryonic development and apoptosis in normal and experimental COCs and embryos by using SsoFast EvaGreen (double stranded DNA specific fluorescence dye) qPCR Super Mix (BIO RAD, USA.).

3.1.3.7. **Electrophoresis unit and gel documentation**

Agarose gel electrophoresis was performed for resolution of PCR products. The electrophoretic unit (Power Pac Basic, BIORAD, USA) included the buffer chamber, safety lid with cables, UV transparent tray, casting trays, comb set and power supply. For analyzing the PCR products and for capturing the images gel documentation system, (Gel doc, BIORAD, Hercules, CA, USA) was used.

3.1.4. **Biologicals**

3.1.4.1. **Ovaries and oocytes**

The ovaries served as the source of immature oocytes, follicular fluid and cumulus cells during the present investigation. The buffalo ovaries were obtained from a nearby abattoir (New Delhi).

3.1.4.2. **Buffalo follicular fluid**

For collection of buffalo follicular fluid (buFF), buffalo ovaries were obtained from Delhi abattoir and were transported to the laboratory at 4 °C within 6 h of collection. Follicular fluid was aspirated from all visible surface follicles (2 to 10 mm in diameter) with a 23-gauge needle. The cellular debris was removed by centrifugation at 10,000 rpm for 30 min at 4 °C. The supernatant was carefully collected and sterilized by filtration through a 0.45 μm filter. The follicular fluid was divided into 1 ml aliquots in micro centrifuge tubes and was stored at -20 °C until further use. The same pool of buFF was used throughout the study.

3.2. **METHODS**

3.2.1. **Preparation of different media**

For details regarding the composition of various media used in the present study, please see ANNEXURE-I
3.2.2. Collection and classification of oocytes

Buffalo ovaries collected from abattoir after slaughter were immediately washed 3-4 times with isotonic saline (32-37 °C) containing a 100 µg/ml streptomycin. The washed ovaries were then put in a thermos flask containing warm saline and antibiotics. The collected ovaries were transported to the laboratory within 6 h of slaughter. In laboratory, the ovaries were rinsed twice, trimmed to remove the extra tissue and washed properly with warm saline containing antibiotics.

Oocytes were collected by aspiration of surface follicles (2-8 mm diameter) with an 18 gauge needle attached to a 10 ml syringe containing aspiration medium (TCM-199+ 0.3% BSA+ 0.68 mM L-glutamine + 50 µg/ml gentamicin sulfate). The contents of syringe, which included the aspirated oocytes, follicular fluid, granulosa cells and other debris, were poured in 100 mm x 100 mm square Petri dishes with 13 mm grid. The oocytes were searched under a zoom stereomicroscope at around 20x magnification. The oocytes were then shifted to 35 mm Petri dishes containing washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 0.68 mM L-glutamine + 50 µg/ml gentamicin sulfate). The aspirated oocytes were graded according to criteria already in use in laboratory:

Usable quality: Compact cumulus-oocyte complexes (COCs) with an unexpanded cumulus mass having ≥2 layers of cumulus cells and with homogenous evenly granular ooplasm.

Unusable quality: Oocytes partially or wholly denuded or with expanded or scattered cumulus cells or with an irregular ooplasm.

Oocytes of only usable quality were used for in vitro maturation.

3.2.3. In vitro maturation of oocytes

The oocytes were washed six times with washing medium (TCM-199 + 10% FBS + 0.81 mM sodium pyruvate + 50 µg/ml gentamicin sulfate), then twice with the IVM medium (TCM-199 + 10% FBS + 10% follicular fluid + 1 µg/ml estradiol-17β + 5 µg/ml pFSH + 0.81 mM sodium pyruvate + 0.68 mM glutamine + 50 µg/ml gentamicin sulfate). For IVM, groups of 18-20 COCs were placed in 100 µl droplets of the IVM medium, overlaid with sterile mineral oil in 35 mm Petri dishes and cultured for 24 h in a humidified CO₂ incubator at 38.5 °C and respective treatment temperatures viz. 40.5° and 41.5 °C.
3.2.4. **In vitro fertilization**

3.2.4.1. Sperm preparation and *in vitro* fertilization

The spermatozoa used for IVF throughout the study were from the same donor tested for IVF earlier. The spermatozoa were prepared for fertilization as described by Chauhan *et al.* (1998). Briefly, two straws of frozen-thawed buffalo semen were washed twice with washing Brackett and Oliphant (BO) medium (BO medium containing 10 µg/ml heparin, 137.0 µg/ml sodium pyruvate and 1.942 mg/ml caffeine sodium benzoate). The pellet was re-suspended in around 0.5 ml of the washing BO medium. The *in vitro* matured oocytes were washed thrice with fertilization BO medium and transferred to 50 μl droplets (18-20 oocytes/droplet) of capacitation and fertilization BO medium (washing BO medium containing 10 mg/ml fatty acid-free BSA). The spermatozoa in 50 μl of capacitation and fertilization BO medium (2-4 million spermatozoa/ml) were then added to droplets containing oocytes, covered with sterile mineral oil and placed in a CO\(_2\) incubator at 38.5 °C for 16-18 h for IVF.

3.2.4.2. *In vitro* culture

After the end of sperm-oocyte incubation, cumulus cells were washed off oocytes by gentle pipetting. The oocytes were then washed several times with modified Charles Rosenkrans medium with amino acids (mCR2aa) containing 0.8% BSA and cultured in this medium for 48 h post insemination. After this, the embryos were shifted to the IVC medium (mCR2aa + 0.6% BSA + 10% FBS) and cultured in 100 μl droplets of this medium on original beds of granulosa cells for up to 9 days post insemination in a humidified CO\(_2\) incubator at 38.5 °C. The medium was replaced with 50% of fresh IVC medium every 48 h. The cleavage rate was recorded on Day 2 post insemination and the percentage of oocytes that developed to 4-cell, 8- to 16-cell, morula and blastocyst stages was recorded on days 3, 4, 5 and 8 post insemination, respectively.

3.2.5. **Assessment of nuclear stage of maturation**

3.2.5.1. Denuding of oocytes

The COCs with expanded cumulus were transferred into 1.5 ml microcentrifuge tube containing 500µL hyaluronidase (0.5mg/ml) in T2 (where T denotes TCM-199 supplemented with 2.0 mM L-glutamine, 0.2mM sodium pyruvate, 50µg/ml gentamicin and following number denotes percentage of FBS) and incubated for 1 min at 38.5 °C. The denuded oocytes were further washed with 0.3% PBS-PVA.
3.2.5.2. Removal of zona pellucida

The washed denuded oocytes were then treated with 0.5ml of 2 mg/ml pronase and incubated for 10 min at 38.5 °C for removal of zona pellucida. Oocytes with undigested or partly digested zona were discarded. Zona free oocytes were incubated in the Hoechst 33342 (bisbenzimide, 1μg/mL) for 20-25 min in dark. The stained oocytes were washed to remove the residual stain and placed on microscopic slides followed by the addition of a few drops of glycerol. Finally, the cover slip was mounted and oocytes were visualized on an inverted fluorescent microscope at a magnification of 20x (Nikon fluorescence microscope, Japan). The oocytes in all the treatment groups were assessed for a discernible polar body (metaphase II).

3.2.5.3. Selection of polar body containing oocytes

The polar body containing oocytes were selected and their numbers counted by observation under inverted fluorescent microscope. Oocytes containing polar body were used for all further experimentation.

3.2.6. Total cell number (TCN) assessment

The blastocysts were washed with 0.3% PBS/PVA several times and then immediately suspended in 0.5% triton-X followed by incubation in Hoechst 33342 (bisbenzimide,1μg/mL) for 45 min in dark.

3.2.6.1. Visualization of slides

The stained blastocysts were placed on microscopic slides followed by addition of a few drops of glycerol and antifade gold solution. Finally, the cover slip was mounted and blastocysts were visualized on an inverted fluorescent microscope at a magnification of 20x (Nikon fluorescence microscope, Japan).

3.2.7. Quantitative expression of genes

3.2.7.1. RNase-free plasticware

RNase-free plasticware was prepared by immersing the tubes and tips in 0.1% DEPC (Diethyl pyrocarbonate) overnight at 37 °C over a magnetic stirrer. The solution was prepared by adding 1.5 ml of DEPC to 1.5 liters of distilled water and then mixing vigorously over a magnetic stirrer. The solution was prepared fresh every time. DEPC is reported to destroy enzymatic activity of ribonuclease by modifying -NH, -SH, -OH group in RNase (Ambion,
USA). DEPC is also a known carcinogen, therefore, extra precaution was taken and gloves were worn every time it was handled. The DEPC solution with immersed materials was stirred 3-4 times so that all tips and tubes were soaked completely in the solution. Next day, the solution was drained off and treated material was dried after wrapping in aluminium foil in hot air oven. Tips and tubes were not touched with anything and gloves were used while handling. After drying, tips were carefully filled in the tip boxes and tubes were filled in jars and then materials were double autoclaved to remove the remaining traces of DEPC which could otherwise interfere with the RT reaction during cDNA synthesis.

3.2.7.2. Preparation of RNase-free solutions

Buffers and solutions are a common source of RNase contamination. DEPC treatment is a most commonly used method for eliminating RNase contamination from water, buffer and other solutions. However, DEPC cannot be used with certain reagents containing primary amine groups (e.g. TAE buffer), secondary or tertiary amines (e.g. HEPES). The amino groups tend to react with and ‘sop up’ the DEPC, making it unavailable for inactivating RNase. To avoid RNase contamination all solutions were prepared using baked glassware and DEPC treated pyrogen-free MilliQ water. RNase-free chemicals and glassware were handled with baked and autoclaved spatula.

3.2.7.3. RNase-free surface

Prior to using laboratory surfaces e.g., work table, bench tops, laminar hoods, centrifuge and electrophoresis tanks, surfaces were decontaminated by wiping with RNase inhibitor solution, RNAZap (Ambion). For total RNA isolation from oocytes and embryos the RNAqueous- Micro Kit (Ambion) was used.

3.2.7.4. RNA isolation Protocol

Total RNA was isolated from embryos by using the RNAqueous-Micro Kit (Ambion, USA) according to manufacturer’s instructions, with some modifications.

Protocol

1) Oocytes were pelleted down by centrifugation. Supernatant was removed and pellet washed with 1x PBS.

2) Pellet was resuspended in 100 µl lysis solution and mixed vigorously by vortexing.
3) 50 µl of absolute ethanol was added and vortexed briefly.
4) The lysate mixture was loaded onto a Micro Filter Cartridge Assembly and centrifuged for 10 s at 13000rpm.
5) 180 µl of Wash solution 1 was added to filter and centrifuged at 13000rpm for 10 s followed by addition of 180 µl of Wash solution 2/3 and centrifuged at 13000rpm for 10 s.
6) The flow-through was discarded from collection tube. Micro Filter Cartridge was placed in same collection tube again and centrifuged at 13000rpm for 1 min to remove residual fluid and dry filter.
7) After this, Micro Filter Cartridge was placed in a fresh 1.5 ml Micro Elution tube. 20 µl of elution solution pre heated to 75 ºC was placed to centre of the filter, incubated at RT for 1 min and then centrifuged at 13000rpm for 30 s.
8) Then RNA was quantified using nanodrop.
9) The genomic DNA contamination was removed by DNase treatment at 37 ºC for 20 min.

3.2.7.5. cDNA synthesis and real time quantification

For cDNA preparation the ‘Revertaid cDNA synthesis kit (Fermentas) was used.

Protocol

For cDNA synthesis, RNA (in 10 ul) was taken in 200µl tube and 1 µl oligo dT (50µM), and nuclease free water was added to make volume up to 12 µl. To denature the RNA and remove secondary structures, the RNAs were heated at 65 ºC for 5 min and the reaction was quenched by placing the tube immediately on ice for 1 min. Then 4 µl 5x RT buffer, 2µl dNTP mix (10mM), 1 µl RNase and 1µl revertaid RT enzyme were added. The reaction was incubated at 42°C for 60 min. The cDNA prepared was stored at -80 ºC until use.

3.2.7.6. Semiquantitative PCR for heat stress, apoptosis, developmental competence and housekeeping genes

The oligonucleotide primers used for these genes and GAPDH amplification were custom synthesized. PCR reactions were set up in a final volume of 25 µl having 10X PCR buffer, 10mM dNTPs, 1.0 U of DNA polymerase and 10pmol/µl each of forward and
reverse primers. In the present study cDNA extracted from oocytes was taken as template for amplification of aforesaid genes.

The PCR cycling conditions for different heat stress, apoptosis, mitochondrial and oxidative stress, developmental competence, metabolism and endolysosomal proteases related genes were 94°C for 3 min, followed by a cycling programme of 94°C for 30 s, X°C (annealing temperature as mentioned for each gene in Table 3.3 for 30 s and 72°C for 30 s for 36 cycles. The reactions were ended with a final extension at 72°C for 10 min. RT negative and PCR negative controls were set for all the PCR reactions to negate the DNA contamination in cDNA templates and for ascertaining the authenticity of PCR amplifications. PCR products were visualized on 2% agarose gel in 1x TAE buffer containing 0.5μg/ml of ethidium bromide and migrated at 100V for 20min. 25 μl of PCR reaction was prepared by adding following components in this order:

Table 3.1: RT PCR components for semiquantitative expression

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PCR Buffer</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>MgCl₂ (2mM)</td>
<td>2.0 μl</td>
</tr>
<tr>
<td>10mM dNTP mix</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Forward primer (10μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Reverse primer (10μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Taq polymerase (5U/μl)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Template</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>15.5 μl</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25 μl</strong></td>
</tr>
</tbody>
</table>

3.2.7.7. Comparative quantification of candidate genes related to heat shock proteins, apoptosis, mitochondrial and oxidative stress, developmental competence, metabolism and endolysosomal proteases in control and treatment groups by Real-Time PCR

Real-time PCR was performed on CFX96 system (Bio-Rad, Hercules, USA) using SYBR green (double-stranded DNA-specific fluorescent dye) master mix from Fermentas.
The reaction was performed in 12 well PCR strips for real time (Biorad) in a final volume of 10 μl.

3.2.8. Standardization of Real Time-PCR

3.2.8.1. Primer concentration

The concentration of primer plays important role in quantitative estimation of transcripts following RT-PCR. Along with exponential amplification of given stretch of nucleic acid some amount of primer dimers also generate and SYBR green cannot differentiate between these two, being double stranded. In present experiment initially 1.0 μl (10μM) reverse and forward primers were used in 10 μl reaction. The same concentration of primers was used for amplification of all genes from their respective primers.

Protocol

For determining the relative mRNA abundance, cDNA of control and treatment groups from was taken. GAPDH was used as reference gene for all experiments. The qRT-PCR reactions were performed using the SYBER green (double stranded DNA-specific fluorescent dye) master mix from BIORAD, each run was performed in duplicate in a 10 μl reaction volume which contained 5 μl fluorescence dye, 2 μl of gene specific primers (forward and reverse) from 10 μM stock and 1 μl template. The final volume was made up with nuclease-free water (Table 3.2). The PCR condition used for all genes was as follows: Initial denaturation at 95 °C for 3 min, 40 cycles (denaturation 95 °C for 10 sec., annealing X for 10 sec., and extension at 72 °C for 10 sec) and melting cycle starting from 65 °C up to 95 °C with a 0.5 °C/sec transition rate. The qRT-PCR specificity was confirmed by analysis of melting curve shown by machine by CFX Manager Software. During data analysis, the \( C_t \) value of housekeeping was subtracted from \( C_t \) value of target gene to obtain change in \( C_t \) (\( \Delta C_t \)). The \( \Delta C_t \) value of target gene sample was subtracted from the calibrator (control) to get \( \Delta\Delta C_t \) values.

To detect the expression of genes, specific primers are essential for amplification of target gene of interest. The gene specific primers were designed with a target to amplify a fragment of around 150-240 bp preferably from the end of cDNA. The primers were designed with highly conserved region of either Bovine or Buffalo sequences using Primer3 Software. (http://www-genome.wi.mit.edu/cgi-bin/prime/primer3-www.cgi.)
### Table 3.2: SYBR green reaction mix for Real time PCR reaction

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X SYBR green mix</td>
<td>5.0 μl</td>
</tr>
<tr>
<td>Forward primer (10 μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Reverse primer (10 μM)</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Template</td>
<td>1.0 μl</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>2 μl</td>
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<tr>
<td><strong>Total</strong></td>
<td><strong>10 μl</strong></td>
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### Table 3.3: Primer sequence for quantitative real time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
<th>Accession No.</th>
</tr>
</thead>
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<tr>
<td>Hsp70.1</td>
<td>F5’TCATCAACGAGCAGCACAGCCTA3’</td>
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<td>103</td>
<td>GU-183097.1</td>
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<tr>
<td></td>
<td>R-5’TTCTGCTGGTGTCACGACTGAC3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp70.2</td>
<td>F-5’AAGCACAAGAGACATTCAGCACC3’</td>
<td>58.5</td>
<td>130</td>
<td>NM_174344.1</td>
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<tr>
<td></td>
<td>R-5’AAGCTGAGAAATCCACGCGCCCT3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp70.8</td>
<td>F5’CGGTGATGCGAAGAAAGCCAGTTT3’</td>
<td>58.0</td>
<td>133</td>
<td>NM_174345.3</td>
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<tr>
<td></td>
<td>R-5’CACCACCATGAAAGCGCAATTTT3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hsp 60</td>
<td>F-5’ACTGGCTCTCTATCTACTC3’</td>
<td>58.0</td>
<td>146</td>
<td>NM_001166610.1</td>
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<td></td>
<td>R-5’TGTCTCAATACACTTCCTTCCC3’</td>
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<tr>
<td>Hsp 10</td>
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<td>56.0</td>
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<td>HSF-1</td>
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<td>58.0</td>
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<td>R-5’GAGATGAGGAAGCTGATGAG3’</td>
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<tr>
<td>Glut-1</td>
<td>F-5’ACAGCCAGCTGAGATAGACT3’</td>
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<td>230</td>
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<tr>
<td></td>
<td>R-5’TGTGGGTGAGAGATCTCTG3’</td>
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<tr>
<td>Glut-3</td>
<td>F-5’CATCCCTGTGCTTTGTCT3’</td>
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<td>NM_174603.3</td>
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<td>R-5’CAGCTTCAACCGACTCTG3’</td>
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<tr>
<td>IGF1R</td>
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<td>R-5’GAATGTCATCTGCTCTTCTG3’</td>
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<td>MnSOD</td>
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<td></td>
<td>R-5’TGTCTGATCTGCTACATGAGCCAG3’</td>
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<tr>
<td>iNOS</td>
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<td>60.0</td>
<td>121</td>
<td>DQ_676956.1</td>
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### Materials and Methods

<table>
<thead>
<tr>
<th>Gene</th>
<th>F- Primer</th>
<th>R- Primer</th>
<th>Tm</th>
<th>GenBank Accession</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dna J</td>
<td>F- 5'ATCTTCATGCGTTGCTGTC3'</td>
<td>R- 5'CAGTGGTAGTGGTGAGG3'</td>
<td>60.0</td>
<td>NM_174068.2</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>F- 5'GCAAGTATTGGTGAGTGG3'</td>
<td>R- 5'ATTGTCCGTAGGAGGCT3'</td>
<td>58.0</td>
<td>HM_630302.1</td>
</tr>
<tr>
<td>Bcl-xl</td>
<td>F- 5'TTGTTGAGGCTTTTCTCCTC3'</td>
<td>R- 5'GATCAAGGCTCTAGGG3'</td>
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<td>Mcl-1</td>
<td>F- 5'TCGGAAAACGCAACATC3'</td>
<td>R- 5'CAGAAGGCAAAAGAAGA3'</td>
<td>58.0</td>
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<tr>
<td>Bax</td>
<td>F- 5'CCTTTTGCTTGCAGGTTCA3'</td>
<td>R- 5'CGCTTCAGCACACTGCT3'</td>
<td>60.0</td>
<td>AJ812564</td>
</tr>
<tr>
<td>Bid</td>
<td>F- 5'CTGTGGAGGAGGACAGG3'</td>
<td>R- 5'GATGGAGGTGCTATCTTTTG3'</td>
<td>60.0</td>
<td>NM_001075446.1</td>
</tr>
<tr>
<td>Caspase 3</td>
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<td>R- 5'CGCAGGCTCTAGGG3'</td>
<td>60.0</td>
<td>HM_630302.1</td>
</tr>
<tr>
<td>Caspase 7</td>
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<td>NM_001077840.1</td>
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<td>R- 5'GGTGTCGCTATCTTTTG3'</td>
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<td>NM_174681.2</td>
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<tr>
<td>ZAR1</td>
<td>F- 5'GCTTCCAGTTCTAGGAGCAG3'</td>
<td>R- 5'TCTCGTAAACCTGGTTAGGCT3'</td>
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<td>NM_001076203.1</td>
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<tr>
<td>Cathepsin B</td>
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<td>R- 5'TGCGCCAGCGCCAGTGG3'</td>
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<tr>
<td>Cathepsin K</td>
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<td>R- 5'CACCACATCTGTGTAAGGAGG3'</td>
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<td>Cathepsin S</td>
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<td>R- 5'CTTGCCATCGTAGTATATATCTGG3'</td>
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<td>Cathepsin Z</td>
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<td>Gapdh</td>
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<td>GU324291.1</td>
</tr>
</tbody>
</table>
3.2.9. Assessment of apoptosis by TUNEL assay

For examining the health of the embryos, the total cell number and level of apoptosis in COCs after maturation were determined by TUNEL staining.

Protocol

1) The COCs/embryos were washed three times with PBS containing 0.3% polyvinyl alcohol (PVA) in 4-well dishes and were fixed in 4% paraformaldehyde for 1 h at room temperature and stored at 4°C until further use.

2) These were then washed three times with PBS + 0.3% PVA to remove any traces of paraformaldehyde after which these were kept at 4°C till start of staining.

3) The COCs/embryos were permeabilized by incubation in 0.5% Triton X-100 for 1 h after which these were incubated with fluorescein isothiocyanate (FITC)-conjugated dUTP and terminal deoxynucleotidyl transferase (TdT) enzyme for 1 h at 37°C in dark.

4) The COCs/embryos were then treated with 50 µg/ml RNase at room temperature for 1 h, and were counterstained with 10 µg/ml propidium iodide for 15 min at 37°C.

5) Along with the treatment groups, positive and negative controls were also processed. For positive controls, COCs/embryos were incubated in DNase solution (100 U/mL) for 20 min at 37°C prior to incubation with FITC-conjugated dUTP and TdT.

6) The stained COCs/embryos were washed with DPBS (Ca²⁺ and Mg²⁺-free) and were mounted on glass slides in 3 µL droplets of antifade solution and were covered with a cover slip.

7) The images were captured at both red and green filters for examining the nuclei and site of apoptosis, respectively. The images generated from red and green filters showing green bodies on exact site of red nuclei were considered as apoptotic cells.

8) Cell counting was performed from digital images obtained on inverted Nikon fluorescence microscope.
Materials and Methods

Calculations

Apoptotic index = Number of TUNEL-positive cells/ Total no. of COCs x 100

In each group, the number of COCs for calculating the apoptotic index was 50.

The total apoptotic indices in blastocysts were calculated as follows:

Apoptotic index = Number of TUNEL-positive nuclei/ Total no. of nuclei x 100

3.2.10 Estimation of cellular stress markers

3.2.10.1. Reactive oxygen species (ROS)

Reactive oxygen species (ROS) were determined in COCs, denuded oocytes (DOs) and cumulus cell mass by “OxiSelect™ Hydrogen Peroxide Assay Kit” (Catalog No. STA-343) from Cell Biolabs, Inc. 7758 Arjons Drive San Diego, CA 92126 USA.

Materials provided: Xylenol Orange Dye, AFS Reagent, Sorbitol Solution, Hydrogen Peroxide, 1000X BHT Solution, TCEP Solution.

Reagent Preparation:

Aqueous working reagent: The working reagent for aqueous assays was prepared by diluting the Xylenol Orange 1:100, Sorbitol 1:40 and the AFS Reagent 1:100 together with deionized water. For 100 assays, 25 ml working reagent solutions: 0.250 ml of Xylenol Orange, 0.625 ml Sorbitol and 0.250 ml AFS Reagent added together and QS with deionized water to 25 ml was prepared. The working reagent was vortexed to make the solution homogenous.

Preparation of standard curve: To prepare the hydrogen peroxide standards, 1:1000 dilution of stock hydrogen peroxide was made in deionized water that resulted in 8.8 mM H₂O₂ solution. The 8.8 mM H₂O₂ solution was used to prepare standards in the concentration range of 0 to 100 μM by further diluting in water.

Assay Protocol:

1) 25 μl of standard or sample was added to the microtiter plate wells.
2) 250 μl of aqueous working reagent was added to each well. The contents were mixed thoroughly and incubated on a shaker for 30 minutes at room temperature.
3) Optical density was taken, using TECAN infinite PRO200 ELISA reader (Tecan Asia Pvt Ltd. Singapore) at 595 nm.
Calculation:

The duplicate readings were averaged for each standard and samples and subtracted the average zero standard optical density. Standard curve was constructed by plotting the mean absorbance for each standard on the y-axis against concentration on x-axis and drawn the best-fit curve through the points on graph. The concentration of hydrogen peroxide within samples was calculated by comparing sample absorbance to the standard curve.

3.2.10.2. Nitric oxide (NO)

Nitric oxide (NO) was determined by “Nitrate/Nitrite Colorimetric Assay kit” (Catalog No. 780001) from Cayman Chemical Company, 1180 East Ellsworth Road Ann Arbor, MI 48108, USA.


Reagent Preparation:

Assay Buffer: The total content of the assay buffer vial was diluted to 100 ml with Milli-Q water.

Nitrate Reductase Enzyme Preparation: The vial was reconstituted with 1.2 ml assay buffer.

Nitrate Reductase Cofactors Preparation: The vial was reconstituted with 1.2 ml assay buffer.

Nitrate Standard: The vial was reconstituted with 1.0 ml assay buffer. Again 0.1 ml of reconstituted Nitrate Standard + 0.9 ml of assay buffer yielded 200µM concentration that was used to prepare 0, 5, 10, 15, 20, 25, 30 and 35 µM concentration.

Assay Protocol:

1) 200 µl of assay buffer was added to blank wells. No other reagent was added to blank wells.
2) 80 µl of sample and standards was added to sample and standard wells.
3) 10 µl of enzyme cofactor mixture was added to each standard and sample well.
4) 10 µl of the nitrate reductase enzyme mixture was added to each standard and sample well.
5) Plate was covered with adhesive strip and incubated at room temperature for one hour.
6) 50 µl each of Griess reagents R1 and R2 was added to each standard and sample well.
7) Optical density was taken after 10 minutes, using TECAN infinite PRO200 ELISA reader (Tecan Asia Pte Ltd. Singapore) at 540 nm.

**Calculation:**

The duplicate readings were averaged for each standard and samples. The average blank optical density was subtracted. The average absorbance of standards as a function of final nitric oxide concentration (µM) was plotted and concentration of the samples was calculated using linear regression equation of standard curve.

**Precision:** Inter-assay and intra-assay coefficient of variation was 3.4% and 2.7% respectively.

**Assay Range:** 2.5–200 µM

### 3.2.10.3. Thiobarbituric acid reactive substances (TBARS)

Thiobarbituric acid reactive substances (TBARS) were determined by “TBARS Assay Kit” (Catalog No. 10009055) from Biovision Inc. 155 S. Milpitas, CA, 95035 USA.

**Materials provided:** Assay plate, Thiobarbituric acid, TBA acetic acid, TBA sodium hydroxide, TBA malondialdehyde standard, TBA SDS solution, color reagent.

**Reagent Preparation:**

**Thiobarbituric acid:** The reagent provided was ready to use.

**TBA acetic acid:** 40 ml of TBA acetic acid was diluted with 160 ml HPLC grade water.

**TBA sodium hydroxide:** 20 ml of TBA NaOH was diluted with 180 ml HPLC grade water.

**TBA Malondialdehyde Standard:** The reagent was ready to use to prepare the standard curve.

**TBA SDS Solution:** The reagent was ready to use.

**Color Reagent:** 530mg of TBA was dissolved in 50ml of TBA acetic acid solution and mixed until the TBA was completely dissolved.
Materials and Methods

Assay Protocol:

1) 100 μl of samples was added to a 5 ml vial

2) **TBA Malondialdehyde Standard**: 0, 5, 10, 20, 40, 80, 200 and 400 μl of the TBA Malondialdehyde standard was added into 5ml vials in duplicate to generate 0, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.5 and 5 μM/vial standard.

3) 100μl of SDS solution was added to each vial and mixed.

4) The vials were placed upright in boiling water for one hour.

5) The reaction was stopped by placing the vials in ice bath for 10 minutes.

6) The vials were centrifuged at 1600x g at 4˚C and incubated at room temperature for 30 minutes.

7) 150 µl of sample (in duplicates) from each vial was added to colorimetric 96-well assay plate.

8) Optical density was taken at 540nm.

**Calculation:**

1) The Average absorbance (Abs) of each sample and standard was taken.

2) The average absorbance of standard A was subtracted from itself and all other standards and samples.

3) The corrected absorbance of standards as a function of final malondialdehyde concentration (µM) was plotted and the malondialdehyde concentration of samples was calculated using linear regression equation of standard curve.

4) Following formula was used to calculate GR activity (nmol/min/ml):

   \[
   MDA \text{ (µM)} = \frac{\text{Corrected absorbance} - y \text{ intercept/slope}}{
   \text{Assay range: } 0 - 50 \text{ µM.}
   \]

3.2.11. **Estimation of Antioxidant enzymes**

3.2.11.1. **Superoxide dismutase**

Superoxide Dismutase was determined by “Superoxide Dismutase Assay kit” (Catalog No.706002) from Cayman Chemical Company, 1180 East Ellsworth Road Ann Arbor, MI 48108, USA.

**Materials provided:** Assay plate, Assay Buffer, Sample Buffer, Radical Detector, SOD Standard and Xanthine Oxidase.
Reagent Preparation:

**Assay Buffer:** 3 ml of concentrate assay buffer was diluted with 27 ml of HPLC-grade water.

**Sample Buffer:** 2 ml of sample buffer concentrate was diluted with 18 ml of HPLC-grade water.

**Radical Detector:** 50 µl of solution + 19.95 ml of Assay Buffer.

**SOD Standard:** Standards differing in SOD activity were prepared - 0, 0.025, 0.05, 0.1, 0.15, 0.2 and 0.25 U/ml by serial dilution with sample buffer.

**Xanthine Oxidase:** 50 µl of enzyme + 1.95 ml of sample buffer.

Assay Protocol:

1) SOD Standard Wells: 200 µl of diluted radical detector and 10 µl of standard was added per well.
2) Sample Wells: 200 µl of diluted radical detector and 10 µl of sample was added to each well.
3) The reaction was initiated by adding 20 µl of diluted xanthine oxidase to all wells and plate was shaked carefully for a few seconds to mix.
4) The plate was incubated on shaker for 20 min at room temperature.
5) Optical density was taken, using TECAN infinite PRO200 ELISA reader (Tecan Asia Pvt Ltd. Singapore) at 460 nm.

Calculation of Results:

1) The average absorbance (Abs) of each standard and sample was calculated.
2) Linearized rate (LR) for all the standards was calculated, for this Abs Std A was divided by Abs of all the standards.
3) Linearized rate (LR) for all the samples was calculated, for this Abs Std A was divided by Abs of all the samples.
4) Linearized SOD standard rate (LR) as a function of final SOD activity (U/ml) was plotted and SOD activity of samples were calculated using linear regression equation of standard curve.

\[
\text{SOD (U/ml)} = \left[\frac{\text{sample LR} - \text{intercept}}{\text{slope}}\right] \times \frac{0.23}{0.01}
\]

**Precision:** The intra-assay coefficient of variation was 3.2% and inter-assay coefficient of variation was 3.7%.
Assay Range: 0.025–0.25 U/ml

Unit: One unit of SOD is the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

3.2.11.2. Catalase

Catalase was determined by “Catalase Assay kit” (Catalog No.707002) from Cayman Chemical Company, 1180 East Ellsworth Road Ann Arbor, MI 48108, USA.

Materials provided: Assay plate, Assay Buffer, Sample Buffer, Formaldehyde Standard, Catalase (control), Potassium Hydroxide, Hydrogen Peroxide, Purpald (chromagen) and Potassium Peroxide.

Reagent Preparation:

Assay Buffer: 2 ml of concentrate assay buffer was diluted with 18ml of HPLC-grade water.

Sample Buffer: 5 ml of sample buffer concentrate was diluted with 45 ml of HPLC-grade water.

Catalase (control): CAT was reconstituted by adding 2 ml sample buffer to the vial and 100 µl of this solution was further diluted with 1.9 ml of sample buffer.

Potassium Hydroxide: 4 ml of cold HPLC-grade water was added to vial and vortexed to yield 10 M solution.

Hydrogen Peroxide: 40 µl of hydrogen peroxide was diluted with 9.96 ml of HPLC-grade water.

Formaldehyde Standard: Standards were prepared of different concentration of 0, 5, 15, 30, 45, 60 and 75 µM by serial dilution with sample buffer.

Assay Protocol:

1) Formaldehyde Standard Wells: 100 µl of assay buffer + 30 µl of methanol + 20 µl of standard (A–G) were added.

2) Positive Control Wells: 100 µl of assay buffer + 30 µl of methanol + 20 µl of CAT control were added.

3) Sample Wells: 100 µl of assay buffer + 30 µl of methanol + 20 µl of sample were added to each well.

4) The reaction was initiated by adding 20 µl of diluted hydrogen peroxide to all wells and mixed carefully for a few seconds.
5) The plate was incubated on shaker for 20 min at room temperature.
6) 30 µl of potassium hydroxide was added to each well to terminate the reaction.
7) 30 µl of purpald (chromagen) was added to each well and incubated the plate on shaker for 10 min at room temperature.
8) 10 µl of potassium periodate was added to each well and incubated the plate on shaker for 5 min at room temperature.
9) Optical density was taken, using TECAN infinite PRO200 ELISA reader (Tecan Asia Pvt Ltd. Singapore) at 540 nm.

**Calculation of Results:**

1) The average absorbance (Abs) of each standard and sample was calculated.
2) The average absorbance of standard A was subtracted from itself and all other standards and samples.
3) The corrected absorbance of standards as a function of final formaldehyde concentration (µM) was plotted and the formaldehyde concentration of samples was calculated using linear regression equation of standard curve.

\[
\text{Formaldehyde (µM)} = \left(\frac{\text{sample absorbance} - \text{intercept}}{\text{slope}}\right) \times 0.17/0.02
\]

\[
\text{CAT Activity (U/ml)} = \left(\frac{\mu M \text{ of sample/20 min}}{\text{sample dilution}}\right)
\]

**Sensitivity:** The sensitivity range of the assay was 2–34 nmol/min/ml.

**Precision:** The intra-assay coefficient of variation was 3.8% and the inter-assay coefficient of variation was 9.9%.

**Unit:** One unit of catalase is the amount of enzyme that will cause formation of 1nmol of formaldehyde per minute at 25 °C.

3.2.11.3. **Glutathione peroxidase (GSH-Px)**

Glutathione peroxidase (GSH-Px) was determined by “Glutathione Peroxidase Assay kit” (Catalog No. 703102) from Cayman Chemical Company, 1180 East Ellsworth Road Ann Arbor, MI 48108, USA..

**Materials provided:** Assay plate, GSH-Px Assay Buffer, GSH-Px Sample Buffer, GSH-Px control, GSH-Px Co-Substrate mixture and GSH-Px Cumene hydroperoxide.
Reagent Preparation:

1) **GSH-Px Assay Buffer**: 3 ml of concentrate assay buffer was diluted with 27 ml of HPLC-grade water.

2) **GSH-Px Sample Buffer**: 2 ml of concentrate sample buffer was diluted with 18 ml of HPLC-grade water.

3) **GSH-Px Control**: 10 µl bovine erythrocyte GSH-Px was added to 490 µl of diluted sample buffer.

4) **GSH-Px Co-Substrate**: The contents of vial were diluted with 2 ml of HPLC-grade water.

Assay Protocol:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Non-enzymatic wells</th>
<th>Positive Control wells</th>
<th>Sample wells wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Buffer</td>
<td>120 µl</td>
<td>100 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Co-Substrate Mixture</td>
<td>50 µl</td>
<td>50 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>GSH-Px Control</td>
<td>–</td>
<td>20 µl</td>
<td>–</td>
</tr>
<tr>
<td>Lysate Sample</td>
<td>–</td>
<td>–</td>
<td>20 µl</td>
</tr>
<tr>
<td>Cumene Hydroperoxide</td>
<td>20 µl</td>
<td>20 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

- Carefully plate was shaken for few seconds to mix well.
- Optical density was taken at five time points every minute interval using TECAN infinite PRO200 ELISA reader (Tecan Asia Pvt Ltd. Singapore) at 340 nm.

Calculation of Results:

1) Change in absorbance ($\Delta A_{340}$) per minute was determined by plotting the absorbance values as a function of time to obtain the slope of the linear portion of the curve.

2) $\Delta A_{340}$/min was determined for non-enzymatic wells and subtracted from sample wells.

3) Following formula was used to calculate GSH-Px activity (nmol/min/ml):

$$GSH-Px \text{ activity (nmol/min/ml)=} \frac{\Delta A_{340/\text{min}}}{0.19 \text{ ml}} \times 0.00373 \mu\text{M}^{-1} \times \frac{\text{Sample dilution}}{0.02 \text{ ml}}$$

**Precision**: The intra and inter-assay coefficient of variation was 5.7% and 7.2% respectively.
Unit: One Unit of GSH-Px is the amount of enzyme that will cause the oxidation of 1nmol of NADPH to NADP⁺ per minute at 25 °C.

3.2.11.4. Glutathione reductase (GSR)

Glutathione Reductase (GR) was determined by “Glutathione Reductase Activity Assay Kit” (Catalog No. K761-200) from Biovision Inc. 155 S. Milpitas Blvd, Milpitas, CA, 95035 USA.

Reagent Preparation:

Catalase: Lyophilized catalase was dissolved with 1 ml assay buffer.

TNB Standard: Lyophilized TNB standard was dissolved with 0.5 ml assay buffer to generate 5 mM TNB Standard.

DTNB Solution: DTNB was dissolved with 0.45 ml assay buffer.

NADPH-GNERAT™: One vial was dissolved with 0.22 ml assay buffer.

GSSG: GSSG was dissolved with 1.3 ml assay buffer.

GSR Positive Control: Lyophilized GSR was dissolved into 100 μl assay buffer.

Assay Protocol:

1. Sample Pretreatment: Samples were treated to destroy GSH before the assay. 5.0 μl 3% H₂O₂ was added to 100 μl sample, mixed and incubated at 25 °C for 5 min. Then 5 μl of catalase was added, mixed and incubated at 25 °C for another 5 min.

2. 50 μl of the pretreated samples was added into a 96-well plate; the final volume was made to 50 μl with assay buffer.

3. TNB Standard: 0, 2, 4, 6, 8, 10 μl of the TNB standard was added into 96-well plate in duplicate to generate 0, 10, 20, 30, 40, 50 nmol/well standard. The final volume was made to 100 μl with assay buffer.

4. 10μl/well positive control was used and adjusted to 50 μl with assay buffer.

5. 50μl of the reaction mix (40 μl GR assay buffer, 2 μl DTNB solution, 2 μl NADPH-GNERAT™ solution, 6 μl GSSG solution) was added to each test sample.

6. Optical density was taken at 405 nm at T1 (reading A₁). The reaction was incubated at 25°C for 10 min, and again measured OD 405 nm at T₂ (reading A₂).

Calculation:

1) ΔA405 nm = A₂–A₁.
2) The TNB standard curve was plotted by applying the $\Delta A_{405\text{nm}}$ to the TNB standard curve to get $\Delta B$ nmol of TNB (TNB amount generated between T1 and T2 in the reaction wells).

3) Following formula was used to calculate GSR activity (nmol/min/ml):

\[
\text{GSR Activity (nmol/min/ml} = \text{mU/mL) = } \frac{\Delta B}{(T_2 - T_1) \times 0.9 \times V} \times \text{Sample dilution factor}
\]

Where: $\Delta B$ was the TNB amount from TNB standard Curve (in nmol)

$T_1$ was the time of first reading ($A_1$) (in min)

$T_2$ was the time of second reading ($A_2$) (in min)

$V$ was pretreated sample volume added into the reaction well (in ml)

$0.9$ was the sample volume change factor during sample pre-treatment procedure.

**Assay range:** 0.1–40 mU/ml

**Unit:** One unit of GSR is the amount of enzyme that generates 1.0 μmol of TNB from DTNB per minute at 25°C.

### 3.2.12. Non-enzymatic antioxidant

#### 3.2.12.1. Glutathione

Glutathione was determined by “Glutathione Assay Kit” (Catalog No. 780001) from Cayman Chemical Company, 1180 East Ellsworth Road Ann Arbor, MI 48108, USA.

**Materials provided:** Assay Buffer, GSSG Standard, GSH Cofactor, GSH Enzyme, GSH DTNB.

**Reagent Preparation:**

**GSH MES Buffer:** 60 ml of buffer (2x) was diluted with 60 ml of HPLC-grade water before use.

**GSH Cofactor Mixture:** The contents of vial were reconstituted with 0.5 ml of water.

**GSH Enzyme Mixture:** The vial contents were diluted with 2 ml of diluted MES Buffer.

**GSH DTNB:** The contents of vial were reconstituted with 0.5 ml of water.
Performing the Assay:

1) 50 µl of standard (tubes A–H) and 50 µl of samples were added in the designated wells on the 96-well plate.
2) Plate was covered with the provided plate cover.
3) Assay cocktail was prepared by mixing the following reagents in a 20 ml vial: MES Buffer (11.25 ml), reconstituted cofactor mixture (0.45 ml), reconstituted enzyme mixture (2.1 ml), water (2.3 ml) and reconstituted DTNB (0.45 ml).
4) 150 µl of freshly prepared assay cocktail was added to each of wells containing standards and samples using a multichannel pipette.
5) The plate was incubated at room temperature for 25 minutes on an orbital shaker.
6) Optical density was taken after 25 minutes, using TECAN infinite PRO200 ELISA reader (Tecan Asia Pte Ltd. Singapore) at 405 nm.

Calculation:

1) The duplicate readings were averaged for each standard and sample and subtracted the average blank optical density.
2) The average absorbance of standards as a function of final concentration of total GSH was plotted and total GSH (µM) concentration of the samples were calculated using linear regression equation of standard curve.

\[ \text{GSH (µM)} = \left( \frac{\text{Sample average absorbance} - \text{y intercept}}{\text{slope}} \right) \times \text{Dilution} \]

Precision: Inter-assay and intra-assay coefficient of variations were 3.6% and 1.6% respectively.

Assay Range: 0–16 µM

3.3. Experimental design

For fulfilling objectives pertaining to determination of the developmental competence, relative mRNA expression analysis and TUNEL assay, the COCs were divided into the following groups.

Control group: IVM, IVF and IVC were carried out at 38.5°C.

Group 2 (T1): IVM was carried out at 40.5 °C for first 12h of \textit{in vitro} maturation and at 38.5 °C for the remaining 12h.

Group 3 (T2): IVM was carried out at 40.5 °C for the entire 24h of maturation.
Group 4 (T3): IVM was carried out at 41.5 °C for the entire 24 h of in vitro maturation and at 38.5 °C for the remaining 12h.

Group 5 (T4): IVM was carried out at 41.5 °C for the entire 24h of maturation

3.3.1. Experiment 1: Isolation and culture of bubaline COCs in vitro

Buffalo ovaries were procured from a nearby abattoir and COCs were collected from the aspirated follicular fluid. COCs were subjected to IVM, IVF and IVC. The collected COCs were subsequently matured at 38.5 °C (control group) and respective treatment groups viz. 40.5° and 41.5 °C for 24 h and after in vitro maturation the COCs were fertilized with frozen semen straws obtained from NDRI semen bank and cultured for 9 days for blastocyst development.

3.3.2. Experiment 2: Assessment of nuclear stage of metaphase II oocytes

COCs of the control and treatment groups were subjected to Hoechst staining for assessing the nuclear stage of maturation. The oocytes having a discernible polar body were taken for subsequent experiments.

3.3.3. Experiment 3: Comparative developmental competence of bubaline COCs in control and treatment groups

COCs of the all the groups were subjected to IVM, IVF and IVC and cleavage rate was recorded on day 2 post insemination. The percentage of oocytes that developed to 4-cell, 8- to 16-cell, morula and blastocyst stages was recorded on days 3, 4, 5 and 8 day post insemination, respectively.

3.3.4. Experiment 4: Assessment of health of embryos

For evaluating the health of embryos, blastocysts of control and treatment groups were taken on day-8 and subjected to Hoechst staining for determining the total cell number (ICM+ trophectoderm).

3.3.5. Experiment 5: Real time PCR analysis of candidate genes

The relative mRNA abundance of genes related to heat shock, apoptosis, mitochondrial and oxidative stress, developmental competence, metabolism and endolysosomal proteases was carried out in oocytes of control and treatment groups after maturation.
3.3.6. Experiment 6: Effect of heat stress on the level of apoptosis in embryos

The apoptotic cell index in COCs after maturation at control and respective temperature conditions was determined by TUNEL assay. Also, the percentage of TUNEL-positive blastomeres was determined in embryos at blastocyst stage.

3.3.7. Experiment 7: Effect of heat stress on the biochemical parameters of COC, DO and cumulus cell mass

The biochemical analysis was carried out separately in COCs, denuded oocytes (DOs) and cumulus cell mass after maturation at 38.5°C (control group), 40.5°C and 41.5°C for first 12h of maturation and subsequently moved to 38.5°C for remaining12 h (i.e. up to 24 h). The experiment was designed as per the following design:

**Group 1 (C₁):** COCs were matured at 38.5°C for complete 24h of maturation.

**Group 2 (T₁):** COCs were matured at 40.5°C for first 12 h of maturation and then matured at 38.5°C for rest of the 12 h.

**Group 3 (T₂):** COCs were matured at 41.5°C for first 12h of maturation and then matured at 38.5°C for rest of the 12h.

**Group 4 (C₂):** COCs were denuded after maturation at 38.5°C for complete 24 h of maturation and subsequently used for biochemical analysis.

**Group 5 (T₁):** COCs were denuded and matured at 40.5°C for first 12 h of maturation and then at 38.5°C for rest of the 12 h. The denuded oocytes were subjected to biochemical analysis.

**Group 6 (T₂):** COCs were denuded and matured at 41.5°C for first 12 h of maturation and then at 38.5°C for rest of the 12 h. The denuded oocytes were subjected to biochemical analysis.

**Group 7 (C₃):** COCs were denuded after maturation at 38.5°C for complete 24 h of maturation and subsequently used for biochemical analysis. Cumulus cell mass was subjected to biochemical analysis.

**Group 8 (T₁):** COCs were denuded and matured at 40.5°C for first 12 h of maturation and then at 38.5°C for rest of the 12 h. Cumulus cell mass was subjected to biochemical analysis.
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**Group 9 (T2c):** COCs were denuded and matured at 41.5 °C for first 12 h of maturation and then matured at 38.5 °C for rest of the 12 h. Cumulus cell mass was subjected to biochemical analysis.

3.4. Statistical Analysis

The data was analyzed using Graph Pad Prism (SPSS Inc. Chicago, IL, USA) after arcsine transformation. The differences between means were analyzed by one way ANOVA followed by Fisher’s LSD test.