III. MATERIALS AND METHODS

All the laboratory works were carried out in an air controlled room (Animal biotechnology laboratory, National Institute of Animal Nutrition and Physiology NIANP, Bangalore) to prevent contamination and spread of microorganisms. The media preparation and oocyte/embryo handling was done under laminar flow (Holten Lamin air biosafety, Heto Holten, Denmark).

3.1 Procurement of chemicals and plastic ware

All media and chemicals were obtained from Sigma Aldrich chemical pvt. Ltd, St. Louis, MO, USA unless otherwise indicated. The 0.22 µm filters were from Millipore Corporation, Bedford, MA, USA. Disposable plastic dishes (large square and 35 mm diameter circular) and plastic tubes were from FALCON, Becton Dickinson Labware, Lincoln Park, NJ, USA.

3.2 Buffaloes for OPU

For ultrasound guided transvaginal aspiration of ovarian follicles for OPU, five healthy, normal cycling non-descriptive buffaloes (*Bubalus bubalis*) between 4 to 6 years of age and with normal reproductive tracts (on rectal and ultrasonographic examination) were used. The buffaloes were maintained under standard feeding and management conditions at the Experimental Livestock Unit (ELU), NIANP, Bangalore-560030.

3.3 Collection and transportation of slaughterhouse ovaries

Ovaries collected from mature, non-pregnant buffaloes (*Bubalus bubalis*) from the Corporation slaughterhouse, Bangalore were brought to the laboratory in normal saline (32 to 33°C) supplemented with gentamicin (50 µg/ml) with in 2 hours of slaughter. Ovaries were washed 3 times in normal saline in the laboratory and extra ovarian tissues were removed.

3.4 Semen
The frozen semen of Murrah buffalo bull No.1427, known for high rates of in vitro fertilization and cleavage was obtained from Central Frozen Semen Production and Training Institute (CFSP & TI), Hessaragatta, Bangalore.

3.5 Stock media

3.5.1 Tissue culture medium-199 (TCM-199)

0.1 gm L-glutamine and 50 mg gentamicin were added to 1000 ml TCM-199 with Earle's salt and 25 mM HEPES filtered through 0.22 \( \mu \)m filter and stored at 4°C. This supplemented TCM-199 was used for preparing different media in the present study.

3.5.2 Brackett - Oliphant (BO) medium

The composition of BO medium was same as described by Brackett and Oliphant (1975) except for inclusion of HEPES buffer, non inclusion of glucose and slight modification in milimolar concentrations of certain chemicals.

**Composition:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>4040 mg</td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate</td>
<td>85 mg</td>
</tr>
<tr>
<td>HEPES</td>
<td>300 mg</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>1552.3 mg</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>197.5 mg</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>217.5 mg</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>70 mg</td>
</tr>
<tr>
<td>Millipore water</td>
<td>500 ml</td>
</tr>
</tbody>
</table>

All the chemicals were dissolved in 500 ml millipore water and 100 \( \mu \)l of 0.5% phenol red and 50\( \mu \)g/ml gentamycin were added.

3.5.3 Follicle stimulating hormone- from porcine pituitary (pFSH)

50U (1 vial) of pFSH were dissolved in 50 ml of normal saline solution to prepare stock solution of pFSH (0.02U /20\( \mu \)l).
3.5.4 Steer serum

Steer serum (SS) was collected by clotting the blood of a bullock, heat inactivated at 56°C for 30 min, filtered (0.22µm), sterilized and stored in 1.5 ml aliquots at -20°C until use. The same pool of serum was used throughout the study.

3.5.5 Basal media

The basal media consisted of TCM-199 supplemented with 10% SS

3.6 Working media for IVM-IVF-IVC

3.6.1 Aspiration medium

The aspiration medium consisted of TCM-199 supplemented with 5% SS

3.6.2 Oocyte maturation medium

The maturation medium consisted of TCM199 + 10 %Steer Serum (SS) + 50 µM of cysteamine + 0.02 U of pFSH / ml.

3.6.3 Working BO medium for sperm preparation (capacitation)

The composition of sperm preparation/capacitation media consisted of:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>BO medium</td>
<td>50 ml</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>6.5 mg</td>
</tr>
<tr>
<td>Heparin</td>
<td>10 µg/ml</td>
</tr>
</tbody>
</table>

3.6.4 Working BO medium for fertilization (fertilization medium)

The fertilization medium was prepared by adding 10 mM of caffeine sodium benzoate and 0.5 % fatty acid free BSA into the working BO medium (4 ml)

3.6.5 Medium for embryo culture (upto 5 days)

The embryo culture medium for first 5 days of culture consisted of TCM-199 supplemented with 10 % SS.

3.6.6 Medium for embryo culture (after 5 days)
The embryo culture medium from 6th day onwards, culture consisted of TCM-199 supplemented with 10 % SS and 0.1% BSA and 1% ITS. All media were checked for pH (7.2 to 7.4) and osmolarity (280 to 310 mosm) before use.

All the above prepared media were filtered through 0.22 µm filter and placed in CO₂ incubator (38.5°C, 5% CO₂ in air and 90-95% relative humidity) for at least 3 hrs for equilibration of pH and temperature before further use.

### 3.7 Fixation and staining of oocyte/zygotes

#### 3.7.1 Mountant/Vaselin-Paraffin wax mixture

**Composition:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaselin</td>
<td>40 gm</td>
</tr>
<tr>
<td>Paraffin wax</td>
<td>2 gm</td>
</tr>
</tbody>
</table>

The above contents were heated to melt, stored in 10 ml syringes and cooled. Blunt 18 gauge needles were attached to the syringes for proper utilization of vaselin-wax mixture.

#### 3.7.2 Fixative

**Composition:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glacial acetic acid</td>
<td>10 ml</td>
</tr>
<tr>
<td>Ethanol</td>
<td>30 ml</td>
</tr>
</tbody>
</table>

The contents (1:3 v/v) were mixed and stored in a sealed container at room temperature.

#### 3.7.3 Aceto-orcein stain stock solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orcein</td>
<td>1 gm</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>45 ml</td>
</tr>
</tbody>
</table>

The mixture was boiled till orcein dissolved in glacial acetic acid.

#### 3.7.3.1 Working solution

The 1% aceto-orcein working solution was prepared by adding 5.5 ml of millipore water in to 4.5 ml of stock solution and filtered through 0.22 µm filter before use.
3.8 Oocyte recovery and grading

3.8.1 Oocyte recovery from the slaughterhouse ovaries

The oocytes were aspirated from surface antral follicles of ≥3mm in diameter using a 20 G hypodermic needle attached to a 5 ml syringe containing 0.5 to 1.0 ml of aspiration medium.

3.8.2 Ovum pick up (OPU) by transvaginal ultrasound guided follicle aspiration

3.8.2.1 Preparation of animals

The animals were restrained in a suitably designed crate, which allowed minimal movement and prepared for OPU by administering 0.02 mg/kg Xylazine HCL (Xylaxin, Indian Immunologicals Ltd., India) i.m. and 10 min later, with 3 to 4 ml 2% lignocaine HCL given epidural (Xylocaine 2%, Astra Zeneca Pharma India Ltd, India).

3.8.2.2 Collection of oocytes

In the preliminary studies, aspiration of follicles from the slaughterhouse ovaries was carried out with four different vacuum pressures (50, 70, 90 or 110 mmHg) to standardize the optimum vacuum pressure for oocyte collection. The vacuum pressure of 110 mmHg (results in a flow rate of 20 to 25 ml/min) resulted in the highest recovery rate of oocytes and the same vacuum pressure was used throughout the study.

The collection medium for OPU consisted of TCM-199 supplemented with 5% steer serum, 2.2 IU/ml heparin and 50 µg/ml gentamicin. OPU was performed using an ultrasound machine (Aloka SSD-500, Tokyo, Japan) with a 5MHz transvaginal transducer having stainless steel dorsal needle guide and collection apparatus (Plate 1 & 2). The collection apparatus consisted of a 18-gauge, 55 cm long sterile needle with an ultrasound echo tip (V-OPAA-1855, Cook Veterinary Products, Queensland, Australia) attached to the aspiration line of 100 cms length (K-OPAL-1100-L, Cook Veterinary Products, Queensland, Australia) and that was connected to 50-ml centrifuge tube (Falcon, Becton Dickinson, New Jersey, USA). After the
manual evacuation of faeces from the rectum the uterus was retracted and the ovary was located with one hand while the other hand guided the ultrasound transvaginal probe through vagina. The probe was inserted so as to lie distal to the cervix, and by its gentle rotation the ovary was imaged and follicles examined. The number and size of follicles in each ovary was determined before puncture. The follicles were classified on the basis of their diameter as small (2 to 5 mm), medium (5 to 9 mm) or large (>9 mm). The follicles of ≥3mm in diameter were considered for aspiration. The needle was passed through the vaginal wall and follicle was brought into the line of the needle by gentle rotation of the ovary and the needle was advanced again. A popping sensation was felt when the needle entered the follicle, and suction was applied immediately and continued until the follicle seen, on the ultrasound screen collapsed completely. During aspiration, the needle was very gently rotated to curette the follicle to facilitate dislodging of the oocyte, if still attached to its wall or trapped in its folds. The follicle contents passed along the collection line and were drawn into the 50-ml centrifuge tubes using a regulated vacuum pump at 110 mm Hg vacuum pressure. The aspiration line was continuously rinsed with collection media during follicle aspiration. The needle was then withdrawn from the ovary, but remained in position exterior to the vaginal wall with the tip still visible on the ultrasound screen for next aspiration. The OPU was conducted from September 2004 to February 2006. Further, the animals were observed for the subsequent period of 3 months to assess the effect of OPU on cyclicity.

3.8.3 Searching of oocytes

The aspirated fluid collected was transferred to a dish and the oocytes were searched under stereozoom microscope (Olympus, Japan), at 110X magnification. All the oocytes were picked up by pipette and placed in 35 mm culture petridishes containing 4 ml of washing medium for grading.

3.8.4 Grading of oocytes

Oocytes derived from slaughterhouse ovaries by manual aspiration and from live animals by OPU were evaluated by visual morphological appearance under stereozoom microscope and graded as per the criteria given below (Gupta et al., 2002b).

**Grade A (Good Quality):** Oocytes with 4-5 layers of cumulus cells with homogenous and evenly granular grey ooplasm (Plate 5).
**Grade B (Fair Quality):** Oocytes not having much compaction, with 2-3 layers of cumulus cells surrounding the zona pellucida and having evenly granular ooplasm (Plate 6).

Grade C (Average Quality): Oocytes with 1-2 layers of cumulus cells or partially denuded with irregular dark ooplasm (Plate 7).

Grade D (Poor Quality): Oocytes without cumulus cells or with highly expanded or scattered cumulus cells and having irregular dark ooplasm (Plate 8).

Only grade A and grade B oocytes were used for *in vitro* maturation.

### 3.9 *In vitro* maturation of oocytes

The oocytes were washed once with the aspiration medium and twice in the medium in which they would be cultured. The washed oocytes were transferred into 50 µl droplets (5-10 oocytes in a group/droplet) of TCM-199 supplemented with 10% SS, 50µM cysteamine and 0.02U pFSH in a 35 mm petridish. The droplets were covered with warm (37-38°C) mineral oil and then cultured in a CO₂ incubator for 24 hours.

#### 3.9.1 Evaluation of oocytes for *in vitro* maturation

The evaluation of oocytes for *in vitro* maturation was based on the visual assessment of the degree of expansion of cumulus cells under steriozoom microscope as per the criteria described by Kobayashi *et. al.*, (1994):

- **Degree 0:** No expansion
- **Degree 1:** Cumulus cells were non-homogenously spread and clustered cells were still observed (Plate 10).
- **Degree 2:** Cumulus cells were homogenously spread and clustered cells were no longer present (Plate 9).

Oocytes with degree 1 and degree 2 expansion were considered as matured and were used for IVF.

### 3.10 Collection and processing of buffalo oviductal epithelial cells

Oviducts were collected from the slaughtered buffaloes that had newly formed Corpora lutea on their ovaries. The oviducts were then washed with normal saline and transferred to container containing DPBS, and brought to the laboratory by
placing in an ice-bucket containing ice cubes to maintain the temperature at around 4°C. In the laboratory, the oviducts were trimmed of excess and surrounding tissue and washed with normal saline. Oviductal epithelial cells were extracted by compressing the duct with a glass slide in a large square culture petridish containing TCM-199 supplemented with 10% SS. The cells were disaggregated into small clusters by flushing 25-30 times through 26-gauge needle and transferred into a 15 ml tube having 14 ml of washing medium (DPBS supplemented with 0.03% BSA) and centrifuged at 3000 X g for 5 minutes. The supernatant was discarded and 14 ml of washing medium was again added and the pellet was mixed and centrifuged again at 3000 X g for 5 minutes. After discarding the supernatant the final pellet was dissolved in 5 ml TCM-199 containing 10% SS in a 35 mm petridish and the cell suspensions were allowed to settle at the bottom and then 20-25 oviductal cells (Plate 23) were picked up and transferred into another petridish with 50 µl droplet of TCM-199 containing 10% SS. The droplet was covered with warm mineral oil and cultured in a CO₂ incubator. A monolayer of the oviductal cells (Plate 24) was observed to be formed on the basement of the culture dish after 3 days of culture.

3.11  In vitro fertilization

3.11.1 Sperm preparation for IVF

For sperm preparation, two straws (0.25 ml/straw; 20 million spermatozoa/straw) were thawed in warm (37°C) water for 1 minute and emptied into a 15-ml tube. Then 14 ml working BO medium was added and centrifuged at 500 X g for 5 minutes and the supernatant was discarded. The process was repeated again and pellet was dissolved in 0.5ml fertilization medium and the sperm concentration was adjusted to 8-10 x 10⁶/ml before inseminating the oocytes.

3.11.2 In vitro fertilization of oocytes

After IVM, the oocytes were washed twice in the fertilization medium and then transferred to 50 µl fertilization droplet containing 8-10 x 10⁶ spermatozoa/ml. The dishes were then incubated in CO₂ incubator for 16 hours. After 16 hours, 40 µl of BO medium was removed from the upper part of the droplet (containing unattached sperm and detached cumulus cells) and the same quantity of TCM-199 supplemented with 10% SS along with 10-15 motile buffalo oviductal epithelial cells were added
into sperm oocyte droplets. The dishes were again incubated in CO₂ incubator for another 24 hours.

3.12 Assessment of *in vitro* fertilization

3.12.1 Assessment of cleavage

Following 40-42 hours after inseminating the oocytes, the presumptive zygotes were evaluated under stereozoom microscope at 110X magnification for evidence of cleavage. Results were recorded in terms of cleavage rate (percentage of oocytes inseminated that were cleaved to 2-cell stage or beyond) and cleaved embryos were selected for *in vitro* culture.

3.12.2 Assessment of fertilization rate

The uncleaved oocytes/zygotes after 42 hours of insemination were washed with TCM-199 supplemented with 10% SS to remove the attached cumulus cells and spermatozoa. They were then transferred to grease-free glass slides and the extra medium was removed with the help of filter paper. Small drops of mountant (Vaseline - wax mixture) were placed on the four corners of the cover slips. The cover slips were placed over the oocytes/zygotes. The slides were then placed in fixative (acetic acid: ethanol 1:3 v/v) in a slide holder for 24 hours. After 24 hours of fixation, oocytes/zygotes were stained with 1% aceto-orcein and were examined immediately under phase contrast microscope with DIC attachment of 400X magnification.

Oocytes with two polar bodies in perivitelline space (Plate 12), with decondensed sperm nucleus and their associated tail (s), pronucleus (ei) in the ooplasm (Plate 13 & 14) and those that cleaved after 40-42 hours of insemination were together designated as fertilized (Nandi *et al.*, 1998). Remaining oocytes were considered unfertilized and oocytes with darkened and uneven ooplasm were considered as degenerated.

3.13 *In vitro* culture of embryos

The embryos (two cell stage or beyond) were removed from the fertilization droplets after 40-42 hours of insemination and were washed by transferring through two to three dishes of TCM-199 supplemented with 10% SS and were further cultured
on the oviductal cell monolayer in CO2 incubator up to 8 days. After 3 days of culture, 40 µl of the culture medium was replaced with fresh medium. Every day, embryos were examined under inverted microscope to evaluate the development. On Day 7, the percentage of good quality transferable embryos (TE) - i.e. tight morulae (TM) and blastocysts (BL) graded 1 and 2 was recorded. All TE yield data reported in this study was from the Day 7 assessment. However, the blastocyst evaluation was reported on Day 8 to assess whether TM-stage embryos, recorded on Day 7 would develop into viable BL during extra 24 hours culture.

The following morphological criteria were employed in the evaluation of buffalo embryonic development *in vitro*.

1) Embryos with 2, 4, 8, 16 blastomeres were termed as **2-cell** (Plate 15), **4-cell**, **8-cell** (Plate 16), **16-cell** (Plate 17), stage, respectively.

2) **Morula**: 32 to 64 blastomeres where the cellular mass occupied most of the perivitelline space (Plate 18).

3) **Blastocyst**: Embryo with fluid-filled cavity or blastocoele, concurrent thinning of the zona-pellucida and having the general appearance of a signet ring (Plate 19).

4) **Hatched blastocyst**: Embryo that had undergone the process of hatching (Plate 20) or had completely shed zona pellucida (Plate 21).

5) **Arrested 2-16 cell embryo**: Embryos at 2-16 cell stage even after 10 days of culture.

All the procedures of IVM-IVF-IVC were carried out in highly sterile conditions under laminar flow cabinet to avoid bacterial/fungal contamination.

### 3.14 Experimental design:

### 3.14.1 Experiment 1. Effect of frequency of OPU on the oocyte recovery and embryo production *in vitro*

The aim of this experiment was to examine the effect of aspiration frequency on the number of follicles available for aspiration, follicles aspirated, oocytes recovered and embryo yield. Ovum pick up was carried out once a week for 16 weeks and twice a week for 8 weeks in each buffalo. Oocytes were then graded and only grade A and grade B were selected for IVEP. Oocytes were cultured in TCM-
199 supplemented with 10% steer serum, 50 μM/ml cysteamine and 0.02 U/ml pFSH for in vitro maturation in CO₂ incubator for 24 hr. The processing of frozen thawed semen was similar to that adopted for insemination of oocytes collected from slaughterhouse ovaries. The oocytes were inseminated and evaluated for cleavage after 40-42 hr post insemination. The cleaved embryos were cultured for a period of 7 days for screening the embryo development.

3.14.2 Experiment 2: Effect of breeding seasons on the transvaginal ultrasound guided oocyte recovery and embryo production in vitro

The aim of this experiment was to examine the effect of breeding season on the number of follicles available for aspiration, follicles aspirated, oocytes recovered and embryo yield. Ovum pick up was carried out once a week for 8 weeks and twice a week for 4 weeks during the low-breeding (March-August) and peak-breeding (September-February) seasons (Shukla et. al., 1973; Chohan 1998). The oocyte grading, maturation, fertilization and embryo culturing was similar to that of experiment 1.

3.14.3 Experiment 3: Effect of superstimulation on the transvaginal ultrasound guided oocyte recovery and embryo production in vitro

The objective of this experiment was to examine the effect of superstimulation during both low and peak breeding seasons on the number of follicles available for aspiration, follicles aspirated, oocytes recovered and embryo yield. All the animals were superstimulated during low and peak breeding seasons. The estrous cycle of the animals was synchronized by administration of 25mg of PGF2α (Lutalyse, Novarties,Mumbai) as intramuscular (i.m.) injection on day –13. Luteolysis was induced by administration of second PGF2α injection on day 0 of the experiment. All the animals received 2500 I.U of PMSG (Folligon,Intervet International, Boxmeer, The Netherlands) i.m. on day –2 and OPU was performed on day 1 and day 4 of the superstimulation protocol. The oocyte grading, maturation, fertilization and embryo culturing was similar to that of experiment 1.
**Superstimulation protocol and OPU**

<table>
<thead>
<tr>
<th>Day of treatments</th>
<th>Hormone used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day (-) 13</td>
<td>25 mg PGF2α i.m.</td>
</tr>
<tr>
<td>Day (-) 2</td>
<td>2500 I.U.PMSG i.m.</td>
</tr>
<tr>
<td>Day 0</td>
<td>25 mg PGF2α i.m.</td>
</tr>
<tr>
<td>Day (+) 1</td>
<td>OPU</td>
</tr>
<tr>
<td>Day (+) 4</td>
<td>OPU</td>
</tr>
</tbody>
</table>

3.14.4 *Experiment 4: Effect of oocyte source on oocyte recovery and in vitro embryo development.*

The aim of the experiment was to examine the effect of oocytes collected from slaughterhouse ovaries by manual aspiration and from live animals by OPU technique on the oocyte recovery and embryo yield. Buffalo ovaries were collected from local abattoir during the same day when the OPU was performed. The protocols and assessment criteria of IVM-IVF-IVC were kept same as described earlier.

3.15 Cryopreservation of embryos by vitrification

Good quality morulae on day 6 (fertilization = day 0), blastocysts on day 7 and expanded blastocysts on day 8 were selected for cryopreservation. All vitrification solutions were prepared in 25mM Hepes-buffered TCM 1999 supplemented with 10% steer serum and 50μg/ml gentamicin (base medium).

3.15.1 *Experiment 1: Effect of cryoprotectants with different exposure time on post thaw survivability of vitrified embryos.*

This experiment was designed to study the effect of three different cryoprotectant solutions with three different exposure times (2, 4 or 6 min) on post thaw survivability of vitrified embryos. A total of 360 good quality morulae and 367 blastocysts were obtained *in vitro* from slaughterhouse-derived oocytes in 25 replicates. Embryos were held at 24°C in 500μl TCM 199 until vitrification. Embryos were vitrified following two step addition of cryoprotectant.
Step 1: Embryos were transferred randomly from holding medium into one of the following vitrification solutions for cryoprotectants exposure for 2, 4 or 6 min.

a. 0% ethylene glycol v/v in basal medium  
b. 10% glycerol + 10% ethylene glycol in basal medium  
c. 10% ethylene glycol + 10% DMSO in basal medium

Step 2: After the initial exposure, the embryos were transferred to a 7 μl drop of the respective vitrification solutions at higher concentration and exposed for 45 sec at 24°C.

a. 40% ethylene glycol v/v in basal medium and 0.3M sucrose  
b. 25% glycerol + 25% ethylene glycol in basal medium and 0.3M sucrose  
c. 25% ethylene glycol + 25% DMSO in basal medium and 0.3M sucrose.

During the second exposure step, embryos in 6 μl of vitrification solution were loaded into 0.25 ml straws inbetween 180 μl of dilution medium (0.5M sucrose in TCM 199). The straw was then heat sealed, and immediately after a lapse of 45 sec, the straws were plunged vertically, sealed end first, into liquid nitrogen to cover the embryos. The rest of the straw was then slowly immersed. After one week of cryopreservation, these straws were warmed in air (24°C) for 10 sec and then in water horizontally at 20°C until ice disappeared. The straw was held at the sealed end and shaken to mix the columns; 30 sec later, embryos were expelled, washed with three drops of culture medium, and within 5 min, placed into embryo culture medium droplet in CO2 incubator. The post thaw in vitro development was studied over a period of 72 hours. Non-vitrified morulae and blastocysts from the same batch of ovaries as those vitrified were cultured for 72 hours after being held at 24°C during the entire vitrification procedure. The number of morulae that could develop up to blastocyst / hatched blastocyst stage and the number of blastocysts that could develop up to expanded blastocyst / hatched blastocyst stage was recorded during the 72 hours of observation.

3.15.2 Experiment 2: Effect cytochalasin-B on the vitrification of embryos.
The aim of the study was to examine the effect of addition of cytochalasin-B (cyto-B) in the vitrification media on post thaw development of vitrified buffalo embryos.

The embryos were incubated in CO₂ incubator for 45 min in TCM 199 containing 10% SS and 7.5 µg/ml cyto-B before vitrification. On the basis of the earlier experiment the vitrification medium used was EG+DMSO as it gave better results. Embryos were vitrified in the absence (control) or presence of cyto-B in two steps as described earlier.

Step 1. 10% ethylene glycol + 10% DMSO in basal medium for 4 min.
Step 2 After the initial exposure time, embryos were transferred to a 7 µl drop of 25% ethylene glycol + 25% DMSO in basal medium and 0.3M sucrose for 45 sec at 24°C

After thawing, cyto-B treated embryos were washed to remove the cyto-B. Blastocyst, expanded blastocyst and hatched blastocyst were evaluated over a period of 72 hours post thaw in vitro culture.

3.16 Selection of oocytes for IVM by brilliant cresyl blue staining test (BCB)

The aim of the experiment was to evaluate the BCB staining test as an indirect measure of oocyte growth in order to select more competent buffalo oocytes for IVEP compared to conventional morphological selection of oocytes. The oocytes were aspirated from slaughterhouse ovaries as described earlier. Grade A and grade B oocytes were washed thrice in mDPBS (DPBS supplemented with 0.4% BSA and gentamicin (50µg/ml)). In the preliminary studies, oocytes were exposed to three different concentrations of BCB (13, 26 or 39 µM) in mDPBS for 90 min at 38.5°C in a CO₂ incubator. The oocytes were then transferred to mDPBS and washed twice. After washing, the oocytes were examined under stereozoom microscope and divided into two groups according to their ooplasm coloration: a) oocytes with blue coloration of the ooplasm, designated as BCB (+) and b) oocytes without blue ooplasm, designated as BCB (-). Based on the preliminary studies, 26 µM of BCB was selected to test the developmental competence of oocytes for IVEP. The BCB (+) and BCB (-) oocytes were evaluated for maturation, fertilization and cleavage and embryo
development. Some of the aspirated oocytes were used for culture without subjecting to BCB (control). Oocytes in a holding control group were incubated in mDPBS for 90 min without BCB staining to exclude the influence of the storage in mDPBS itself.

A total of 567 oocytes, over 8 replicates, were randomly distributed in the four maturation groups. A sample of oocytes (N=297) were fixed 24 hr post-IVM for the assessing maturation status and the remaining (N=270) were inseminated and then fixed to evaluate pronuclear formation, incidence of polyspermy and asynchronous fertilization. In next experiment, a total of 818 oocytes, over 15 replicates (sometimes 2 droplets per day) were randomly assigned to the four different experimental maturation groups and then fertilized and cultured in order to assess embryo development.

3.17 *In vitro* developmental competence of oocytes

The aim of the experiment was to examine the developmental competence of oocytes collected from slaughterhouse ovaries with different morphofunctional state. The paired ovaries were collected individually from the slaughtered buffaloes and divided into 5 groups on the basis of the following criteria (according to their morphofunctional state):

Group 1: Ovaries with corpus haemorrhagicum, many follicles of varying diameter and without dominant follicle, (CH-No DF)
Group 2: Ovaries with mature functional corpus luteum, many follicles of varying diameter and with dominant follicle, (CL-DF)
Group 3: Ovaries with mature functional corpus luteum, many follicles of varying diameter and without dominant follicle, (CL- NO DF)
Group 4: Ovaries with regressing corpus luteum, many follicles of varying diameter and with dominant follicle, (RCL-DF)
Group 5: Ovaries with out any luteal structures and with small follicles, (Anestrus)
The dominant follicle was characterized as the one which has more than 10 mm diameter and exceeding the diameter of other subordinate follicles. (Baruselli et al., 1997).

The oocytes were aspirated from each group of the ovaries and subjected to in vitro maturation, fertilization and subsequent embryo culture.

### 3.18 Effect of antioxidants on IVEP

The aim of the study was to examine the effects of three antioxidants (taurine, cysteamine, and melatonin) on maturation, fertilization, cleavage of buffalo oocytes and subsequent embryo development in vitro. The oocytes were aspirated from slaughterhouse ovaries as described earlier and grade A and grade B oocytes were cultured in the following media:-

(i) TCM 199 + 0.02 U pFSH +10% SS,
(ii) TCM 199 + 0.02 U pFSH +10% SS + 1 mM Taurine,
(iii) TCM 199 + 0.02 U pFSH +10% SS + 3 mM Taurine,
(iv) TCM 199 + 0.02 U pFSH +10% SS + 6 mM Taurine,
(v) TCM 199 + 0.02 U pFSH +10% SS + 50 µM Cysteamine,
(vi) TCM 199 + 0.02 U pFSH +10% SS+ 100 µM Cysteamine,
(vii) TCM 199 + 0.02 U pFSH +10% SS + 10 µM Melatonin,
(viii) TCM 199 + 0.02 U pFSH +10% SS+ 50 µM Melatonin and
(ix) TCM 199 + 0.02 U pFSH +10% SS+ 100 µM Melatonin.

Oocytes were inseminated in vitro for evaluation of rates of fertilization and cleavage. The cleaved embryos were cultured up to morula /blastocyst stage in the same formulations of media except for use of FSH during oocyte maturation.

### 3.19 Statistical analysis

The statistical software “Graph pad Prism” (Graph Pad Inc., U.S.A.) was used for the statistical analysis. The percentage values were subjected for arcsine transformation before applying statistical analysis. The difference between the mean
values of all parameters were tested by ‘ t ’ test wherever the comparison was made between two treatment groups and by Analysis of variance followed by Bonneforni multiple comparison test wherever the comparison was made among more than two groups. Differences between the mean values were considered significant when the probability values were less than 0.05.