II. REVIEW OF LITERATURE

2.1 IVEP technology: Present status in buffaloes.

Fertilization is a complex process resulting from the union of the spermatozoa and oocyte. This signals the start of the transition of the oocyte into an embryo. In vitro fertilization (IVF) provides the ability for the union of gametes during karyogamy and early embryo development to occur in a controlled laboratory environment. The main focus of research in buffalo IVEP has been to solve the problems of the low rate of embryos developing to the blastocyst stage (Gasparrini, 2002).

The integration of in vitro embryo production into conventional breeding programme could increase the rate of genetic improvement by 20 to 25% (Nicholas, 1996). In vitro Production of embryos (IVP) is instrumental in the progress of related areas like sperm sexing (Schenk et. al., 1999), embryo sexing (Bredbacka et. al., 1995), cryopreservation of gametes and embryos (Vajta, 2000), sperm injection (Sutter et. al., 2000) and emerging biotechniques like transgenesis (Eyestone, 1999), chimera production (Suzuki, 2001) and cloning (Westhusin et. al., 2001). The combined use of IVEP, gender sorted sperm-cells and embryo transfers to produce offspring of a predicted sex are the most sought after reproductive technique of all time. In spite of numerous protocols adopted for the production of embryos in vitro, the rate of transferable embryo yield remains low [cattle: 30-60%; Holm et. al., (1999); buffalo: 15-30%; Gasparrini (2002), Nandi et. al., (2002)].

The common method of IVP of embryos involves culturing of primary oocytes aspirated from tertiary follicles for maturation (IVM), fertilizing the secondary oocytes with capacitated spermatozoa (IVF) and culturing of the resulting embryos until morulae and blastocysts stage (IVEC), which can be transferred non-surgically to the recipients (ET) or cryopreserved for future use.

Since the birth of the first buffalo calf from IVP embryo (Madan et. al., 1991), various researcher’s demonstrated the effects of different protocols and media conditions on oocyte and embryo development. However, practical use of IVP
embryos was limited due to high cost and low overall efficiency under field conditions. Though well advanced and already in commercial applications in cattle, *in vitro* embryo production systems in buffaloes are comparatively more sub-optimal and require substantial improvements (Palta and Chauhan, 1998; Gasparrini, 2002; Nandi *et. al.*, 2002). Approximately 80% of buffalo oocytes fail to develop to blastocyst, a high percentage of blastocysts are incapable of developing to term and the current oocyte or embryo culture conditions seem to be sub optimal (Nandi *et. al.*, 2002). The present scenario of different aspects of buffalo IVEP is tabulated below.

<table>
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<tr>
<th>Aspects of IVEP</th>
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<tr>
<td>Good quality oocyte recovery (per ovary)</td>
<td>0.4</td>
<td>Totey <em>et. al.</em>, (1992)</td>
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<td>2.4</td>
<td>Kumar <em>et. al.</em>, 1997</td>
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<td><em>In vitro</em> maturation rate (%):</td>
<td>60-85, &gt;90; 92-95</td>
<td>Nandi <em>et. al.</em>, (2001a); Gupta <em>et. al.</em>, (2001b)</td>
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<td>Gasparrini <em>et. al.</em>, (2006)</td>
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<td><em>In vitro</em> fertilization rate (%):</td>
<td>60-70; 78-84</td>
<td>Nandi <em>et. al.</em>, (1998); Gupta <em>et. al.</em>, (2001b)</td>
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<td>Chohan and Hunter (2003a)</td>
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<td>Cleavage rate (%):</td>
<td>40-50, 46-67, 55-78</td>
<td>Chauhan <em>et. al.</em>, (1998b); Nandi <em>et. al.</em>, (2001b)</td>
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<td>Gasparrini <em>et. al.</em>, 2003, 2006</td>
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<tr>
<td>Pregnancy rate (%):</td>
<td>25</td>
<td>Madan <em>et. al.</em>, (1994a); Galli <em>et. al.</em>, (1998)</td>
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<td></td>
<td>16</td>
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<td>Calf production (%):</td>
<td>10.5</td>
<td>Madan <em>et. al.</em>, (1994b); Chauhan <em>et. al.</em>, (1997)</td>
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<td></td>
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<td>Hufana-Duran <em>et. al.</em>, 2004</td>
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### 2.2 IVEP using oocytes from slaughterhouse ovaries

#### 2.2.1 Retrieval of oocytes

Abattoir derived ovaries provide an economic and abundant source of oocytes. Though several methods of oocyte recovery (aspiration of follicles, slicing of ovaries, and puncture of follicles) are available, aspiration is the most widely accepted technique owing to its speed of operation (Das *et. al.*, 1996). The overall yield of acceptable quality oocytes per ovary in cattle (10, Gordon and Lu, 1990) was much
higher than that obtained in buffalo (0.4, Totey et al., 1992; Madan et al., 1994b; 0.6, Das et al., 1996; 1.76, Samad et al., 1998; Nandi et al., 2000a). Low oocyte yield in buffalo may be due to i) considerable low number of primordial follicles reserve [12,000 in Surti buffalo (Danell, 1987) and 19,000 in Nilli- Ravi buffalo (Samad and Nasseri, 1979] as compared to 1,50,000 in cattle (Erickson, 1966), ii) low number of antral follicles at all stages of estrous cycle (Ty et al., 1989 and Kumar et al., 1997), iii) a high incidence of deep atresia (Ocampo et al., 1994) and iv) slaughtering of sub fertile, unproductive, aged and poor body condition animals.

2.2.2 In vitro maturation of oocytes

In conventional in vitro maturation studies, oocytes were cultured in groups in 50-100 μl droplets of TCM-199 supplemented with serum and gonadotrophins at 38.5°C under 5% CO2 in air for 24 hours (Palta and Chauhan, 1998; Gupta et al., 2002) and with several other additives. The maturation rate in vitro was assessed by various methods like staining of the oocytes (M-II stage), identification of extruded first polar body in the perivitelline space and degree of expansion of cumulus cell mass (Raghu et al., 2002a). Use of chemically defined media is now being tested for oocyte culture as it avoids the possible effects of unknown components in biological fluids. Different free radical scavengers like taurine (Hardy, 1997), cysteamine (Gasparrini et al., 2000, 2003, 2006), melatonin (Ishizuka et al., 2000; Poleszczuk et al., 2004) and others were also being examined for their efficacy in oocyte or embryo culture media.

2.2.3 In vitro fertilization

In vitro matured buffalo oocytes were co-incubated with frozen-thawed in vitro-capacitated spermatozoa in Tyrode’s modified medium (TALP, Totey et al., 1996) or Brackett and Oliphant (BO, Nandi et al., 1998) medium for fertilization. Although fresh semen gave better fertilization rates than frozen-thawed semen (Totey et al., 1992), the practicality of using fresh buffalo semen in IVF was negligible due to changes in buffalo semen quality with season.

2.2.4 In vitro embryo culture

The different systems widely employed for the in vitro culture of the embryos were: (a) culturing of the cleaved embryos in the ligated oviducts of rabbit and sheep
(Totey et al., 1992); (b) culturing cleaved embryos in vitro in complex medium (TCM-199, B2, Minimum Essential Medium) supplemented with serum and somatic cells viz; oviductal epithelial cells, cumulus cells, buffalo rat liver cells (Gasparrini, 2002); c) culturing cleaved embryos in vitro in semi-defined media (Raghu et al., 2002a) and d) culturing cleaved embryos in vitro in defined media (Boni et al., 1999).

2.3 IVEP using oocytes recovered from live Buffaloes (Ovum pick up)

Ovarian follicular development in buffaloes has not been studied in detail. On the basis of histological evaluation, Danell, (1987) reported 12,636 primordial follicles in the ovaries of cyclic buffalo heifers and the incidence of atresia of follicles was more (66%) than that of in cattle (50 %). In addition, buffalo ovaries contain only about 20% of the number of antral follicles found in cattle ovaries (48 vs. 233; Ty et al., 1989). Various workers have reported seasonality of breeding in buffaloes and summer was associated with lower preovulatory oestradiol concentration (Shafie et al., 1982), which might indicate lower rate of follicular development. Karaivanov (1986) reported seasonal difference in response to superovulation (SO) treatment in Bulgarian Murrah buffaloes with higher response in summer than in spring. In another study in Murrah buffaloes, Taneja et al., (1991) observed no seasonal difference in the SO response in terms of number of CL or embryos recovered. Further studies by Taneja et al., (1995) assessed the follicular dynamics in superovulated Murrah buffaloes through ultrasound scanning. They found greater number of small and medium-sized follicles available before the start of the superovulatory treatment during the summer season than during the wet cool season. There was no significant difference between the summer and wet cool seasons in the number of corpora lutea detected by rectal palpation (2.8 ± 0.7 vs. 2.2 ±0.6) and the yield of embryos on day 6 (0.2± 0.2 vs. 0.6 ± 0.2). However, summer season was found to be detrimental to embryo quality, as none of the embryos recovered were transferable (Taneja et al., 1995).

Ovaries from slaughtered animals represent an important source of gametes that can be successfully used for IVEP. Usually majority of buffaloes slaughtered are
either sub-fertile, unproductive, aged or with poor body condition. The use of ovaries of such slaughtered buffaloes as oocyte source may have poor impact on genetic improvement.

Oocytes from the valuable live donors are being recovered by transvaginal ultrasound guided oocyte collection (Ovum pick-up, OPU). This technique allows repeated retrieval of oocytes from the superior female animals, which can be used for IVEP to maximize the contribution of genetically superior females in a breeding programme. The OPU has been performed even in clinically sub fertile, infertile cattle (Looney et al., 1994; Hasler et al., 1995) and buffaloes (Manik et al., 2002).

The technique of transvaginal ultrasound guided follicular aspiration as a method for recovery of oocytes was first described by Wikland et al., (1987) in humans. The technique was first employed in non-stimulated cows weekly once or twice by Pieterse et al., (1988). It was performed over a period of three months with no adverse effects on their future fertility (Pieterse et al., 1991). Repeated use of the OPU technique yielded a large quantity of meiotically competent oocytes from the individual bovine donors (Pieterse et al., 1988, Kruip et al., 1993, Bungartz et al., 1995). These oocytes were suitable for in vitro embryo production programs that supplied transferable embryos and live calves (Kruip et al., 1993, Looney et al., 1994).
Following successful trials of OPU in cattle, efforts were made to exploit the technique in anoestrus buffaloes (Boni et. al., 1994). The OPU technique has been successfully applied in mature buffaloes (Boni et. al., 1995; Pavasuthipaisit et. al., 1995; Kitiyanant et. al., 1995; Boni et. al., 1996; Galli et. al., 1998, 2001; Manik et. al., 2002), prepubertal buffaloes (Presicce et. al., 2002) and buffalo calves (Techakumphu et. al., 2004). However, the success in terms of available follicles for aspiration and quality of oocytes suitable for IVEP was low.

The average yield of oocytes from non-stimulated buffaloes by OPU technique was 1.33 per ovary per collection in Mediterranean buffaloes (Boni et. al., 1994), of these only 31.3% were suitable for in vitro embryo production. In another study on OPU in cycling swamp buffaloes, Pavasuthipaisit et. al., (1995) recorded an average of 9.8±2.4, 6.0±1.5 and 7.7±1.9 follicles available for aspiration with a recovery of 5.4±1.5, 3.0±1.4 and 4.3±1.1 oocytes on day 1 or 2, 8 or 9 and 15 or 16 respectively of the estrous cycle. In a study in Mediterranean buffaloes, average of 5.48 follicles were available for aspiration, with the oocyte recovery rate of 49.4%, of which only 53.5% were suitable for IVEP. An average of 2.71 oocytes were collected per animal, when twice a week schedule was followed (Boni et. al., 1996). The percentage recovery of grade A (>1 layer of cumulus cells), grade B (1 layer of cumulus surrounding more than 70% of the oocyte surface), grade C (very few corona radiata cells or expanded cumulus cells or relatively naked oocyte) and grade D (degenerated oocyte) oocytes were 23.5, 30.0, 36.9 and 9.6% respectively.

In another study in Murrah buffaloes with reproductive problems (Manik et. al., 2002), the mean number of follicles available on twice a week aspiration was 5.1, with oocyte recovery rate of 35% (56 oocyte/165 follicles), of which only 43% (24/56) were suitable for in vitro embryo production. The mean numbers of oocytes recovered and suitable for in vitro embryo production per animal per session were 1.7 and 0.7 respectively. However OPU did not significantly affect the subsequent population of follicles or their size.

In river buffaloes, Huang et. al., (2005) carried out OPU at 4-day intervals and recovered 66 oocytes per 100 follicles aspirated, of which 91.1% (oocytes with at least one layer of granulosa cells) were suitable for IVM. In a recent report, in cyclic
Murrah buffaloes (Gupta et al., 2006) the mean number of small, medium, large and total follicles per OPU session once a week was 2.2 ±0.3, 0.6 ±0.2, 0.9± 0.1 and 3.7± 0.3 respectively. A mean number of 3.0±0.3 follicles were punctured and 2.0±0.3 oocytes recovered per animal per session, with a recovery rate of 68%. The oocytes of grade A and B together and grade C and D together were 59 and 41% respectively.

2.3.1 Factors affecting OPU-IVP

The success of OPU is measured by recovery rate of oocytes and expressed as a percentage of the number of follicles aspirated in cattle (Pieterse et al., 1988). The oocyte recovery rate is influenced by variety of factors such as, aspiration vacuum pressure, hormonal pre-treatment of animals (Pieterse et al., 1992; Meinjtes et al., 1993; Bungartz et al., 1995), frequency of puncture (Gibbons et al., 1994; Broadbent et al., 1997; Garcia and Salaheddine 1998; Goodhand et al., 1999), stage of the estrous cycle (Vos et al., 1994) and experience of the operator (Scott et al., 1994). These factors make it difficult to compare recovery rate of oocytes; since different researchers follow different procedures. However contemporary studies are lacking in buffaloes, with information available on these factors being very sparse. The physiological state of the ovary seems to influence the quality of oocytes more than the days of postpartum in buffaloes (Boni et al., 1997). The OPU conducted on 6th day of cycle resulted in an average of 46.8% good quality COC, while OPU on days 7-11 yielded only 25% good quality COC (Boni et al., 1997)

2.3.1.1 Aspiration vacuum pressure

The available information regarding the most appropriate vacuum pressure for follicular aspiration is contradictory. The aspiration vacuum pressure is expressed in millimeters of mercury and has varied between 40 and 400 mmHg (Pieterse et al., 1988). The exact aspiration vacuum at the tip of the needle depends on the construction of the OPU device, the length and diameter of the tubing system and the diameter of the needle (Bols et al., 1995). In hormone (FSH) stimulated prepubertal swamp buffaloes, Techakumphu et al., (2005) collected oocytes with three vacuum pressures (90, 100 or 120 mmHg). The vacuum pressure used for the oocyte retrieval technique influenced the oocyte recovery rate, but not the oocyte quality.
2.3.1.2 OPU session interval

When OPU was performed effectively, all follicles of >2-3 mm in diameter were aspirated, which subsequently induces growth of new follicles of 2-3 mm over the following days. The interval between two OPU sessions significantly affects quality and quantity of oocytes (Gibbons et. al., 1994; Broadbent et. al., 1997; Garcia and Salaheddine 1998; Goodhand et. al., 1999) and embryo production rate (Hanenberg et. al., 1997) in cattle. In buffaloes, Boni et. al., (1996) performed OPU twice weekly, every 3 to 5 days. The interval between two consecutive OPU (intersession interval) did not affect either the number of aspirated follicles or the number of oocytes collected. The increase in the intersession interval in buffalo caused an increase in the incidence of large follicles and decrease in number of small follicles and the quality of oocytes collected.

2.3.1.3 Pre-stimulation with Gonadotropins (FSH/eCG)

Over the years, several superstimulation protocols have been applied to the oocyte donors, prior to OPU in cattle (Vos et. al., 1994; Bungartz et. al., 1995; Bols et. al., 1995; Goodhand et. al., 1999) and in buffaloes (Boni et. al., 1994, Promdireg et. al., 2000, Promdireg et. al., 2005). The OPU is currently applied in superstimulated, as well as non-superstimulated animals. The advantage of superstimulation was obvious with more follicles to aspirate, more oocytes retrieved, more embryos produced and less work as compared to non-superstimulated OPU. But the reports of the effects superstimulation on embryo production have been conflicting in cattle (Bungartz et. al., 1995; Blodin et. al., 1996; Goodhand et. al., 1999).

The effect of gonadotropin pre-stimulation on oocyte competence depends on the stimulation procedure, type of gonadotropin used, time period between last FSH injection and oocyte retrieval (coasting period, Hendriksen et. al., 2000). When heifers were treated for 60 hour with PMSG until ovariectomy, oocytes from ≥ 8mm follicles yielded higher blastocyst rate than oocytes from 5-8mm follicles from the same ovaries (41 vs 25%). The difference was apparent in both cleavage and post-cleavage development. The competence of the oocytes from ≥ 8mm follicles from 60
h PMSG-stimulated heifers did not differ from that of oocytes from ≥ 11mm follicles from 104 h PMSG/PG-stimulated heifers (41 vs 39% blastocyst), indicating that prolonged treatment did not improve the proportion of competent oocytes of the largest follicles (Hendriksen et. al., 2000). The FSH superstimulated pre-pubertal swamp buffaloes (Techakumpu et. al., 2000) showed more significant ovarian response than PMSG treatment (13.9±8.6 vs. 5.9±3.3 follicles recorded by laparotomy) and there was no difference in the type/quality of oocytes retrieved between FSH and PMSG treatments.

In buffaloes, FSH priming 24 h before OPU increased both the number of punctured follicles (6.17 vs 1.33) and the number of oocytes collected (3.00 vs. 1.33) as well as the quality of the oocyte (55.6% vs. 31.3%: Boni et. al., 1994). In another study, OPU in PMSG treated swamp buffalo heifers, an average of 6.6±1.1 follicles with a diameter of 7.1±4.1 mm was recorded (Promdireg et. al.,2000). In this study, the mean number of oocytes collected per animal was 4.1±2.0, with a recovery rate of 63%, of which 17.2% oocytes had single layer of cumulus cells, 10.3% partial cumulus cells, 48.3% denuded oocytes, 3.5% expanded cumulus oocytes and 20.7% only zona pellucida. The OPU in PMSG treated prepubertal Mediterranean Italian buffalo calves (Presicce et. al., 2002) did not increase the number of ovarian antral follicles available for aspiration compared to the control group, but resulted in higher number of medium and large follicles and expanded oocytes.

In a recent report of OPU in superstimulated (FSH) cycling and lactating postpartum swamp buffaloes (Promdireg et. al., 2005), the number of follicles aspirated (7.2±3.7 and 9.0±3.2) and oocytes recovered (3.7±2.7 and 5.9±3.5, respectively) were not significantly different. In non-stimulated cycling and lactating postpartum swamp buffaloes animals, the number of follicles aspirated (2.12±1.4 and 1.4±0.7) and oocytes recovered (1.4±1.3 and 0.7±0.8), respectively was also not significantly different. FSH administration was shown to increase the number of aspirated oocytes in both cycling and lactating postpartum buffaloes (Promdireg et. al., 2005).

2.3.2 Efficiency of OPU - IVEP
The production of transferable embryos from OPU-IVM-IVF-IVC in buffaloes is 1.8-2.8 fold less than that in cattle, yet it is about 2-3 times more than that produced using the in vivo embryo production system (MOET) in buffaloes (Aboul-Ela 2000). The first report on the normal development of the OPU-IVP embryos to term in Italian buffaloes was reported by Galli et al. (1998). In swamp buffaloes, Kitiyanant et al. (1995) reported 87.5% in vitro maturation, 67.8% cleavage and 26.7% morula/blastocysts from grade 1 oocytes recovered through ultrasound guided OPU. In a study in cyclic Mediterranean buffaloes with long postpartum period (>500 days), Boni et al. (1996) reported 55.6% cleavage rate and 16.7% expanded blastocyst yield. In another study in cyclic Mediterranean buffaloes with short postpartum period the IVEP efficiency in terms of morulae and blastocyst was 18.3% (Boni et al., 1997). A study in Italian Mediterranean buffaloes (Galli et al., 2001) reported 40.1% cleavage rate and 0.7 freezable embryos per OPU session. Neglia et al., (2002) reported first pregnancies in buffaloes established from in vitro produced vitrified blastocysts from OPU derived oocytes. A total of 21 blastocysts were produced in vitro, using 245 COCs. In another study, Neglia et al., (2003) reported a higher Blastocyst yield (29.7% vs 19.9%) and a lower proportion of embryos arrested at tight morula stage (11.1% vs 22.3%) from OPU derived oocytes as compared to oocytes derived from abattoir ovaries. Recently, Huang et al., (2005) reported 14.8% blastocyst yield using OPU oocytes from river buffalo. Even though, transferable embryo production efficiency is considered to be less than half that expected in cattle, it can be deduced that in a year's time with an OPU interval of 3-4 days, it may be possible to expect an average yield of about 15.7 to 34.6 transferable embryos per buffalo (Zicarelli, 1997).

2.4 Cryopreservation of in vitro produced embryos

The aim of cryopreservation is to preserve embryos in a suspended animation, from which they can be revived after a short or long period of storage to continue their normal development. A major obstacle in large-scale commercial application of IVEP in buffaloes was the lack of a suitable cryopreservation method. In view of the fact that the development and commercial application of standard programmable embryo freezing methodologies, many new embryo cryopreservation technologies had been developed in the last few years. The most notable alternative to programmable embryo freezing had been the application and development of
vitrification technology for cryopreserving embryos. Conventional slow-rate programmable freezing and vitrification of embryos had given the Veterinarians, the scientists and the producers, alternatives in their herd reproduction practices. Rall and Fahy, (1985) introduced vitrification as a method to cryopreserve mammalian embryos in the absence of ice. Vitrification is a physical process by which a solution is transformed into a stable amorphous glass by rapid cooling, bypassing ice crystal formation while maintaining the properties of the liquid in a solidified form (Rall, 1987). The application of vitrification under field conditions reduced the equipment needed and the technical skill required and provided considerable saving on cost and time per embryo transferred (Van Wagendonk-de Leeuw et al., 1997). A modified vitrification procedure called Open Pulled traw (OPS) vitrification was developed subsequently (Vajta et al., 1999).

Cryoprotectants, the organic solutes that help to protect cellular organelles during cryopreservation, can also damage the cytoskeleton system as they can be toxic and cause disruptive osmotic damage to the cell. Cellular disruptions, particularly to the cytoskeleton and mitochondria of embryos, during cryopreservation were studied by Dobriansky et al., (1996). Although many cryoprotectants function to depolymerize the microfilaments and microtubules prior to cryopreservation, they might cause irreparable damage to cytoskeletal components. Not all cryoprotectants were consistent in their interaction with cellular organelles and the cytoskeleton of embryos across species. Cryoprotectants generally used for vitrification of embryos fall into two categories namely intracellular and extracellular. Of the intracellular cryoprotectants, which were of low molecular weight permeates all cells of the embryo; glycerol and Dimethyl sulfoxide (DMSO) were initially most commonly used. The extracellular cryoprotectants are larger molecules, such as sugars and proteins (e.g., sucrose, hyaluronic acid, BSA). The mechanism by which such molecules provide cryoprotection is not well understood. BSA appears to protect the embryo in the immediate post-thaw phase by helping to stabilize the cell membrane (Rall, 1987).

Embryo survival depends on type of cryoprotectants, species, and stage of development, as well as the culture systems used for IVEP. Ultrastructure of cryopreserved blastocysts derived by in vivo and in vitro methods revealed that the
embryo morphology was affected by exposure to cryoprotectants or cryopreservation (Fair et al., 2001). The porcine embryos are very sensitive to cooling as they have fragile plasma membrane (Dobrinsky et. al., 2000). To prevent or to overcome cytoskeletal disruption of the porcine embryos during and after cryopreservation, a cytoskeletal stabilizer, cytochalasin, was added in the vitrification solutions (Dobrinsky et. al., 2000). The morulae or the early blastocysts did not survive cryopreservation either with or without cytochalasin. However, vitrification under the influence of cytochalasin improved the survival rates of expanded and hatched blastocysts.

During the past decade, ethylene glycol (EG) had been effectively employed as a cryoprotectant for cattle embryo preservation (Saha et. al., 1996; Saha and Suzuki 1997; Bai et. al., 2000; Ponsart et. al., 2000). The EG was proved to be nontoxic for murine embryos (Valdez et. al., 1992). Bai et. al., (2000) reported a high survival rate and hatching rate after freezing embryos in 1.8 M EG + 0.05 M sucrose. Ponsart et. al., (2000) recorded a pregnancy rate of 55.4% following the transfer of bovine embryos cryopreserved with EG and 47.2% with glycerol + sucrose combination. Improvement of embryo survival after freezing could be achieved by changing the conditions of their culture, selecting embryos based on the kinetics of their development and changing standard cryopreservation procedures (Rizos et. al., 2001). The resulting pregnancy rates in cattle for both conventional cryopreservation (45.1%) and vitrification (44.5%) were nearly identical (Gordon, 2003).

Cryopreservation techniques for preserving buffalo oocytes (Das et. al., 1999; Dhali et. al., 2000), spermatozoa (Fabbrocini et. al., 2000) and in vivo (Apimeteetumrong et. al., 1998) and in vitro produced embryos (Nandi et. al., 2003; Hufana-Duran et. al., 2004) have been employed. However, intrinsic biological problems like high chilling sensitivity and high lipid content in buffalo oocytes and/or embryos have impeded the progress in cryopreservation. Information on cryopreservation of in vitro produced buffalo embryos by vitrification and programmable freezing is scarce. Cryopreservation of in vitro-produced buffalo embryos using slaughterhouse derived oocytes was still in infancy stage where the embryo survivability rates were compromised (Nandi et. al., 2002). Nandi et. al., (2003) reported higher hatching rates in programmable frozen blastocysts derived
from culturing cleaved embryos in defined medium (mSOF) than in the complex co-culture system (30.4 vs 20.1). In another study in buffaloes, the viability of *in vitro*-derived vitrified-thawed buffalo embryos in terms of *in vitro* hatching rate and birth of live calves were 83.10 % and 10.91 % respectively (Hufana-Duran *et. al.*, 2004). A major limiting factor in buffalo embryo cryopreservation is the poor freezability of *in vitro* produced embryos, which may be related to their high lipid content (Gasparrini 2002). From the above review, it is evident that there is scope to improve the techniques for better embryo yield and cryopreservation of *in vitro* produced buffalo embryos.

### 2.5 Selection of developmentally competent oocytes

Majority of the buffalo oocytes fail to develop to morula-blastocyst stage following *in vitro* maturation, fertilization, and culture. The sub optimal culture conditions might be a contributing factor. Immature oocytes obtained from buffaloes with reduced reproductive performance or buffaloes slaughtered at the end of their reproductive life were heterogeneous in quality and with low developmental competence.

The selection of oocytes for *in vitro* embryo production (IVEP) was made on the basis of compaction of cumulus corona investment and the homogeneity of ooplasm (Chauhan *et. al.*, 1998a). Selection of oocytes based only on morphological criteria may result in low maturation rate and the overall embryo yield, since some of the oocytes might have already started degenerating by the time they were retrieved from the ovaries. It is essential to know the viability of oocytes before using them for IVEP. Staining of oocytes with trypan blue (0.05%) for 2 minutes was one such technique generally used to differentiate live and dead buffalo oocytes without adversely affecting the maturation, fertilization and subsequent embryonic development (Gupta *et. al.*, 2002a). With the aim of establishing a non-invasive method for selecting more homogeneous and competent oocytes, the brilliant cresyl blue staining (BCB) had been used. After staining, the oocytes with blue coloration of the ooplasm were designated as BCB (+) and those without blue coloration were designated as BCB (-). The brilliant cresyl blue (BCB) assay was found to be useful to select more competent oocytes for *in vitro* embryo production in pig (Ericsson *et. al.* *et.*
pre pubertal goats (Rodriguez-Gonzales et. al., 2002 and 2003; Urdaneta et. al., 2003), heifers (Pujol et. al., 2000, 2004) and cows (Alum et. al., 2005).

In a study in prepubertal goats, Rodriguez-Gonzales et. al.,(2002) recorded 29.4% oocytes to be BCB (+) with a mean diameter of 136.6±6.3 µm which was higher than that of BCB (-) oocytes (125.5±10.2µm). The percentage of BCB (+) oocytes (81.4%) reaching the M-II stage was higher than those of BCB (-) oocytes (52.5%). Normal fertilization rate of BCB+ oocytes was also higher (23.5%) as compared to BCB (-) oocytes (8.2%). Further, the percentage of total embryos undergoing development beyond 8-cell and the morulae / blastocyst stage were higher in the BCB (+) oocytes (41.3 and 12.0%, respectively) as compared to 21.3 and 3.6%, respectively for BCB (-) oocytes. In another study in prepubertal goats, Rodriguez-Gonzales et. al., (2003) found that the addition of cysteamine to the culture medium improved the fertilization rate of BCB (+) oocytes (40%) than those of BCB (-) oocytes (21%) and control oocytes (22%). The percentage of morulae plus blastocysts was higher in the BCB (+) group than in the BCB (-) group (23.8 vs. 5.1%). In a similar study in prepubertal goats (Urdaneta et. al., 2003), the addition of 50 µM and 100 µM of cysteamine to culture medium did not affect the proportion of total embryos obtained from BCB (+) oocytes (35.89% and 38.29%), but was significantly different in BCB (-) oocytes (34.23% and 29.04%, respectively).

A study in heifers (Pujol et. al., 2004) showed significantly higher diameter (152.6±5.8) of the BCB (+) oocytes than BCB (-) oocytes (147±5.9µm). The percentage of BCB (+) oocytes reaching the blastocyst stage was also significantly higher than those of BCB (-) and control oocytes (12.3, 1.6, and 5.2%, respectively). A recent study in cattle (Alum et. al., 2005) revealed a significantly higher maturation rate of BCB (+) oocytes (72.5%) than BCB (-) oocytes (58.1%) and also the BCB (+) oocytes yielded significantly higher proportion of blastocysts (34.1%) than BCB (-) oocytes (3.9%).

2.7  *In vitro* developmental competence of oocytes collected from ovaries with different morphofunctional state
The in vitro developmental competence of buffalo oocytes was influenced by biological factors like oocyte diameter and follicle size (Tasripoo and Kamonpatana 1997; Nandi et. al., 2000a, b, Raghu et. al., 2002b). However, the determination of these parameters is labour intensive and hardly compatible with routine production of a high number of embryos. Aspiration of oocytes from slaughtered buffalo ovaries without having a corpus luteum (CL) yielded more oocytes with higher developmental competence (Das et. al., 1996; Kumar et. al., 1997; Singla et. al., 1999; Nandi et. al., 2000a,b) but in cattle, quality oocytes could be obtained from ovaries in the luteal phase and from ovaries bearing the corpus luteum (Boediono et. al., 1995). In contrast, Gupta and Sarma (2001) reported no significant effect of CL on buffalo oocyte recovery. In buffaloes, the fertilization and cleavage remained unaffected under in vitro conditions for the oocytes recovered from ovaries with and without CL (Sajjan-Singh et. al., 2001).

The morphological evaluation of the ovary might be a useful method in predicting in vitro developmental potential of bovine oocytes (Lauria et. al., 1996; Gandolfi et. al., 1997. Varisanga et. al., 1998). Ovaries with a dominant follicle (DF, > 10 mm diameter) or with more than ten follicles of 2-5 mm diameter and no dominant follicle (DF) yielded higher quality oocytes than ovaries with fewer than ten follicles and no DF, with or without CL (Lauria et. al., 1996). In another study in cattle (Gandolfi et. al., 1997), oocytes recovered from ovaries with a follicle of >10 mm diameter and, other 10 follicles of 2 to 5 mm diameter resulted in higher maturation and blastocyst yield when compared to those recovered from the ovaries with less than 10 follicles of 2 to 5 mm diameter and no follicle of >10 mm.

On the contrary, presence of a dominant follicle in either one or both ovaries of pairs had a negative effect on in vitro developmental competence of bovine oocytes (Varisanga et. al., 1998). Results of the studies on ovarian morphology and stage of cycle supported the concept that the intraovarian environment to which oocytes were exposed could play a major role in determining their developmental competence (Varisanga et. al., 1998, Goto et. al., 1990). Shen and Lee (1999) and Hagemann (1999) reported that higher quality oocytes could be aspirated from small antral follicles in the absence of a dominant follicle. The developmental competence was significantly greater in oocytes collected during phases of follicular growth than
during phases of follicular dominance (Hagemann et. al., 1999 a, b). The developmental competence of oocytes collected by OPU during growth phase of the first follicular wave before dominant follicle selection was significantly higher (Machatkova et. al., 2000). The developmental competence of COCs appeared to be influenced by the presence of a dominant follicle (DF at the non-growing phase, Steenweg et. al., 2000). A positive relationship existed between early follicular regression and oocyte competence (Vassena et. al., 2003) and the morphologic characteristics of oocyte quality were not predictive in identifying competent oocytes. However, the maturational and developmental competence of immature oocytes was not reported to be affected by the phase of folliculogenesis in cattle (Chian et. al., 2002). Oocyte morphology and competence changed during the various phases of subordinate follicle development (Salamone et. al., 1999). Another study (Jewgenow et. al., 1999a, b) examined the developmental competence of good-quality oocytes in relation to the degree of apoptosis evident in the follicle wall and other ovarian features and it was concluded that the degree of apoptosis did influence the developmental competence, even of good-quality oocytes. The IVM rates (Chohan and Hunter, 2003b) of the oocytes recovered from different reproductive status in bovines ranged from 89.8% to 95.4% (follicular, metestrus and diestrus stages of the estrous cycle and in anestrus). However, the fertilization rates were lower for oocytes from the diestrus phase (72.4%) than from the other phases (range: 81.1 to 86.6%). Oocytes, recovered during the metestrus phase of the estrous cycle resulted in the highest cleavage rate (60.0%), while oocytes from the diestrus phase had the poorest embryonic development (39.8%; p<0.05). Majority of the embryos from all reproductive phases showed a developmental arrest around 8-cell stage. The developmental competence of oocytes from pregnant and anestrus animals was lower than that from the other reproductive stages.

2.8 Oxidative stress in oocyte-maturation and embryo-development

The major factors limiting the commercial use of IVEP in buffalo were low number of oocytes recovered, low cleavage rates and poor success in cryopreservation of IVP embryos. It was likely that buffalo oocytes/embryos are particularly sensitive to oxidative stress due to their high lipid content (Boni et. al., 1992). Reactive oxygen species (ROS) production is a normal process of cell metabolism. In vitro environments usually increased production of ROS, which often is implicated as the
main cause of cell damage. Ample evidence support that the ROS in oocyte-maturation and embryo development culture affect the IVP of cattle and buffalo embryos (De Matos and Furnus 2000; Gasparrini et. al., 2000; Gasparrini et. al., 2003).

Glutathione (GSH) was synthesized during oocyte in vitro maturation (IVM) in mouse (Calvin et. al., 1986), hamster (Perreault et. al., 1988), pig (Yoshida et. al., 1993), cattle (Miyamura et. al., 1995) and buffaloes (Gasparrini et. al., 2003). During maturation of the oocyte in the ovary, GSH content increased as the time of ovulation approached (Perreault et. al., 1988) forming a reservoir pool which would protect the cell in the later stages of post fertilization development (Telford et. al., 1990). Different free radical scavengers like taurine, EDTA, cysteamine, melatonin, hypotaurine were being used in oocyte culture media in different species with varying results. Funahashi et. al.,(1996) has reported that the presence of organic osmolytes such as taurine and sorbitol at 6 and 12 mM in maturation medium containing 68.49 or 92.40 mM NaCl increased pig oocyte glutathione content. In a study by Harris et. al., (2005) on nutrient concentrations in biological fluids, taurine, glycine, alanine, glutamine and glutamate were found to be the major amino acids detected and their concentrations differed in the follicular fluid, oviductal fluid and uterine fluid with higher levels in the oviductal fluid. In addition, the follicular fluid and reproductive tract nutrient profiles differed from those of murine maturation, fertilization and embryo culture media. In humans, taurine, hypotaurine and transferrin were mainly found in the tubal and follicular fluid where they protect the embryo from ROS (Guerin et. al., 2001)

It was demonstrated that low molecular weight thiol compounds such as β-mercaptoethanol and cysteamine enhanced cysteine-mediated GSH synthesis in bovine embryos (Takahashi et. al., 1993). An increase of intracytoplasmic GSH concentration caused by the addition of β-mercaptoethanol and cysteamine to the culture medium was found to be beneficial for the development of 6-8-cells stage bovine embryos to the blastocyst stage. It was demonstrated that cysteamine supplementation during in vitro maturation (IVM) improved embryo development by increasing glutathione synthesis in several species. An improved developmental competence of oocytes matured in the presence of cysteamine was also recorded in
buffalo (Gasparrini et al., 2003). Furthermore, the overall poor embryo development recorded in the presence of Buthionine sulfoxide (BSO), a specific inhibitor of GSH synthesis, demonstrated that the GSH concentration during buffalo IVM was critical for further development. Use of cysteamine in the buffalo oocyte and embryo culture media were reported to increase the intracellular glutathione synthesis, which in turn protects the cells from oxidative stress, thus increasing the blastocyst yield (Gasparrini et al., 2001).

Melatonin, a potent scavenger of reactive oxygen species (ROS) had been tested in the promotion of mouse (Ishizuka et al., 2000) and bovine (Poleszczuk et al., 2004) embryo development in vitro. Melatonin was investigated as a drug to improve oocyte quality in human patients failing to get pregnant in earlier IVF cycles because of poor quality oocytes (Takasaki et al., 2003). A significant reduction in the number of degenerate oocytes was reported, and the number of fertilized embryos increased. Increased follicular concentrations of melatonin reduced lipid peroxide concentration and might prevent the DNA damage. In bovines, a beneficial effect of melatonin was observed at $10^{-4}$ M concentration, in the embryo culture media (Poleszczuk et al., 2004).

By the foregoing review, of literature available on the works relating to in vitro production of embryos in buffaloes, it is apparent that much of the studies have been conducted using oocytes from the abattoir derived ovaries and the sparse studies with OPU have yielded limited success. Detailed studies on factors affecting OPU-IVEP are lacking. Much of transfers have been with fresh, non-cryopreserved embryos since good success with cryopreservation of buffalo embryos is yet to be achieved with further work in this aspect. Hence, the present studies were taken up with over all objective of improving the OPU-IVEP and cryopreservation in buffaloes.