1. INTRODUCTION

The prehistoric remains in the Shivalik hills of Punjab suggest that buffalo has been an important animal species since that time with traceable history of domestication as old as 2500 B.C. in China and Indian subcontinent (Sidhu and Guraya, 1985). The estimated world buffalo population is 185.29 million, spread in around 42 countries, of which 179.75 million (97%) are in Asia (FAO, 2008). Buffalo is the skeletal base of Indian dairy industry with a population of 105.1 millions, which is 56.7 percent of the total world buffalo population which includes almost all the finest breeds of this species. During the last 10 years, the world buffalo population increased by approximately 1.49% annually, which is 1.53% in India and 1.45% in Asia (FAO, 2008). Inspite of its enormous economic contribution, buffalo has remained largely untouched by science and its innovations largely due to the fact that maximum buffalo population and almost all its finest breeds are found in under developed or developing countries.

Inherent reproductive problems like low germ cell reserve, delayed puberty, poor expression of oestrus, early embryonic mortality, longer inter-calving period and seasonal variation in fertility are major limitation of buffalo farming. Artificial insemination has been routinely used for maximum utilization and propagation of superior germplasm of male but genetics of superior female is not yet completely utilized. Embryo transfer technology provides an effective tool for maximum utilization of superior germplasm in females, but the application of superovulation and embryo transfer in buffaloes has only limited success (Madan et al., 1996). Therefore, there is an increasing interest in laboratory production of embryos through in vitro fertilization (IVF) for rapid and maximum dissemination of superior genetic potential in female buffaloes.

Following the birth of Louise Brown, the first IVF baby in 1978, (Steptoe and Edwards, 1978), there was drastic increase in research efforts to develop similar techniques for the bovine. In vitro embryo production technologies have been developed using ovaries collected from the abattoir as oocyte donors and therefore, had less impact on genetic improvement. The advent of transvaginal ultrasound guided ovum pick up (OPU), a non-invasive
and repeatable procedure for recovering large quantity of meiotically competent oocytes from antral follicles of genetically superior live animals has boosted *in vitro* embryo production (IVEP) programme.

These methods represent an important tool to link IVEP technologies to animal breeding. Ovum pick-up (OPU) technique combined with IVEP has greater potential than multiple ovulation embryo transfer (MOET) to improve the genetic progress through maternal lineage. Collection of oocytes by means of ultrasound guided follicular aspiration can be regularly performed with success in prepubertal and adult buffaloes under various physiological and pathological conditions (Boni *et al*., 1996; Manik *et al*., 2002; Techakumphu *et al*., 2005).

Although viable buffalo blastocysts were produced from abattoir ovaries using protocols that have been developed for cattle, the rate of transferable embryo yield remains low (15-30%, Chauhan *et al*., 1997; Palta and Chauhan 1998; Gupta *et al*., 2001; Nandi *et al*., 2002). Though *in vitro* produced embryos have been used for producing blastocysts, pregnancies and calf birth in buffalo (Madan *et al*., 1994a; Galli *et al*., 2001; Gasparriini *et al*., 2003, 2006; Hufana-Duran *et al*., 2004; Prasad *et al*., 2013), but the success rate is still lower as compared to cattle. Limited knowledge about the developmental competence of *in vitro* produced embryos, role of reactive oxygen species (Sadeesh *et al*., 2014) and other factors affecting embryo quality like apoptosis (Gonzalez, 2009) may be a major hurdle in achieving optimum success rate and wide application of these techniques at field level.

Reactive oxygen species (ROS), derived from oxygen metabolism, are apparently involved in the two cell block of embryos (Nasr-Esfahani *et al*., 1990). Bovine embryos cultured *in vitro* under low oxygen tension (Takahashi *et al*., 2000) have been reported to show higher developmental rates than those cultured under high oxygen tension, indicating more oxidative damage in oxygen rich culture conditions, perhaps due to increased accumulation of reactive oxygen species in the cytoplasm of developing embryo (Kwon *et al*., 1999). Generation of free radicals is an inevitable consequence of oxidative reactions and to ameliorate the deleterious effect of free radicals, cellular protective processes have evolved
including the use of “antioxidants” or free radical scavengers”. The later prevent cellular damage caused by free radical reactions with essential biochemical components by interfering with inactivation of enzymes and alterations of membranes and DNA (Yu, 1994).

Apoptosis can result in poor embryonic development or may even cause arrest of embryonic development prior to compaction. Induction of apoptosis at early stages of embryo development is ultimately associated with poor pregnancy outcomes (Heilig et al., 2003). Apoptosis predominately affects the inner cell mass of the blastocysts, the incidence being higher in in vitro produced embryos as compared to their in vivo counterparts (Byrne et al., 1999). Possible causes of apoptosis in pre-implantation embryos includes chromosomal and nuclear abnormalities, impaired developmental potential, imbalance of growth or survival factors such as Insulin like growth factor (IGF-I), suboptimal culture conditions, ultra violet radiation, heat shock and reactive oxygen species (Yang et al., 1998; Betts and King, 2001; Hardy and Spanos, 2002; Jousan and Hansen, 2004).

Therefore, research needs to be directed towards formulating strategies to combat the deleterious effects of oxidative damage and apoptosis in in vitro embryo production by incorporating suitable antioxidants and anti-apoptotic factors in embryo culture medium. Keeping in view very limited reports or no report of supplementation of the anti-apoptotic factors and antioxidant, the research work was designed with the following objectives-

1. To study the developmental competence of in vitro produced buffalo embryos derived from abattoir ovaries and ultrasound guided transvaginal ovum pick up (OPU).
2. To study the effect of reactive oxygen species on oocyte maturation and in-vitro fertilization.
3. To study the effect of apoptosis on oocyte maturation and in-vitro fertilization.
4. To design strategies to augment the developmental competence using anti-apoptotic factors and antioxidants.
2. REVIEW OF LITERATURE

2.1 In vitro embryo production (IVEP) in buffaloes

Rabbit was the first mammalian species in which live offspring was produced by in vitro fertilization (Chang, 1959). But in India, since the birth of the first buffalo calf from in vitro produced embryo (Madan et al., 1991), the technique has incited interest in various research groups of the country to explore its potential for salvage of reproductive functions of elite animals and conservation of species on the verge of extinction by integrating it with conventional breeding programme to enhance the genetic improvement which has been reported upto 20 to 25% in cattle (Nicholas, 1996). Though in vitro embryo production is commercial in cattle but the technique is still sub optimal in buffaloes with developmental competence of less than 20 % blastocysts production and further failure of blastocysts to develop to term (Manjunatha et al., 2008). Besides the limitation of higher cost of in vitro embryo production which is approximately two folds as compared to multiple ovulation and embryo transfer (MOET) is also major hurdle in field application of this technique in India. Inspite of all these factors, it has attained popularity and relevance worldwide with production of approximately 4,51,000 in vitro produced embryos and nearly 3,40,000 embryo transfers during the year 2010 with Brazil emerging as the global leader with 59 % of worlds total IVEP and 77 % of transfers of in vitro produced embryos (Stroud, 2011).

2.1.1 IVEP using oocytes from abattoir ovaries

Abattoir ovaries still forms the major source of in vitro produced embryos in India because of the easy and economic availability and considerably good retrieval rate of the oocytes (Agrawal et al., 1995). The major components of the IVEP using abattoir ovaries are discussed below.

2.1.1.1 Retrieval of oocytes

Abattoir derived ovaries are till date the most convenient and economic source for retrieval of oocytes in abundant numbers and hence has acquired edge over the other methods for oocyte retrieval like slicing of ovaries and transvaginal ultrasound guided ovum pick in routine IVF set up. There is very low primordial follicle reserve in buffaloes as compared to cattle.
Primordial follicle reserve as low as 19,000 in Nilli Ravi (Samad and Nasseri, 1979) and 12,000 in Surti buffaloes (Danell, 1987) has been reported against 1,50,000 in cattle. Low primordial follicle reserve besides higher incidence of follicular atresia (Ocampo et al., 1994) and low number of antral follicle reserve at all stages of oestrus cycle (Kumar et al., 1997) and slaughtering of sub fertile, unproductive, aged and poor body condition animals may be the reason of lower yield of oocytes in buffaloes as compared to cattle (Nandi et al., 2000a).

2.1.1.2 In vitro maturation of oocytes

For in vitro maturation, oocytes were cultured in groups in droplets of TCM-199 supplemented with serum and gonadotrophins at 38.5°C under 5% CO₂ tension for 24 hours (Madan et al., 1994b; Palta and Chauhan, 1998) and with several other additives. Other media like Weymouth media has also been used for in vitro maturation (Ravindranath et al., 2003). The maturation rate (M-II stage) in vitro was assessed by various methods like staining of the oocytes, identification of extruded first polar body in the perivitelline space and degree of expansion of cumulus cell mass. Use of chemically defined media to enhance maturation rate and reduce the effect of metabolites and free radical scavengers has also been attempted in the past (Gasparrini et al., 2003; Poleszczuk et al., 2004; Konard et al., 2013).

2.1.1.3 In vitro 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. Insemination of in vitro matured buffalo oocytes is done with frozen-thawed in vitro-capacitated spermatozoa in Tyrode’s modified medium (TALP, Totey et al., 1996) or Brackett and Oliphat (BO, Nandi et al., 1998) medium followed by gamete co-incubation for 18 h.

2.1.1.4 In vitro embryo culture

To date there are various systems available for in vitro culture of zygotes. These includes co-culture with various types of cells such as bovine oviduct epithelial cells (BOEC) (Eyestone and First, 1986), cummulus cells or
tropoblastic vesicles, established cell line, buffalo rat liver (BRL) cells or vero cells etc. The trend is changed toward the use of the chemically defined media like modified synthetic oviductal fluid (mSOF), CR1a, chatot ziomek bavister (CZB) medium (Chatot et al., 1989), hamster embryo culture medium-6 (HECM-6) etc., of which SOF medium is used very commonly by different laboratories and workers. Secretions of the female reproductive tract have several amino acids that can be used as energetic substrate by the embryo and they can also reduce the stress and cell fragmentation caused by *in vitro* embryo culture (Donnay et al., 1997). Lonergan et al. (1999) observed that culturing bovine oocytes in SOF and in SOF plus BSA using 5 percent oxygen compared to 20 per cent increased the blastocyst yield on Day 8. These defined media generally require low oxygen tension (5 per cent) to yield higher blastocyst rate (Vanroose et al., 2001). The use of amino acids in serum-free culture media improves embryo development, probably through an antioxidant action, controlling pH and osmolarity (Lee et al., 2004). Higher developmental rates of cleavage and blastocyst were also recorded using Research vitro cleave (RVCL) as compared to mCR2 and mSOF medium in buffalo (Shah et al., 2008) and cloned and parthenogenetic embryos in goats (Jena et al., 2012), which further improved by sequential culture in RVCL media for 72 h followed by blastocyst medium (Gopalakrishna et al., 2014; Kumar, 2014).

### 2.2 IVEP using transvaginal ultrasound guided ovum pick up (OPU) derived COCs

Majority of buffaloes slaughtered are either sub-fertile, unproductive, aged or with poor body condition and most importantly may bear poor genetic potential for production traits. Therefore, the technique of ultrasound guided transvaginal Ovum pick up (OPU) initially developed for human (Wikland et al., 1987) was subsequently modified to be adapted in cattle (Pieterse et al., 1988). This technique allows repeated oocyte retrieval from genetically superior live animals which can be used for IVEP programme to maximize the contribution of such animals in genetic improvement programme. Once weekly or twice weekly oocyte retrieval can be done with this technique up to three months continuously without any adverse effect on fertility (Pieterse et al., 1988; Pieterse et al., 1991), yielding meiotically competent oocytes from
individual bovine donor (Kruip et al., 1993) and ultimately leading to production of transferable embryos and live calves (Kruip et al., 1993; Looney et al., 1994). The most important advantage of this technique is that it can be applied to subfertile and infertile animals (Looney et al., 1994; Hasler et al., 1995), pregnant cows (Meinjtes et al., 1995), buffaloes (Das et al., 1996) and juvenile or prepubertal heifers (Galli et al., 2001).

Following successful application of this technique in cattle, it was subsequently applied in mature buffaloes (Galli et al., 2001; Manik et al., 2002) and prepubertal buffalo heifers (Presicce et al., 2002) and calves (Techakumphu et al., 2004) with low success rate in terms of follicles available for aspiration and quality of oocytes retrieved.

In riverine buffaloes, Huang et al. (2005) recovered 66 oocytes per 100 follicles aspirated in OPU sessions performed at 4 days interval, of which 91.1% were suitable for IVM. In cyclic buffaloes an average 2.0±0.3 oocytes were retrieved per animal per session, with a recovery rate of 68% and mean number of 3.0±0.3 punctured follicles. The oocytes of grade A and B together and grade C and D together were 59 and 41%, respectively.

Various factors including vacuum pressure (Techakumphu et al., 2005), Gonadotropin superstimulation (Promdireg et al., 2005; Chaubal et al., 2006) and session interval (Manjunatha, 2006) affects the oocyte recovery rate in ultrasound guided OPU. The advantage with this technique is that it can be performed for a long period without negative effects on the animal’s health, well-being and subsequent fertility. The developmental competence of oocytes retrieved from live animals by ultrasound guided OPU was reported to be significantly better than abattoir ovaries both in terms of quality of oocytes retrieved and embryos produced (Gupta et al., 2008; Manjunatha et al., 2008). Another substantial advantage of this approach is the possibility to obtain cumulus oocyte complexes (COCs) from donors during the first trimester of their pregnancy (Takuma et al., 2010). In India, a landmark was, however, attained with the birth of first buffalo calf by OPU-IVF named ‘Saubhagya’ at Govind Ballabh Pant University of Agriculture and Technology, Pantnagar (Prasad et al., 2013) which opened new frontiers in genetic improvement of buffaloes.
2.2.1 Efficiency of ovum pick-up for oocyte retrieval and IVEP

The developmental competence of OPU derived embryos has been extensively studied in buffaloes and *in vitro* maturation rate of 87.5%, cleavage rate of 67.8% and blastocyst rate of 26.7% was recorded in swamp buffaloes (Kitiyanant et al., 1995). However, lower competence of OPU oocytes was recorded in cyclic Mediterranean buffaloes having long postpartum period (>500 days) with 55.6% cleavage rate and 16.7% expanded blastocyst yield (Boni et al., 1996). In another study in cyclic Mediterranean buffaloes with short postpartum period the IVEP efficiency in terms of morula and blastocyst rate was 18.3% (Boni et al., 1997). Even though, transferable embryo production efficiency in buffaloes is considered to be less than half of 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 et al., 1997).

The success story of ultrasound guided OPU in buffaloes can be stated to be initiated with normal development of the OPU-IVP embryos to term in Italian buffaloes (Galli et al., 1998) and since then the technique emerged as a potential technique for incorporation with conventional breeding programmes for genetic improvement. 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). In a 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. Similar findings have been reported in river buffaloes in India (Manjunatha et al., 2008). The technique has been extensively reported as a source of developmentally competent oocytes in various breeds of buffaloes including Murrah (Gupta et al., 2008; Manjunatha et al., 2008), swamp (Duran, 2008; Baruselli et al., 2010) and Italian Mediterranean (Di Francesco et al., 2011).
2.3 Predictors/reflectors of development competence

Majority of the buffalo oocytes fail to develop to morula or blastocyst stage following in IVEP mainly because of sub optimal culture and selection of oocytes with compromised development competence. 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 IVEP was made on the basis of compaction of cumulus corona investment and the homogeneity of ooplasm (Chauhan et al., 1998b). 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. Hence, 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., 2002). Besides various cellular predictors of developmental competence like glucose-6-phosphate dehydrogenase activity, reactive oxygen species and oocyte and embryonic apoptosis are used successfully in conjunction with morpho-physical criteria like oocyte diameter, follicular activity of the source ovary and stage of oestrus cycle of the donor to predict the quality and development competence of the retrieved oocytes.

2.3.1 Morphofunctional state of the ovary and oocyte

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; Lakhera, 2015).

2.3.1.1 Oocyte diameter

The bovine oocytes of diameter of 110-120 µm and those greater than 120 µm developed at greater proportion to M-II stage than the oocyte of the smaller diameter, which remained at the germinal vesicle stage of the nuclear maturation (Fair, 1995; Hyttel et al., 1997). The bovine oocytes acquired the ability to complete nuclear maturation at a diameter of
approximately 110 µm. The oocytes gradually acquire competence to undergo meiotic maturation and sustain embryonic development after reaching a diameter between 110-120 µm (Raghu et al., 2002).

2.3.1.2 Effect of presence of corpus luteum

Various biological factors affect the in vitro developmental competence of buffalo oocytes including oocyte diameter and follicle size (Tasripoo and Kamonpatana, 1997) and stage of oestrus cycle (Kumar et al., 1997). However, the determination of these parameters is labour intensive and practically cannot be studied in routine commercial embryo production. Oocyte retrieval from abattoir ovaries of buffaloes bearing corpus luteum resulted in higher percent oocyte recovery with greater developmental competence (Kumar et al., 1997; Singla et al., 1999; Nandi et al., 2000a,b). However, contradictory report of no significant effect of CL on buffalo oocyte recovery (Gupta and Sharma, 2001) and fertilization and cleavage rate (Sajjan-Singh et al., 2001) are also on records. Buffalo ovaries without corpus luteum also resulted in greater number of observed follicles, aspirated follicles, COCs and number of grade I and II COCs than their counterparts with corpus luteum (Manjunatha et al., 2009; Khandoker et al., 2011). Similar findings were also recorded in cattle (Lakhera, 2015).

2.3.1.3 Effect of follicular activity

Ovaries with a dominant follicle (> 10 mm) 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). Similar results were obtained in terms of maturation and blastocyst rate in cattle (Gandolfi et al., 1997). On the contrary, 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). Varisanga et al. (1998) further established the role of intra ovarian environment to which oocytes are exposed on their developmental competence (Varisanga et al., 1998). 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 is influenced by the presence of a dominant follicle at the non-growing phase, (Steenweg et al., 2000). A positive relationship existed between early follicular regression and oocyte competence (Vassena et al., 2003). Absence of corpus luteum has also been associated with better in vitro maturation in cattle (Lakhera, 2015).

2.3.2 Cellular predictors/ reflectors of developmental competence

The effect of various cellular predictors of developmental competence of IVP embryos is discussed in the following section.

2.3.2.1 Glucose-6-phosphate dehydrogenase (G-6-PDH) activity

The limited blastocyst rate of 20-30 % after IVM and IVF, indicates insufficiency morphological evaluation in isolation for assessment of developmental competence of oocytes (De Loos et al., 1992). Therefore, Brilliant cresyl blue (BCB) staining has been used as a non-invasive method for selecting more homogeneous and competent oocytes. BCB stain is an electron acceptor, which can be used to semi quantitate the level of glucose-6-phosphate dehydrogenase (G6PDH) activity in the oocytes, by modification of a visual color (Tian et al., 1998). G6PDH is a component of the pentose phosphate pathway (PPP), which provides ribose phosphate for nucleotide synthesis and formation of fatty acids. The BCB test is based on the ability of G6PDH to reduce BCB stain from blue to colourless (Rodriguez-Gonzalez et al., 2002). The brilliant cresyl blue (BCB) assay was found to be useful to select more competent oocytes for in vitro embryo production in pre pubertal goats (Rodriguez-Gonzales et al., 2002 and 2003; Urdaneta et al., 2003), heifers and cows (Pujol et al., 2004). The percentage of morulae plus blastocysts was higher in the BCB+ group than in the BCB– group (23.8 vs. 5.1%). 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 (Alm \textit{et al}., 2005) revealed a significantly higher maturation (72.5 vs 58.1\%) and blastocyst rate (34.1 vs 3.9\%) of BCB\(^+\) oocytes than BCB\(^-\) oocytes. After staining, oocytes that have completed growth phase and low G6PDH activity take blue coloration of the ooplasm and were designated as BCB\(^+\) whereas growing oocytes with high G6PDH activity were without blue coloration were designated as BCB\(^-\) (Opiela \textit{et al}., 2010). Therefore, assessment of G6PDH activity by BCB staining can provide a non invasive cellular predictor of development competence of oocytes (Mirshamsi \textit{et al}., 2013; Mohammadi-Sangcheshmeh \textit{et al}., 2014; Shabankareh \textit{et al}., 2014).

2.3.2.2 Oxidative stress in oocyte-maturation and embryo-development

Buffalo oocytes/embryos are particularly sensitive to oxidative stress due to their high lipid content (Boni \textit{et al}., 1992) and hence show low development competence under \textit{in vitro} conditions. Reactive oxygen species (ROS) production is a normal process of cell metabolism. There are ample evidence to 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 \textit{et al}., 2000; Gasparrini \textit{et al}., 2003; Bain \textit{et al}., 2011).

2.3.2.3 Apoptosis

Apoptosis is the normal or programmed cell death (Kerr \textit{et al}., 1972) occurring spontaneously in cells and is required for the normal development and maintenance of tissue homeostasis. Apoptosis is associated with a series of morphological changes like separation of the dying cell from its adjoining cells, shrinkage and condensation of cytoplasm, increased cell density, compaction of cytoplasmic organelles, compaction of chromatin and its aggregation into large compact granular masses on the inner nuclear membrane, nuclear condensation and indentation of nuclear and cytoplasmic membranes. The nucleus undergoes fragmentation and fragments of the nuclear membrane form apoptotic bodies which may contain nuclear
fragments. The apoptotic cells are either dispersed in the intercellular space, extruded from the tissue, or are phagocytosed by the neighbouring cells (Willie, 1997).

Several hallmarks of apoptosis have been detected on mammalian embryos affecting pre and post implantation development (Jurisicova et al., 1998a,b). Embryos with higher incidence of apoptosis and DNA fragmentation are associated with lower developmental competence (Byrne et al., 1999). Though apoptosis during early embryonic development has been reported to eliminate abnormal cells, but above a certain threshold apoptosis can have detrimental effect on subsequent embryo development (Hardy, 1999).

The possible causes of apoptosis in preimplantation embryos include suboptimal culture conditions (Hardy, 1999) and hyperglycemia leading to excess glucose concentration and hence down regulation of glucose transporters (GLUT) resulting in low intracellular glucose concentration (Chi et al., 2000a). This decrease in glucose utilization by the blastocysts is associated with increased apoptosis (Chi et al., 2000b). Various chromosomal and nuclear abnormalities such as multiploidy and mosaicism, impaired developmental potential, imbalance between growth and survival factors such as IGF-I, IGF-II and TGF-α (Byrne et al., 2002; Makarevich and Markkula, 2002) and exposure to damaging factors such as reactive oxygen species, UV irradiation and heat shock (Paula-Lopez et al., 2012; Jousan and Hansen, 2004) are also associated with increased incidence of apoptosis. In vivo produced embryos have lesser incidence of apoptosis as compared to in vitro produced embryos (Rubio Pomar et al., 2005).

2.3.3 Effect of reactive ROS on developmental competence of IVP embryos

Increase in hydrogen peroxide production and associated risk from reactive oxygen species (ROS) in response to stress during culture is deleterious for embryonic development (Nasr-Esfahani et al., 1990). The 2-cell block observed in mouse embryos cultured in vitro is associated with a rise in ROS whereas no such effect is observed in embryos collected in vivo.
(Noda et al., 1991). Also the deleterious effects of ROS during oocyte maturation may alter the embryo development (Blondin et al., 1997). Oxidative stress in the bovine embryo leads to DNA damage (Takahashi et al., 2000) and enhance the demand for antioxidant enzymes to maintain homeostatic control, which may further compromise developmental potential (Orsi and Leese, 2001). The implications of impaired metabolic activity within embryos for fetal and postnatal development are potentially serious, but direct consequences are yet to be explored. Elevated glucose levels can lead to suppression of insulin and glucokinase expression, decreased mitochondrial function, increased ROS formation, and accelerated apoptosis, as well as activation of common stress activated signaling pathways, which could readily influence proliferative, metabolic, and neuroendocrine axes during later development (Evans et al., 2002; Robertson et al., 2003). For example, one major intracellular target of hyperglycemia is the transcription factor nuclear factor-kB (NF-kB), which in turn can regulate the expression of diverse growth factors, cytokines, and adhesion molecules, all of which have the potential to modulate the phenotypic response to early embryo environment (Fleming et al., 2004). A significantly deleterious effect of oxidative stress on development competence has been reported in the past which could be ameliorated by incorporation of antioxidants and growth factors during embryo culture (Bain et al., 2011; Sadeesh et al., 2014).

2.3.4 Effect of apoptosis on developmental competence of IVP embryos

Apoptosis affects mainly the inner cell mass of the blastocysts (Brison and Schultz, 1997) resulting in poor embryonic development or even arrest of embryonic development (Chi et al., 2000a). The incidence of apoptosis at early embryonic life is often associated with arrest of embryonic development prior to compaction and ultimately poor pregnancy outcomes (Pinto et al., 2002; Heilig et al., 2003). Post-implantation development potential of embryo is dependent on incidence of apoptosis during pre-implantation embryonic stage. Also a higher incidence of apoptosis is found on inner cell mass (ICM) of in vitro embryos as compared to their in vivo produced counterparts. These are plueripotent cells which form the fetus and hence their damage may impact the developmental competence (Maddock-
Hyttel et al., 2003). Early apoptosis in bovine oocytes has been reported to be associated with development competence (Aziz et al., 2014).

2.4 Ameliorating deleterious effects of oxidative stress and apoptosis

2.4.1 Oxygen tension

Oxygen tension in the oviduct is approximately one quarter to one third of atmospheric oxygen tension (Mastroianni and Jones, 1965, Mass et al., 1976) and hence the media incubated at 37 °C at atmospheric oxygen contains the oxygen concentration of 224 µmol/l which is considerably higher than physiological oxygen concentration of approximately 10 µmol/l within the cell (Jones, 1985). Oxidases are oxygen dependent and hence hyperoxic conditions may increase their activity resulting in increase in the concentration of O$_2^-$ within the cell. Reducing the oxygen concentration enhances the embryo development and allows to overcome the 2 cell block (Pabon et al., 1989) because the ROS concentration in bovine embryos increases during culture at higher oxygen concentration (Nagao et al., 1994). Significant improvement in maturation rate, blastocyst rate and pregnancy outcomes was recorded in in vitro embryo production carried out at 5% oxygen tension as compared to 20% oxygen tension in bovines (Leivas et al., 2006; Bain et al., 2011). Further association of oocyte density in the culture and oxygen tension was also recorded (Giotto et al., 2015).

2.4.2 Glutathione (GSH)

Glutathione, is the main non enzymatic defense system against ROS in embryos. It is a tripeptide of glycine, cysteine and glutamine, is an important regulator of REDOX status and has been detected in reproductive tract fluid. A higher concentration of GSH has been reported in mature hamster oocytes as compared to immature oocytes (Perreault et al., 1988) which provides the supporting evidence of its synthesis during the process of oocyte maturation (Yoshida et al., 1993). The higher concentration of GSH in Oocytes (Yoshida et al., 1993) is highly correlated to early embryonic development (Takahashi et al., 1993). It plays a role in reducing the environment in oocytes and embryos and is also the substrate of glutathione peroxidase (GPX), which is a vital antioxidant enzyme. Inhibition of GSH
synthesis has been reported to be associated with DNA damage and its depletion causes an increase in H$_2$O$_2$ concentration and DNA lesions in bovine embryos (Takahashi et al., 1993). Glutathione concentrations measured throughout pre-implantation embryo development decrease as early cleavage proceeds (Gardiner and Reed, 1995). Pre-implantation stage embryos have little capacity for GSH synthesis (Gardiner and Reed, 1995). However, increasing intracellular concentrations of reduced glutathione, especially during oocyte maturation, are associated with both improved fertilization and subsequent embryo development in vitro (Abeydeera et al., 1999; de Matos and Furnus, 2000). GSH has been reported to play a major role in regulating ROS concentrations within the cytoplasm, both directly as a free-radical scavenger, and as a substrate with NADPH for detoxifying ROS using GSH peroxidase (Guerin et al., 2007). Appropriate GSH status is also usually achieved by the inclusion of its constituent amino acids, cysteine, glycine and glutamine, within the incubation medium.

2.4.3 Insulin like growth factor (IGF-I)

The effect of IGF-I on embryo development are mediated by stimulation of protein synthesis through the IGF-I receptors (Harvey and Kaye, 1991) and they include an increase in glucose uptake and expression and translocation of GLUT-1 (Pantaleon and Kaye, 1996). Incorporation of 100 ng/ml IGF-I in TCM-199 medium supplemented with granulose cell and oestrus cow serum lead to significant increase in percent cleavage, balstocyst and hatched blastocysts in bovine (Palma et al., 1997) and buffaloes (Pawshe et al., 1998). Similar beneficial effects of IGF-I supplementation has also been reported by others (Byrne et al., 2002; Demeestere et al., 2004). Its specific effect on pre-implantation embryo development is mediated through its receptors and through signaling pathways distinct from the proliferative action (Foulstone et al., 2005). It has been reported to reduce the proportion of apoptotic cells in pre-implantation embryos induced by heat shock and increased total cell count (Block, 2007). It acts as a survivor factor for bovine pre-implanataion embryo by inducing a multitude of cellular changes including those that maintain competence for embryo development. The anti apoptotic action of IGF-I are mediated through AKT via PI3K pathway, while the
proliferative action involves the MAPKK pathway that allows the embryos to develop to blastocyst stage independent of their action to block apoptosis (Jousan and Hansen, 2007). In buffaloes, supplementation IGF-I (100ng/ml) coupled with antioxidants cysteamine (50µM) in maturation medium (TCM-199) significantly improved maturation, cleavage and blastocyst rate (Singhal et al., 2009).

2.4.3 Epidermal growth factor (EGF)

Epidermal growth factor added to IVC media, promotes nuclear maturation of oocytes in multiple species including rat, mouse, bovine, porcine, and human. Morrish et al. (1987) concluded that EGF causes morphological differentiation of human trophoblasts. According to Lonergan et al. (1996), addition of EGF, irrespective of concentration, to TCM-199 media stimulated cumulus cell expansion and significantly increased the proportion of oocytes attaining metaphase II. Maturation in the presence of EGF significantly increased the proportion of embryos at the 5 to 8-cell stage (52%) and increased the proportion of d 8 blastocysts by 30% when compared to the controls. Sirisathien et al. (2003) also demonstrated that addition of 5 ng/mL of EGF significantly increased the percentage of blastocysts (50%) developing from 4-cell embryos when compared to the control medium (36.5%). Similar beneficial effect has also been reported by Singhal et al. (2009). Similar findings have also been recorded in feline (Fujihara et al., 2014).

2.4.3 n-Propyl gallate

The n-Propyl gallate is a food additive with antimicrobial and antioxidant properties (Gailani and Fung, 1984). It not only increases the activity of Catalase peroxidase and Glutathione reductase enzymes but also acts against the gram +ve and gram –ve bacteria, reduces the psychrotrophs, coliforms and fecal coliforms (Gailani and Fung, 1984). It exerts significant beneficial effect on semen preservation due to its antimicrobial (Gailani and Fung, 1984) and antioxidant properties (Bains et al., 1996). It has more oxygen scavenging capacity than Vitamin C and also protects the cells against superoxide radical damage and prevents lipid peroxidation to maintain sperm plasma membrane integrity (Bains et al., 1996). The beneficial effect as antioxidant may have been enhanced due to its antimicrobial properties as
the contamination of semen samples by microbes may lead to the generation of reactive oxygen species thereby reducing the motility and increasing the membrane damage (Wang et al., 1997). Significant beneficial effect of 15 μM n-Propyl gallate on bubaline sperm motility at refrigeration temperature (Sharma, 1998) and cryopreservation (Shukla and Misra, 2005) has been reported in the past. Though no reported evidence of its beneficial effect on IVEP could be traced but its antimicrobial and antioxidant activities promises its role as a potential candidate for incorporation in embryo culture media.
3. MATERIALS AND METHODS

3.1 Animals/ source of oocytes

The oocytes were recovered from 6 genetically superior healthy Murrah buffaloes of 3-6 years age, maintained at livestock farm, Adhartal by ultrasound guided trans-vaginal ovum pick up (OPU) and from ovaries procured from local abattoir. The experimental animals had normal reproductive tract on per rectal and ultrasonographic examination and were maintained under identical and optimal conditions of feeding and management throughout the study period.

3.2 Materials Used

3.2.1 Plastic wares, chemicals and media

The sources of procurement of plastic wares, chemicals and media etc. used for the experiments are summarized in the table 01.

Table 01: Source of plastic wares, chemicals and media

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Particulars</th>
<th>Name of companies</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Petri dishes</td>
<td>Becton Dickinson, NJ, USA</td>
</tr>
<tr>
<td></td>
<td>Four well embryo culture dish</td>
<td>Nunc, Thermo Fisher Scientific, Rochester, NY, USA</td>
</tr>
<tr>
<td>2</td>
<td>Polypropylene tubes</td>
<td>Nunc, Thermo Fisher Scientific, Rochester, NY, USA</td>
</tr>
<tr>
<td>3</td>
<td>Syringe filters (0.22µm)</td>
<td>Nalgene Corporation, Bedford, USA</td>
</tr>
<tr>
<td>4</td>
<td>Pasture pipettes (230mm)</td>
<td>Brand Co., Wertheim, Germany</td>
</tr>
<tr>
<td>5</td>
<td>Microtips</td>
<td>Axygen Scientific, California, USA</td>
</tr>
<tr>
<td>6</td>
<td>Chemicals and media for cell culture and embryo culture</td>
<td>Sigma Chemical Co., St. Louis, MO, USA, unless otherwise indicated</td>
</tr>
<tr>
<td>7</td>
<td>Research vitro cleave media (RVCL)</td>
<td>Cooks, Queensland, Australia</td>
</tr>
<tr>
<td>8</td>
<td>Tissue culture medium-199 and defined fetal bovine serum</td>
<td>Hyclone laboratories, South Logan, Utah, USA</td>
</tr>
</tbody>
</table>
### 3.2.2 Major equipments used in research work

Equipments used in the study are given in Table 02.

#### Table 02: List of major equipments/instruments, their sources and use

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Equipments</th>
<th>Supplier</th>
<th>Use</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Zoom stereomicroscope</td>
<td>NIKON, Japan, Model- SMZ-64</td>
<td>To search the oocytes and evaluate the quality of embryos.</td>
</tr>
<tr>
<td>2</td>
<td>Fluorescence inverted microscope</td>
<td>Leica, Germany, Model- DMIL-LED</td>
<td>For fluorescence microscopy</td>
</tr>
<tr>
<td>3</td>
<td>Inverted microscope</td>
<td>Nikon ECLIPSE-Ti with thermo plate (Tokat Hit), Japan</td>
<td>To observe oocytes and embryo development.</td>
</tr>
<tr>
<td>3</td>
<td>pH meter</td>
<td>Eutech Instruments, Cyberscan, Malaysia</td>
<td>To adjust the pH of media</td>
</tr>
<tr>
<td>8</td>
<td>Hera cell 150l CO₂ incubator</td>
<td>Thermo Fisher Scientific, USA</td>
<td>To maintain the humidified environment, temperature and CO₂ level for culture of oocytes and embryos.</td>
</tr>
<tr>
<td>10</td>
<td>Bio safety cabinet</td>
<td>Thermo Scientific 1300 series, A2, USA</td>
<td>To maintain the sterile environments during media preparation, in vitro fertilization embryo culture</td>
</tr>
<tr>
<td>11</td>
<td>Centrifuge machine</td>
<td>Eppendorf</td>
<td>Used for sperm processing</td>
</tr>
<tr>
<td>12</td>
<td>Ultrasound machine</td>
<td>Aloka, Japan, Model- SSD500</td>
<td>Used to visualize follicles for transvaginal ovum pick up</td>
</tr>
<tr>
<td>13</td>
<td>Suction assembly</td>
<td>Cook® Queensland, Australia</td>
<td>For application of negative pressure for oocyte aspiration using transvaginal OPU</td>
</tr>
<tr>
<td>14</td>
<td>Test tube heater</td>
<td>Cook® Queensland, Australia</td>
<td>For maintaining temperature of oocytes and media during OPU</td>
</tr>
<tr>
<td>15</td>
<td>Bench-top CO₂ incubator (Minc)</td>
<td>Cook® Queensland, Australia</td>
<td>For embryo culture</td>
</tr>
</tbody>
</table>

### 3.4 Collection of oocytes

The cumulus oocyte complexes used for various experiments in the present study were derived from the ovaries procured from local abattoir or from live donors using transvaginal ultrasound guided ovum pick up (OPU).
3.4.1 Oocyte aspiration from abattoir ovaries

Buffalo ovaries were collected from large animal slaughterhouse, Jabalpur (M.P.) in sterile warm normal saline (37°C) supplemented with antibiotics (Appendix I) and transported to the laboratory within 1-2 hrs of slaughter. The extraovarian tissue attached to the ovaries was excised off and the ovaries were washed 3 times with normal saline and 2 times with dulbecco’s phosphate buffer saline (DPBS) followed by brief exposure to 70% ethanol for 8-10 sec and finally rinsed with DPBS (Apéndix I).

The cumulus oocyte complexes (COCs) were aspirated from 5-10 mm sized ovarian follicles by syringe aspiration method using 20 gauge needles. Aspirated material was transferred in 60mm size searching dish containing DPBS. COCs were searched under stereo-zoom microscope and were placed in 35 mm petri dish containing 50µL DPBS drops. The oocytes were washed 4 times each in DPBS and washing media and finally transferred to 50µL of maturation media.

3.4.2 Oocyte aspiration by transvaginal ultrasound guided Ovum pick-up (OPU) from live animals

The aspiration of oocytes from live donors was done by transvaginal ultrasound guided ovum pick up (OPU) method as described by Pieterse et al. (1988). OPU was performed using an ultrasound machine (SSD 500; Aloka, Japan) equipped with a 5 MHz transvaginal OPU probe fitted with a needle guide (Plate 1a). For follicle aspiration, a 490 mm long single lumen 17 Gauge puncture needle (Cova needle ‘Type A’, Misawa Medical Industry Co., Ltd., Tokyo, Japan) connected to a 15 ml Falcon collection tube and to a suction assembly (Cooks, Australia) was used. The aspiration system was attached to a vacuum pump producing a pressure of 100 mm Hg and a fluid flow rate of 10-15 ml/minute.

A total of 68 sessions of OPU were carried out. Immediately prior to OPU session, back racking was done to empty the rectum followed by administration of epidural anesthesia using 5-7 ml of Lignocaine Hydrochloride 2% solution (Xylocaine®; AstraZeneca Pharma India Limited, India). Subsequently, the perineum was properly scrubbed and cleaned with
soap and water and wiped dry. Well lubricated OPU probe was introduced per vaginally after fixing ovaries by another hand introduced per rectally. Ovaries were imaged on the screen and structures like follicles or corpus luteum were recorded. The puncture needle was guided through the vaginal wall and the tip of the needle was placed into the follicles under visual control on a monitor. A puncture line on the screen was used to facilitate the exact positioning of needle tip and follicles (Plate 1b). When inside a follicle, the needle was twisted until all visible fluid was sucked out. The aspiration system was rinsed with DPBS with 0.3% bovine serum albumin (BSA), 50 µg/ml gentamicin and 20 µg/ml heparin sodium (oocyte collection medium) before and during the follicle aspiration. All visible follicles larger than 2 mm in diameter were successively punctured and aspirated. Before puncture, the size of each follicle was measured and categorized as small (2-7 mm) or large (> 7 mm). The aspirated follicular fluid was transferred into a 90 mm petridish and COCs were recovered under a stereomicroscope within 15 min. The COCs were classified into four morphological categories based on number and density of cumulus cell layers as well as colour and granulation of ooplasm. COCs were subsequently processed for in vitro embryo production (IVEP).

Initially the animals were subjected to once weekly OPU sessions (n=34) for a period of four consecutive weeks after induction of new cycle by injection of PGF$_2$α at 11 days interval. The first OPU session was initiated after 24 h of second PGF$_2$α. Similarly, twice a week OPU sessions were also performed. After four weeks of use in OPU, animals were provided rest of 3 weeks followed by induction of a new cycle in similar manner and subsequent OPU sessions.

Comparisons of COCs grades between once weekly and twice weekly ovum pick up sessions and between abattoir ovaries and ovum pick up was drawn.

3.5 Grading of Immature oocytes

Isolation and grading of oocytes was carried out under stereo zoom microscope in a 90 mm petridish. Recovered oocytes were graded from I to IV as per the following criteria:
Grade-I (good quality): Oocytes having compact cumulus oocyte complex (COC) with more than 4-5 layers of complete ring cumulus cells and homogeneous granular gray cytoplasm.

Grade-II (fair quality): Cumulus oocyte complex with 2-3 layers of cumulus cells surrounding the zona pellucida with evenly granular ooplasm.

Grade-III (average quality): Oocytes with 1-2 layers of complete or incomplete ring of cumulus cells with irregular dark ooplasm.

Grade-IV (poor quality): Oocytes without cumulus cells and having irregular dark ooplasm.

Effect of grade of oocytes on development competence was studied by performing IVM, IVF and IVC of different grades of oocytes in separate droplets of the respective media. Maturation rate, cleavage rate and blastocyst rate of each grade of oocyte was recorded.

3.5 *In vitro* maturation of oocytes

All the recovered oocytes were washed three times in *in vitro* maturation medium (TCM-199; Appendix II). The 50 μl droplets of maturation medium TCM-199 medium were prepared in petri dish (35mm) and oocytes were transferred in groups of 25-30 oocytes per droplet. The droplets were then covered with sterile pre-equilibrated (38.5°C) mineral oil (Sigma M5310) and incubated in CO₂ incubator with >95% humidity, 5% CO₂ at 38.5°C for 24 hrs.

3.5.1 Assessment of oocytes for *in vitro* maturation

Following two methods were carried out for assessment of IVM oocytes:

i. Cumulus cell expansion

ii. Identification of polar body

(i) Cumulus cell expansion

Maturation of oocytes was classified on the visual assessment of the degree of cumulus cells expansion according to the procedure described by Hunter and Moor (1987). The assessment of matured oocytes was done in the following way:
Grade-A: Full cumulus cell expansion was characterized by adhering nature of cumulus cells and enlargement of the cumulus mass to at least 300µm away from zona pellucida.

Grade-B: Moderate cumulus cell expansion, at the most 150-200µm away from zona pellucida.

Grade-C: Slight expansion of cumulus cells, characterized by cumulus cells being tightly adhering to the zona pellucida.

(ii) Identification of polar body under light microscope

Matured oocytes were collected in eppendorf tubes and vortexed in 0.1% hyaluronidase (Sigma H4272; Appendix III) in DPBS (without Ca$^{++}$ and Mg$^{++}$) for one minute followed by centrifugation at 1000 rpm for one minute. The denuded oocytes were kept in 35 mm petri dish and observed under stereomicroscope. The oocytes having one extruded small body (polar body) between peri-vitelline membrane and zona pellucida were considered as mature oocyte and were further subjected to Hoechst 33342 staining for confirmation of polar body.

3.5.2 Hoechst 33342 staining for confirmation of polar body

Stock solution was prepared as 1 mg/ 1ml DPBS+ 20% glycerol. From this 15µl was taken and added in 985µl DPBS containing 20% glycerol and aliquots of 50µl were made and stored at -20°C. The aliquots were stored in dark vials to protect from light (kept away from direct light sources).

The matured denuded oocytes were washed five times in DPBS and subsequently were placed in small droplet of Bis benzimide H33342 trihydrochloride (Hoechst stain 33342; Sigma B2261, Appendix IV) for 10-15 min and observed under fluorescent microscope. The presence of polar body was confirmed by white fluorescence.

3.6 In vitro fertilization process

In vitro fertilization was done by using ejaculated sperms as well as epididymal sperms.

3.6.1 Semen collection

Bracket and Olliphant (BO) media was used for sperm preparation for IVF. The composition of BO media is given in Appendix V.
Buffalo epididymis was collected from large Animal Slaughterhouse, Jabalpur (M.P.) in sterile warm normal saline (37°C) and transported to the laboratory within 1-2 hrs of slaughter. The epididymis was washed 3 times with normal saline and twice with DPBS, followed by brief exposure of 70% ethanol for 10-15 sec and finally rinsed with DPBS. The epididymal sperms were harvested by multiple incisions on caudae epididymis and collected into DPBS in 15 ml centrifuge tube and immediately centrifuged at 1000 rpm for 8 min. The supernatant was removed, leaving 1ml sperm pellet.

Ejaculated semen was obtained by semen collection from 3 healthy buffalo bulls maintained at Livestock Farm, Nanaji Deshmukh Veterinary Science University using sterile goat artificial vagina using method described by Shukla (2002). The semen of the three bulls was randomly assigned to different IVF trials without bias.

3.6.2 Semen evaluation

Immediately after collection, the semen volume, color, concentration, morphology, initial and gross motility of sperms were assessed.

3.6.2.1 Mass motility

Mass motility of undiluted semen was observed under phase contrast microscope (100x) on a glass slide kept at 37°C and classified as per vigour of swirls and wave formation and graded as below:

<table>
<thead>
<tr>
<th>Number of swirls (Per-second)</th>
<th>Motility (%)</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 to 5</td>
<td>80 and above</td>
<td>+ 4</td>
</tr>
<tr>
<td>3 to 4</td>
<td>70 and above</td>
<td>+ 3</td>
</tr>
<tr>
<td>2 to 3</td>
<td>60 and above</td>
<td>+ 2</td>
</tr>
<tr>
<td>1 to 2</td>
<td>50 and above</td>
<td>+ 1</td>
</tr>
</tbody>
</table>

3.6.2.2 Individual motility

Individual motility of diluted semen was observed under phase contrast microscope (400x) fitted with stage biotherm (37°C) and classified as described below:
<table>
<thead>
<tr>
<th>S. No</th>
<th>Motility pattern</th>
<th>Motility of spermatozoa (%)</th>
<th>Grade</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Forward and progressive</td>
<td>80 per cent and above</td>
<td>excellent</td>
</tr>
<tr>
<td>2.</td>
<td>Forward and progressive</td>
<td>70 per cent and above</td>
<td>very good</td>
</tr>
<tr>
<td>3.</td>
<td>Forward and progressive</td>
<td>60 per cent and above</td>
<td>good</td>
</tr>
<tr>
<td>4.</td>
<td>Forward and progressive</td>
<td>50 per cent and above</td>
<td>fair</td>
</tr>
<tr>
<td>5.</td>
<td>Circular or static movement</td>
<td>below 50 per cent</td>
<td>poor</td>
</tr>
</tbody>
</table>

### 3.6.2.3 Sperm preparation/ capacitation

The sperms obtained from both the sources were processed by washing in BO medium (Brackett and Oliphant, 1975; Appendix V) using the following protocol:

- The 0.2ml of semen pellet was distributed in 5-6 eppendorf tubes (1.5ml).
- One ml of equilibrated BO-A medium was added to each of the tubes and kept in the CO₂ incubator for 1h at 38.5˚C for swim up.
- After swim up, the top 0.7ml medium from all these tubes were pooled into a sterile 15ml centrifuge tube and made final volume upto 10ml and centrifuged @ 1000 rpm for 8 min.
- Supernatant was discarded and the sperm pellet was again re-suspended with 10ml equilibrated BO-B medium having 0.3% BSA, followed by centrifugation @ 1000 rpm for 8 min.
- Supernatant was discarded again and the sperms were again re-suspended in 5ml of fertilization medium, having heparin and 0.6% BSA.
- Supernatant was discarded and the sperm pellet was transferred in 2 ml tube to make final volume upto 1.5ml, followed by centrifugation @ 1000 rpm for 5 min.
- With a fine bore of glass Pasteur pipette, sperm pellet was loosen and kept in incubator for 30-40 min.
3.6.3 Oocytes preparation

The matured oocytes of each source were collected from IVM drops and transferred in 35mm dish containing 50µl drops of fertilization medium covered with mineral oil. The oocytes were washed 2-3 times in the fertilization drops and finally incubated @ 15-20 oocytes/drop in CO₂ incubator at 38.5°C and 5% CO₂ for at least 30 min before IVF.

3.6.4 In vitro fertilization

In vitro fertilization was done in fertilization medium having 50 µg/ml heparin and 0.6% BSA. The matured oocytes were washed thrice in fertilization medium and transferred into the final fertilization drop (50µl), prepared in 35mm petri dish, covered with sterile mineral oil. The final concentration of sperm used for IVF was adjusted @ 1 million sperms/ml. The matured oocytes were co-incubated with capacitated sperms at 5% CO₂ at 38.5°C for 18 hrs.

3.6.5 Embryo culture

After 18 hrs of co-incubation, the oocytes of each source were partially denuded using 0.1 % hyaluronidase (Appendix III) and cultured in Research vitro cleave (RVCL) medium supplemented with 1% BSA upto 6th d. For embryo culture, the presumptive zygotes were washed six times and transferred in 500µl pre-equilibrated RVCL medium in a well of 4 well dishes (Nunc) covered with 500 µl mineral oil and incubated in CO₂ incubator at 38.5°C and 5% CO₂ and 20% O₂ tension upto 6th day.

3.7 Assessment of effect of various morpho-functional and cellular predictors of developmental competence of buffalo oocytes

The effect of various morpho-functional and cellular predictors on developmental competence of buffalo oocytes was assessed as per the under mentioned procedure.

3.7.1 Assessment of effect of various morpho-functional parameters on developmental competence of buffalo oocytes

The oocytes collected were classified according to the stage of estrus cycle (Early luteal phase, luteal and follicular phase), size of follicle (2-7
mm: small and >7mm large), oocyte diameter (< 120µm, 120-150µm and > 150µm) and grade of oocyte (Grade I, II, III and IV). The effect of season (Rainy, Winter, Summer) was also assessed. The effect of each class on follicular activity (follicles per ovary), oocyte retrieval (COCs per ovary and Oocyte recovery rate), oocyte quality (grading of oocytes, in vitro maturation (cytoplasmic and nuclear maturation) was assessed and compared. Separate experiments were also conducted to study the effect of different morpho-functional parameters on developmental competence of buffalo oocytes and parameters like cleavage rate, morula rate and blastocyst rate was recorded and compared in each group.

3.7.2 Assessment of effect of cellular predictors on developmental competence of oocytes

The effect of following cellular reflectors on developmental competence of buffalo oocytes was assessed as per the methods described below.

3.7.2.1 Glucose -6-Phosphate dehydrogenase activity (G6PDH assay)

To study the effect of G6PDH on developmental competence, the recovered immature oocytes of all grades were classified on the basis of size of source follicle (Small and Large). These were subjected to 26µM Brilliant cresyl blue (BCB, Sigma, B-5388) for 90 min at 38.5°C and 5% CO₂ under humidified atmosphere of incubator as per the procedure described by Ghanem et al. (2007). The stained oocytes were washed in DPBS and examined under a inverted microscope and categorized into two groups according to their degree of cytoplasmic staining:

- Oocytes with low G-6PDH activity
  - Any degree of blue colouration in the cytoplasm (BCB⁺) – Fully grown immature oocytes.
- Oocytes with high G-6PDH activity
  - No visual blue colouration – Growing immature oocytes.

These BCB stained COCs were subsequently subjected to IVEP to study their developmental competence.
3.7.2.2 Reactive oxygen assay in matured oocytes/embryos

The matured oocytes were incubated with 10µM H2DCFDA (2’,7’-dichlorodihydro fluorescein diacetate) dye (Invitrogen) for 30 min in dark at 39°C temperature. After incubation, the oocytes were washed 3 times with PBS and immediately examined under a fluorescence microscope with excitation and emission filters of 450-480nm and 515nm, respectively (Hwang et al., 2012). The fluorescent images were recorded at JPEG files using a digital camera and the intensity of fluorescence in each oocyte/embryo was analyzed using image J software 1.37. The fluorescence intensity was recorded as pixel/oocyte or embryos.

3.7.2.3 Apoptosis

The apoptosis of matured oocytes of both sources viz., abattoir and ovum pick-up, were subjected to apoptotic assay using ApoDETECT™ ANNEXINV-FITC Kit (Invitrogen™, Cat- 33-1200) as per method described by Aziz et al. (2014) with slight modifications. Briefly, the oocytes of either sources were washed in ice cold PBS (pH-7.4) and were subsequently suspended in 500µl of 1X binding buffer kept in a well of 4 well plate. Oocytes were then transferred into a second well containing 10µl of Annexin V-FITC +190µl 1x binding buffer and incubated for 10 min at room temperature. These oocytes were washed with 1X binding buffer and finally resuspended in 10µl propidium iodide (20µg/ml) + 190µl of binding buffer.

The oocytes were analyzed under fluorescence microscope.

3.7.2.4 Differential cell count

The blastocysts were incubated in 500µl of solution I (Appendix VI) for 10-15 sec. The blastocyst are immediately transferred into 500µl of solution-II (Appendix VI) and incubated at 4°C overnight. The stained blastocysts were observed at wavelength 330-380nm and barrier filter 420nm and cells of inner cell mass and trophectoderm were counted.

The inner cell mass was stained as blue fluorescence and trophoectoderm took tinged orange stain.
3.8 Effect of supplementation of antioxidants and antiapoptotic factors in culture media

The oocytes retrieved from abattoir ovaries were randomly assigned to supplementation of antioxidant and antiapoptotic factors (Table 3) in RVCL media and subsequently cultured for seven days.

After 72 hours of co-culture the cleavage rate was recorded and on seventh day of co-culture blastocyst rate, ROS assay, apoptosis assay and differential cell count of inner cell mass and trophectoderm was carried out to determine the effect of antioxidant and antiapoptotic factors on developmental competence of in vitro produced buffalo embryos.

3.9 Embryo transfer

The in vivo developmental competence was assessed by non surgical transfer of in vitro produced embryos in synchronized recipients. Eight healthy Murrah buffaloes aged 2-5 years were used as recipients for embryo transfer. The estrus of the recipients was synchronized so that they show behavioural estrus at ± 12-16 hours of the time of IVF, using two injections of Cloprostenol sodium (Clostenol, Zydus Animal Health Limited, India). The behavioural estrus was confirmed by rectal examination.

On the day of embryo transfer corpus luteum was assessed and the recipient was administered epidural anaesthesia using 5-7 ml Lignocaine hydrochloride 2% (Xylocaine, AstraZeneca, India), immediately after backracking to empty the rectum of dung. The perineum was scrubbed and cleaned with soap followed by mild antiseptic solution.

Grade I embryos (morula/ early blastostostyst) were transferred to synchronized recipients after loading in radiated mini embryo transfer straws (IMV, France). The straw containing embryo was subsequently mounted on embryo transfer gun (IMV, France). The embryo was gently transferred to the tip (anterior one third part) of the uterine horn, epsilateral to the ovary bearing corpus luteum.

3.4.9. Pregnancy Diagnosis

Pregnancy diagnosis was performed after 2 months of embryo transfer using portable ultrasound machine (Exago, ECM, France).
Statistical Analysis

The data were analyzed using one way analysis of variance (ANOVA) and correlation analysis using standard statistical software (Systat, version 11).

Table 03: Supplementation of antioxidant and antiapoptotic factors in culture medium

<table>
<thead>
<tr>
<th>Groups</th>
<th>Additives</th>
<th>Source</th>
<th>Replicates</th>
<th>Nos. Of COCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>5 % O2 tension</td>
<td>-</td>
<td></td>
<td>356</td>
</tr>
<tr>
<td>T2</td>
<td>n-Propyl gallate (15 µM)</td>
<td>Sigma (Catalogue no 48710)</td>
<td></td>
<td>329</td>
</tr>
<tr>
<td>T3</td>
<td>Glutathione (1 mM)</td>
<td>Sigma (Catalogue no G4251)</td>
<td></td>
<td>346</td>
</tr>
<tr>
<td>T4</td>
<td>Epidermal growth factor (EGF) (10 ng ml⁻¹)</td>
<td>Sigma (Catalogue no E4127)</td>
<td>11</td>
<td>336</td>
</tr>
<tr>
<td>T5</td>
<td>Insulin like growth factor-I (IGF-I) (100 ng ml⁻¹)</td>
<td>Sigma (Catalogue no I8779)</td>
<td></td>
<td>342</td>
</tr>
<tr>
<td>Control</td>
<td>20 % O2 tension</td>
<td>-</td>
<td></td>
<td>332</td>
</tr>
</tbody>
</table>
EXPERIMENTAL DESIGN

Oocyte Retrieval

Aspiration technique (slaughter house ovaries)

Follicular size
Presence of CL
Oocyte diameter
G6PDH activity

Ultrasound guided transvaginal ovum pick up (OPU)

In vitro maturation

Cumulus expansion
Polar body extrusion
Nuclear maturation by Hoechst 33342 stain
ROS assay
Apoptosis assay

In vitro capacitation of spermatozoa

In vitro fertilization

In vitro culture

Cleavage rate
Blastocyst rate
ROS
Apoptosis
Differential cell count

Study of developmental Competence of buffalo oocytes
# EXPERIMENTAL DESIGN

<table>
<thead>
<tr>
<th>Groups</th>
<th>Additives</th>
<th>Replicates</th>
<th>Nos. Of COCs</th>
<th>Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>5 % O2 tension</td>
<td></td>
<td>356</td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>n-Propyl gallate (15 µM)</td>
<td></td>
<td>329</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>Glutathione (1 mM)</td>
<td>11</td>
<td>346</td>
<td>ROS assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Apoptosis</td>
</tr>
<tr>
<td>T4</td>
<td>Epidermal Growth Factor (EGF) (10 ng ml⁻¹)</td>
<td></td>
<td>336</td>
<td>Differential cell count</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cleavage rate</td>
</tr>
<tr>
<td>T5</td>
<td>Insulin like Growth Factor-I (100 ng ml⁻¹)</td>
<td></td>
<td>342</td>
<td>Blastocyst rate</td>
</tr>
<tr>
<td>Control</td>
<td>20 % O2 tension</td>
<td></td>
<td>332</td>
<td>Differential cell count</td>
</tr>
</tbody>
</table>

Study of effect of antioxidant and antiapoptotic factors
4. RESULTS

The present study was conducted in three phases I, II and III to study the developmental competence of in vitro produced (IVP) buffalo embryos, effect of ROS and apoptosis on in vitro embryo production (IVEP) and effect of antioxidants and anti apoptotic factors on IVEP.

Phase I: Retrieval rate and developmental competence of oocytes retrieved by transvaginal ultrasound guided ovum pick-up (OPU) and aspiration from abattoir ovaries.

Phase II: The effect of apoptosis and reactive oxygen species (ROS) on in vitro embryo production.

Phase III: The effect of various antioxidants and anti apoptotic factors to ameliorate the deleterious effects of apoptosis and ROS.

A series of experiments were conducted in different phases and the results of the experiments are presented below.

4.1 Oocyte retrieval

Two different techniques of oocyte retrieval viz. aspiration from abattoir ovaries and oocyte retrieval from live donors using OPU were used for oocyte retrieval (Table 4 to 15).

4.1.1 Oocyte retrieval by ultrasound guided ovum pick up

Oocyte retrieval by OPU was done to study the recovery rate, effect of donor, collection frequency and comparative developmental competence with abattoir derived ovaries (Plate 2 to 5).

4.1.1.1 Recovery rate by OPU

Transvaginal ultrasound guided ovum pick-up technique was performed in six genetically superior Murrah buffaloes. A total of 68 OPU sessions were performed and the results are tabulated in table 4 and 5.

During ultrasound scanning prior to the OPU session, the total numbers of large and small follicles present on both the ovaries of each donor were recorded. The mean total numbers of follicle, number of small and large follicle were recorded to be 5.56±0.14, 3.15±0.08 and 2.41±0.10, respectively
with an average of 2.78±0.07 follicles per ovary (Table 4; Figure 1). There
was no statistically significant difference between different oocyte donors in
the number of follicles recorded and follicles per ovary in the present study.
Out of the scanned follicles, an average of 3.85±0.10 follicles was punctured
for aspiration of oocytes (Table 5).

Table 4: Follicular activity in buffaloes undergoing transvaginal ultrasound guided ovum pick-up

<table>
<thead>
<tr>
<th>Oocyte donors</th>
<th>Replicates</th>
<th>No. of small follicles</th>
<th>No. of large follicles</th>
<th>Total No. of follicle recorded</th>
<th>Follicles per ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>11</td>
<td>3.27±0.14</td>
<td>2.73±0.14</td>
<td>6.00±0.19</td>
<td>3.00±0.10</td>
</tr>
<tr>
<td>D2</td>
<td>11</td>
<td>3.09±0.21</td>
<td>2.45±0.25</td>
<td>5.54±0.34</td>
<td>2.77±0.17</td>
</tr>
<tr>
<td>D3</td>
<td>12</td>
<td>3.08±0.19</td>
<td>2.50±0.23</td>
<td>5.58±0.31</td>
<td>2.79±0.16</td>
</tr>
<tr>
<td>D4</td>
<td>13</td>
<td>3.17±0.17</td>
<td>2.25±0.18</td>
<td>5.42±0.26</td>
<td>2.71±0.13</td>
</tr>
<tr>
<td>D5</td>
<td>12</td>
<td>3.25±0.18</td>
<td>2.50±0.23</td>
<td>5.75±0.30</td>
<td>2.88±0.15</td>
</tr>
<tr>
<td>D6</td>
<td>9</td>
<td>3.00±0.26</td>
<td>2.00±0.26</td>
<td>5.00±0.47</td>
<td>2.50±0.24</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>3.15±0.08</td>
<td>2.41±0.10</td>
<td>5.56±0.14</td>
<td>2.78±0.07</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

In the present study an average of 1.96±0.10 cumulus oocyte complexes (COCs) were retrieved by OPU technique with 0.98±0.05 COCs per ovary (Figure 2a). The oocyte retrieval rate in the present study was 50.46±2.75 (Figure 2b). There was no significant difference between oocyte donors in terms of number of COCs retrieved, COCs per ovary and oocyte retrieval rate in the present study (Table 5).

Table 5: Oocyte retrieval by transvaginal ultrasound guided ovum pick-up

<table>
<thead>
<tr>
<th>Oocyte donors</th>
<th>Replicates</th>
<th>Total no. of follicles aspirated</th>
<th>No. of COCs retrieved</th>
<th>COCs per Ovary</th>
<th>Oocyte recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>11</td>
<td>4.00±0.13</td>
<td>2.09±0.21</td>
<td>1.05±0.11</td>
<td>54.39±5.70</td>
</tr>
<tr>
<td>D2</td>
<td>11</td>
<td>4.00±0.27</td>
<td>2.00±0.26</td>
<td>1.00±0.13</td>
<td>43.79±6.42</td>
</tr>
<tr>
<td>D3</td>
<td>12</td>
<td>4.08±0.29</td>
<td>2.00±0.28</td>
<td>1.00±0.14</td>
<td>49.03±5.62</td>
</tr>
<tr>
<td>D4</td>
<td>13</td>
<td>3.67±0.19</td>
<td>2.00±0.21</td>
<td>1.00±0.10</td>
<td>54.86±5.07</td>
</tr>
<tr>
<td>D5</td>
<td>12</td>
<td>3.92±0.23</td>
<td>2.17±0.21</td>
<td>1.08±0.10</td>
<td>55.56±4.44</td>
</tr>
<tr>
<td>D6</td>
<td>9</td>
<td>3.40±0.34</td>
<td>1.60±0.27</td>
<td>0.80±0.13</td>
<td>45.17±6.03</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>3.85±0.10</td>
<td>1.96±0.10</td>
<td>0.98±0.05</td>
<td>50.46±2.75</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference
4.1.1.2 Frequency of OPU sessions

To study the effect of frequency of OPU sessions on oocyte retrieval in Murrah buffaloes, the oocyte donors were subjected to once weekly OPU sessions (n=34) for a period of four weeks after induction of a new cycle followed by a rest of three weeks before starting fresh OPU session on the same animal. Similarly, twice a week OPU sessions (n=34) were also performed. The results are tabulated in table 6 and 7.

**Table 6:** Effect of frequency of ovum pick-up sessions on follicular activity

<table>
<thead>
<tr>
<th>Frequency of sessions</th>
<th>Replicates</th>
<th>No. of small follicles</th>
<th>No. of large follicles</th>
<th>Total no. of follicles recorded</th>
<th>Follicles per ovary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once weekly</td>
<td>34</td>
<td>3.14±0.11</td>
<td>2.48±0.13</td>
<td>5.63±0.18</td>
<td>2.84±0.09</td>
</tr>
<tr>
<td>Twice weekly</td>
<td>34</td>
<td>3.15±0.10</td>
<td>2.33±0.13</td>
<td>5.49±0.19</td>
<td>2.74±0.09</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The average number of small, large and total follicles recorded in once weekly OPU sessions in the present study were 3.14±0.11, 2.48±0.13 and 5.63±0.18, respectively with an average of 2.84±0.09 follicles per ovary. In twice weekly sessions also the similar results were obtained with 3.15±0.10, 2.33±0.13 and 5.49±0.19 small, large and total follicles, respectively with an overall average of 2.74±0.09 follicles per ovary (Figure 3).

There was no significant difference between large, small and total follicle and follicles per ovary between once weekly and twice weekly OPU sessions. The mean number of follicles aspirated in once weekly (3.97±0.15) were also not significantly different with twice weekly (3.73±0.14) OPU sessions (Table 7; Figure 4a).

**Table 7:** Effect of frequency of ovum pick-up sessions on oocyte retrieval

<table>
<thead>
<tr>
<th>Frequency of OPU sessions</th>
<th>Replicates</th>
<th>Follicles aspirated</th>
<th>COCs retrieved</th>
<th>COCs per Ovary</th>
<th>Oocyte recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once weekly</td>
<td>34</td>
<td>3.97±0.15</td>
<td>1.91±0.14</td>
<td>0.96±0.07</td>
<td>47.90±3.30</td>
</tr>
<tr>
<td>Twice weekly</td>
<td>34</td>
<td>3.73±0.14</td>
<td>2.00±0.13</td>
<td>1.00±0.06</td>
<td>52.93±3.20</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference
An average of 1.91±0.14 COCs were retrieved in once weekly OPU session with 0.96±0.07 COCs per ovary and oocyte recovery rate of 47.90±3.30 against respective values 2.00±0.13, 1.00±0.06 and 52.93±3.20, in twice weekly OPU sessions (Figure 4b). There was no significant difference in mean number of COCs retrieved, COCs per ovary and oocyte recovery rate between once and twice weekly OPU sessions.

4.1.1.3 Effect of retrieval techniques on oocyte retrieval

In the comparative study of two retrieval techniques viz. transvaginal ultrasound guided ovum pick-up and aspiration from abattoir derived ovaries (Table 8; Figure 5), a significantly (p<0.01) higher average COCs per ovary was recorded on aspiration from abattoir derived ovaries (3.24±0.08) as compared to OPU (0.98±0.05). Similarly oocyte recovery rate was also recorded to be significantly (p<0.01) higher in abattoir derived ovaries as compared to ultrasound guided OPU (67.26±0.28 vs. 50.42±2.53%). However, on in vitro maturation of retrieved oocytes, the maturation rate was significantly (p<0.01) higher in OPU derived COCs (87.50±4.34) as compared to abattoir derived ovaries (84.91±0.44).

Table 8: Effect of retrieval technique on oocyte recovery and in vitro maturation

<table>
<thead>
<tr>
<th>Oocyte retrieval technique</th>
<th>Replicates</th>
<th>No. of follicles aspirated</th>
<th>No. of COCs retrieved</th>
<th>COCs per ovary</th>
<th>Oocyte recovery rate (%)</th>
<th>COCs showing polar body (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPU</td>
<td>68</td>
<td>262</td>
<td>133</td>
<td>0.98±0.05</td>
<td>50.42±2.53</td>
<td>87.50±4.34</td>
</tr>
<tr>
<td>Abattoir ovaries</td>
<td>53</td>
<td>5341</td>
<td>3593</td>
<td>3.24±0.08</td>
<td>67.26±0.28</td>
<td>84.91±0.44</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.1.2 Effect of various morpho-functional parameters on oocyte retrieval from abattoir ovaries

Different experiments were conducted to study the effect of various morpho-functional parameters on oocyte retrieval from abattoir derived ovaries. The results are present in the following section (Table 9 to 11).
4.1.2.1 Effect of follicular size on oocyte retrieval from abattoir derived ovaries

A total of 1347 large (>7 mm) and 988 small (2-7 mm) follicles were aspirated to study the effect of follicular size on oocyte retrieval. Mean number of large follicles per ovary (3.07±0.12) were observed, which was significantly (p<0.01) higher as compared to small follicles (2.25±0.07). The mean number of COCs per ovary retrieved from the large follicles (1.92±0.08) were also significantly (p<0.01) higher as compared to small follicles (1.17±0.24). The oocyte recovery rates of follicles retrieved from large and small follicle, in the present study was 62.49±0.84 and 52.00±0.66% respectively, the value being significantly higher in large follicles than small follicles (Table 9; Figure 6b).

Table 9: Effect of follicle size on oocyte retrieval from abattoir ovaries

<table>
<thead>
<tr>
<th>Follicle size</th>
<th>Replicates/No. of ovaries</th>
<th>No. of follicles aspirated</th>
<th>Follicles per ovary</th>
<th>Total no. of COCs retrieved</th>
<th>COCs per ovary</th>
<th>Oocyte recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>15/439</td>
<td>1347</td>
<td>3.07±0.12</td>
<td>843</td>
<td>1.92±0.08</td>
<td>62.49±0.84</td>
</tr>
<tr>
<td>Small</td>
<td>15/439</td>
<td>988</td>
<td>2.25±0.07</td>
<td>514</td>
<td>1.17±0.24</td>
<td>52.00±0.66</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.1.2.2 Effect of phase of oestrus cycle on oocyte retrieval from abattoir derived ovaries

The stage of oestrus cycle of the abattoir derived ovaries was classified on the basis of presence of a developing small corpus luteum (early luteal), fully developed large corpus luteum (luteal) and a dominant follicle of > 7 mm without corpus luteum or a regressing corpus luteum (follicular phase). To study the effect phase of oestrus, 1339 follicles from 406 ovaries in follicular phase, 1191 follicles from 371 ovaries in early luteal phase and 1121 follicles from 233 ovaries in luteal phase were aspirated. A total of 913, 831 and 758 COCs were retrieved respectively from follicular, early luteal phase and luteal phase ovaries (Table 10). The mean number of follicles per ovary in follicular, early luteal phase and luteal phase of oestrus cycle was recorded to be 3.34±0.12, 3.23±0.13 and 4.87±0.32, respectively (Figure 7a). Though, there was no significant difference between the mean number of follicles per
ovary in ovaries in follicular and early luteal phase, a highly significant difference (p<0.01) was recorded between ovaries in luteal phase of the cycle with that in follicular phase and early luteal phase. Similar trend was observed in COCs retrieved per ovary where mean number of COCs retrieved per ovary were not significantly different in follicular (2.28±0.09) and early luteal (2.26±0.11) phase, whereas a significantly higher number of COCs per ovary were retrieved during luteal phase (3.29±0.23) as compared to the other two phases.

Table 10: Effect of phase of oestrus cycle on oocyte retrieval from abattoir ovaries

<table>
<thead>
<tr>
<th>Phase of estrus cycle</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of follicles aspirated</th>
<th>Follicles per ovary</th>
<th>Total no. of COCs retrieved</th>
<th>COCs per ovary</th>
<th>Oocyte recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>13/406</td>
<td>1339</td>
<td>3.34±0.12</td>
<td>913</td>
<td>2.28±0.09</td>
<td>68.22±1.40</td>
</tr>
<tr>
<td>EarlyLuteal</td>
<td>13/371</td>
<td>1191</td>
<td>3.23±0.13</td>
<td>831</td>
<td>2.26±0.11</td>
<td>69.74±1.44</td>
</tr>
<tr>
<td>Luteal</td>
<td>13/233</td>
<td>1121</td>
<td>4.87±0.32</td>
<td>758</td>
<td>3.29±0.23</td>
<td>67.47±0.62</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The oocyte recovery rate during follicular (68.22±1.40), early luteal (69.74±1.44) and luteal (67.47±0.62) phases were not significantly different (Figure 7b).

4.1.2.3 Effect of season on oocyte retrieval from abattoir derived ovaries

The effect of season on COCs was studied in three different seasons of the year viz. rainy, summer and winter. A total of 439, 376 and 310 ovaries for rainy, summer and winter seasons were collected and a total of 2079, 1469 and 1793 follicle were aspirated in the respective seasons (Table 11).

Table 11: Effect of season on oocyte retrieval from abattoir ovaries

<table>
<thead>
<tr>
<th>Season</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of follicles aspirated</th>
<th>Follicles per ovary</th>
<th>Total no. of COCs retrieved</th>
<th>COCs per ovary</th>
<th>Oocyte recovery rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainy</td>
<td>20/ 439</td>
<td>2079</td>
<td>4.75±0.05</td>
<td>1380</td>
<td>3.15±0.05</td>
<td>66.33±0.50</td>
</tr>
<tr>
<td>Summer</td>
<td>18/ 376</td>
<td>1469</td>
<td>3.90±0.08</td>
<td>986</td>
<td>2.62±0.06</td>
<td>67.14±0.46</td>
</tr>
<tr>
<td>Winter</td>
<td>19/ 310</td>
<td>1793</td>
<td>5.76±0.12</td>
<td>1227</td>
<td>3.94±0.09</td>
<td>68.35±0.27</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference
The average number of follicles per ovary in the present study were significantly (p<0.01) higher in winter (5.76±0.12) as compared to rainy (4.75±0.05) and summer (3.90±0.08) seasons (Figure 8a). Likewise, a highly significant (p<0.01) difference was also observed in the average number of follicles per ovary between summer and rainy seasons, indicating the effect of season on follicular activity. The number of COCs retrieved per ovary also followed a similar trend with significantly higher number of COCs retrieved per ovary during winter (3.94±0.09) as compared to rainy (3.15±0.05) and summer (2.62±0.06) seasons. A highly significant (p<0.01) difference between the number of COCs per ovary between rainy and summer season was also observed.

Though the oocyte recovery rate (%) was significantly (p<0.01) higher in winter (68.35±0.27) as compared to rainy (66.33±0.50) but the difference was non significant between winter and summer (68.35±0.27 vs. 67.14±0.46) and summer and rainy seasons (67.14±0.46 vs. 66.33±0.50) (Figure 8b).

4.1.3 Effect of various morpho-functional parameters on quality of abattoir derived COCs

Different experiments were performed to study the effect of various morpho-functional parameters on the quality of retrieved oocytes. The abattoir derived COCs were isolated and graded immediately after retrieval by aspiration technique. Following isolation the oocytes were graded as Grade I to IV depending on the layers of cumulus cells (Plate 6a). Grade I and II oocytes were processed for in vitro maturation (IVM) and in vitro fertilization (IVF) where as grade III and IV COCs were clubbed to study the quality of retrieved oocytes but were not used for IVM and IVF.

4.1.3.1 Effect of follicle size on oocyte quality

In the present experiment the mean per cent grade I, II and grade III-IV COCs clubbed together in the large follicles were recorded to be 35.74±0.56, 45.37±0.66 and 18.79±0.48%, respectively. The respective figures for the small follicles were 32.49±0.73, 41.82±0.61 and 25.59±1.20% (Table 12). The proportion of grade I and II COCs were significantly (p<0.01)
higher in large follicles as compared to the small follicles suggesting the better quality of COCs retrieved from large follicles. The vice versa was true for grade III-IV COCs (Figure 9).

Table 12: Effect of follicle size on oocyte quality of the retrieved oocytes

<table>
<thead>
<tr>
<th>Follicle size</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of follicles aspirated</th>
<th>Total no. of COCs retrieved</th>
<th>Grade I (%)</th>
<th>Grade II (%)</th>
<th>Grade III &amp; IV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>15/ 439</td>
<td>1347</td>
<td>843</td>
<td>35.74±0.56</td>
<td>45.37±0.66</td>
<td>18.79±0.48</td>
</tr>
<tr>
<td>Small</td>
<td>15/ 439</td>
<td>988</td>
<td>514</td>
<td>32.49±0.73</td>
<td>41.82±0.61</td>
<td>25.59±1.20</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.1.3.2 Effect of phase of oestrus cycle on oocyte quality

The phase of oestrus cycle was ascertained by the presence of either small developing corpus CL (early luteal phase), fully mature large corpus luteum (luteal phase) or a dominant follicle without corpus luteum or with a regressing corpus luteum (follicular phase). The respective per cent mean oocytes of grades I, II and III-IV in follicular; early luteal and luteal phase of oestrus cycle were recorded to be 33.04±0.51, 43.61±0.71 and 23.23±0.80; 31.73±0.79, 41.68±0.76 and 26.59±1.39; 33.19±1.00, 41.66±0.72 and 25.15±1.49, respectively (Table 13). No significant difference was recorded in grades of COCs between different phases of oestrus cycle in the present study. This indicates that oocyte retrieval can be done throughout the cycle irrespective of the presence or absence of CL for in vitro embryo production (Figure 10).

Table 13: Effect of phase of oestrus cycle on oocyte quality

<table>
<thead>
<tr>
<th>Phase of estrus cycle</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of follicles aspirated</th>
<th>Total no. of COCs retrieved</th>
<th>Grade I COCs (%)</th>
<th>Grade II COCs (%)</th>
<th>Grade III-IV COCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>13/406</td>
<td>1339</td>
<td>913</td>
<td>33.04±0.51</td>
<td>43.61±0.71</td>
<td>23.23±0.80</td>
</tr>
<tr>
<td>Early luteal</td>
<td>13/371</td>
<td>1191</td>
<td>831</td>
<td>31.73±0.79</td>
<td>41.68±0.76</td>
<td>26.59±1.39</td>
</tr>
<tr>
<td>Luteal</td>
<td>13/233</td>
<td>1121</td>
<td>758</td>
<td>33.19±1.00</td>
<td>41.66±0.72</td>
<td>25.15±1.49</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference
4.1.3.3 Effect of season on oocyte quality

In the present study the number of grade I COCs retrieved were significantly \((p<0.01)\) higher in winter \((36.32\pm0.64)\) as compared to rainy \((32.55\pm0.64)\) and summer \((31.07\pm0.49)\) seasons. The difference between the per cent grade I oocytes retrieved during summer and rainy seasons was, however, not statistically significant. Similar trend was also observed in grade II oocytes where a significantly higher proportion of COCs were retrieved during winter \((45.05\pm0.70)\) as compared to rainy \((42.78\pm0.58)\) and summer \((41.04\pm0.42)\) seasons (Table 14; Figure 11).

### Table 14: Effect of season on oocyte quality

<table>
<thead>
<tr>
<th>Season</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of follicles aspirated</th>
<th>Total no. of COCs retrieved</th>
<th>Grade I COCs (%)</th>
<th>Grade II COCs (%)</th>
<th>Grade III &amp; IV COCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainy</td>
<td>20/ 439</td>
<td>2079</td>
<td>1380</td>
<td>32.55(^{a})±0.64</td>
<td>42.78(^{a})±0.58</td>
<td>24.60(^{a})±1.08</td>
</tr>
<tr>
<td>Summer</td>
<td>18/ 376</td>
<td>1469</td>
<td>986</td>
<td>31.07(^{a})±0.49</td>
<td>41.04(^{a})±0.42</td>
<td>27.90(^{a})±0.79</td>
</tr>
<tr>
<td>Winter</td>
<td>19/ 310</td>
<td>1793</td>
<td>1227</td>
<td>36.32(^{b})±0.64</td>
<td>45.05(^{b})±0.70</td>
<td>18.54(^{b})±0.46</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The proportion of grade III-IV COCs retrieved during summer \((27.90\pm0.79)\) were, however, significantly \((p<0.01)\) higher as compared to rainy \((24.60\pm1.08)\) and winter seasons \((18.54\pm0.46)\). The per cent grade III-IV COCs retrieved during rainy seasons were also significantly \((p<0.01)\) higher as compared to winter seasons. The results indicate significantly higher quality of COCs were retrieved during winter followed by rainy and summer seasons.

4.1.3.5 Effect of diameter on oocyte quality

To study the effect of diameter of oocyte on its quality, immediately after retrieval the diameters of the collected COCs were measured and were classified as large (> 150 µm), medium (120-150 µm) or small (< 120 µm) groups. The per cent of grade I, II and grade III-IV COCs in different diameter categories were recorded.

The percentage of grade I COCs was significantly \((p<0.01)\) higher in large oocytes \((35.79\pm0.69)\) as compared to medium \((31.09\pm0.61)\)
and small (32.60±0.57) groups. Also a significant (p<0.05) difference was observed in grade I COCs retrieved from small and medium oocyte diameter group. Significantly (p<0.05) higher per cent of grade II COCs were observed in large oocytes (44.50±0.78) as compared to medium (41.41±0.56) and small (42.84±0.66) oocytes (Table 15; Figure 12). The difference between the later two was, however, non significant.

Table 15: Effect of diameter on oocyte quality

<table>
<thead>
<tr>
<th>Oocyte diameter groups</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of oocytes retrieved</th>
<th>Grade I COCs (%)</th>
<th>Grade II COCs (%)</th>
<th>Grade III-IV COCs (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>16/ 268</td>
<td>1015</td>
<td>35.79±0.69</td>
<td>44.50±0.78</td>
<td>19.70±0.91</td>
</tr>
<tr>
<td>Medium</td>
<td>16/ 339</td>
<td>969</td>
<td>31.09±0.61</td>
<td>41.41±0.56</td>
<td>27.50±1.06</td>
</tr>
<tr>
<td>Small</td>
<td>16/ 353</td>
<td>1069</td>
<td>32.60±0.57</td>
<td>42.84±0.66</td>
<td>24.47±0.89</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

In the present study, a significantly (p<0.01) higher proportion of grade III and IV COCs were recorded in oocytes with medium diameter (27.50±1.06) as compared to large (19.70±0.91) and small (24.47±0.89) diameter groups. The difference between the later two was also significant (p<0.01).

4.2 In vitro maturation of oocytes

The retrieved oocytes were graded and subjected to in vitro maturation. The cytoplasmic maturation was assessed by cumulus expansion (Plate 6b) and nuclear maturation (Plate 6c and d) by visualization of polar body. The results of different experiments to study the effect of various morpho-functional parameters on in vitro maturation of COCs are presented in table 16 to 20.

4.2.1 Effect of follicle size on in vitro maturation of abattoir derived COCs

Out of total 843 and 514 COCs retrieved from large and small follicles respectively, 674 and 380 (grade I and II) were cultured for in vitro maturation. Cytoplasmic maturation revealed 45.93±0.42, 39.21±0.37 and 14.86±0.63 per cent grade A, B and C cumulus expansion respectively in
large follicle group (Table 16; Figure 13a). The respective grade A, B and C cumulus expansion in small follicle group was recorded to be 47.01±0.58, 37.83±1.04 and 15.30±0.57 per cent. No significant difference was recorded between cumulus expansion in in vitro matured COCs retrieved from large and small follicle groups. The nuclear maturation in COCs retrieved from large follicles (88.03±0.49) was, however, significantly (p<0.01) higher as compared to those retrieved from small follicles (82.15±0.47) indicating comparatively better maturation of COCs from large follicles (Figure 13b).

### Table 16: Effect of Follicle size on in vitro maturation of oocytes

<table>
<thead>
<tr>
<th>Follicle size groups</th>
<th>Replicates / no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>No. of COCs set for IVM</th>
<th>Cumulus expansion/ cytoplasmic maturation (%)</th>
<th>COCs showing polar body (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Large</td>
<td>15/ 239</td>
<td>843</td>
<td>674</td>
<td>45.93±0.42</td>
<td>39.21±0.35</td>
</tr>
<tr>
<td>Small</td>
<td>15/ 331</td>
<td>514</td>
<td>380</td>
<td>47.01±0.58</td>
<td>37.83±1.04</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.2.2 Effect of phase of oestrus cycle on in vitro maturation of abattoir derived COCs

The effect of different phases of oestrus cycle was observed on in vitro maturation. The cytoplasmic maturation depicted by cumulus expansion of grade A, B and C in COCs retrieved from ovaries in follicular phase was recorded to be 47.81±0.54, 38.06±0.93 and 14.28±0.55%, respectively (Table 17). The respective values of the same parameter in early luteal and luteal phase were 47.40±0.57, 36.95±0.86 and 15.65±0.64; 47.26±0.38, 37.23±0.63 and 15.51±0.79 (Figure 14a). No significant difference between the cumulus expansion of different grades was observed between different phases of oestrus cycle.

The per cent nuclear maturation assessed by extrusion of first polar body revealed significantly (p<0.01) higher proportion of COCs with polar body in luteal phase (86.54±0.53) as compared to follicular (80.86±0.23) or early luteal phase (83.76±0.48) of oestrus cycle (Figure 14b). The difference between nuclear maturation in COCs retrieved during early luteal and follicular phase was also significant (p<0.05).
Table 17: Effect of phase of oestrus cycle on \textit{in vitro} maturation of oocytes

<table>
<thead>
<tr>
<th>Phase of estrus cycle</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>No. of COCs cultured</th>
<th>Cumulus expansion/ cytoplasmic maturation (%)</th>
<th>COCs showing polar body (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Follicular</td>
<td>13/ 406</td>
<td>913</td>
<td>700</td>
<td>47.81±0.54</td>
<td>38.06±0.93</td>
</tr>
<tr>
<td>Early luteal</td>
<td>13/ 371</td>
<td>831</td>
<td>612</td>
<td>47.40±0.57</td>
<td>36.95±0.86</td>
</tr>
<tr>
<td>Luteal</td>
<td>13/ 233</td>
<td>758</td>
<td>574</td>
<td>47.26±0.38</td>
<td>37.23±0.63</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.2.3 Effect of season on \textit{in vitro} maturation of abattoir derived COCs

In the present experiment, the grade A cumulus expansion of COCs during rainy (47.65±0.31), summer (47.31±0.54) and winter (46.40±0.41) seasons did not differ significantly (Table 18; Figure 15a). Whereas grade B cumulus expansion was significantly (p<0.01) higher during winter season (39.39±0.36) as compared to rainy (36.01±0.42) and summer (37.75±0.93) seasons. The difference between the later two was also significant. Grade C cumulus expansion was recorded to be lower during winter (14.21±0.60) as compared to rainy (16.34±0.58) and summer (15.04±0.52) seasons, the difference was highly significant (p<0.01). There was also a significant (p<0.05) difference between the grade C cumulus expansion of rainy and summer season.

Table 18: Effect of season on \textit{in vitro} maturation of oocytes

<table>
<thead>
<tr>
<th>Season</th>
<th>Replicates / no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>COCs set for IVM</th>
<th>Cumulus expansion / cytoplasmic maturation (%)</th>
<th>COCs showing polar body (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Rainy</td>
<td>18/ 376</td>
<td>986</td>
<td>714</td>
<td>47.65±0.31</td>
<td>36.01±0.42</td>
</tr>
<tr>
<td>Summer</td>
<td>20/ 439</td>
<td>1380</td>
<td>1041</td>
<td>47.31±0.54</td>
<td>37.75±0.93</td>
</tr>
<tr>
<td>Winter</td>
<td>19/ 310</td>
<td>1227</td>
<td>1000</td>
<td>46.40±0.41</td>
<td>39.39±0.36</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference
A significantly (p<0.01) higher nuclear maturation (% COCs showing extrusion of 1st polar body) was observed during winter (88.34±0.48) as compared to rainy (84.18±0.55) and summer (82.30±0.49) seasons. The nuclear maturation was also significantly (p<0.01) higher in rainy (84.18±0.55) as compared to summer (82.30±0.49) season (Figure 15b).

### 4.2.4 Effect of oocyte diameter on *in vitro* maturation of abattoir derived COCs

The assessment of cytoplasmic expansion in oocytes of different diameters (low, medium and high) revealed no significant difference in grade A cumulus expansion between oocytes of large (46.14±0.41), medium (47.92±0.33) and small (47.31±0.55) diameters (Table 19; Figure 16a). A highly significant (p<0.01) difference between the oocytes of large (38.65±0.37) and medium (35.91±0.52) and between medium (35.91±0.52) and small (37.96±0.92) diameters was, however, recorded in grade B cumulus expansion. The grade C cumulus expansion of medium (16.17±0.53) was significantly (p<0.01) higher as compared to oocytes of small (14.85±0.60) diameter. No significant difference was, however, recorded between oocytes of large (15.21±0.62) and small (14.84±0.60) and large and medium oocyte diameters.

<table>
<thead>
<tr>
<th>Oocyte diameter groups</th>
<th>Replicate s/ no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>No. of COCs set for IVM</th>
<th>Cumulus expansion/ cytoplasmic maturation (%)</th>
<th>Oocytes showing polar body (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Large</td>
<td>16/ 268</td>
<td>1015</td>
<td>818</td>
<td>46.14±0.41</td>
<td>38.65±0.37</td>
</tr>
<tr>
<td>Medium</td>
<td>16/ 339</td>
<td>969</td>
<td>705</td>
<td>47.92±0.33</td>
<td>35.91±0.52</td>
</tr>
<tr>
<td>Small</td>
<td>16/ 353</td>
<td>1069</td>
<td>809</td>
<td>47.31±0.55</td>
<td>37.96±0.92</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The nuclear maturation depicted by extrusion of 1st polar body in oocytes of large diameter (88.49±0.46) was significantly (p<0.01) higher as compared to the oocytes of medium (83.78±0.53) and small diameters.
(81.76±0.43). A highly significant (p<0.01) difference was also recorded between oocytes of medium and small diameters (Figure 16b).

4.3 Developmental competence of in vitro produced (IVP) embryos

The developmental competence of in vitro produced embryos derived from both OPU and abattoir ovaries was studied and the results are presented in table 20 to 28.

4.3.1 Developmental competence of OPU derived IVP embryos

OPU was performed on Six Murrah buffaloes and the COCs retrieved were subjected to IVM-IVF procedures (Plate 7 and 8). The developmental competence of OPU derived IVP embryos are presented in table 20.

4.3.1.1 Effect of oocyte donors on developmental competence of OPU derived embryos

The maturation (%) of the six Murrah buffaloes D1, D2, D3, D4, D5 and D6 were 90.91±6.10, 90.91±9.09, 87.50±8.97, 87.50±8.97, 87.90±8.97, 90.00±10.00 with an overall mean value of 88.24±3.65. There was no significant difference between oocyte donors in maturation per cent of the oocytes retrieved by OPU (Table 20; Figure 17).

The cleavage (%) of Murrah buffaloes D1 (68.18±12.20), D2 (68.18±12.20), D3 (70.83±11.45), D4 (70.83±12.99), D5 (70.83±13.00) and D6 (76.67±13.19) were not significantly different. Similar trend was also observed for morula (50.00±11.68, 45.45±10.56, 50.00±9.13, 45.83±12.99, 50.00±10.66 and 51.67±13.02% for D1, D2, D3, D4, D5 and D6, respectively) and blastocyst (27.27±10.38, 27.27±7.87, 33.33±7.11, 29.17±7.43, 25.00±7.54 and 28.33% for D1, D2, D3, D4, D5 and D6, respectively). The overall Morula (%) and blastocyst rate in the present study was recorded to be 48.01±4.54 and 28.43±3.35, respectively. There was no difference between donors in the morula or blastocyst (%) in the present study.
Table 20: Developmental competence of oocytes retrieved by transvaginal ultrasound guided OPU

<table>
<thead>
<tr>
<th>Oocyte donors</th>
<th>No. of oocytes retrieved</th>
<th>COCs cultured</th>
<th>Maturation %</th>
<th>Cleavage Rate (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>23</td>
<td>17</td>
<td>90.91±6.10</td>
<td>68.18±12.20</td>
<td>50.00±11.68</td>
<td>27.27±10.38</td>
</tr>
<tr>
<td>D2</td>
<td>20</td>
<td>18</td>
<td>90.91±9.09</td>
<td>68.18±12.20</td>
<td>45.45±10.56</td>
<td>27.27±7.87</td>
</tr>
<tr>
<td>D3</td>
<td>24</td>
<td>20</td>
<td>87.50±8.97</td>
<td>70.83±11.45</td>
<td>50.00±9.13</td>
<td>33.33±7.11</td>
</tr>
<tr>
<td>D4</td>
<td>24</td>
<td>18</td>
<td>87.50±8.97</td>
<td>70.83±12.99</td>
<td>45.83±12.99</td>
<td>29.17±7.43</td>
</tr>
<tr>
<td>D5</td>
<td>26</td>
<td>19</td>
<td>87.50±8.97</td>
<td>70.83±13.00</td>
<td>50.00±10.66</td>
<td>25.00±7.54</td>
</tr>
<tr>
<td>D6</td>
<td>16</td>
<td>15</td>
<td>90.00±10.00</td>
<td>76.67±13.19</td>
<td>51.67±13.02</td>
<td>28.33±10.84</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>133</strong></td>
<td><strong>107</strong></td>
<td><strong>88.24±3.65</strong></td>
<td><strong>70.59±5.05</strong></td>
<td><strong>48.01±4.54</strong></td>
<td><strong>28.43±3.35</strong></td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.3.1.2 Effect of frequency of sessions on developmental competence of OPU derived embryos

To study the effect of OPU sessions on in vitro embryo production (IVEP) 34 sessions each of once weekly or twice weekly frequency were conducted after induction of a new cycle by double injection protocol of PGF$_2$α.

The average number of COCs retrieved by OPU in the present study was 2.00±0.14 (Table 21). The respective averages for once weekly and twice weekly OPU sessions were recorded to be 1.91±0.14 and 2.00±0.13. There was no significant difference between numbers of COCs retrieved by different frequencies of OPU sessions. Average number of good quality oocytes fit for maturation culture (grade I and II) was recorded to be 1.56±0.11 with the respective averages of 1.54±0.11 and 1.57±0.11 for once weekly and twice weekly OPU sessions, respectively. No significant difference in frequency of OPU sessions was recorded for the numbers of COCs cultured.

The overall cytoplasmic maturation in COCs retrieved by 68 sessions of OPU was recorded to be 87.50±5.42. As evident from the table, the cytoplasmic maturation of the oocytes retrieved by twice weekly (89.28±5.14) OPU session was significantly (p<0.05) higher as compared to once weekly (85.71±5.72) OPU session.
Table 21: Effect of frequency of OPU sessions on developmental competence of oocytes

<table>
<thead>
<tr>
<th>Session</th>
<th>Replicates</th>
<th>Average no. of COCs retrieved</th>
<th>Average no. of COCs cultured</th>
<th>Maturation (%)</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Once weekly</td>
<td>34</td>
<td>1.91±0.14</td>
<td>1.54±0.11</td>
<td>85.71±5.72</td>
<td>61.43±7.23</td>
<td>44.12±5.49</td>
<td>24.14±4.81</td>
</tr>
<tr>
<td>Twice weekly</td>
<td>34</td>
<td>2.00±0.13</td>
<td>1.57±0.11</td>
<td>89.28±5.14</td>
<td>76.80±7.14</td>
<td>51.79±6.62</td>
<td>29.72±4.73</td>
</tr>
<tr>
<td>Total</td>
<td>68</td>
<td>2.00±0.14</td>
<td>1.56±0.11</td>
<td>87.50±5.42</td>
<td>69.12±7.26</td>
<td>48.01±6.06</td>
<td>26.93±4.74</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The overall cleavage rate of in vitro fertilized OPU oocytes was recorded to be 69.12±7.26%, with cleavage rate of 61.43±7.23 and 76.80±7.14%, respectively in oocytes retrieved in once weekly and twice weekly OPU sessions. Significantly (p<0.05) higher cleavage was recorded in twice weekly OPU sessions as compared to once weekly OPU session.

In the present study the overall average of morula and blastocyst rate was recorded to be 48.01±6.06 and 26.93±4.74, respectively. The per cent morula (44.12±5.49 vs. 51.79±6.62) and blastocysts (24.14±4.81 vs. 29.72±4.73) in once weekly and twice weekly OPU sessions were significantly (p<0.05) different from each other (Figure 18).

4.3.1.3 Effect of oocyte retrieval technique on developmental competence of IVP embryos

The effect of the two retrieval techniques viz. OPU and aspiration from abattoir ovaries on developmental competence of buffalo embryos was studied by culturing 106 and 2755 COCs each from OPU and abattoir ovaries, respectively. Though the maturation rate of OPU derived COCs (87.50±4.34) was numerically higher as compared to COCs retrieved by aspiration from abattoir ovaries (84.90±0.44), but the difference was non significant (Table 22). Also no significant difference was observed in cleavage...
of OPU derived oocytes (69.12±5.82%) as compared to abattoir derived oocytes (65.59±0.60%).

Table 22:  Effect of retrieval technique on developmental competence of oocytes

<table>
<thead>
<tr>
<th>Retrieval techniques</th>
<th>Replicates / COCs cultured</th>
<th>Maturation (%)</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocysts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPU</td>
<td>68/ 106</td>
<td>87.50±4.34</td>
<td>69.12±5.82</td>
<td>48.01±4.89</td>
<td>28.43±3.80</td>
</tr>
<tr>
<td>Abattoir ovaries</td>
<td>53/ 2755</td>
<td>84.90±0.44</td>
<td>65.59±0.60</td>
<td>36.89±0.67</td>
<td>19.04±0.49</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

In the present study, significantly (p<0.01) higher proportion of OPU derived COCs reached the morula stage (48.01±4.89%) as compared to abattoir derived COCs (36.89±0.67%). Similarly blastocyst rate was also significantly (p<0.01) higher in OPU derived COCs (28.43±3.80) as compared to abattoir derived (19.04±0.49) COCs in the present study, which indicates better developmental competence of the OPU derived oocytes (Figure 19).

4.3.2 Effect of follicle size on developmental competence of IVP embryos

The effect of follicular size on developmental competence of buffalo oocytes is presented in table 23 and Figure 20. The cleavage rate of COCs retrieved from large follicles (70.19±0.99%) was significantly (p<0.01) higher as compared to the COCs retrieved from small follicles (62.63±0.40). Similar trend was also observed in case of morula (42.05±0.70 vs. 33.08±0.92) and blastocyst per cent (23.83±1.05 vs. 15.59±0.46). A highly significant (p<0.01) difference in developmental competence in terms of morula and blastocyst per cent was recorded in COCs retrieved from large and small follicles.
Table 23: Effect of follicle size on developmental competence of in vitro produced (IVP) embryos

<table>
<thead>
<tr>
<th>Follicle size</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>No. of COCs set for culture</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>12/ 231</td>
<td>744</td>
<td>576</td>
<td>70.19±0.99</td>
<td>42.05±0.70</td>
<td>23.83±1.05</td>
</tr>
<tr>
<td>Small</td>
<td>12/ 263</td>
<td>830</td>
<td>613</td>
<td>62.63±0.40</td>
<td>33.08±0.92</td>
<td>15.59±0.46</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.3.3 Effect of phase of oestrus cycle on developmental competence of IVP embryos

A total of 838, 776 and 701 COCs were retrieved respectively in follicular, early luteal and luteal phase of oestrus cycle. Out of the retrieved COCs, 643 (for follicular phase), 575 (for early luteal phase) and 527 (for luteal phase) were set for in vitro maturation followed by in vitro fertilization and culture. Results of the effect of phase of oestrus cycle on developmental competence are presented in table 24 and figure 22.

Table 24: Effect of phase of oestrus cycle on developmental competence of in vitro produced (IVP) embryos

<table>
<thead>
<tr>
<th>Phase of estrus cycle</th>
<th>Replicates / no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>No. of COCs set for culture</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follicular</td>
<td>12/ 367</td>
<td>838</td>
<td>643</td>
<td>61.92±0.60</td>
<td>33.14±0.63</td>
<td>14.75±0.25</td>
</tr>
<tr>
<td>Early luteal</td>
<td>12/ 340</td>
<td>776</td>
<td>575</td>
<td>63.22±0.54</td>
<td>34.05±1.28</td>
<td>17.13±0.31</td>
</tr>
<tr>
<td>Luteal</td>
<td>12/ 217</td>
<td>701</td>
<td>527</td>
<td>69.43±0.92</td>
<td>42.53±0.58</td>
<td>22.51±0.55</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The cleavage rate of the COCs retrieved from the ovaries in luteal phase of the oestrus cycle (69.43±0.92) was significantly (p<0.05) higher as compared to the follicular (61.92±0.60) and early phase (63.22±0.54). There was, however, no significant difference between the cleavage rate of the COCs retrieved during follicular and early luteal phase of the cycle. Similar trend was also observed in morula (%) where a highly significant (p<0.01) difference was observed between luteal (42.53±0.58) and
follicular (33.14±0.63) and early luteal phase (34.05±1.28) of oestrus cycle. No significant difference between follicular and early luteal phase was, however, observed.

A significantly (p<0.01) higher blastocyst rate in COCs retrieved during luteal phase of the cycle (22.51±0.55) as compared to follicular (14.75±0.25) and early luteal (17.13±0.31) phase was observed. The blastocyst rate of COCs aspirated during early luteal phase of the oestrus cycle was also significantly (p<0.01) higher as compared to follicular phase of the cycle. This indicates that the developmental competence was significantly higher during luteal phase followed by early luteal and follicular phase of the cycle.

4.3.4 Effect of season on developmental competence of IVP embryos

The effect of season on developmental competence of COCs studied revealed significantly (p<0.01) higher cleavage during winter (70.70±1.00) as compared to rainy (64.52±0.47) and summer (62.07±0.43) seasons (Table 25). Though higher cleavage rate was observed in rainy season as compared to summer, the difference was not significant.

Table 25: Effect of season on developmental competence of *in vitro* produced (IVP) embryos

<table>
<thead>
<tr>
<th>Season</th>
<th>Replicates/ no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>No. of COCs set for culture</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainy</td>
<td>17/ 316</td>
<td>841</td>
<td>612</td>
<td>64.52±0.47</td>
<td>35.39±0.91</td>
<td>17.92±0.52</td>
</tr>
<tr>
<td>Summer</td>
<td>15/ 373</td>
<td>1178</td>
<td>880</td>
<td>62.07±0.43</td>
<td>33.06±0.75</td>
<td>15.75±0.46</td>
</tr>
<tr>
<td>Winter</td>
<td>15/ 240</td>
<td>931</td>
<td>756</td>
<td>70.70±1.00</td>
<td>42.69±0.35</td>
<td>23.28±0.41</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The proportion of morula developed from COCs cultured during winter season (42.69±0.35) was significantly (p<0.01) higher as compared to summer (33.06±0.75) and rainy (35.39±0.91) seasons. The difference between the later two was, however, not statistically significant. Similarly the
blastocyst rate of COCs retrieved during winter season (23.28±0.41) was significantly (p<0.01) higher as compared to the rainy (17.92±0.52) and summer (15.75±0.46) seasons with no statistically significant difference the later two (Figure 22).

4.3.5 Effect of oocyte diameter on developmental competence of IVP embryos

In the present study, the large oocytes (69.21±0.67) were associated with significantly (p<0.01) higher cleavage rates as compared to oocytes of medium (64.47±0.62) and small (62.10±0.48) diameters (Table 26; Figure 23). Likewise the difference between the cleavage rate of oocytes with medium and small diameters was also highly significant (p<0.01). The proportion of oocytes developing to the morula stage in large oocytes (41.43±0.75) was significantly greater than oocytes of medium (36.28±0.69) and small (32.25±0.83) groups. Per cent morula in oocytes of medium and small diameters was also significantly (p<0.01) different.

Table 26: Effect of oocyte diameter on developmental competence of oocytes

<table>
<thead>
<tr>
<th>Oocyte diameter groups</th>
<th>Replicate s/ no. of ovaries</th>
<th>No. of COCs retrieved</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>16/ 268</td>
<td>1015</td>
<td>69.21±0.67</td>
<td>41.43±0.75</td>
<td>23.23±0.45</td>
</tr>
<tr>
<td>Medium</td>
<td>16/ 339</td>
<td>969</td>
<td>64.47±0.62</td>
<td>36.28±0.69</td>
<td>17.70±0.39</td>
</tr>
<tr>
<td>Small</td>
<td>16/ 353</td>
<td>1069</td>
<td>62.10±0.48</td>
<td>32.25±0.83</td>
<td>15.18±0.37</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

Similar trend was also observed in case of blastocysts rate in large oocyte group (23.23±0.45) which was significantly (p<0.01) higher as compared to medium (17.70±0.39) and small (15.18±0.37) oocyte groups, the difference between the later two was also significant (p<0.01).

4.3.6 Effect of grades of oocytes on developmental competence of IVP embryos

Effect of various grades of COCs was studied and the results are presented in table 27 and figure 24. The cytoplasmic maturation was significantly (p<0.01) higher in grade I (88.57±0.64) and grade II (84.26±0.97) COCs as compared to grade III (79.36±1.35) COCs. NO significant difference
between the rate of cytoplasmic maturation in grade I and grade II COCs was recorded in the present study.

The cleavage, morula and blastocyst rate in grade I oocytes (71.40±0.64, 43.85±1.20 and 25.77±1.73) and grade II (68.32±0.99, 39.54±1.51 and 23.75±1.55) were significantly (p<0.01) higher as compared to grade III (61.69±0.93, 34.05±0.86 and 14.30±0.66, respectively) COCs. No significant difference between grade I and grade II COCs in cleavage, morula and blastocyst rates was observed.

Table 27: Effect of grades of COCs on developmental competence of *in vitro* produced (IVP) embryos

<table>
<thead>
<tr>
<th>Grades of COCs</th>
<th>Replicates/ no. of COCs cultured</th>
<th>Cytoplasmic maturation (%)</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>11/ 332</td>
<td>88.57±0.64</td>
<td>71.40±0.64</td>
<td>43.85±1.20</td>
<td>25.77±1.73</td>
</tr>
<tr>
<td>Grade II</td>
<td>11/ 356</td>
<td>84.26±0.97</td>
<td>68.32±0.99</td>
<td>39.54±1.51</td>
<td>23.75±1.55</td>
</tr>
<tr>
<td>Grade III</td>
<td>11/ 346</td>
<td>79.36±1.35</td>
<td>61.69±0.93</td>
<td>34.05±0.86</td>
<td>14.30±0.66</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.3.7 Effect of source of spermatozoa on developmental competence of IVP embryos

Two different sources of spermatozoa i.e. ejaculated and epididymal sperms were processed and used for *in vitro* fertilization. Their effect on developmental competence of IVP embryos was studied and results are summarized in table 28 and figure 25.

Cleavage (%) of COCs in *in vitro* fertilization with ejaculated and epididymal spermatozoa was recorded to be 70.23±0.71 and 69.15±0.78% respectively, which was not significantly different. Similar trend was also recorded in morula (42.35±1.67 and 39.63±1.36) and blastocyst rate (24.05±1.21 and 20.28±1.09) using ejaculated and epididymal spermatozoa, respectively for *in vitro* fertilization. Therefore, both ejaculated and epididymal spermatozoa can be used for *in vitro* fertilization with equally good results in terms of developmental competence.
Table 28: Effect of source of spermatozoa on developmental competence of in vitro produced embryo

<table>
<thead>
<tr>
<th>Source of sperm</th>
<th>Replicates</th>
<th>No. of COCs set for culture</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ejaculated Semen</td>
<td>11</td>
<td>164</td>
<td>70.23±0.71</td>
<td>42.35±1.67</td>
<td>24.05±1.21</td>
</tr>
<tr>
<td>Epididymal sperm</td>
<td>11</td>
<td>166</td>
<td>69.15±0.78</td>
<td>39.63±1.36</td>
<td>20.28±1.09</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

4.4 Cellular predictors of developmental competence of IVP embryos

The effect of various cellular predictors viz. glucose-6-phosphate dehydrogenase activity (G6PDH), reactive oxygen species (ROS), apoptosis and differential cell count on developmental competence of IVP embryos were studied and the results are presented in the following section.

4.4.1 Effect of G6PDH activity on developmental competence of IVP embryos

The proportion of G6PDH activity in large and small follicles was assessed by brilliant cresyl blue (BCB) staining (Table 29; Plate 9). The overall average of BCB⁺ (inactive G6PDH) and BCB⁻ (active G6PDH) COCs in the present study was recorded to be 51.68±1.25 and 48.32±1.25%, respectively (Figure 26 a,b). The BCB⁺ COCs in large follicle (56.42±0.47) was significantly (p<0.01) higher as compared to small follicles (45.82±0.56). The reverse was observed in BCB⁻ COCs where significantly (p<0.01) higher proportion of BCB⁻ COCs was recorded in small follicle (54.18±0.56) as compared to large follicles (43.58±0.47).

Table 29: Glucose-6-phosphate dehydrogenase (G6PDH) activity in immature cumulus oocyte complexes

<table>
<thead>
<tr>
<th>Class of follicles</th>
<th>Replicates</th>
<th>No. of ovaries</th>
<th>BCB⁺</th>
<th>BCB⁻</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>No. of COCs</td>
<td>BCB⁺ (%)</td>
</tr>
<tr>
<td>Large</td>
<td>22</td>
<td>441</td>
<td>350</td>
<td>56.42⁺±0.47</td>
</tr>
<tr>
<td>Small</td>
<td></td>
<td></td>
<td>231</td>
<td>45.82⁺±0.56</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>581</td>
<td>51.68⁺±1.25</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significant difference (p<0.05)

BCB⁺: COCs showing positive reaction to brilliant cresyl blue (inactive G-6-PDH)

BCB⁻: COCs showing negative reaction to brilliant cresyl blue (active G-6-PDH)
The effect of G6PDH activity on developmental competence of abattoir oocytes is presented as Table 30 and figure 27. The cytoplasmic maturation was recorded to be significantly higher in BCB+ COCs aspirated from large follicles (85.79±0.98) as compared to BCB- COCs (81.30±0.60). Similar trend was also observed in COCs retrieved from small follicles where significantly higher BCB+ oocytes (79.94±1.27) were recorded as compared to BCB- oocytes (73.59±0.84).

**Table 30: Effect of glucose-6-phosphate dehydrogenase (G6PDH) activity on developmental competence of oocytes**

<table>
<thead>
<tr>
<th>Type of follicles</th>
<th>G-6-PDH activity</th>
<th>No. of COCs</th>
<th>Cytoplasmic maturation (%)</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large</td>
<td>BCB+</td>
<td>350</td>
<td>85.79±0.98</td>
<td>67.03±0.90</td>
<td>46.86±1.40</td>
<td>24.87±1.09</td>
</tr>
<tr>
<td></td>
<td>BCB-</td>
<td>269</td>
<td>81.30±0.60</td>
<td>62.01±1.13</td>
<td>36.27±0.95</td>
<td>15.41±1.26</td>
</tr>
<tr>
<td>Small</td>
<td>BCB+</td>
<td>231</td>
<td>79.94±1.27</td>
<td>60.42±1.61</td>
<td>38.96±1.22</td>
<td>18.49±1.47</td>
</tr>
<tr>
<td></td>
<td>BCB-</td>
<td>273</td>
<td>73.59±0.84</td>
<td>54.99±2.73</td>
<td>36.62±1.75</td>
<td>15.12±1.25</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

BCB+ : COCs showing positive reaction to brilliant cresyl blue (inactive G-6-PDH)

BCB- : COCs showing negative reaction to brilliant cresyl blue (active G-6-PDH)

Though no significant difference was observed between BCB+ (67.03±0.90) and BCB- (62.01±1.13) COCs retrieved from large follicles but the proportion of morula developed from BCB+ COCs (46.86±1.40%) was significantly (p<0.01) higher as compared to BCB- COCs (36.27±0.95). Similar trend was also observed in blastocyst rate where significantly (p<0.01) higher blastocyst rate was observed in BCB+ (24.87±1.09) than BCB- (15.41±1.26). The per cent morula and blastocyst rates in BCB+ and BCB- COCs from small follicles were not significantly different.

**4.4.2 Effect of ROS on developmental competence of IVP embryos**

The intensity of ROS (pixels/COC) in immature COCs of grade I, II and III were recorded to be 16.45±1.41, 20.40±0.63 and 24.62±0.33,
respectively (Table 31). Significantly (p<0.05) higher ROS was recorded in grade I COCs as compared to grade II and grade III COCs (Figure 28; Plate 10). A highly significant difference was also observed in ROS intensity of grade II and grade III, oocytes.

Similarly, a significant (p<0.05) difference between ROS intensity of grade I (15.01±1.18) and grade II (19.19±0.84) in vitro matured (IVM) COCs was recorded in the present study. The difference between grade I (15.01±1.18) and grade III (24.61±1.36) in vitro matured COCs was also significant (p<0.01). A significantly (p<0.01) higher ROS intensity was also recorded in grade II (19.19±0.84) as compared to grade III (24.61±1.36) IVM COCs.

**Table 31: Reactive oxygen species (ROS) in immature, mature oocytes and embryos**

<table>
<thead>
<tr>
<th>Grades of COCs</th>
<th>Immature COCs (pixels/COCs)</th>
<th>In vitro matured COCs (pixels/COCs)</th>
<th>IVP embryos (pixels/embryo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>16.45^A±1.41</td>
<td>15.01^A±1.18</td>
<td>16.27^A±1.90</td>
</tr>
<tr>
<td>Grade II</td>
<td>20.40^A±0.63</td>
<td>19.19^A±0.84</td>
<td>20.44^A±0.27</td>
</tr>
<tr>
<td>Grade III</td>
<td>24.62^A±0.33</td>
<td>24.61^A±1.36</td>
<td>24.64^A±0.74</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference

The ROS intensity (pixels/embryo) in IVP embryos (morula and blastocyst clubbed together) derived from grade I, grade II and grade III COCs was recorded to be 16.27±1.90, 20.44±0.27 and 24.64±0.74, respectively. A significantly (p<0.01) higher ROS intensity was recorded in IVP embryos derived from grade I COCs as compared to those derived from grade II and grade III COCs. Also a highly significant (p<0.01) difference was recorded between ROS intensity of IVP embryos produced from grade II and grade III COCs.

No significant difference between ROS intensity at different stages of in vitro embryo production (IVEP) i.e. immature COCs, IVM COCs and IVP embryos was however, recorded within different grades of COCs.
4.4.3 Effect of apoptosis on developmental competence of IVP embryos

The incidence of apoptosis was significantly (p<0.01) higher in grade III (13.35±0.59) immature COCs as compared to grade II (10.56±0.92) and grade I (7.29±0.65) COCs (Figure 29; Plate 11 and 12). Similarly, the IVM COCs of grade III (17.30±0.89) were also associated with significantly (p<0.01) higher incidence of apoptosis as compared to grade II (13.43±0.86) and grade I (9.54±0.84). A highly significant (p<0.01) difference was also recorded between grade II and grade III IVM oocytes (Table 32).

Table 32: Incidence of apoptosis (%) in immature and in vitro matured oocytes and IVP embryos

<table>
<thead>
<tr>
<th>Grades of COCs</th>
<th>Immature COCs</th>
<th>In vitro matured COCs</th>
<th>IVP embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grade I</td>
<td>7.29aA±0.65</td>
<td>9.54aA±0.84</td>
<td>10.14aA±1.07</td>
</tr>
<tr>
<td>Grade II</td>
<td>10.56bA±0.92</td>
<td>13.43bA±0.86</td>
<td>14.23bA±1.39</td>
</tr>
<tr>
<td>Grade III</td>
<td>13.35cA±0.59</td>
<td>17.30cBC±0.89</td>
<td>18.43cC±1.07</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference.

The IVP embryos from grade III (18.43±1.07) COCs were also associated with significantly (p<0.01) higher incidence of apoptosis as compared to those derived from grade I (10.14±1.07) COCs. A significant difference between IVP embryos produced from grade I and grade II and between those produced from grade II and grade III was also recorded in the present study.

4.5 In vivo developmental competence of IVP embryos (Conception rate)

In vivo developmental competence of in vitro produced embryos was assessed in terms of conception rate through embryo transfer of in vitro produced in to synchronized recipients. A total of 8 embryo transfers were done and successful clinical pregnancy was established in one recipient (MB 231). The pregnancy completed 5 months with normal fetal development and is continuing till date (Plate 13). The conception rate following embryo transfer of in vitro produced embryo was 12.5% in present study.
4.6 Effect of antioxidant and antiapoptotic factors on developmental competence of IVP embryos

In the present study the effect of antioxidant and antiapoptotic factors on developmental competence of IVP buffalo embryos was assessed and the results are tabulated in table 3 and figure 30 a-d.

The cleavage rate (%) of embryos cultured in 20% oxygen tension (control), 5% oxygen tension, glutathione (1 mM), epidermal growth factor (10 ng ml⁻¹), Insulin like growth factor – I (100 ng ml⁻¹) and n-propyl gallate (15 µM) groups was recorded to be 65.46±0.36, 71.17±0.37, 73.41±0.37, 76.73±0.74, 77.72±0.61 and 69.83±1.50%, respectively. The cleavage rate of embryos cultured in 5 % oxygen tension, glutathione, EGF, IGF-I and n-PG was significantly higher (p<0.01) as compared to control. Also significantly (p<0.01) higher cleavage rate was observed in EGF and IGF-I groups as compared to 5% oxygen tension and n-PG. No significant difference was, however, observed between cleavage rate of embryo culture in 5% oxygen tension and glutathione supplementation; 5% oxygen tension, glutathione and n-PG supplementation; Glutathione, EGF and IGF-I supplementation.

The proportion of oocytes developing to morula stage was significantly (p<0.01) lower in control (38.24±0.72) as compared to 5% oxygen tension (43.31±0.74), glutathione (44.60±0.71), EGF (48.10±0.54) and IGF-I (50.11±1.00) groups. No significant difference between control and n-PG supplementation (40.11±1.27) was, however, observed in the present study. Similarly, there was no significant difference in morula (%) between 5% oxygen tension and glutathione and between EGF and IGF-I.

The blastocyst rate (%) in control (20.09±0.60) was significantly lower than that of 5% oxygen tension (25.23±1.29) and with glutathione (28.46±1.37), EGF (33.88±0.63), IGF-I (34.96±0.92) supplementation. No significant difference between control and n-PG supplementation was, however, recorded in the present study. Also the blastocyst rate in EGF and IGF-I group was significantly (p<0.01) higher as compared to glutathione supplementation and embryo culture at 5% oxygen tension. There was,
however, no significant difference between the blastocyst rate in EGF and IGF-I supplementation.

4.7 Effect of antioxidant and antiapoptotic factors on reactive oxygen species in IVP embryos

The intensity of reactive oxygen species (ROS) expressed as pixels per embryo in IVP embryos in the present study was significantly (p<0.01) higher in control (19.12±1.76) as compared to embryos cultured in 5% oxygen tension (14.15±0.45) and with supplementation of glutathione (12.90±0.51), EGF (12.48±0.68), IGF-I (12.32±0.35) and n-PG (12.34±0.64). Also the intensity of ROS in embryos cultured in 5% oxygen tension was significantly higher as compared to other groups with supplementation of antioxidant or anti apoptotic factors. No significant difference in ROS intensity between embryos cultured in glutathione, EGF, IGF-I and n-PG was, however, detected in the present study.

4.8 Effect of antioxidant and antiapoptotic factors on apoptosis of IVP embryos

The incidence of apoptosis (%) was significantly (p<0.01) higher in control (9.73±2.45) as compared to embryos cultured in 5% oxygen tension (4.76±1.10), glutathione (2.08±0.81), EGF (1.20±0.65), IGF-I (0.90±0.62) and n-PG (3.56±1.22) groups (Table 33). The embryos cultured in 5% oxygen tension also depicted significantly (p<0.01) higher incidence of apoptosis as compared to those cultured with supplementation of glutathione, EGF and IGF-I. No significant difference was, however, recorded for 5% oxygen tension and n-PG groups. Also there was no statistically significant difference between the incidence of apoptosis in embryos cultured with supplementation of glutathione, EGF and IGF-I.

4.9 Effect of antioxidant and antiapoptotic factors on differential cell count of IVP blastocysts

The differential cell count of embryo in control and with supplementation of different antioxidant and anti apoptotic factors revealed significantly (p<0.05) lesser number of cells in the inner cell mass (ICM) of the hatched blastocyst in control (61.89±1.60) as compared to embryos cultured
in 5% oxygen tension (65.59±1.01) and with supplementation of glutathione (65.59±0.91), EGF (66.70±1.02), IGF-I (79.18±0.56) groups (Table 33; Plate 14). No significant difference between the control and n-PG treated (63.00±1.38) could, however, be recorded in the present study. Also significantly higher cells in ICM were recorded in IGF-I treatment as compared to the other treatment groups. There was, however, no statistically significant difference between the cell numbers of ICM with that of the embryos cultured in 5% oxygen tension and with supplementation of glutathione and EGF.

The total blastocyst cell number (TCN) recorded in control (153.12±3.45), embryos cultured in 5% oxygen tension (154.06±3.04) and with supplementation of glutathione (152.76±2.91), EGF (152.47±3.03) and n-PG (153.06±3.36) were not significant different in the present study. A significantly (p<0.01) higher total cell number in blastocyst obtained after in vitro culture in media supplemented with IGF-I (176.00±1.10) as compared to control and other treatment groups was, however, observed in the present study.

The ratio of the cell number in ICM and TCN was not significantly different between control (40.44±3.45) and embryos cultured in n-PG supplemented media (41.23±3.36). A highly significant difference between the ICM: TCN ratio was, however, recorded in IGF-I treated group (45.00±1.10) and control (40.44±3.45) and other treatment groups viz. 5% oxygen tension (42.66±3.04), glutathione (43.04±2.91), EGF (43.97±3.03) and n-PG (41.23±3.36). There was however, no significant difference between control and n-PG supplemented group.
Table 33: Effect of antioxidant and antiapoptotic factors on development competence of *in vitro* produced (IVP) embryos

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Replicates/ No. of COCs set for IVF</th>
<th>Cleavage (%)</th>
<th>Morula (%)</th>
<th>Blastocyst (%)</th>
<th>ROS (pixels/embryos)</th>
<th>Apoptosis (%)</th>
<th>ICM</th>
<th>Total cell no. (TCN)</th>
<th>ICM:TCN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=17)</td>
<td>17/ 516</td>
<td>65.46a ±0.36</td>
<td>38.24a ±0.72</td>
<td>20.09a ±0.60</td>
<td>19.12a ±1.76</td>
<td>9.73a ±2.45</td>
<td>61.89a ±1.60</td>
<td>153.12a ±3.45</td>
<td>40.44a ±3.45</td>
</tr>
<tr>
<td>5% Oxygen (n=17)</td>
<td>17/ 544</td>
<td>71.17bd ±0.37</td>
<td>43.31b ±0.74</td>
<td>25.23b ±1.29</td>
<td>14.15b ±0.45</td>
<td>4.76b ±1.10</td>
<td>65.59b ±1.01</td>
<td>154.06a ±3.04</td>
<td>42.66b ±3.04</td>
</tr>
<tr>
<td>Glutathione (n=17)</td>
<td>17/ 537</td>
<td>73.41bc ±0.37</td>
<td>44.60b ±0.71</td>
<td>28.46c ±1.37</td>
<td>12.90c ±0.51</td>
<td>2.08c ±0.81</td>
<td>65.59b ±0.91</td>
<td>152.76a ±2.91</td>
<td>43.04bc ±2.91</td>
</tr>
<tr>
<td>EGF (n=17)</td>
<td>17/ 518</td>
<td>76.73c ±0.74</td>
<td>48.10c ±0.54</td>
<td>33.88d ±0.63</td>
<td>12.48c ±0.68</td>
<td>1.20c ±0.65</td>
<td>66.70b ±1.02</td>
<td>152.47a ±3.03</td>
<td>43.97bc ±3.03</td>
</tr>
<tr>
<td>IGF-I (n=17)</td>
<td>17/ 534</td>
<td>77.72c ±0.61</td>
<td>50.11dc ±1.00</td>
<td>34.96d ±0.92</td>
<td>12.32c ±0.35</td>
<td>0.90c ±0.62</td>
<td>79.18c ±0.56</td>
<td>176.00b ±1.10</td>
<td>45.00c ±1.10</td>
</tr>
<tr>
<td>n-PG (n=17)</td>
<td>17/ 499</td>
<td>69.83ad ±1.50</td>
<td>40.11a ±1.27</td>
<td>22.95a ±0.76</td>
<td>12.34c ±0.64</td>
<td>3.56bc ±1.22</td>
<td>63.00ab ±1.38</td>
<td>153.06a ±3.36</td>
<td>41.23a ±3.36</td>
</tr>
</tbody>
</table>

Different superscript within a column indicate significance of difference
5. DISCUSSION

5.1 Oocyte retrieval by transvaginal ultrasound guided ovum pick up (OPU)

A total of 68 sessions of OPU in 6 elite Murrah buffaloes were carried out for ooocyte retrieval. The overall mean total numbers of follicles recorded in the present study was 5.56±0.14, out of which mean number of large and small follicles were 3.15±0.08 and 2.41±0.10, respectively with an average of 2.78±0.07 follicles per ovary. The mean total number of follicles recorded (5.56±0.14) and punctured (3.85±0.10) per session in the present study were comparable to those reported in riverine (Manjunatha, 2006, Gupta et al., 2006) and Mediterranean (Boni et al., 1996) buffaloes. However, our values were higher than those recorded in Indian buffaloes with reproductive problems (Manik et al., 2002). One of the potential applications of OPU is retrieval of oocytes in animals with reproductive problems. However, as reported by Manik et al. (2002), reproductively compromised buffaloes may be associated with less follicular activity or impaired folliculogenesis and hence may be associated with reduced retrieval rate. The use of cyclic healthy buffaloes in the present study may be the reason for higher follicular activity and hence higher number of follicles available for aspiration. The number of follicles available for aspiration and number of follicles aspirated in the present study was, however, higher as compared to those recorded in swamp buffaloes (Promdireg et al., 2005). The differences may be due to different genetic make up of the animals which might be associated with lower follicular activity and retrieval rate. However, oocyte recovery (50.46±2.75%) in the present study was in agreements with the previous reports in swamp (Promdireg et al., 2005), Mediterranean (Boni et al., 1996) and riverine buffaloes (Manjunatha, 2006). The recovery rates were, however, lower than that reported by Huang et al., 2005 in another study on swamp buffaloes.

There was no significant difference between oocyte donors in the mean number of follicles recorded, follicles punctured and follicles per ovary per session which corroborate with the findings of previous studies.
(Gupta et al., 2006; Manjunatha et al., 2007; Manjunatha et al., 2009). Non-significant variation between oocytes donors may be due to selection of healthy donors with optimum body condition and maintenance of all the experimental animals on ideal and optimum conditions of feeding and management throughout the study period.

5.2 Effect of frequency of OPU sessions on oocyte retrieval and developmental competence

In the present study the mean number of total follicles available for aspiration and follicles aspirated per session in once weekly (5.63±0.18 and 3.97±0.15, respectively) and twice weekly (5.49±0.19 and 3.73±0.14, respectively) OPU session were not significantly different. The number of COCs retrieved in once weekly (1.91±0.14) and twice weekly (2.00±0.13) sessions, were also not significantly different. But the weekly oocyte retrieval in twice weekly OPU session was higher as compared to once weekly session. Similar finding have also been reported by Manjunatha (2006) in riverine buffaloes. Twice weekly OPU sessions at an interval of three to five days have also been reported by Boni et al. (1996). The inter session interval did not affect either the number of aspirated follicles or the number of oocyte collected and hence equally good results in terms of oocyte retrieval per session were obtained in both the approaches. The oocyte recovery per week in twice weekly OPU sessions was almost two folds as compared to once weekly OPU.

Extensive literature is available on the mechanics of OPU and the relationship between the frequency of OPU and oocyte production in cattle. Most of the studies have demonstrated an advantage of twice weekly as compared to once weekly OPU (Garcia and Salaheddine, 1998; Goodhand et al., 1999; Hasler, 1998). Hasler (1998) has reported 571 oocytes from a single donor in 167 OPU sessions on primarily a once per week collection schedule. Although twice weekly collections have the potential for at least doubling this production, however, repeated OPU on the same donor should be viewed with some caution. It has been reported by Hasler (2003) that repeating the number of sessions in the same donor for prolonged period reduces the recovery of oocytes.
In the present study, twice a week aspiration resulted in a significantly higher per cent maturation (89.28±5.14 vs. 85.71±5.72), cleavage (76.80±7.14 vs. 61.43±7.23) and blastocyst (29.72±4.73 vs. 24.14±4.81) per cent. Studies indicate that twice a week OPU could be done for extended period of time without any 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. Further, twice a week OPU resulted in a higher number of embryos produced per buffalo per unit of time as compared to once a week OPU and best results were obtained when a 3 and 4 days or 2 and 5 days interval was maintained between OPU sessions in cattle (Merton et. al., 2003). In line with our results, a higher percentage of oocytes that reached metaphase II stage was reported in twice weekly (65.7%) as compared to once-weekly (51.4%) OPU sessions (Yindee et al., 2011). Also, the percentage of oocytes that reached the metaphase II stage was higher when oocytes were collected twice weekly, indicating improved quality of oocytes in this group. Though the values in this report were comparatively higher but the difference was non significant. In cattle, the morphological quality and developmental competence of oocytes in terms of rate of blastocyst production is also higher when OPU is performed twice weekly compared to one-weekly OPU (Lopes et al. 2006). This may be attributed to the hypothesis that a dominant follicle emerging approximately 3 days after OPU will exert a negative effect on developmental competence of oocytes of subordinate follicles and thus the oocytes collected during weekly sampling will have reduced developmental competence (Merton et al. 2003).

No effect of the frequency of oocyte collection on oocyte nuclear morphology was observed. Abnormalities in spindle formation and improper chromosome alignment can frequently be observed following *in vitro* maturation of oocytes and are incompatible with normal embryonic development in once weekly OPU. This may deteriorate the oocyte quality and thus effect the *in vitro* maturation (Christopikou et al. 2010).
5.3 Effect of retrieval techniques on oocyte retrieval and developmental competence

In the present study the average number of COCs retrieved per ovary (3.24±0.08) and oocyte recovery rate (67.26±0.28) was significantly higher (p<0.01) in slaughterhouse ovaries as compared to OPU (0.98±0.05 and 50.42±2.53), because the oocyte recovery is directly proportional to the number of surface follicles on the ovaries. Similar findings were reported by Manjunatha (2006) in riverine buffaloes. In OPU, follicles are determined by the resolution of the ultrasound machine (Merton et al., 2003) and only follicles larger than 2 mm can be aspirated. In contrast visible follicles of all sizes can be punctured in slaughterhouse ovaries, leading to greater retrieval rate. Also the postmortem changes lead to loosening of attachment of follicles with COCs thus the oocyte with complete cumulus investments are easily aspirated (Mullart et al., 1999) whereas in OPU manipulation of COCs by OPU needle is required for loosening the COCs for better recovery.

The maturation (%) was significantly higher in oocytes derived from OPU (87.50±4.34) as compared to those derived from abattoir ovaries (84.90±0.44). Higher oocyte maturation and developmental competence has also been reported in OPU derived oocytes in cattle (Neglia et al., 2003; Karadjole et al., 2010). This can perhaps be attributed to the fact that OPU results in resetting of the follicular population and subsequently increasing the follicular wave frequency (Boni et al., 1996). Also there is reduced incidence of follicular atresia as the follicles are punctured before they become atretic (Boni et al., 1996).

Further in slaughterhouse ovaries the retrieved oocytes may undergo a longer time from collection of ovaries to laboratory processing as compared to OPU. Possibly, buffalo oocytes may be more affected by cellular damage due to autolytic changes during prolonged period after excision of ovaries (Neglia et al., 2003). A contradictory report of higher maturation rate in cattle was, however, reported by Presicce et al. (2011). This may be due to difference in species of animal used for experimentation.
Significantly (p<0.01) higher developmental competence as depicted by per cent morula and blastocyst was observed in OPU derived oocytes as compared to abattoir derived oocytes. These data were also subsequently confirmed in riverine buffaloes by various workers who reported both higher blastocyst yields (30.6±4.3 vs 18.5±1.8) and hatching rates (52.8±4.2 versus 40.2±4.4) following embryo vitrification from OPU-derived compared to abattoir-derived buffalo oocytes (Manjunatha et al., 2008). A higher developmental competence in OPU-derived oocytes has been reported as compared to abattoir-derived COCs in Italian Mediterranean buffaloes (Neglia et al., 2003; Duran, 2008; Di Francesco et al., 2012).

The improved embryo yield may be due to the OPU-induced modification of the follicular dynamics. Resetting the follicular population twice weekly results in increased follicular wave frequency and hence may lead to aspiration of follicles before they become atretic thus improving the oocyte quality. On the contrary, a heterogeneous oocyte population is recovered from pooled ovaries of slaughtered buffaloes. In addition, it is worth reminding that buffaloes are usually slaughtered when they are old and/or infertile.

It is speculated that the better developmental competence of OPU vs abattoir oocytes is related to the shorter exposure to environmental stress. Indeed, abattoir-derived oocytes spend a longer time between excision of ovaries from the peritoneal cavity and laboratory processing and are probably affected by cellular damages due to autolytic processes, especially when they reside in excised ovaries for prolonged periods. It follows that, in the latter case, another important factor to consider is the time interval between ovary collection and processing in the laboratory.

Although oocyte quality seems to improve at the shorter time interval between animal slaughter and laboratory processing (3 h compared to 4, 5 and 6 h), as depicted by the higher percentage of grade A and B oocytes together with the lower incidence of degenerated oocytes. However, the cleavage and blastocyst rates were not affected by extending the time interval up to 6 h (Di Francesco et al., 2012).
5.4 Effect of various morpho-functional parameters on oocyte retrieval and developmental competence of abattoir derived oocytes

The effect of various morpho-functional parameters on oocyte retrieval and developmental competence of abattoir derived oocytes is discussed in the following section.

5.4.1 Effect of follicular size on oocyte retrieval and developmental competence

In the present study the mean number of large follicles per ovary (3.07±0.12) was significantly higher (p<0.01) as compared to small follicles (2.25±0.07). The oocyte recovery rate from large follicles (62.49±0.84%) was significantly higher as compared to the small follicles (52.00±0.66%). Several studies have suggested that the chance of recovering an oocyte decreases with smaller follicular size (Scott et al., 1989; Wittmaack et al., 1994; Rosen et al., 2009), and our findings are consistent with the above reports.

In the present study significantly higher maturation, cleavage, morula and blastocyst rates in COCs retrieved from large follicles as compared to small follicles was recorded. As per the previous reports the most important criteria for oocyte selection is the size of follicles (Pavlok et al., 1992; Longergan et al., 1994). As the size of the follicle reflects upon the developmental competence of the oocytes produced by it (Vatzias and Hagen, 1999). It has been reported in bovines that the oocytes acquire developmental competence when the follicles grow from small to antrum size (Hendriksen et al., 2000). The oocyte first acquires competence to develop into a blastocyst in an in vitro system at a follicular size of 2 or 3 mm (Pavlok et al., 1992; Blondin et al., 1995). This coincides with the completion of most of its RNA synthesis and growth (Fair et al., 1995; Hyttel et al., 1997). The competence to undergo meiotic maturation up to metaphase II is attained some what earlier than developmental competence (Blondin et al, 1995; Fair et al., 1995; Arlotto et al., 1996). The follicle size at which developmental competence is achieved depends on its ability to respond to FSH stimulation with rapid growth. In the mouse this ability coincides with a change in the
intracellular signal transduction of FSH action and not with changes in numbers of FSH receptors (Richards, 1994). Therefore, the acquisition of developmental competence of COCs might depend on changes both in the oocyte and in the cumulus cells.

Like present study significantly higher developmental competence of oocytes isolated from large size follicles has been reported in bovine (Shabankareh et al., 2014). Also a relationship between follicles and oocyte diameter has been reported in the past (Romanguera et al., 2010).

In agreement to our findings, several research groups have reported that oocytes derived from larger follicles (>4, 6 or 8mm) resulted in higher percentage of blastocysts than those from smaller follicles (Tan and Lu, 1990; Lonergan, et al., 1994; Fair et al., 1995; Hagemann, 1999). Lonergan et al. (1994) reported a blastocyst rate of 66% for oocytes from >6mm follicles, while a blastocyst rate of 34% was obtained with oocytes from 2-6mm follicles. Though these studies corroborate with the significantly higher developmental competence of oocytes from large follicles in the present study but the higher blastocyst (%) as compared to our experiment may be because of difference in species and environmental conditions. The developmental competence of small follicles (<2mm) was compromised due to the fact that they have not yet completed growth and RNA synthesis and, therefore, were not competent (Tan and Lu, 1990; Pavlok et al., 1992). Blondin and Sirard (1995) found no significant difference in competence between oocytes from 3-5mm and >5mm follicles. Similarly, Hagemann et al. (1999a) found no difference in competence between oocytes from 3-5mm and 6-8mm follicles. Further, oocytes of dominant follicles( >13mm) yielded a blastocyst rate of 65% vs 44% for oocytes from 3-8mm follicles obtained during growth phases.

The better developmental competence of large follicles (>7mm) recorded in the present study may be due to the association of large follicles with higher differentiation occurring in advanced stages of follicular development, expression of LH receptors by granulosa cells, decrease of IGF-binding protein, increase of IGF-I within the follicular fluid, increased expression of growth factors such as TGF-β, activin and inhibin. This occurs
simultaneously along with ultrastructure changes within the oocyte and cumulus cells (Hendriksen et al., 2000).

5.4.2 Effect of phase of oestrus cycle on oocyte retrieval and developmental competence

In the present study a significantly (p<0.01) higher mean number of follicles per ovary was recorded during luteal phase (4.87±0.32) as compared to follicular (3.23±0.13) and early luteal phase (3.34±0.12). The mean number COCs retrieved per ovary in follicular (2.28±0.09) and early luteal (2.26±0.11) phase and luteal phase (3.29±0.23) also depicted similar trend. The per cent nuclear maturation assessed by extrusion of first polar body revealed significantly (p<0.01) higher proportion of COCs with polar body in luteal phase (86.54±0.53) as compared to follicular (80.86±0.23) or early luteal phase (83.76±0.48). Changes in size of the follicle with the stages of oestrus cycle has been reported in past due to growth and atresia of the follicles. Significantly higher population of oocytes from small and medium size follicles increases the number of morphologically normal and meiotically competent oocytes between early and mid luteal phase, with maximum being reached at late luteal or early follicular phase of the cycle (Machatkova et al., 2008). Thereafter these numbers begin to decrease and hence higher recovery of oocytes during luteal phase in our study confirms the finding reported in various mammalian species (Schnurrbusch et al., 1990; Machatkova et al., 2008). The higher meiotic competence may be due to the fact that during luteal phase (particularly late luteal phase), selection for growth and maturation of presumptive preovulatory follicles begins from medium follicles, while smaller subordinate follicles become atretic (Ratky et al., 2005). During follicular phase the oocytes are basically retrieved from large follicles as the number of small and medium follicles rapidly decline. In our study also significantly lesser number of oocytes were recovered during follicular phase which could be attributed to the above fact.

Significantly higher developmental competence in terms of cleavage, morula and blastocyst per cent during luteal phase (69.43±0.92, 42.53±0.58 and 22.51±0.55) as compared to early luteal (63.22±0.54, 34.05±1.28 and 17.13±0.31) and follicular phase (61.92±0.60, 33.14±0.63 and
14.75±0.25) was recorded in the present study. The developmental competence in terms of blastocyst production in in vitro embryo production set up depends not only on the intrinsic oocyte quality but also on IVP procedures and sperm quality. Ovarian follicles in buffalo develop in waves similar to cattle, except the absence of four waves (Baruselli et al., 1997). Only scanty literature is available on the effect of phase of oestrus cycle on developmental competence in buffaloes for direct comparison of our findings (Manjunatha, 2006). It could be speculated that atresia of subordinate follicles mainly occurs during the dominance phase of the follicular wave, and hence oocytes retrieved from atretic follicles in dominance phase (which corresponds to the follicular phase) may be responsible for compromised developmental competence. In confirmation to our findings, higher developmental competence of oocytes retrieved during phase of follicular growth (corresponding to luteal phase of oestrus cycle) as compared to phase of dominance (corresponding to follicular phase) was also reported in bovines (Hagemann et al., 1999a). This might be associated with higher level of apoptosis in subordinate follicles of 6-8mm and hence leading to reduced developmental competence of oocytes retrieved from such follicles (Hagemann et al., 1999b). Similar negative effect of presence of dominant follicles on in vitro developmental competence of bovine oocytes has also been described by other research groups (Varisanga et al., 1998; Hagemann et al., 1999b), which might be attributed to intraovarian environment to which the oocytes were exposed (Goto et al., 1990; Varisanga et al., 1998). Contrary to our findings Chian et al. (2002) reported that the developmental competence of bovine oocytes from small follicles was not affected by the presence of a dominant follicle or phase of folliculogenesis.

5.4.3 Effect of season on oocyte retrieval and developmental competence

The follicles per ovary (5.76±0.12), COCs retrieved per ovary (3.94±0.09) and oocyte recovery rate (68.35±0.27) was significantly higher during winter season as compared to respective values during summer (3.90±0.08, 2.62±0.06 and 67.14±0.46) and rainy seasons (4.75±0.05, 3.15±0.05 and 66.33±0.50) seasons. In contrast to our findings, the oocyte
number recovered per ovary and the number and incidence of good quality oocytes were not affected by season in Italian Mediterranean buffaloes (Gasparrini et al., 2000; Di Francesco et al., 2012), which may be due to difference of breeds and different agro-climatic conditions. However, Manjunatha et al. (2008) reported higher oocyte retrieval during peak breeding season which corresponds to the cooler months in Indian riverine buffaloes. This is in agreement to our findings wherein higher oocyte retrieval was recorded during winter season. This is further confirmation of the fact reported in the past that though breeding in Indian buffaloes occurs round the year, but seasonal variation in sexual rhythm is observed (Taneja et al., 1995). Many reports from other part of the world also support our findings, where lowest level of sexual activity was observed during summer months.

In the present study significantly higher grade I (36.32±0.64) and grade II (45.05±0.70) oocytes were recovered during winter as compared to their respective values during rainy (32.55±0.64 and 42.78±0.58) and summer (31.07±0.49 and 41.04±0.42) season. The proportion of grade III oocytes was significantly higher in summer (27.90±0.79) followed by rainy (24.60±1.08) and winter (18.54±0.46) season. This is in agreement with the findings of Nandi et al. (2001) in buffaloes who also retrieved significantly higher proportion of grade I and grade II oocytes in cool environmental temperature as compared to hot environmental temperature. Lower yield of usable oocytes (grade I and II) in the present study during hot summer season may be due to heat stress which alters the endocrine patterns and reduces follicular development. This heat induced decrease in usable oocytes may be due to a series of cellular alterations that affects nuclear and cytoplasmic compartments of the bovine oocytes (Paula-Lopes et al., 2012), leading to retrieval of higher proportion of grade I and II (usable oocytes) during winter months / cooler part of the year.

In the present study significantly higher developmental competence of IVP buffalo embryos in terms of blastocysts production rate was recorded during winter (23.28±0.41) as compared to summer (15.75±0.46) and rainy (17.92±0.52) seasons. The cleavage and Morula per cent also followed the same trend. Our findings were in conformity to
significantly higher developmental competence recorded in Indian (Manjunatha et al., 2009), Italian Mediterranean (Di Francesco et al., 2011; Di Francesco et al., 2012) and Egyptian (Khairy et al., 2007) buffaloes during cooler part of the year. Nandi et al. (2001) observed no difference in proportion of morula and blastocysts derived from matured oocytes during cold and hot climate, which contradicts our findings. The author suggested that the non significant variation between morula and blastocyst rate may be due to production of heat shock protein by embryos in response to thermal stress (Putney et al., 1988), which provided protection against heat stress during summer season (Ealy et al., 1992; Ryan et al., 1992). But the higher proportion of degenerated oocytes and embryos during hot climate, points towards lower developmental competence which is in agreement to our findings. In Indian riverine buffaloes, reared under farm and field condition, breeding activity is observed round the year but the sexual rhythm vary with seasons, with a selective preference towards the cooler months of the year for breeding activity (Taneja et al., 1995). Lower reproductive efficiency is also observed in buffaloes reared in other parts of the world during summer month indicating lower in vivo developmental competence of naturally ovulated oocytes.

5.4.4 Effect of Oocyte diameter on developmental competence of IVP embryos

To study the effect of diameter of oocyte on its quality, immediately after retrieval the diameters of the collected oocytes were measured and they were classified as large (> 150µm, medium (120-150µm) or small (<120µm) groups on the basis of diameter. The developmental competence of large oocytes group was significantly (p<0.01) higher as compared to the medium and small size oocytes as depicted by significantly higher cleavage, morula and blastocyst rates. The results are in agreement to the previous findings in goats (Anguita et al., 2007), cattle (Otoi et al., 1997) and buffaloes (Raghu et al., 2002; Yousaf and Chohan, 2003; Duran, 2008). Oocyte diameter is a positive indicator of developmental competence of oocytes. Only smaller proportion of small size oocytes (<120µm) developed to MII stage, demonstrating reduced maturation capacity. The potential to
acquire full maturation and developmental competence was associated with increase in oocyte diameter with most of the large (>150µm) oocytes showing optimum maturation and subsequent embryonic development. Most of the smaller oocytes failed to show optimum developmental competence and were at degenerating stage. The high incidence of degeneration in smaller oocytes in cattle was characterized by cessation RNA synthesis (Fair et al., 1996) that represents a stable storage of macromolecules essential for resumption of meiosis and early embryonic development (De Smedt et al., 1994).

Most of the literature in this regard points towards the fact that larger oocytes are associated with increased developmental competence but few authors also stated that oocytes larger than 145µm or smaller than 110µm are associated with the increased incidence of degeneration in swamp buffaloes (Duran, 2008). These authors have associated this fact with ultrastructure study and observed that these oocytes had no microvilli projecting towards the corona radiata except for only a few areas with the presence of few gap junctions, which points towards the degenerative process and lack of communication with the surrounding corona cells. However, in Indian buffaloes, oocytes of size >145µm were reported to be associated with greater developmental potential similar to the findings in the present study (Raghu et al., 2002). These variations in findings may be due to different breeds in question. But the findings of Crozet et al. (2000) in cattle corroborate our findings that the developmental competence of the oocytes is directly proportional to their diameter and in order to achieve complete developmental competence oocytes should have attained a diameter of atleast <120µm.

5.4.5 Effect of grade of oocytes on developmental competence of IVP embryos

Significantly higher developmental competence of grade I and II COCs was recorded in terms of cleavage, morula and blastocyst rates as compared grade III, which indicates that oocyte morphology is a positive indicator of developmental competence. Similar results depicting higher maturation per cent (Suzuki et al., 1992) and developmental competence (Duran, 2008) has also been reported in buffaloes. Similar findings have also
been reported in bovines (Kakkassery et al., 2010). No significant difference between developmental competence of grade I and II oocytes were recorded in the present study, which corroborate with the findings of Kakkassery et al. (2010) in cattle who also reported no significant difference between the maturation percent in grade I and II oocytes. Oocytes surrounded by compact cumulus (grade I) were mostly at germinal vesicle stage while those with loose cumulus were mostly at germinal vesicle breakdown stage, and those devoid of cumulus were degenerating (De Loos et al., 1989). Germinal vesicle breakdown was observed in all oocyte categories confirming the report that mammalian oocytes removed from their follicular environment spontaneously resume meiosis (Pincus and Enzmann, 1935). Also cumulus cell controls meiosis since compounds which elevate intracellular cAMP levels delay meiotic resumption in cumulus-enclosed oocytes, but not in denuded oocytes (Dekel et al., 1984).

Cumulus cells have been considered to play an important role in oocyte maturation by keeping the oocytes under meiotic arrest, inducing meiotic resumption and by supporting cytoplasmic maturation. These functions have been attributed to their gap junctions and their specific metabolizing capabilities (Tanghe et al., 2002). Physical contact between oocytes and cumulus cells has been considered necessary for the transfer of nutrients and factors essential for oocyte development (Albertini et al., 2001). Cumulus cells communicate to the oocyte across zona pellucida through corona radiata cells, which penetrate the zona pellucida and form gap junctions with oolemma. These intercellular communications allow metabolic transfer as molecules of small molecular weight help in nutrition of oocytes, which ultimately plays a vital role in oocyte growth and maturation (Buccione et al., 1990; Armstrong et al., 1996). Staigmiller and Moor (1984) postulated that granulosa cells provide energy substrate, some amino acids, nucleotides and phospholipid precursors to the oocyte, that generate some interactional signals. These signals influence the nucleus and direct the synthesis of certain structural proteins and maturation specific proteins. Dissociated cumulus cells as seen in the case of grade III oocytes have also been
reported to produce paracrine factors, which resume meiosis in denuded oocytes (Downs, 1989). Raghu et al. (2002) reported that removal of cumulus cells perturbed the cytoplasmic maturation and hence developmental competence was reduced, which explains the significantly lower maturation and developmental competence in grade III oocytes in the present study.

So the present study reaffirms the fact that cumulus oocyte complex morphology has a definite bearing on the in vitro maturation and developmental competence. Oocytes with multiple layers of cumulus cells (three or more layers) had better developmental competence as compared to the denuded oocytes or oocytes with lesser cumulus investment (less than three layers). Oocytes with more than three complete layers of cumulus cells (grade I and II) are best suited for in vitro maturation and fertilization in buffaloes.

5.4.6 Effect of source of spermatozoa on developmental competence of in vitro produced embryos

Epididymal sperm provides a last chance to conserve the germplasm of superior male. The use of bovine epididymal sperm in IVF was first reported by Ball et al. (1983) wherein fresh epididymal sperms derived from slaughterhouse bull testes used for IVF resulted in formation of male and female pronuclei in 40% oocytes. Pavlok et al. (1988) reported a fertilization rate of 72% for epididymal and 54% for ejaculated bull sperms in IVF. These reports established the epididymal sperms to be a viable source of sperms in IVF.

In the present study, the overall embryonic developmental rates of oocytes fertilized with ejaculated and epididymal spermatozoa was recorded to be almost similar between the two groups. A reasonably good cleavage (69.15±0.78), morula (39.63±1.36) and blastocyst (20.28±1.09) rates were obtained by use of epididymal sperm. However, there was no significant difference between developmental competence of oocytes fertilized with epididymal and ejaculated spermatozoa. Though no report for direct comparison of the results is available in buffaloes (Kumar et al., 2014) also recorded no significant difference between blastocyst rate obtained after in
vitro fertilization with ejaculated and epididymal spermatozoa in caprine. In feline also structural and fertilizing potential has been reported in epididymal spermatozoa stored at refrigerated temperature for up to 7 days (Buarpung et al., 2015).

Thus, epididymal spermatozoa can be effectively used for IVF and comparable results as in case of ejaculated spermatozoa can be obtained. This will prove an important innovation in conservation of germplasm of slaughtered buffalo bulls.

5.5 Cellular predictors of developmental competence of in vitro produced embryos

The effect of glucose-6-phosphate dehydrogenase activity, reactive oxygen species and apoptosis as predictors of developmental competence of in vitro produced buffalo embryos was studied and the results are discussed in the following section.

5.5.1 Glucose-6-phosphate dehydrogenase (G6PDH) activity and follicular size

The G6PDH activity was assessed by brilliant cresyl blue (BCB) staining, which is an electron acceptor and can be used to semi quantitate the level of G6PDH activity in the oocytes (Tian et al., 1998). G6PDH is a component of pentose phosphate pathway which provides ribose phosphate for nucleotide synthesis and the formation of fatty acids. The BCB test is based on the ability of G6PDH to reduce BCB stain from blue to colorless (Rodriguez-Gonzalez et al., 2002).

In the present study, the G6PDH activity in large and small follicles in abattoir derived ovaries was assessed by BCB staining. The proportion of BCB⁺ oocytes (inactive G6PDH) in large and small follicles recorded in the present study was 56.42±0.47 and 45.82±0.56 (%), respectively with a significant (p<0.01) difference between the G6PDH activity of oocytes derived from large and small follicles. The BCB⁺ oocytes indicate that they have completed their growth phase and could be used for in vitro embryo production (IVEP). These findings are in agreement with the findings reported earlier in bovine heifers (66%: Pujol et al., 2004; 58: Alm et al.,
2005), cows (56.34%; Shabankareh et al., 2014) and buffaloes (57%; Manjunatha et al., 2007; 53.9%; Heleil and Fayed, 2010). The values obtained in the present study were, however, either lower (81% in swine; Roca et al., 1998; 70% in bovine; Opiela et al., 2008) or higher (30.1% in caprine; Rodriguez-Gonzalez et al., 2002) than those recorded in other species. The difference in G6PDH activity of the present study from these reports may be either because of different selection criteria after BCB staining or may be due to species difference. 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 stained oocytes among laboratories.

The significant difference of G6DPH activity between small and large follicles in BCB− (active G6DPH) and BCB+ (inactive G6DPH) reaffirms the previous findings that follicular size can be used as criteria for selection of developmentally competent oocytes in various animal species (Pavlok et al., 1992; Crozet et al., 2000; Manjunatha et al., 2007; Held et al., 2012; Mohammadi-Sangcheshmeh et al., 2013; Mohammadi-Sangcheshmeh et al., 2014).

**Effect of Glucose-6-phosphate dehydrogenase (G6PDH) activity on developmental competence of in vitro produced buffalo embryos**

In the present study the per cent cytoplasmic maturation in BCB+ oocytes aspirated from large follicles (85.79±0.98) was significantly higher (p<0.01) as compared to BCB− (81.30±0.60) oocytes. Similarly BCB+ (inactive G6PDH) oocytes (79.94±1.27) also showed significantly higher per cent maturation as compared to BCB− (active G6PDH) oocytes (73.59±0.84) derived from small follicles. The results are in line with the earlier reports in various species of farms animals including caprine (Urdaneta et al., 2003), bovine (Alm et al., 2005) and bubaline (Manjunatha et al., 2007). Similar trend was also observed in cleavage, morula and blastocyst per cent in oocytes derived from large and small follicles indicating higher developmental competence in BCB+ as compared to BCB−. Thus higher developmental competence was observed in oocytes with low G6PDH activity. This is in
agreement with the previous reports in various species viz. equine (Mohammadi et al., 2011), swine (Roca et al., 1998; El Shourbagy et al., 2006; Wongsrikeao et al., 2006), caprine (Rodriguez-Gonzalez et al., 2002; Rodriguez-Gonzalez et al., 2003; Urdaneta et al., 2003), bovine (Pujol et al., 2004, Alm et al., 2005; Bhojwani et al., 2007; Shabankareh et al., 2014) and bubaline (Manjunatha et al., 2007; Duran, 2008; Heleil and Fayed, 2010). It has been reported in the past that the oocytes that complete their growth shows full competence for meiotic maturation. As a result of incomplete cytoplasmic maturation the BCB⁺ oocytes did not acquire full developmental competence (Susor et al., 2007).

The developmental competence expressed in terms of cleavage, morula and blastocyst per cent of the oocytes derived from large follicles was significantly (p<0.01) higher as compared to oocytes derived from small follicles. Therefore, it could be concluded that the developmental competence of oocytes depends on size of the follicle from which it is obtained (Vatzias and Hagen., 1999) and hence the size of the follicle is the most important criteria for oocyte selection (Longergan et al., 1994).

Although the classification of oocyte quality by morphological characteristics could provide valuable information for the preselecteion of oocytes with higher developmental competence, this method is not precise. The high association of G6PDH activity with developmental competence recorded in the present study points towards the fact that it represent a molecular and subcellular predictor for developmental competence of oocytes in buffaloes as has also been reported in the past for cattle (Shabankareh et al., 2014) and buffaloes (Manjunatha et al., 2007; Heleil and Fayed, 2010). Immature oocytes with low G6PDH activity (BCB⁺) are presumed to have completed their growth phase where oocytes with high G6PDH activity (BCB⁻) are still in their growing phase (Opiela et al., 2010). A high proportion of oocytes with low G6PDH activity progressed to diakinesis stage whereas immature oocytes with high G6PDH activity are with higher probability of being retarded in development (Torner et al., 2008), which may be attributed to the fact that most of the BCB⁺ oocytes are normally derived from fully grown follicles and have undergone better cytoplasmic maturation during the
final phases of folliclogenesis (Mohammadi-Sangcheshmeh et al., 2011). Also the previous studies demonstrated that BCB⁺ oocytes contained comparatively higher number of mitochondrial DNA copies and had a greater diameter with higher cytoplasm volume (Pujol et al., 2004), which was positively correlated with fertilization and developmental competence (El Shourbagy et al., 2006). The BCB⁻ oocytes have lower transcript of genes responsible for mitochondrial biosynthesis (Opiela et al., 2010) and shows delayed onset of expression of some vital proteins for embryonic development (Spikings et al., 2007).

Thus on a hypothetic time line, growing oocytes (high G6PDH activity) are developmentally retarded as compared to mature oocytes with low G6PDH activity.

5.5.2 Effect of reactive oxygen species (ROS) on developmental competence of in vitro produced embryos

Significantly (p<0.05) higher ROS was recorded in grade I COCs as compared to grade II and grade III immature COCs in the present study. Similarly a significantly lower (p<0.05) ROS intensity was recorded in grade I (15.01±1.18) as compared to grade II (19.19±0.84) and grade III (24.61±1.36) in vitro matured (IVM) COCs. Further, significantly (p<0.01) higher ROS intensity was recorded in IVP embryos derived from grade I (16.27±1.90) COCs as compared to those derived from grade II (20.44±0.27) and grade III (24.64±0.74) COCs.

Handling or maturation of oocytes or embryo culture in a high atmospheric oxygen and glucose concentration results in an increased ROS levels (Thompson et al., 2000), causing cell membrane lipid peroxidation (Nash-Esfahani et al., 1990; Noda et al., 1991), DNA fragmentation and influences RNA transcription and protein synthesis (Takahashi et al., 2000). These cellular changes lead to in vitro developmental blocks and early embryonic death (Noda et al., 1991; Goto et al., 1993). Thus, a modification of IVM culture system for reduced ROS activity is necessary. IVM medium supplementation with antioxidants has been demonstrated to stimulate the synthesis of GSH and decreased the ROS levels in oocytes (Wu et al., 2011).
Contradictory to our finding, no effect of higher $O_2$ and hence increased ROS was reported in the past (Khurana and Niemann 2000). These discrepancies could be due to effect of oxygen tension and the glucose concentration of the various culture systems (Karja et al., 2006; De Castro e Paula and Hansen, 2007).

**5.5.3 Effect of apoptosis on developmental competence of *in vitro* produced embryos**

The incidence of apoptosis was significantly ($p<0.01$) higher in grade III immature COCs (13.35±0.59), *in vitro* matured COCs (17.30±0.89) and IVP embryos derived from grade III COCs (18.43±1.07) as compared to the incidence for grade II (10.56±0.92; 13.43±0.86 and 14.23±1.39) and grade I (7.29±0.65; 9.54±0.84 and 10.14±1.07, respectively). Literature for direct comparison of our findings could not be traced in buffaloes but similar findings in immature bovine oocytes were reported by Aziz et al. (2014).

It is generally accepted that the COCs derived from non atretic follicles show compact cumulus cell layers with a homogeneous oocyte cytoplasm (de Wit et al., 2000) and hence higher developmental competence (Lee et al., 2001; Corn et al., 2005; Yuan et al. 2005). Nevertheless, some studies provided conflicting results concluding that COCs showing mild signs of atresia lead to higher blastocyst rates in cows (de Wit et al., 2000; Feng et al., 2007). The incidence of apoptosis in mature and immature oocytes was similar to the previous reports (Li et al., 2009; Anguita et al., 2007). Immature oocytes with compact and not dense cumulus cells (grade II), and oocytes with thin or little remnants of cumulus cell layers (grade III) showed a significantly high rate of early apoptosis compared to oocytes with compact and dense cumulus cells (grade I) in the present study. The occurrence of apoptosis in grade III oocytes was twice as high as in grade I. All groups of oocytes analyzed showed heterogenous ooplasm i.e. one of the characteristics of early signs of atresia. These oocytes may be at earlier stages of apoptosis before DNA breakdown since they are the last compartment to be affected by apoptosis during atresia of antral follicle (de Wit et al., 2000). However, it is still not known whether oocytes with compact
and more layers of cumulus cells surrounding the oocytes such as grade I come from late atretic follicles.

During preimplantation embryo development, 15-50% of embryos succumb to death, largely due to unknown factors (Warner, 1998). However, apoptosis has been shown to have a crucial role during preimplantation development to eliminate cells with abnormal (chromosomal and nuclear abnormalities) or inappropriate developmental potential with a reduction in cell numbers of inner cell mass (Fabian et al., 2005). In addition, apoptosis has been shown to play a role in mouse and human embryo fragmentation at the 1 to 2 cell stage (Jurisicova et al., 1998a; O’Neill, 1998). It is likely that apoptosis at this stage may serve to eliminate embryos that have failed to activate the embryonic genome competently or in a timely manner (Kim et al., 2008). At the blastocyst stage, apoptosis is prevalently confined to the ICM lineage. In vivo derived mouse blastocysts showed approximately 10% of dead cells in the ICM in contrast to less than 3% in the trophoectoderm (Byrne et al., 1999). Apoptosis at this stage may serve to regulate the cell population, as mitotic cell division is still occurring. Further, ICM cells that retain the potential to form trophoectoderm cells after blastocyst expansion are also eliminated by apoptosis. Hence apoptosis at this stage may function to eliminate damaged, developmentally incompetent, or excess cells (Byrne et al., 1999).

5.6 In vivo developmental competence of IVP embryos (Conception rate)

One successful pregnancy was established using IVP embryos obtained from abattoir derived male and female germplasm out of eight embryos transferred to synchronized recipients. The conception rate was observed to be 12.5%. The quantum of data is not sufficient to compare our results with other studies. Successful production of calf has also been reported in the past in Murrah (Madan et al., 1991), Swamp (Duran, 2008) and Italian Mediterranean buffaloes (De Fransesco, 2008) using IVF embryos derived from abattoir ovaries and ejaculated semen. No report of establishment of pregnancy using IVP embryo produced from gametes from
both slaughtered male and females, could however be traced for direct comparison.

5.7 Effect of antioxidant and antiapoptotic factors on developmental competence of IVP embryos

A highly significant beneficial effect of embryo culture in reduced oxygen tension (5%) as compared to atmospheric oxygen (20%) was recorded in the present study. Also significantly lower apoptosis, reactive oxygen species and cell number in inner cell mass and total cell numbers were recorded in the present study. These results are consistent with previous reports in bovine (Takahashi et al., 1996a; Van Soom et al., 2001; Leivas et al., 2006; Balasubramanian et al., 2007; Goovaerts et al., 2009) and porcine (Kitagawa et al., 2004) embryos. Balasubramanian et al. (2007) also reported higher numbers of cell of inner cell mass of blastocysts cultured at 5% oxygen tension. Whereas contradictory reports of no beneficial effect of reduced oxygen tension (Khurana and Neimann, 2000) is also on records. However, no report in buffaloes for direct comparison could be traced.

The concentration of oxygen in bovine reproductive tract of females is approximately 5% (Mastroianni and Jones, 1965). Higher levels (20%) of oxygen are toxic for different type of mammalian cells, including oocytes and spermatozoa, probably due to formation of free radicals which cause severe cell damage by oxidation, enzyme inactivation and DNA damage (Umaoka et al., 1992).

A significantly higher total cell number was reported in in vivo produced bovine (Gjorret et al., 2003; Balasubramanium et al., 2007; Goovaerts et al., 2009) and porcine (Kitagawa et al., 2004) embryos as compared to IVP embryos. This may be due to reduced oxidative stress of in vivo produced embryos due to in vivo culture in fallopian tube at 5% oxygen tension. In the present study, significantly lower intensity of ROS in embryos cultured at 5% O₂ and subsequently lower apoptosis and higher the total cell number of blastocysts corroborate with the above findings. In agreement with our observations, it has been stated that the slow rate of development of IVP embryos cultured at atmospheric oxygen when compared to those produced in vivo at 5% oxygen may be responsible for this characteristic lower cell
number (Rizos et al., 2002). It was hypothesized that cell division progresses more efficiently in *in vivo* produced embryos at reduced oxygen tension.

The intensity of reactive oxygen species was higher in embryos cultured at 20% oxygen tension as compared to 5% oxygen tension. Similar reports on porcine (Kitagawa *et al*., 2004) and murine embryos (Goto *et al*., 1993; Kwon *et al*., 1999) are also on records. The H$_2$O$_2$ (ROS) releases OH$^-$ which is highly reactive with other molecules in the cytoplasm and causes cell injury. The H$_2$O$_2$ functions in the regulation of gene expression, and its concentration is regulated by a number related oxydases (Guerin *et al*., 2007). The activities of oxidases are known to be dependent on environmental O$_2$ concentration, and optimal concentrations for their activity are usually higher than those *in vivo* (Halliwell, 1978). Two major enzymes, NADPH oxidase and Xanthine oxidase, produced ROS in the embryo (Nasr-Esfahani and Johnson, 1991; Manes and Lai, 1995; Kelly *et al*., 2001), perhaps the high H$_2$O$_2$ content in embryos cultured under 20% O$_2$ is due to production of higher level of H$_2$O$_2$ by these oxidases at higher oxygen concentration.

A highly significant effect of glutathione (1 mM) on development competence of IVP buffalo embryos was recorded in the present study. A significantly higher (p<0.01) per cent cleavage, morula and blastocyst in RVCL media supplemented with glutathione (73.41±0.37, 44.60±0.71 and 28.46±1.37) as compared to control (65.46±0.36, 38.24±0.72 and 20.09±0.60) was recorded. Glutathione is a tripeptide thiol synthesized by glutamic acid, cysteine and glycine in the pathway of γ-glutamyl cycle (Cornell and Meister, 1976; Cheong *et al*., 2009) and is present in either reduced (GSH) or oxidized (GSSG) form. GSH acts as a strong reducing agent and also serve as electron donor for glutathione peroxidase. It might have caused improved developmental competence of buffalo IVP embryos as compared to the control by maintaining equilibrium in the redox state to protect the cell against the harmful effects caused by oxidative stress. Besides the protective effects of GSH may also have been manifested by its role in DNA synthesis (Fidelus *et al*., 1987), transcription (Bergelson *et al*., 1994), cell cycle (Messina and Lawrence, 1989), cytokine activity (Liang *et al*., 1989) and apoptosis (Chiba *et al*., 1996).
Embryonic development is also affected by intra and extra cellular redox state correlated with GSH synthesis (Takahashi et al., 1993; Gardiner et al., 1998) as well as DNA synthesis (Takahashi et al., 1996b). For an effective embryo culture, the maintenance of intracellular redox state by increasing GSH level is very important.

In the present study significantly beneficial effect of IGF-I (100 ng ml$^{-1}$) on developmental competence of embryos as compared to the control, was recorded which is in agreements to the previous works in buffaloes (Duran, 2008; Singhal et al., 2009) and cattle (Wasielak and Bogacki, 2007).

Insulin and insulin-like growth factor-I are small mitogenic polypeptides with structural and functional homologies to each other. In addition to their broad range of effects on a number of cells, they have been demonstrated to be expressed in a developmentally regulated manner during preimplantation embryo development (Schultz and Heyner, 1993). The insulin family of growth factors exerts this biological action by binding to specific cell surface glycoprotein receptors.

Duran, 2008 reported that IGF-I, IGF-II and insulin receptors are preferentially localized in almost all stages from 2 cell to the blastocyst stage of pre-implantation buffalo embryos and the development of embryos into blastocysts was significantly increased with IGF-I supplementation (Chandra et al., 2012). Several reports are available on the effect of addition of insulin or insulin-like growth factors to medium for culture of preimplantation mammalian embryos and the stimulatory effect on embryo metabolism and cellular proliferation in developing embryos of mouse (Harvey and Kaye, 1988; Heyner et al., 1989; Gardner and Kaye, 1991; Kaye et al., 1992), cow (Zhang et al., 1991) and pig (Lewis et al., 1992) was observed. However Lee and Fukui (1995) did not observe any improvement in bovine embryonic development although, there have been reports that IGF-I and its receptor gene are present in cattle embryos. The dose of IGF-I used may not have been optimal for stimulation of embryonic development in the above study (Rappolee et al., 1990).
Many growth factors have both growth-promoting and growth-inhibiting activities, depending on the biological context. Cellular response to exogenous growth factors in vitro is dose dependent and it varies in different species. For example, Harvey and Kaye (1992) have demonstrated that a concentration of IGF-I as low as 0.1 to 1.0 ng/ml results in cellular proliferation of mouse embryos.

Sometimes embryos have to be cultured singly or in small groups especially in case of OPU derived embryos. However, it has been shown in the mouse and cow that only small percentages of embryos cultured singly develop to blastocysts when compared with those incubated in groups (mouse, 49% vs. > 80%; cow, 2.9% vs. 30.7%) (Paria and Dey, 1990; Goovaerts et al., 2009). Furthermore, previous studies have also demonstrated that individually cultured cat (Spindler and Wildt, 2002) and cow embryos (Carolan et al., 1996) fail to reach the compaction stage. To date, several strategies have been established to overcome the poor developmental rate of singly cultured embryos (Goovaerts et al., 2009). These include growth factor supplementation in in vitro culture in conditioned medium derived from group-embryo culture and co-culture with autologous cumulus cells (Paria and Dey, 1990; Fujita et al., 2006; Goovaerts et al., 2009; Goovaerts et al., 2010; Goovaerts et al., 2011). Growth factors such as epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) have been shown to enhance blastocyst development in a single-embryo culture system in the mouse and cow (Paria and Dey, 1990; Lim and Hansel, 1996; O’Neill, 1997).

The total cell number (TCN), and inner cell mass (ICM) and ICM:TCN ratio significantly (p<0.01) improved following in vitro culture in medium supplemented with IGF-I (100 ng ml⁻¹) in the present study. As reported earlier in mouse, IGF-I increases the total cell number in blastocysts by increasing the ICM (Harvey and Kaye, 1992; Smith et al., 1993). The similar trend was also observed in the present study. Although the levels of IGF-I present in the reproductive tract of mouse is not known, but recent analysis indicates that there is about 30 ng/ ml IGF-I in porcine oviductal fluid. It has been also demonstrated that the cleavage rate of pig oocytes was
markedly increased in a dose dependant manner by addition of IGF-I during oocyte maturation (Xia et al., 1994).

In the present study, epidermal growth factor (EGF) significantly (p<0.01) increased the number of embryos reaching cleavage (76.73±0.74 Vs. 65.46±0.36%), morula (48.10±0.54 Vs. 38.24±0.72%) and blastocyst (33.88±0.63 Vs. 20.09±0.60%). Similar results were also reported in buffaloes (Duran, 2008; Purohit et al., 2005), cattle (Sirisanthien et al., 2013), goats (Palanisammi et al., 2014) and cats (Thongkittidilok et al., 2015). Although the physiological significance of EGF is not yet fully understood, it is possible that EGF plays a regulatory role in follicular development in a paracrine/autocrine manner, or it might be one of the signaling factors for resumption of meiosis in oocytes (Coskun et al., 1991). The improvement in the developmental competence after addition of this combination was also observed in mouse cumulus-oocyte complexes in vitro (Downs, 1989). Similar information was obtained in oocytes from the mouse follicles using a whole follicle culture system (Boland and Gosden, 1994), as well as in sheep oocytes (Guler et al., 2000).

Similarly, cumulus expansion was the distinct morphological effect exhibited by the addition of EGF and was more striking when the combination of these compounds was used in the IVM medium (Goovaerts et al., 2009). This means that cumulus expansion plays an important role in the process of fertilization by enhancing sperm capacitation and increasing sperm motility (Schroeder and Eppig, 1984) which may explain the improved cleavage rate in the present experiment.

Although evidence have accumulated concerning the ability of gonadotrophins and growth factors to increase proportions of buffalo oocytes developing to blastocyst stage, but little is known about the validity of the cell number as indicator of the viability of in vitro developed pre-implantation embryos. Totey et al. (1996) reported that slow developing blastocysts are of lower quality and have fewer cell numbers than their fast developing counterparts. Significantly higher cell number of inner cell mass (ICM) of blastocysts was observed in embryos cultured in media containing 10 ng ml⁻¹ EGF as compared to control in the present study. The reason for an increase
in cell number of blastocyst in EGF group is not clear but previous results have indicated that the mitogenic actions of FSH and EGF have been documented during follicular development in mice (Boland and Gosden, 1994) and bovine (Stewart et al., 1996). They have associated these observations to DNA synthesis which culminates in DNA duplication followed by cell proliferation.

EGF has been found in small and medium preantral follicles as well as in small antral follicles of hamster ovaries (Roy and Greenwald, 1990). It stimulates DNA synthesis and cell proliferation in pig granulosa cells while inhibiting differentiation. EGF also shortens the time required for germinal vesicle breakdown (GVBD) and increases the percentage of oocytes undergoing GVBD in mice (Downs, 2001), rat (Dekel and Sherizly, 1985), cow (Sanbuissho and Threlfall, 1990), human (Das et al., 1994) and pig (Coskun and Lin, 1993). It has been demonstrated (Harper and Brackett, 1993) that EGF has a physiological role in regulating bovine oocyte maturation. However, EGF induced oocyte maturation is not mediated by a cAMP second-messenger system that mediates gonadotropin-induced oocyte maturation. EGF-induced oocyte maturation is probably mediated through the protein kinase-C pathway (Coskun et al., 1995). In one study it was observed that EGF either alone or together with IGF-I stimulates cumulus expansion and both growth factors, acting alone or in conjunction enhance nuclear maturation in the oocyte surrounded by compact cumulus cells in bovine (Lorenzo et al., 1994). The interaction between EGF and IGF-I with follicle stimulating hormone during the in vitro maturation of bovine oocytes enhances subsequent blastocyst formation (Harper and Brackett, 1993).

Addition of growth factors from the insulin gene family of peptides and the EGF gene family, to the culture medium of early murine embryos results in a broad range of effects. This includes stimulation of RNA and protein synthesis, increase in the rate of cell division, increase in cell number of blastocyst and increase in the percentage of cultured embryos that hatch from the zona pellucida (Harvey and Kaye, 1988; Heyner et al., 1989; Wood and Kaye, 1989; Harvey and Kaye, 1990; Paria and Dey, 1990; Rappolee et al., 1990; Harvey and Kaye, 1991). It has also been
demonstrated in porcine and bovine that growth factors, including EGF and IGF-I, improve embryonic development after IVF, when added during IVM (Xia et al. 1994).

It has been known for many years that preimplantation embryos grow more rapidly when cultured in group than individual embryos (Wiley et al., 1995). Addition of EGF to the culture medium (Paria and Dey, 1990) can compensate for crowding to a remarkable degree. It has been recently reported (Das et al., 1994) that heparin binding-EGF (HB-EGF) enhance embryo cell number and increase the hatching incidence of individually cultured embryos. Since EGF is present in oviductal fluid (Dardik et al., 1992), the current hypothesis is that the EGF receptor present on the apical embryonic surface interacts with this maternal EGF to promote growth and developmental processes of the embryos in vivo. It has also been shown (Wood and Kaye, 1989) that EGF stimulates uptake of (3 H)-leucine during morula stage and later stages, with most of the incorporation occurring in the trophoderm cells. Since the trophoderm cells actively transport salts into the blastocoele, it is of interest to this discussion of the EGF receptor (Dardik and Schultz, 1991). All these observations are consistent with the hypothesis that the internally facing EGF receptors interact with embryo-derived TGF-α to promote the epithelial duties of the trophoderm during blastocoele formation and blastocoele expansion (Wiley et al., 1995). In terms of the functional effect of IGF-I in development, it has been shown (Harvey and Kaye, 1992) that the inclusion of exogenous IGF-I in the culture medium of preimplantation mouse embryos results in the acceleration of blastocyst development and enhanced protein synthesis. However, no acceleration of development was observed (Heyner et al., 1993), but there was a significant increase in the number of hatched blastocysts when embryos were cultured for 48 h in a medium containing 40 ng/ml IGF-I. The effect of IGF-I, IGF-II and insulin, on cell number in the murine blastocyst was compared and it was observed that all the three factors caused an increase in the number of inner cell mass (Rappolee et al., 1992). IGF-I and IGF-II have also been detected in uterine flushing medium by RIA analysis and this maternal insulin from the fallopian tube and uterine fluid is taken up by insulin receptors present on
preimplantation embryo and internalized by receptor-mediated endocytosis (Heyner et al., 1989). Insulin and IGF-I stimulate metabolism and growth of preimplantation embryos. As insulin shows its effect at very low concentrations, it has been suggested that its effects are mediated by its own receptors (Gardner and Kaye, 1991). It was concluded that IGF-I and EGF at physiological concentrations stimulate preimplantation mouse embryos, indicating an important role for both these growth factors in early development.
6. SUMMARY, CONCLUSION AND SUGGESTIONS FOR FURTHER WORK

6.1 Summary

The developmental competence of *in vitro* produced (IVP) buffalo embryos obtained from transvaginal ultrasound guided ovum pick-up (OPU) and abattoir derived oocytes was studied in the present research work besides the effect of reactive oxygen species (ROS) and apoptosis on developmental competence. The strategies to augment *in vitro* embryo production with incorporation of antioxidant and antiapoptotic factors were also attempted. A total of 68 sessions of OPU at different interval/ frequency viz, once weekly (n=34) and twice weekly (n=34) were conducted in 6 genetically superior Murrah buffaloes to study the developmental competence of OPU derived COCs. An average of 1.96±0.10 cumulus oocyte complexes (COCs) with oocyte recovery rate of 50.46±2.75 was retrieved per OPU session. The mean number of surface follicles (5.63±0.18 vs. 5.49±0.19) recorded and oocyte recovery rate (47.90±3.30 vs 52.93±3.20) was not significantly different between once and twice weekly OPU sessions. Significantly (p<0.05) higher developmental competence of COCs retrieved in twice weekly OPU session, in terms of maturation (89.28±5.14), cleavage (76.80±7.14), morula (51.79±6.62) and blastocyst (29.72±4.73) was recorded as compared to their respective values of 85.71±5.72, 61.43±7.23, 44.12±5.49 and 24.14±4.81 per cent in once weekly OPU session. A significantly (p<0.01) higher morula (48.01±4.89) and blastocyst (28.43±3.80) per cent was recorded in OPU derived *in vitro* produced (IVP) embryos as compared to those derived from abattoir ovaries (36.89±0.67 and 19.04±0.49 per cent, respectively), indicating better developmental competence of OPU derived IVP embryos.

In abattoir derived embryos the effect of various morpho-functional and cellular parameters was studied. The oocyte retrieval in terms of COCs per ovary (1.92±0.08) and developmental competence in terms of blastocyst (23.83±1.05%) was significantly (p<0.01) higher in COCs derived from large follicles as compared to their respective values of 1.17±0.24 and
15.59±0.46% in COCs derived from small follicles. The phase of oestrus cycle of the donor animals also significantly (p<0.01) affected the oocyte recovery and developmental competence (blastocyst per cent), the respective values being significantly higher during luteal phase (3.29±0.23 and 22.51±0.55) as compared to follicular (2.28±0.09 and 14.75±0.25) and early luteal (2.26±0.11 and 17.13±0.31%) phase of oestrus cycle. Winter season was observed to be optimum for in vitro embryo production with significantly higher (p<0.01) COCs retrieved per ovary (3.94±0.09) and blastocyst per cent (23.28±0.41) as compared to summer (2.62±0.06 and 15.75±0.46) and rainy seasons (3.15±0.05 and 17.92±0.52%).

Also significantly higher blastocyst rate was observed in oocytes of large diameter group (23.23±0.45) as compared to small (15.18±0.37) and medium (17.70±0.39) groups. Oocytes of grade III (14.30±0.66) had significantly lower developmental competence as compared to grade I (25.77±1.73) and grade II (23.75±1.55) oocytes.

The source of spermatozoa did not significantly affect the developmental competence of abattoir derived IVP embryos which established the potential for use of epididymal spermatozoa for IVF with equally good efficiency as ejaculated spermatozoa.

The glucose-6-phosphate dehydrogenase (G6PDH) activity was assessed by brilliant cresyl blue (BCB) staining and expressed as BCB⁺ (inactive G6PDH) and BCB⁻ (active G6PDH) based on reaction to the dye. The developmental competence (maturation, morula and blastocyst per cent) was significantly (p<0.01) higher in BCB⁺ COCs from large follicles (85.79±0.98, 46.86±1.40 and 24.87±1.09) as compared to BCB⁻ (81.30±0.60, 36.27±0.95 and 15.41±1.26%, respectively). Similar trend was also recorded in BCB⁺ and BCB⁻ oocytes derived from small follicles.

The study of in vivo developmental competence of in vitro produced embryos resulted in establishment of successful clinical pregnancy in a synchronized recipient buffalo with a conception rate of 12.5%.
A significantly (p<0.01) higher ROS intensity was recorded in IVP embryos derived from grade I COCs as compared to those derived from grade II and grade III COCs. The IVP embryos from grade III (18.43±1.07) COCs were also associated with significantly (p<0.01) higher incidence of apoptosis as compared to those derived from grade I (10.14±1.07) and grade II (14.23±1.39) COCs.

The effect of various antioxidant and antiapoptotic factors was studied. A highly significant (p<0.01) beneficial effect of treatments in terms of cleavage, morula and blastocyst per cent viz. embryo culture at 5 % oxygen tension (71.17±0.37, 43.31±0.74, 25.23±1.29), supplementation of 1 mM glutathione (73.41±0.37, 44.60±0.71, 28.46±1.37), 10 ng ml⁻¹ EGF (76.73±0.74, 48.10±0.54, 33.88±0.63) and 100 ng ml⁻¹ IGF-I (77.72±0.61, 50.11±1.00, 34.96±0.92) in embryo culture medium was recorded as compared to control (65.46±0.36, 38.24±0.72 and 20.09±0.60, respectively). The incidence of apoptosis and intensity of reactive oxygen species was also significantly (p<0.01) reduced and differential cell count of inner cell mass and total cell number in hatched blastocysts was significantly increased (p<0.01) in the mentioned treatment groups as compared to control.
6.2 Conclusions

1. The developmental competence of OPU derived oocytes was significantly higher as compared to abattoir derived oocytes.

2. Twice weekly OPU sessions were significantly better in terms of weekly oocyte retrieval and developmental competence as compared to once weekly OPU sessions. Thus the twice weekly OPU protocol can be effectively used for oocyte retrieval for in vitro embryo production.

3. Various morpho-functional and cellular parameters had a significant impact on developmental competence of IVP embryos.

4. Epididymal spermatozoa can also be efficiently used for IVF in buffaloes and thus have a role in conservation of livestock.

5. Higher intensity of ROS and higher incidence of apoptosis at oocyte and embryonic stage was associated with reduced developmental competence.

6. The developmental competence of in vitro produced embryos was significantly improved with culture of embryos at reduced oxygen tension (5% O₂) or with supplementation of glutathione (1 mM), EGF (10 ng ml⁻¹) or IGF-I (100 ng ml⁻¹) in research vitro cleave (RVCL) media.
6.3 **Suggestions for further work**

1. The developmental competence of OPU derived oocytes may be studied following hormonal superstimulation of oocyte donors to increase the oocyte recovery.

2. Comparative study of expression pattern of some developmentally important genes in OPU and abattoir derived oocytes may be done.

3. Time lapse imaging of developmentally important events may be done to establish the proportion of early and delayed developing *in vitro* produced embryos.

4. *In vivo* studies of developmental competence of *in vitro* produced embryos may be done on a larger scale.


FAO. (2008). Data Bank, Italy.


APPENDIX-I

Normal saline solution for transport of ovaries

<table>
<thead>
<tr>
<th>NSS for washing &amp; transportation of ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Penicillin</td>
</tr>
<tr>
<td>Streptomycin</td>
</tr>
<tr>
<td>Milli Q H₂O upto</td>
</tr>
</tbody>
</table>

Dulbecco's phosphate buffer saline

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Components</th>
<th>100ml</th>
<th>200ml</th>
<th>500ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>NaCl</td>
<td>0.8</td>
<td>1.6</td>
<td>4.0</td>
</tr>
<tr>
<td>02</td>
<td>KCl</td>
<td>0.02</td>
<td>0.04</td>
<td>0.1</td>
</tr>
<tr>
<td>03</td>
<td>Na₂HPO₄·H₂O</td>
<td>0.217</td>
<td>0.434</td>
<td>1.085</td>
</tr>
<tr>
<td>04</td>
<td>KH₂PO₄</td>
<td>0.02</td>
<td>0.04</td>
<td>0.1</td>
</tr>
<tr>
<td>05</td>
<td>Glucose</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>06</td>
<td>Na-Pyruvate</td>
<td>0.0036</td>
<td>0.0072</td>
<td>0.018</td>
</tr>
<tr>
<td>07</td>
<td>Gentamicin</td>
<td>0.004</td>
<td>0.008</td>
<td>0.02</td>
</tr>
<tr>
<td>08</td>
<td>PVA</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>09</td>
<td>CaCl₂·H₂O</td>
<td>0.01325</td>
<td>0.0265</td>
<td>0.06625</td>
</tr>
<tr>
<td>10</td>
<td>MgSO₄·H₂O</td>
<td>0.0121</td>
<td>0.0242</td>
<td>0.0605</td>
</tr>
</tbody>
</table>

Dissolve the first eight chemicals in three-fourths of the batch amount of de-ionized water. When completely dissolved, pour the contents into a carboy. Rinse flask with desired amount of distilled water and pour into carboy. Be sure not to add too much water.

Weigh out the CaCl₂ and MgSO₄ and add these to the remaining one fourth of de-ionized water. When completely dissolved add to the carboy. This procedure must be followed explicitly, otherwise the calcium phosphate will precipitate and the batch must be thrown out.

The pH should be near 7.2-7.5. Osmolarity should be between 280 – 310. Filter with 0.22 μ filter and store at 4°C.
APPENDIX- II

Oocyte washing medium (1ml)

TCM – 199 : 924µl  
Fetal bovine serum : 75µl  
Gentamicin sulphate : 1µl

Maturation medium

TCM - 199 : 900µl  
FBS : 75µl  
FSH : 10µl  
LH : 10µl  
Estradiol : 1µl  
Sodium pyruvate : 1µl  
Gentamicin sulphate : 1.0µL

β-Estradiol (Sigma E8875) : Dissolved in absolute alcohol @ 1 mg/ml and aliquots of 5µl were prepared and stored at -20°C.

FSH (Sigma F2293) : 2.5 unit/ml normal saline  
LH (Sigma L5269) : 2.5 unit/ml normal saline

Sodium pyruvate (Sigma P4562): 250mM solution of sodium pyruvate was prepared in ultrapure double distilled water. Aliquots of 5µl were prepared and stored at -20°C.

Gentamicin sulphate (Sigma G1264): 50mg of gentamicin was dissolved in 1ml of double distilled water. Aliquots of 5 µl were prepared and stored at -20°C.
Hyaluronidase solution for denudation of oocytes

Stock solution

Hyaluronidase (Sigma H4272) - 30mg
PBS - 3ml

For working solution, 50µl of stock was dissolved in 450µl of PBS to make 0.1% Hyaluronidase.
Hoechst 33342 stain for staining of polar body

Stock solution is prepared as 1 mg/ 1ml DPBS+ 20% glycerol. From this 15µl was taken and added in 985µl DPBS+ 20% glycerol and made aliquots of 50µl and stored at -20°C (kept away from direct light sources).
**APPENDIX - V**

**Preparation of Fertilization Medium (BO Media)**

<table>
<thead>
<tr>
<th>1. BO (STOCK SOLUTION)</th>
<th>2. Working BO Media</th>
<th>25 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BO Media A</strong></td>
<td>BO A</td>
<td>19 ml</td>
</tr>
<tr>
<td>(pH 7.3)</td>
<td>BO B</td>
<td>06 ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>Na- Pyruvate</td>
<td>3.4 mg</td>
</tr>
<tr>
<td>430 mg</td>
<td>Caffeine Na Benzoate</td>
<td>9.7 mg</td>
</tr>
<tr>
<td>KCl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.7 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$ dihydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.7 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$ hexahydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.9 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH$_2$PO$_4$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8.4 mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenol Red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibiotic (Gentamicin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milli Q H$_2$O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BO Media B</strong></td>
<td>Working BO</td>
<td>4.5 ml</td>
</tr>
<tr>
<td>20 ml</td>
<td>BSA</td>
<td>50 mg</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>258.7 mg</td>
<td>Heparin (10 µg/ml)</td>
<td>0.5 ml</td>
</tr>
<tr>
<td>Phenol Red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µl</td>
<td>(pH 7.2 - 7.3)</td>
<td></td>
</tr>
<tr>
<td>Antibiotic (Gentamicin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 µl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milli Q H$_2$O</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ml</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Filter the working BO medium and fertilization medium with 0.22 micron syringe filter.

Incubate the working BO and fertilization medium in CO$_2$ incubator at 38.5 °C, 5 % CO$_2$ for 3 hrs.
Differential staining of embryos

Chemicals required:

**Solution I (10ml):**

- Triton X (1%) - 100µl
- Propidium iodide - 01mg
- DPBS - 9.9 ml

**Solution II:**

Fixative solution of 100% ethanol with 25µg/ml Hoechst 33342 stain

**Protocol:**

The blastocysts are incubated in 500µl of solution I for 10-15 sec. The blastocyst are immediately transferred into 500µl of solution-II and incubated at 4°C overnight. The stained blastocysts are observed at wavelength 330-380nm and barrier filter 420 nm.

The inner cell mass will be stained as blue fluorescence and trophoectoderm will take tinged orange stain.