V. DISCUSSION

The exploration of standard protocols of MOET followed in cattle met with limited success in buffaloes and therefore many workers utilized IVM and IVF techniques for embryo production and transfer. However, developmental blocks at 2-cell and 8-cell stages lowered the overall transferable embryo yield (Madan et al., 1996). Majority of the research on IVEP in buffaloes was carried out using the oocytes harvested from slaughterhouse ovaries (Nandi et al., 2002; Gasparrini, 2002). On the other hand, better utilization of individual buffaloes with high genetic merit can be achieved through repeated transvaginal ovum pick up (OPU), which had become more manageable through transvaginal ultrasound guided follicle aspiration (Kruip et al., 1993; Kitiyanat et al., 1995). OPU-IVEP is considered as alternative to MOET for commercial application in cattle, particularly due to unpredictable response to superovulation (Gordon, 1996).

The use of OPU and IVEP may represent a valid approach to speed up the genetic improvement by decreasing the generation interval. OPU can be applied to superior donors of all ages starting from two months-old calves to very old cows and even pregnant animals, without any side effects on the donor's reproductive career (Galli et al., 2001). The disease transmission, that is possible with slaughterhouse ovaries, can be avoided by employing OPU. Ovum pick-up (OPU) technologies have been used for repeated harvesting of oocytes from either gonadotropin stimulated or unstimulated donor animals. After the initial development of the OPU technique (Pieterese et al., 1991), many studies were undertaken with incremental improvements in bovines. However, in buffaloes the technology is yet reach viable level of improvement.

The present studies were focused on the effect of aspiration frequency, breeding season and hormonal prestimulation on the number of follicles available for aspiration, follicles aspirated, oocytes recovery, oocyte quality and in vitro embryo production.
5.1 OPU-IVEP

5.1.1 Effect of frequency of OPU on the number of follicles available for aspiration, follicles aspirated and oocytes recovered.

Compared to aspiration once a week, aspiration twice a week resulted in a significantly higher number of small, medium, large, total number of follicles recorded and follicles aspirated (Table 1). These findings were similar to that reported in cattle (Broadbent et. al., 1997; Goodhand et. al., 1999), wherein increasing the aspiration frequency from once to twice per week approximately doubled the total number of follicles observed and aspirated per week. The mean number of follicles available for aspiration and follicles aspirated in the present study were comparable to that reported in Mediterranean buffaloes (Boni et. al., 1996), Indian buffaloes with reproductive problems (Manik et. al., 2002) and in cyclic Murrah buffaloes (Gupta et. al., 2006). However, follicles available for aspiration and follicles aspirated were much lower than those observed in cattle (Fry et. al., 1997). A significantly higher number of oocytes retrieved per buffalo on two aspirations per week compared to once per week in the present study was similar to that reported in cattle (Hanenberg and van Wagendonk-de Leeuw 1997).

The aspiration rate was significantly high with aspiration frequency of once a week than that observed for twice a week in the present study. The aspiration rate was lower when compared to the other studies in buffaloes (Boni et. al., 1996; Manik et. al., 2002; Gupta et. al., 2006). The difference may be due to the fact that only the follicles of ≥3 mm diameter were aspirated out of the total follicles recorded in the present study. The cattle oocyte also acquired competence to develop into a blastocyst in an in vitro system when recovered from follicles of 2 or 3 mm diameter (Pavlok et. al., 1992; Blondin and Sirard 1995). The competence to undergo meiotic maturation up to metaphase II was attained somewhat earlier than developmental competence (Fair, 1995; Blondin and Sirard 1995; Arlotto et. al., 1996), the smaller group of follicles included 2 mm follicles that were yet to complete their growth and RNA synthesis were also not competent. The aspiration frequency did not
significantly affect the oocyte recovery rate in the present study, which was similar to that reported in cattle (Goodhand et. al., 1999).

5.1.2 Effect of frequency of OPU on oocyte quality and *in vitro* embryo production

In the present study, aspiration twice a week resulted in a significantly higher number of culturable oocytes (grade A and B) collected per week per buffalo compared to once a week aspiration, but aspiration frequency did not significantly affect the transferable embryo production rate (Table 2). Studies indicate that twice a week OPU could be done for extended period of time without any long-detrimental effects on the donor cow’s fertility (Chastant-Maillard et. al., 2003). Similarly in cattle, Gibbons et. al., (1994) reported that the aspiration frequency did not affect the developmental competence of oocytes in terms of morulae and blastocysts yield. Twice aspiration per week resulted in a significant increase in the cleavage rate, 8-cell, 16-cell embryos and transferable embryos per buffalo per week in the present study. Similarly, twice a week OPU resulted in a higher number of embryos produced per cow per time unit compared to once a week OPU and best results were obtained when a 3 and 4-day or 2 and 5 day interval was maintained between OPU sessions in cattle (Merton et. al., 2003; Hanenberg et. al., 1997).

In the present study, transferable embryos produced per buffalo with once and twice aspiration per week was lower compared to cattle heifers (Goodhand et. al., 1999), but embryo production rate was almost similar.

5.1.3 Effect of breeding season on the number of follicles available for aspiration, follicles aspirated and oocytes recovered.

The possible reasons for poor population of recruitable follicles in buffaloes are small size reserves of the primordial follicles, small percentage of easily mobilizable primordial follicles amongst the reserves, reduced growth rate of the growing follicles and high rate of atresia (Aboul-Ela 2000). A complex dependency of
bovine reproduction on soil, plant and climatic factors was reported (Predojevic et al., 1988), particularly in tropical and subtropical parts of the world. It is unclear whether seasonal breeding pattern is a genetic characteristic of buffalo or a effect of environmental factors. However, evidence suggested a strong influence of biometeorological factors (day length, ambient temperature, relative humidity and rain fall) on the endocrine system of buffaloes (Shah, 1988). Although buffaloes are polyestrous, they exhibit a distinct seasonal variation in display of estrus, conception rate, and calving rate (Ahmad et al., 1980; Madan 1988; Shah 1988; Singh et al., 1989; Singh and Nanda 1993). The conception rates were lower between February and August (Madan 1988).

In the present study, OPU once a week during peak breeding season (September-February) resulted in significantly higher number of medium, large and total number of follicles recorded and follicles aspirated per week per buffalo in comparison to those observed in low breeding season (March-August). However, the number of small follicles did not vary significantly during both low and peak breeding season indicating normal follicular turnover irrespective of the season (Table 3). Similar report was made in buffaloes by recording the follicles present on the slaughterhouse ovaries during different periods of the year (Rajesha et al., 2001). The lower number of medium, large and total number follicles during low breeding season in the present study may be due to lower circulating concentrations of FSH (Janakiraman et al., 1980; Razdan et al., 1982) and LH (Rao and Pandey 1983; Aboul-Ela and Barkawi 1988) during dry hot (summer) than wet cool (winter) months. Aspiration twice a week during peak breeding season resulted in a significantly higher number of small, medium and total number of follicles recorded and aspirated and oocytes recovered per week per buffalo when compared to low breeding season. These results indicated that the follicular turnover would be higher during the peak-breeding season. Results of the present study also indicated that the ovarian follicular activity varied significantly during the low and peak breeding season in buffaloes. This was in agreement with Madan and Raina (1984); Vale et al., (1990) and Rajesha et al., (2001).

In the present study, season did not significantly affect the number of large follicles, aspiration rate and oocyte recovery rate when the OPU was performed twice
a week. Aspiration of follicles larger than 2 mm diameter using OPU lead to a new follicular wave surge and the follicles reached full development within 3 days. The faster dynamics of the follicular wave induced by OPU caused the rapid turnover of the follicular population and decreased the occurrence of follicular atresia (Boni et al., 1995).

5.1.4 Effect of breeding season on oocyte quality and in vitro embryo production

The present study showed that season had no effect on the different grades of oocytes collected, oocytes cultured, cleavage rate, number of embryos that progressed to 8 cell, 16 cell, and transferable stage embryos when the OPU was performed once a week (Table 4). Contrary to these findings, Nandi et al., (2001a) collected significantly higher number of culturable oocytes and total oocytes from the slaughterhouse ovaries during the cool period than in the hot period of the year. The average ambient temperature reported by Nandi et al., (2001a) was 1-10°C in cool season and >30°C in hot season. The variations of oocyte quality might be due to extreme temperature differences. However, the ambient temperature recorded during the present study was between 13-30°C in cool season and >30-37°C in hot season. The dominant follicle was reported to have a negative effect on the oocyte quality and competence (Hendricksen et al., 2000). This might cause the low quality of oocytes recovered when OPU was conducted once in a week. When twice aspiration per week was performed, the possibility of development of a dominant follicle was minimum. Similarly in cattle, Merton et al., (2003) reported higher-quality oocytes aspirated from small antral follicles in the absence of a dominant follicle. The cleavage, morulae and blastocyst rate were not significantly affected by external environmental temperature as the in vitro embryo culture was conducted under controlled conditions. In the present study, season did not significantly affect the transferable embryo production rate when the aspiration was performed either once or twice per week. Since the superovulatory response in buffaloes depended on the various intrinsic and extrinsic factors, results of the present study demonstrated that OPU-IVEP technology could be an alternative to MOET technology in buffaloes even during non-breeding season.
5.1.5 Effect of breeding season on the number of follicles available for aspiration, follicles aspirated and oocyte recovery in superstimulated buffaloes.

The primary cause for lower oocyte recovery in buffaloes, compared with that of cattle, was lower follicular population of all sizes (Aboul-Ela, 2000). Although PMSG has been used for superovulation in buffaloes, the response in terms of the number of corpora lutea and the recovery of transferable embryos per flush was highly variable and was very low (Misra et al., 1990; Misra et al., 1994; Madan et al., 1996). The reason for the high variability in the number of transferable embryos, which had also been observed in cattle are not fully understood. Some authors have attributed this variability to the production of secondary follicles after ovulation of the first, due to long half-life of PMSG. Lower growth of the follicles after recruitment during low breeding season might be due to lower levels of circulating concentrations of FSH (Janakiraman et al., 1980; Razdan et al., 1982;) and LH (Rao and Pandey 1983; Aboul-Ela and Barkawi 1988). In the present study the number of small, medium, large follicles, follicles aspirated, aspiration rate, oocyte recovered and oocyte recovery rate in PMSG treated buffaloes were similar irrespective of season (Table 5). Since, seasonal effect was observed in non stimulated buffaloes it implies that the effect of season on follicular development could be overcome by using exogenous gonadotropins.

The overall total number of follicles aspirated after PMSG superstimulation in the present study was comparable to that of FSH superstimulated Mediterranean Italian buffalo (Boni et al., 1994), PMSG treated swamp buffalo heifers (Promdireg et al., 2000) and in cycling and lactating postpartum Swamp buffaloes with FSH stimulation (Promdireg et al., 2005). The mean number of total oocytes recovered was also comparable to that reported in Italian buffaloes (Boni et al., 1994), in swamp buffalo heifers (Promdireg et al., 2000) and in cycling and lactating swamp buffalo (Promdireg et al., 2005).

5.1.6 Effect of breeding season on oocyte quality and in vitro embryo production in superstimulated buffaloes
Various workers have reported seasonality of breeding in buffaloes (Ahmad et al., 1980; Madan, 1988; Shah, 1988; Singh et al., 1989; Singh and Nanda 1993). In the present study, the season did not significantly affect the different grades of oocytes recovered, oocyte cultured, cleavage rate, 8 cell, 16 cell and transferable stage embryos in PMSG superstimulated donors (Table 6). While in vivo, Taneja et al., (1995) observed a significant effect of season on the embryo yield and quality in MOET in Indian buffaloes with yield of 0.2± 0.2 and 0.6 ± 0.2 embryos during dry, hot season and wet, cool season respectively (Taneja et al., 1995). These results again demonstrated that OPU-IVEP could be adopted as an alternate technology to MOET in buffaloes during both low and peak breeding season.

5.1.7 Effect of oocyte source on oocyte recovery and in vitro embryo production

Application of MOET technology in buffalo had a limited success due to poor response to superovulation resulting in low recovery rate and low number of embryo production (Karaivanov 1986; Ismail et al., 1992; Misra et al., 1994). The application of OPU-IVEP in cattle began with the objective to produce embryos and calves from genetically superior cow that did not respond to superstimulation with hormones as part of MOET, that had blocked oviducts; or to produce offspring from pregnant and juvenile animals (Gasparrini, 2002). The main advantage of OPU-IVEP over MOET is the much more consistent and higher production of offspring’s, thus greatly enhancing the planning and breeding efficiency schemes (Lohuis, 1995; Van Arendonk and Bijma 2003). The ovum pickup technology has been used to harvest oocytes repeatedly from either gonadotropin stimulated or unstimulated donor animals. Merton et al., (2003) reported that frequency of OPU affected both the quality and quantity of oocytes in cattle. Oocyte quality is, in turn, a key determinant of the embryo development during subsequent in vitro production.

In the present study, the in vitro embryo production efficiency remained almost similar irrespective of the source of oocyte i.e., abattoir or OPU, or treatment or no treatment with PMSG, or frequency of collection i.e., one or twice a week (Table 7; Fig 4). Since the oocyte recovery was directly related to the number of available follicles on the ovary, in the present study, the culturable oocytes recovered per ovary was significantly higher in PMSG-stimulated ovaries per OPU session than
the slaughterhouse ovaries and non-stimulated ovaries per OPU session. The total oocytes recovered per ovary in slaughterhouse ovaries by manual aspiration and in PMSG stimulated ovaries per OPU session were significantly higher than that of non-stimulated ovaries per OPU session. When OPU was performed without PMSG stimulation, the oocyte recovery per session was significantly more by once a week OPU protocol than twice a week protocol. But, when it was calculated per week basis it was vice versa. Because when it was calculated per week, in two OPU per week protocol the oocyte recovery of both the sessions of a particular week was added. Using abattoir buffalo ovaries, Totey et al., (1992) reported a recovery of 0.7 total and 0.4 culturable oocytes per ovary after aspiration of over 12,000 ovaries and Madan et al., (1994a) obtained a recovery of 0.4 culturable quality oocytes per ovary from 4600 ovaries. The recovery of oocytes by OPU in non-stimulated buffaloes on once/twice aspiration per week in the present study was similar to that obtained through manual aspiration of follicles using syringe needle from abattoir buffalo ovaries (Totey et al., 1992; Madan et al., 1994a). This confirms the efficiency of the OPU employed in the present study for collection of oocytes from live buffaloes.

The greater mechanical disruption, which oocytes undergo during OPU, might result in an improper evaluation of their quality, which is probably underestimated compared with abattoir-derived oocytes. However, the OPU technique results in resetting the follicular population and subsequently increasing the follicular wave frequency (Boni et al., 1996). The occurrence of follicular atresia is highly reduced. In fact follicles are punctured before they become atretic. However, in the present study, the embryo production efficiency was not significantly different for the oocytes derived by OPU from live buffalo and abattoir ovaries. On contrary, Neglia et al., (2003) reported significantly higher transferable embryos from the oocytes derived by OPU in live animals than manual aspiration in abattoir ovaries. Oocytes recovered from the slaughterhouse ovaries may undergo a longer time interval between collection of ovaries from the slaughtered animals and laboratory processing. On the contrary, oocytes recovered by OPU, which are immediately and properly processed on the spot and then moved to the laboratory for further processing, develop in greater proportions to morula and blastocyst stages. Possibly, buffalo oocytes may be more affected or more quickly affected by cellular damage due to autolytic processes after residing for a prolonged period in excised ovaries (Neglia et al., 2003). In the present
study, oocytes were recovered and processed from the abattoir ovaries within 2 hours after slaughtering the animal.

MOET results of various studies in buffaloes indicated that one of the disadvantages of the use of PMSG is the relatively higher percentage of unovulated follicles and poor embryo quality than with the use of FSH (Karaivanov 1986; Ismail et al., 1992; Misra et al., 1994). In OPU-IVEP practice, the number of follicles available for aspiration and OPU interval determines the source of oocytes. The population of oocytes used in OPU-IVEP is more homogeneous compared to MOET, due to repetitive sessions resulting in the elimination of dominant and atretic follicles. Production of embryos from slaughterhouse ovaries begins with the oocytes collected from follicles in every possible phase of development and from all sizes visible on the surface of ovary. In contrast to MOET, COCs collected by OPU originate from follicles that lack dominance and have not undergone final preovulatory development. Final maturation has to take place in vitro. In cattle, Gibbons et al., (1994) found no effect of aspiration frequency or FSH treatment on the percentage of oocytes developing to morulae and blastocysts. Similarly in the present study, the percentage of oocytes developing to morulae and blastocysts were not affected by aspiration frequency or PMSG treatment (Table 7; Fig 4).

5.2 Embryo cryopreservation
5.2.1 Effect of cryoprotectants and exposure time

Vitrification has not been widely adopted by embryo transfer practitioners for commercial use in cattle and buffalo (Vajta, 2000; Gasparini, 2002; Nandi et al., 2002; Hasler, 2003). In the present study morulae and blastocysts were vitrified in either ethylene glycol (EG), glycerol+ethylene glycol (G+EG) or ethylene glycol+DMSO (EG+DMSO) with exposure time of 2, 4 or 6min at room temperature. The best survival rate (post thaw hatching rate) for morulae was achieved after vitrification with EG exposure time of 4min, G+EG exposure time of 2 min and EG+DMSO exposure time of 2 and 4 min. Further the best survival rate (post thaw hatching rate) for blastocysts were achieved after vitrification with EG exposure time of 4min, G+EG exposure time of 4 min and EG+DMSO exposure time of 2,4 or 6 min (Table 8). These findings revealed that a simple two-step addition of
cryoprotectant for vitrification appears to be efficacious, indicating that techniques could potentially reduce the overall cost of cryopreservation of embryos without compromising *in vitro* viability.

Vitrification requires high levels of cryoprotectants to achieve glass transition. A successful vitrification procedure requires optimization of cryoprotectant concentration, one or two cryoprotectants and exposure time. The cryoprotectant ethylene glycol has minimal toxicity and is more permeable to most embryos than glycerol, DMSO, propylene glycol (Voelkel and Hu, 1992; Kasai, 1996; Massip, 2001). In the present study, survival rate of morulae and blastocyst was lower after vitrification by using ethylene glycol alone as a cryoprotectant with shorter and longer exposure time (Equilibration time). However, several reports are available for various species (mice, rabbits, horse, cattle, marsupials: Kasai, 1997; buffalo: Hufana-Duran et al., 2004), of using ethylene glycol as the lone cryoprotectant in vitrification solution (EFS40: ethylene glycol, 40%, v/v; ficoll, 18%, w/v; sucrose, 0.3M).

According to Isachenko et al., (1994) a cryoprotectant like glycerol with less permeability has primary protective action on cytoplasmic membranes whereas a more permeable agent like ethylene glycol protects the intracellular structures. Similarly in the present study, blastocysts were vitrified by exposing for 4 min in the vitrification solution containing glycerol and EG showed better hatching rates after post-thaw culture (Table 8). Cryoprotectant mixtures like EG and DMSO may have some advantages over solutions containing only one penetrating cryoprotectant (Crister et al., 1997; Vajta et al 1999; Paynter et al., 1999). The ability of embryos to hatch after 48 hours post-thaw culture, improved when two (EG, DMSO) or three (EG, DMSO, BD) permeating cryoprotectants were included in the vitrification media rather than the single permeating cryoprotectant like EG (Pugh et al., 2000). The use of two permeable cryoprotectants rather than a single cryoprotectant such as EG, enables the use of lower concentration of each of them in the vitrification solution thereby reducing cryoprotectant toxicity. Composition of the vitrification solution (permeating cryoprotectants and non-permeating macromolecules or saccharides) is among the factors influencing cryosurvival of embryos. Incorporation of DMSO into ethylene glycol containing medium has at least two advantages; vitrification is facilitated because of the greater glass-forming characteristics of DMSO (Ali and
Shelton 1993) and the permeability of each of the cryoprotectant is enhanced by the presence of the other (Vicente and Garcia-Ximenez, 1994). Similarly in the present study, blastocysts vitrified in the solution containing EG and DMSO resulted in better hatching rates after post-thaw culture when compared to other vitrification solutions. Vitrification requires high levels of cryoprotectant to achieve glass transition. A successful vitrification procedure requires optimization of cryoprotectant concentration, exposure time and exposure temperature. As indicated by the results, the present study has not been able to develop the most appropriate combination of these factors for vitrification of in vitro produced buffalo morulae. Vitrification of in vitro produced morulae might be more successful using systems with more rapid cooling such as open pulled straws, cryoloops, etc.

The protocols used for vitrification technique in this study for cryopreservation of in vitro produced buffalo embryos were effective up to 22 and 45% hatching on post-thawing and in vitro culture of morulae and blastocysts. This methodology offers the possibility to introduce affordable genetically superior offspring and increase the widespread adoption of embryo transfer technology in buffaloes. However, the results of this work require verification by embryo transfer.

5.2.2 Effect cytochalasin-B (cyto-B) on vitrification of embryos.

To prevent or overcome cytoskeletal disruption of the embryos during and after cryopreservation, the cytoskeletal stabilizer (cyto-B) was added in the vitrification solution. In the present study, the percentage of tight morulae progressed to hatched blastocyst on vitrification with cyto-B was significantly higher and tight morulae, blastocyst and expanded blastocysts progressed to hatching blastocyst stage was non-significantly higher than control (Table 9; Fig 5).

There is a complex network of protein constituents, actin (MF) and tublin (MT), distributed throughout the cytoplasm (Albertini et. al., 1987; Dobrinsky et. al., 1996). These constituents impart three-dimensionality and mechanical strength to the surface of a cell and provide a system of fibers that regulate cell polarity, cell shape, cell movement, and the plane of cell division. Actin and tublin filaments bind variety of accessory proteins that enables them to participate in distinct functions in
different regions of a cell, including the plasma membrane. Thus, this highly
organized network of filaments forms an internal framework to stabilize the large
volume of cytoplasm within the cell (Dobrinsky et al., 2000).

An intact cytoskeleton is essential for cytokinesis and karyokinesis, and if it is
irreversibly disrupted, the mitotic cell cycle will cease, junctional complexes may be
compromised, and solute transport systems would be affected. Maintaining the
integrity of the cytoarchitecture within an embryo during cryopreservation is of the
utmost importance. To minimize cytoskeletal disruption of the embryos during and
after cryopreservation, cytoskeletal stabilizers are added in the vitrification solution.
Cytoskeletal stabilizers such as the cytochalasins have been used extensively in
reproductive biotechnology for studies dealing with micromanipulation of embryonic
development (McGrath and Scolter 1983; Surani et al., 1984). In the present study,
the percentage of tight morulae progressing to hatched blastocyst stage on vitrification
with cyto-B was significantly higher and tight morulae, blastocyst and expanded
blastocysts that progressed to hatching blastocyst stage was higher than control,
though non-significant.

5.3 **Oocyte developmental competence**

5.3.1 **Brilliant cresyl blue (BCB) staining test for selection of oocytes for IVEP.**

In the present study, about 57% of the grade A and grade B oocytes recovered
from the slaughterhouse ovaries had a blue coloration after staining with BCB,
indicating that they finished their growth phase and could be used for IVEP. Previous
reports using the BCB test in pig oocytes employed a concentration of 13μM BCB
(Ericsson et al., 1993; Roca et al., 1998). In the present study 26μM BCB was used
to stain the oocytes as this concentration resulted in higher rate of BCB (+) oocytes
without apparent loss of viability. The same concentration was used for the oocytes
from prepubertal goats (Rodriguez-Gonzales et al., 2002, 2003 and Urdaneta et al.,
2003), heifers (Pujol et al., 2000, Pujol et al., 2004) and cow (Alum et al., 2005).
Tiffin et al., (1991) reported that BCB stain could be used effectively in the study of
embryo metabolism without being lethal after exposure with 26μM BCB. The
percentage of BCB (+) oocytes (57%) obtained in the present study, employing 26μM
BCB, was almost similar to those reported in heifers (62%; Pujol et al., 2000, 66%;
Pujol et al., 2004) and cow oocytes (58%; Alum et al., 2005) and lower than porcine oocytes (91%; Ericsson et al., 1993, and 81%; Roca et al., 1998). The percentage of BCB (+) oocytes reported in oocytes recovered from 2-month-old goats was 29.4% (Rodriguez-Gonzales et al., 2002). These authors also reported that the BCB staining was influenced by oocyte morphology, thus differences in morphological selection criteria might be associated with the differences observed in percentage of BCB staining oocytes among laboratories.

The oocyte diameter is a determinant factor in acquiring meiotic competence (Cattle: Hyttel et al., 1997; Buffalo: Raghu et al., 2002b). The BCB (+) oocytes showed a significantly higher diameter in porcine (Roca et al., 1998), prepubertal goats (Rodriguez-Gonzales et al., 2002, 2003; Urdaneta et al., 2003) and heifers (Pujol et al., 2000; Pujol et al., 2004). The diameter of oocytes of BCB (+) and BCB (−) oocytes in the present study was 144.4 μm and 136.8 μm respectively. The volume of BCB (+) and BCB (−) oocytes was 1.58 X 10^6 μm^3 and 1.35 X 10^6 μm^3 respectively. Both the diameter and volume of BCB (+) oocytes were significantly more than that of BCB (−) oocytes.

In the present study, the maturation rate was significantly higher in BCB (+) than BCB (−) oocytes and it was neither affected by exposure to BCB nor by holding in mDPBS in comparison to the control. Similar findings have been reported in goats (Rodriguez-Gonzales et al., 2002, 2003; Urdaneta et al., 2003), heifers (Pujol et al., 2000; Pujol et al., 2004) and cow oocytes (Alum et al., 2005). The low maturation rate of BCB (−) oocytes could be due to the incomplete or abnormal cytoplasmic maturation, which would explain the high rates of Polyspermic fertilization (16.23%) and lower percentage of 2 pronuclei (55.5%) compared to that of BCB (+) oocytes (Table 10).

Oocytes recovered from the ovaries of slaughtered animals are heterogeneous (Carolan et al., 1994; Roca et al., 1998), coming from follicles in different stages of growth and atresia. In buffaloes, using morphological criteria such as compaction of cumulus -corona investment and the homogeneity of ooplasm, it was possible to obtain fertilization rates of about 60-70% (Chauhan et al., 1998a) and cleavage rates of 40-50%(Chauhan et al., 1998a). In the present study the high 2 pronuclei
formation and cleavage rates was observed in BCB (+) oocytes compared to BCB (-) oocytes. Similarly in pig, the BCB (+) oocytes had higher rates of sperm penetration than BCB (-) oocytes (Roca et al., 1998). The rate of G6PDH activity was negatively associated with the percent oocyte maturation, fertilization and development of male and female pronuclei (Ericsson et al., 1993). Immature oocytes were known to synthesize a variety of proteins (Wassarman, 1988) and the most common was glucose-6-phosphate dehydrogenase (G6PDH). This enzyme was active in the growing oocyte, but had decreased activity in oocytes that had attained their growth phase. The BCB staining was based on the capability of the glucose-6-phosphate dehydrogenase (G6PDH) to convert the BCB stain from blue to colorless (Ericsson et al., 1993). The cytoplasm of fully grown oocytes turns blue because they do not reduce BCB to a colorless compound and this reaction is considered as BCB (+).

The total percentage of blastocyst development rate in the BCB exposed group (BCB- and BCB + combined; 19.5%) was not different from that in control group, indicating that exposure to BCB did not affect the blastocyst development rate. The present study showed that the BCB test selected more competent oocytes for embryo development than conventional morphological selection. Thus, the percentages of BCB (+) oocytes, which develop to blastocyst stage, were significantly higher than the other groups. The blastocyst development rate (5.12%) was very low in BCB (-) oocytes compared to other groups. Similar findings were also reported in heifers (1.6%; Pujol et al., 2004) and cows (3.9%; Alum et al., 2005) in BCB (-) oocytes.

The present study showed that the BCB staining of cumulus-oocyte complexes from buffaloes before in vitro maturation may be used effectively to select buffalo oocytes for further development and that this process did not have any negative effect on blastocyst development. Hence, BCB staining could be useful in increasing the efficiency of blastocyst production in standard IVF procedures in buffaloes.

5.3.2 Ovarian morphofunctional state on oocyte developmental competence

The present study indicated the possibility of predicting the developmental competence of oocytes on the basis of the morphofunctional state of the paired ovaries collected from the slaughtered individual buffaloes. The developmental
competence of oocytes is of paramount importance in determining the success of in vitro embryo production (Sirard and Blondin 1996). Buffalo oocytes were conventionally selected for embryo production on the basis of compaction of cumulus-corona investment and the homogeneity of ooplasm (Chauhan et al., 1998a). Therefore, to collect good oocytes from ovaries derived from a slaughterhouse, it was recommended to collect in pairs and select ovaries with corpus haemorrhagicum to collect oocytes from follicles at the follicular growth phase of first wave. Oocytes collected from the follicles in the growth phase of the second wave from the ovaries with a functional corpus luteum and only one large follicle (possibly dominant follicle), showed higher developmental competence (Nagai, 2001). In contrast, in some of the studies, the presence or absence of a dominant follicle did not affect the subsequent embryo development in culture (Smith et al., 1996) and the oocytes obtained from later cycles were more developmentally competent than those from first wave (Machatkova et al., 2000).

The competence of an oocyte to yield a blastocyst within an in vitro production system not only depends on intrinsic factors, but is also related to IVP procedures, and sperm and oocyte quality. The COC was the last part of the follicle that was affected by follicular atresia (Kruij and Dieleman, 1982; Wurth and Kruij, 1992). The ovarian follicles in buffalo develop in waves similar to those observed in cattle (Fortune et al., 1988; Baruselli et al., 1997). Unlike that in cattle (Sirois and Fortune 1988) there were no 4 wave cycles in buffalo (Baruselli et al., 1997); most had 2 or 3 wave cycles. Since no published literature is available in buffaloes on the effect of the stages of estrous cycle/ phases of folliculogenesis/follicular atresia/presence or absence of dominant follicle on the oocyte developmental competence in vitro, the findings of the present study was compared with information available for cattle. Atresia mainly occurs during the dominance phase of the follicular wave; it might be possible that oocyte competence would be affected during this phase as well. Indeed, higher blastocyst rates were obtained with oocytes from >3mm follicles collected during phases of follicular growth (Days 2 and 10 of the cycle) than with oocytes from >3mm follicles collected during phases of follicular dominance (Days 7 and 15) respectively (45 vs 36%, Hagemann et al., 1999b). The proportion of apoptotic cells was higher during dominant than during growth phases. In the dominant phases, the highest level of apoptosis was found in subordinate
follicles of 6-8 mm while oocytes from such follicles yielded the lowest blastocyst rates (Hagemann et. al., 1999a).

A negative effect of the DF on the competence of oocytes from the subordinate follicles had also been described by Hagemann et. al., (1999a). In the present study, the developmental competence of the oocytes collected from the ovaries having DF (group 2 and group 4) was significantly lower. The proportion of oocytes that developed to transferable embryos was higher in the absence of a DF (group1 and group 3; 27% and 24%, respectively) than in the presence of a DF (group 2 and group 4; 16% and 15% respectively). The presence of a dominant follicle in either one or both ovaries had a negative effect on in vitro developmental competence of bovine oocytes (Varisanga et. al., 1998). The results of the present study also supported the concept that the, intraovarian environment to which oocytes were exposed could play a major role in determining their developmental competence (Goto et. al., 1990; Varisanga et. al., 1998). Similarly low blastocyst rates had also been reported when OPU was performed once a week in comparison with OPU every 3-4 days (Goodhand et. al., 1999; Hanenberg et. al., 1997). Presumably, the higher frequency of OPU prevented the establishment of a DF. Contrary to the above observations and findings of the present study, Chian et. al., (2002) reported that the developmental competence of bovine oocytes from the small follicles was not affected either by the presence of a dominant follicle or by the phase of folliculogenesis.

On the other hand, the presence of corpus luteum in the pair of ovaries seemed to indicate that the animal was apparently cycling (as in the group 1-4 ovaries in this study). Although the precise endocrine control of the patterns of follicular recruitment, selection, growth and regression particularly during the early and mid-luteal phase of the estrus cycle were not fully understood. The reason for the superiority of the ovary pairs with CL over those of the without CL were not clear.

5.3.3 Antioxidants on oocytes maturation and embryo development

Buffalo oocytes/embryos are particularly sensitive to oxidative stress, due to their high lipid content (Boni et. al., 1992). Many different systems of free radical
scavengers have been investigated during the last few years for in vitro culture of mammalian embryos. In the present study, the effects of antioxidants (taurine, cysteamine, and melatonin) at different concentrations in IVM and in vitro culture of embryo (IVC) medium were evaluated and an increase in in vitro embryo production efficiency was achieved (Table 13).

Taurine is a ß-amino acid present in oviductal and uterine fluids of different species such as rabbit (Leese et. al., 1979) and mouse (Dumoulin et. al., 1992b). It has a number of different mechanisms of action including osmoregulation, calcium modulation, phospholipid interactions, membrane protein receptor interactions, and as the product of its precursor, hypotaurine, antioxidation (Huxtable, 1992; Timbrell et. al., 1995). In the present study, taurine at 1.0 mM concentration in the culture media resulted in a significantly higher rate of transferable embryos. Similarly the positive effect of taurine and hypotaurine on embryo development have been reported in different species including mouse (Dumoulin et. al., 1992a,b), pig (Reed et. al., 1992), cow (Liu and Foote, 1995; Fujitani et. al., 1997; Guyader-joly et. al., 1998), rabbit (Li et. al., 1993) and hamster (Bavister and McKiernan, 1993). In contrast, Devreker and Hardy (1997) found that supplementation of defined media like CZB or KSOM with 5 mM taurine had no significant effect on the proportion of mouse embryos developing to the blastocyst stage and total cell numbers in high oxygen tension environment. Similarly Choi et. al., (1998) reported no effect of taurine (0.01 to 10mM) on development of mouse embryos in KSOM. Thus, although mammalian gametes (Gwatkin 1983) and embryos (Van Winkle and dickinson 1995) seem capable of taurine uptake, the culture system in which it was used (i.e., defined media vs coculture with somatic cells) may likely play an important role in determining its efficiency.

Glutathione (GSH) is the major non-protein sulphydryl compound in the mammalian cells, which plays a critical role in protecting the cells from oxidative damages. In addition to this role, several functions have been described such as amino acid transport, DNA and protein synthesis and reduction of disulfides (Lafleur et. al., 1994). In the mouse GSH was also involved in different embryonic events, including cell proliferation and differentiation at later preimplantation stages and, as a constituent of co-enzymes, it contributed to energy metabolism. GSH synthesis was
critical for the acquisition of developmental competence of oocytes at cytoplasmic levels (Eppig 1996). Furthermore, De Matos et. al., (1997) suggested that the measurement of GSH levels at the end of IVM might represent a reliable indicator of the cytoplasmic maturation.

It was demonstrated that the intracytoplasmic glutathione concentration varied during preimplantation development of the in vitro produced bovine embryos, lowest levels of GSH were found in 2-8 cells embryos and the highest in the hatched blastocysts (Lim et. al., 1996). Interestingly de novo synthesis of GSH began to increase at the 8-16 cells stage, which coincides with the onset of activation of embryonic genome (Telford et. al., 1990). Bovine zygotes were less resistant than 8-16 cells embryos to oxidative stress caused by H2O2 (Morales et. al., 1999). All these findings show that bovine embryos were capable of synthesizing GSH after the activation of the embryonic genome and that a low intracytoplasmic concentration of GSH may be correlated to the 8-cell block. In the mouse, a 10-fold decrease of the GSH content had been reported throughout embryo development from the unfertilized oocytes to the blastocyst stage (Gardiner and Reed 1994). These results suggest that adequate amounts of GSH needed to be synthesized during in vitro maturation of oocytes in order to support development up to the stage at which embryos acquire biosynthetic ability.

In the present study, addition of cysteamine in culture medium resulted in significant enhancement of the maturation, fertilization and cleavage rate and embryo development. Similar findings in buffaloes were reported by Gasparrini et. al., (2000, 2003, 2006). The GSH biosynthesis, through the γ-glutamylcysteine synthetase cycle, depended on the availability of cysteine in the extracellular environment. Cysteine was very unstable and rapidly oxidized to cystine in the culture medium. The lack of cysteine in the medium due to auto-oxidation to cystine may result in GSH synthesis impairment in vitro (Sagara et. al., 1993). Low molecular weight thiol compounds such as cysteamine reduce cystine to cysteine, promoting cysteine uptake in mammalian cells (Bannai 1984) and therefore enhancing GSH synthesis.

In the present study the in vitro system was improved by incorporating melatonin in the culture medium. Similarly the positive effect of melatonin on in vitro
embryo production was also reported in mouse (Ishizuka et al., 2000) and bovine (Poleszczuk et al., 2004). Melatonin has been found to be a free radical scavenger and as an antioxidant, it was effective in protecting nuclear DNA, membrane lipids and possibly cytosolic proteins from oxidative damage. Melatonin had also been reported to alter the activities of various enzymes, which improve the total antioxidative defense capacity of the organism, i.e., superoxide dimutase, glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and nitric oxide synthase (Reiter, 1998).

**The silent findings of the different experiments conducted during the study are as follows:**

1) Aspiration twice a week resulted in a significantly higher number of small, medium, large and total number of follicles counted, follicles aspirated and oocytes recovered per buffalo per week compared to aspiration once a week.

2) Twice aspiration per week resulted in a significantly higher number of culturable oocytes and transferable embryos per buffalo per week compared to aspiration once a week.

3) Maximum oocytes (2.75 oocytes per buffalo per week) were recovered when OPU was conducted twice a week during peak breeding season.

4) Maximum transferable embryos (0.6 embryos per buffalo per week) were produced *in vitro*, when OPU was conducted twice a week during peak breeding season.

5) Seasonal effect on oocyte recovery and transferable stage embryo yield *in vitro* could be overcome by using exogenous gonadotropins in buffaloes.

6) The oocyte source did not significantly affect the percentage of embryos that progressed to 8 cell, 16 cell and to transferable embryo stage.

7) The best survival rates (hatching rate) for morulae were achieved after vitrification with EG exposure time of 4, G+EG with exposures time of 2 min and EG+DMSO with exposure time of 2 and 4 min.

8) The best survival rate (hatching rate) for blastocysts were achieved after vitrification with EG with exposure time 4 of min, G+EG with exposure time of 4 min and EG+DMSO with exposure time of 2, 4 or 6 min.

9) The percentage of tight morulae progressing to hatched blastocyst stage on vitrification with cytochalasin-B was significantly higher and tight morulae,
blastocyst and expanded blastocysts that progressed to hatching blastocyst stage was non-significantly higher than control.

10) About 57% of grade A and grade B oocytes recovered from the slaughterhouse ovaries were BCB (+), indicating that they finished their growth phase and could be used for IVEP.

11) The diameter and volume of BCB (+) oocytes were significantly more than that of BCB (−) oocytes.

12) Higher 2 pronuclei formation and cleavage rates was observed in BCB (+) oocytes compared to BCB (−) oocytes.

13) The percentages of BCB (+) oocytes, which developed to blastocyst stage were significantly higher than the other groups.

14) The cleavage rate of oocytes collected from ovaries with corpus luteum and dominant follicle was significantly lower than those collected from ovaries with corpus haemorrhagicum and without dominant follicle.

15) The transferable embryo production rate of oocytes collected from the ovaries with corpus haemorrhagicum / luteum and no dominant follicle was significantly higher than those obtained from ovaries with corpus luteum / regressing corpus luteum and dominant follicle and from ovaries with out any luteal like structures and with small follicles.

16) Taurine at 1mM concentration in the culture medium resulted in a significantly higher rate of transferable embryos.

17) Addition of cysteamine or melatonin in culture medium resulted in significant enhancement of the maturation, fertilization and cleavage rate and embryo development.

It is suggested that further studies are required on stage of estrus cycle during OPU session, coasting period (time period from gonadotrophin stimulation and aspiration) on in vitro embryo production. Detail studies on cellular injuries at the molecular level during cryopreservation using various cryoprotectants needs to be investigated. Mechanism of actions of various antioxidants on ameliorating the cell toxicity needs to be studied.