



Factors influencing *in-vitro* embryo production efficiency of caprine oocytes: A review

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ABSTRACT

Pre-implanted growth and development of embryo is deficient in many aspects as is evidenced by the great difficulty in growing embryos *in-vitro*, while maintaining viability as shown by development of early embryos *in-vitro* generally delayed or completely blocked at 8–16 cell stage embryo and hardly small percentage of the *in-vitro* fertilized embryo develop to morula and blastocyst, which is probably due to minor physio- chemical variation and lack of certain factors in the culture system. *In-vitro* development of mammalian embryos usually remains inferior to development than *in-vivo* produced embryos. The mechanism regulating the *in-vitro* acquisition of developmental competence of oocytes is still obscure. Therefore it is needed to standardize the culture conditions that mimic *in-vivo* embryo development. To know the exact mechanism of developing embryos, there should be a defined system that caters the needs of developing embryos. The current review describes the criteria and factors affecting *in-vitro* maturation, fertilization and embryo development in goat.

Key words: Embryo development, Fertilization, *In-vitro* maturation, Pre-implanted growth

Recent reproductive technologies are creating more successful live offspring of mammalian species of superior merit within a short period to reduce the gap between the demand and production of animals and conserve them as well. It is necessary to augment the reproductive rate of low productive animals which is not possible through the conventional breeding programs. Efficient *in-vitro* techniques for oocyte maturation and fertilization in small ruminants like sheep and goats are more important than in large ruminants, due to their short generation interval, prolificacy, economical rearing, better visibility and large pronuclear size for biotechnological experiments, viz. gene transfer, cloning and stem cell research (Kharche *et al.* 2009). Number of embryos required for recent biotechnological approaches obtained by super-ovulation is limited and expensive due to unpredictable superovulatory responses which reduce embryo recovery rate. *In-vitro* embryo production technique provides large scale, low cost economical production of early and late stage embryos required for gene integration and cloning, expected to make efficient utilization of a huge number of ova left in ovaries. *In-vitro* embryo development

technology also opened up several possibilities for accelerating genetic gain, enhancing production potential in animals, allow more offspring to be obtained from selected parents to ensure genetic diversity and may reduce the interval between the generations. Although recent efforts in caprine *in-vitro* embryo production system have led to advances in many steps, the efficiency of *in-vitro* production is still low with regards to obtaining the fully matured oocytes, capacitation of spermatozoa and culture of embryos *in-vitro* as only 30% of oocytes develop into blastocysts, probably because the *in-vitro* environment cannot mimic *in-vivo* environments and results in embryos with altered morphology and gene expression. Therefore it is needed to standardize the culture conditions that mimic *in-vivo* embryo development. IVF is based on salvaging immature oocytes from the ovaries of slaughtered animals, their maturation, fertilization and culture *in-vitro*. Studies involving examination of *in-vitro* culture environment of oocytes and embryos have been carried out by many researchers, still there are many aspects regarding the effect of *in-vitro* culture system on embryo development and phenotypic features observed in offspring generated by *in-vitro* embryo production. The improvement of caprine culture system is highly desirable in terms of the production of pre-implantation stage embryos for both biotechnological studies and embryo transfer technique (Gordon 1991). The

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improvement of *in-vitro* culture systems are important for production of embryos with high developmental competence that are used in agricultural and biomedical research, and animal biotechnology (Hansen and Block 2004). This review discusses factors affecting *in-vitro* maturation, fertilization and embryo development potential.

Sources and collection of oocytes: Individual effect of oocyte donor on oocyte developmental competence should be taken into consideration for an *in-vitro* embryo production program. Oocytes can either be retrieved from slaughter house ovaries within stipulated time or from the live animals by different techniques. Superovulation (hormonal treatment) of donor goat is routinely done to increase number of ova released by the ovary. Laparoscopic ovum pick up is one of the best techniques because of less adhesion problems compared to laparotomy or surgical oocytes collection from live animals. The cost of oocyte retrieval from live animal is high due to unpredictable results and low oocytes quantity. Therefore slaughter house ovaries are attractive alternative source for oocyte retrieval as they are less expensive and most abundant source of immature oocytes for large scale production of caprine embryos because the goat is the main meat source in many countries.

Time taken for transportation of ovary in appropriate environment is important factor for *in-vitro* embryo production program. Lesser time taken between the slaughter and transport of ovaries is always preferred. Time interval between collection of ovaries and harvesting of oocytes also vary from 1 hour (Pugh *et al.* 1991) to 3–4 h (Kharche *et al.* 2008b) without any detrimental effects at appropriate temperature. Goat oocytes are particularly sensitive to the temperature. Exposure of the follicle enclosed cumulus oocytes complex to 20°C prior to culture resulted in 11.5% of oocytes exhibiting an abnormal meiotic spindle (De-Smedt *et al.* 1992). Most of the researchers observed a temperature range of 30–37°C during transport of ovary to be the optimum for IVMFC of mammalian oocytes.

The potential application of *in-vitro* technology for maturation, fertilization and culture of domestic animals oocyte partially depends on the development of reliable, repeatable and efficient techniques for recovery of oocytes from genetically valuable animals (Baldassare *et al.* 1994). The obvious advantage of oocyte recovery technique is speed of operation, quality of oocytes, and quantity of oocytes. Therefore different techniques of oocyte collection are employed in order to obtain maximum oocytes of culturable quality. Follicle puncture, Ovary Slicing and follicle aspiration are routinely used techniques for recovery of oocytes from slaughter house ovaries. However different views come from different researchers. Goat ovaries are relatively smaller in size therefore aspiration of follicular oocytes is difficult (Pawshie *et al.* 1994). Therefore slicing and puncture techniques are most common in terms of obtaining good quality and quantity of oocytes. However

follicle puncture after aspiration increased the overall rate of oocytes recovery in goat as compare to sheep (Bonde *et al.* 2000). Oocytes recovery is significantly higher in puncture and slicing method than by the aspiration method (Wani *et al.* 2000). Datta *et al.* (1993) and Mogas *et al.* (1992) also support finding that by slicing technique maximum number of oocytes could be obtained. Yadav *et al.* (2007) compared different oocytes collection techniques with respect to time and found that ovary puncture technique is best for recovery of maximum number of good quality oocytes in minimum time. Debris of ovarian tissue comes during slicing which may cause problems in oocyte searching therefore time taken by slicing technique is comparatively more than puncture technique which is also supported by Wani *et al.* (1999). Although most of the ovary contains corpus luteum but it is suggested to select ovary with smaller size of corpus luteum as oocyte recovery rate may get reduced if the ovary has a large corpus luteum because the follicular development is restricted due to occupation of the great portion of the ovary by corpus luteum.

Selection of oocytes for in-vitro maturation: Oocyte is a gamete that contributes not only half of the genetic material but also practically all of the cytoplasm to the zygote, supplying the transcripts and proteins necessary for early embryonic development (Schultz 2002). It is obvious that oocyte quality is essential for embryonic development before and after genome activation considering the fact that the appropriate embryonic genome activation is a fundamental key for the subsequent embryo development (Comargo *et al.* 2006). The efficiency of *in-vitro* embryo production profoundly influenced by the number and quality of oocytes which successfully complete maturation. The ability to identify good quality oocytes prior to *in-vitro* culture is the important consideration for *in-vitro* embryo production system. The existence of a healthy population of somatic cells surrounding the oocytes is mandatory to facilitate the transport of nutrients and signals into and out of the oocytes (Moor and Seamash 1986).

Selection criteria of cumulus oocyte complexes are extremely important for successful maturation *in-vitro*. Morphology of the cytoplasm and cumulus cell investment surrounding oocytes is the primary criteria for the grading and selection of oocytes for IVM. Normal oocytes should have cumulus cell investment surrounding the zona pellucida (ZP), absence of cracked zona pellucida and absence of vesicle in ooplasm. Presence of more and compact layers of cumulus cells is considered better (Rahman *et al.* 2008). An important role of cumulus cells is to provide nutritional support to the developing oocytes (Buccione *et al.* 1990). Retrieved oocytes could be graded as excellent (A), good (B), fair (C) and poor (D) quality depending upon the cumulus investment and cytoplasmic distribution. Excellent (A), Oocytes with more than 4 layers of bunch of compact cumulus cells mass with evenly granulated cytoplasm. Good

(B), oocytes with at least 2–4 layers of compact cumulus cell mass with evenly granulated cytoplasm. Fair (C), oocytes with at least 1 layer of compact cumulus cell mass with evenly granulated cytoplasm. Poor (D), oocytes with no cumulus cells or incomplete layer of cumulus cell or expanded cells and having dark or unevenly granulated cytoplasm (Kharche *et al.* 2008b). It is desirable to select A and B quality oocytes for IVMFC. In the series of reproductive technologies, mounting evidence that oocytes quality profoundly affects embryo development program that drives the continued search for reliable predictors of oocytes developmental competence.

In-vitro maturation: *In-vitro* maturation of oocyte is an assisted reproductive technology that enables oocytes to be matured *ex vivo*. Like *in-vivo*, both nuclear and ooplasmic maturation is required to ensure normal fertilization and embryo development *in-vitro*. In all mammalian species, nuclear maturation can be achieved when the oocytes are removed from the antral follicles and are cultured *in-vitro* (Thibault *et al.* 1987). Nuclear maturation refers to the progression to the metaphase II. The time required for *in-vitro* maturation of goat oocytes is 27 h. Maturation can be judged directly by staining their nuclear and chromatin structure and/ or by the ability of the oocytes to be fertilized. Cytoplasm of the oocyte may play a crucial role in assembling the correct metabolic environment for production of sufficient energy for cellular functions during maturation, cleavage and blastocyst formation (Teotia *et al.* 2001). However ooplasmic changes occur during oocytes maturation are still difficult to evaluate. Cytoplasmic maturation refers to the other maturation events that prepare the oocytes for fertilization and pre-implantation development (Eppig 1996). Degree of cumulus cells expansion can be used as a morphological indicator for maturation of oocytes. So, it can be said that expanded cumulus cells indicates mature and good quality oocytes while a compact cumulus cells characterizes immature oocytes. When fully grown oocytes are released from their follicles to the culture medium they resume meiosis spontaneously in maturation medium. The reduced development of *in-vitro* derived zygote in goats suggest that the conditions of IVM do not support cytoplasmic maturation, so it is very important that the improvement of the *in-vitro* maturation systems for oocytes aimed at defining *in-vitro* conditions that are more similar to the *in-vivo* environment (Wang *et al.* 2007). Only a small proportion of goat oocytes selected for *in-vitro* oocyte maturation (IVM) can successfully complete cytoplasmic maturation and support embryonic development. Incomplete IVM of oocytes undergo incomplete exocytosis of the cortical granule that could be related to polyspermy (Cran and Cheng 1986). Therefore developing appropriate IVM culture conditions that can mimic *in-vivo* culture condition is essential. To produce goat blastocysts more efficiently *in-vitro*, it is necessary to identify factors required for oocyte maturation.

Factors affecting in-vitro maturation of goat oocytes: *In-vitro* maturation of oocytes is an integral part of *in-vitro* culture system (Yadav *et al.* 2010) as the events occur during *in-vitro* maturation affects not only *in-vitro* maturation but also *in-vitro* fertilization and subsequent embryo development. Oocytes resume and complete meiosis spontaneously *in-vitro* in a wide variety of culture media containing serum, hormones and mixture of energy substrates. It is possible that an oocyte which reaches M-II (Nuclear maturation) may not have the potential to develop to blastocyst stage and subsequent pregnancy. Thus maturation media is critical in terms of providing correct environment for oocytes to provide nuclear and cytoplasmic maturation. Therefore, selection of protein supplements, growth factors, antioxidants and hormones play an important role in *in-vitro* fertilization and subsequent *in-vitro* embryo development. In our previous experiment (Yadav *et al.* 2010) combined studies was conducted on these parameters, where TCM-199 medium containing 10% calf serum and 3 mg/ml BSA used as base medium for control and compared the effect of Epidermal Growth Factor, insulin, β -mercaptoethanol and gonadotropin hormones. The results suggest that supplementation of hormones with EGF in base medium significantly enhances the *in-vitro* maturation rate of caprine oocytes. The most commonly used system for the maturation of oocytes outside the follicle is TCM-199 supplemented with FSH, LH, estradiol and 10% FCS. Various factors such as follicle size, hormones, serum, growth factors, vitamins, amino acids, follicular fluid or cells in the IVM medium, age of the donor goat and the culture conditions are involved for successful IVM of goat oocytes.

Effect of follicle size: Studies have established a relationship between follicle size and oocyte competence, the competence increases as the follicle enlarges (Lonergan *et al.* 1994, Kruip *et al.* 2000). But the recovery of culturable oocytes is higher in medium size follicles than small and large size follicles (Yadav *et al.* 2007, Das *et al.* 1996). Recovery of more culturable oocytes from medium sized follicles as compare to small follicles may be due to these follicles exposed to better hormone stimuli and growth factors (Lonergan *et al.* 1992). Crozet *et al.* (1995) found a significant difference in the percentage of blastocysts obtained from the oocytes recovered from follicles of 2–3 mm (6%), follicles of 3.1–5 mm (12%), follicles >5 mm (26%) and from ovulated oocytes (41%). Indeed, follicles with the same diameter can be found at diverse stages of the estrous cycle and can be either undergoing growth or atresia. Therefore, the developmental stage of follicles may considered as more important factor than their diameter. However the oocyte with compact cumulus cells is primary factor involved in the criteria for the selection of oocytes for maturation. Moderate size follicles releases good quality oocytes i.e. oocyte with compact cumulus cells and homogenous cytoplasm that have ability to be matured *in vitro* properly in compare to very

small and large sized follicles as discussed above.

Effect of follicular cells and follicular fluid: Maturation of oocytes is influenced by many factors, including presence of follicular cells. The relationship between the oocytes and follicle is provided by cumulus cell processes, which penetrates the zona pellucida and provide a route for direct intercellular communication between the somatic cells and the oocytes (Wani 2002). During follicle development, the oocyte acquires the competence to undergo meiotic maturation by an interaction between the oocyte and the theca and granulosa cells (Miyano 2003) and accumulates transcripts and proteins that will guide the maturation, fertilization, and initiate embryo development (Kruip *et al.* 2000). The follicular cells provide nutritive compounds essential for oocyte maturation. Apart from their nutritive role, follicular cells also produce instructional signals for the synthesis of several structural proteins (Osborn and Moor 1982) and maturation proteins (Crossby *et al.* 1981). It has been shown that granulosa cell interactions with cumulus oocyte complexes (COC) during IVM are involved in imparting developmental competence to the maturing caprine oocytes. These cells initiate protein and/or polypeptide synthesis, which render the cytoplasm competent to assume normal cooperation with the male genome (Thibault *et al.* 1987). Granulosa cell monolayer (GCM) delays maturation of goat oocytes for a few hours, but increases the maturation percentage significantly (Tyagi *et al.* 1997). GC monolayers are also suitable to overcome the arrest at initial stages of embryo development (Teotia *et al.* 2001). Follicle health seems to be related to oocyte quality since oocytes from follicles in the advanced stage of atresia are more compromised than oocytes from follicles in the early stage of atresia (Hendriksen *et al.* 2000, Nicholas *et al.* 2005). Follicular aspirates from large follicles shows loose granulosa cells and their follicular fluid coagulates after sometime and form gel like material which causes problem in oocyte recovery, whereas small and medium sized follicle aspirates have compact and heavy granulosa cell masses and expected to release good quality oocytes (Kharche *et al.* 2008b). Granulosa cells get attached to the surface of the petridish in culture drop and spread all over as elongated cells and projected cellular sprouts referred as granulosa cell monolayer. *In-vitro* embryo production rate can be improved by incorporating granulosa cell monolayer during maturation and fertilization that could be due to the additional support in nutritional requirements or generation of specific signals to the developing oocytes (Staigmiller and Moor 1984). Granulosa Cell monolayer supports cytoplasmic maturation of growing caprine oocytes, which is evident by a better maturation rate, active fertilization, an improved cleavage rate and subsequently a higher rate of morula formation (Teotia *et al.* 2001, Kharche *et al.* 2008b). It is also known that somatic counterparts are also required for the transmission of certain amino acids, nucleotides, certain

phospholipid precursors (Wani 2002) and growth factors to the oocytes. Therefore, the oocyte maturation quality is, related to its follicular cells and environment together. Supplementation of IVM medium with follicular fluid from non atretic or gonadotropin stimulated large follicles had some beneficial effect on goat oocytes (Martino *et al.* 1995a, Cognie *et al.* 2004). This beneficial effect on goat oocytes maturation may be due to the presence of growth factors, hormones and intraovarian peptides in more physiological proportions in follicular fluid (Cognie *et al.* 2004). Follicular fluid is composed of transudates from the serum through the blood-follicle barrier (Edwards 1974) but it also contains locally produced molecules. Supplementation of the oocyte maturation medium with 10% follicular fluid (FF) or serum is commonly practiced. During maturation, the cumulus cells undergo a cyto-skeletal rearrangement which is a prerequisite for the deposition of the cumulus matrix (Zhuo and Kimata 2001). In turn, the cumulus cells start the production of hyaluronan (HA), which is the main component of cumulus expansion. It needs several hyaluronan binding proteins (HABPs) to form the HA-rich viscoelastic matrices in the extra cellular space. For *in-vitro* cumulus expansion, the use of serum or FF as a transudate of serum is required to retain HA in a stable complex, which suggests the presence of those hyaluronan binding proteins (HABPs) in serum and FF (Zhuo and Kimata 2001, Rodgers *et al.* 2003). Since the composition of FF reflects changes in the secretory processes of the granulosa and theca cells in the follicle, the source of FF may affect IVM and cumulus expansion of caprine oocytes. It has been suggested that some factors in FF from ovarian follicles at later stages of follicular development may play an important role during oocyte maturation *in-vitro* (Algriany *et al.* 2004). Bovine follicular fluid from follicles of 15 mm or greater contains stimulatory substances that during *in-vitro* maturation promotes the potential of bovine follicular oocytes to develop to blastocysts and hatched blastocysts (Elmleik *et al.* 1995). Singh *et al.* (2010) reported almost similar maturation rate in media containing serum or follicular fluid. However it was lower than that of medium containing combination of serum and hormones together. But the embryo development was better from the oocytes matured in medium containing follicular fluid. Investigations are needed to elucidate the biochemical and functional nature of the Caprine follicular fluid specific factors involved in oocytes maturation.

Effect of donor age: Several studies have reported lower embryo developmental potency of oocytes from prepubertal oocytes than adult goat oocytes. It was reported by researchers that supplementation of hormones and proteins significantly increase maturation rate in adult goats as compared to pre pubertal goats. Oocytes derived from prepubertal does had high rate of polyspermy (Izquierdo *et al.* 2002), failure of sperm head decondensation, formation of male pronucleus and low blastocyst production. Singh *et*

al. (2009) conducted a study on *in-vitro* maturation, fertilization and cleavage rate of pre-pubertal and pubertal goat oocytes and reported almost similar maturation rate in both groups on the morphological basis. But higher cleavage rate of pre-pubertal goat oocytes (42.20%) was obtained as compared to pubertal goat oocytes (39.80%). Glutathione (GSH) during IVM and addition of thiol compounds increases this GSH synthesis. Similarly the addition of cysteamine to the maturation medium improves embryo development from pre-pubertal goat oocytes although all other thiol compounds i.e. cysteamine, b-mercaptoethanol, cysteine and cystine used in this study increased intracellular GSH content (Rodriguez Gonzalez *et al.* 2003). Researchers are trying to provide excellent *in-vitro* maturation potential to prepubertal goats, which are generally slaughtered at abattoirs. The reasons for the low embryonic development competence of oocytes from pre-pubertal animals are still unknown and have been attributed to insufficient, delayed or abnormal nuclear and ooplasmic maturation (Damiani *et al.* 1996, Looney *et al.* 1995).

Effect of hormones: Hormonal stimulation of oocytes during the course of *in-vitro* maturation is of paramount importance in achieving nuclear and cytoplasmic maturation which is essential for the preparation of oocytes for fertilization (Stubbing *et al.* 1988). Gonadotropins are the primary regulatory hormones for *in-vitro* maturation of mammalian oocytes. The inclusion of gonadotropins in IVM medium is reported to enhance oocytes quality and developmental potential by possible alterations in metabolic process (Brackett and Zulke 1993). The beneficial effect of gonadotropins in the IVM medium proved to be more pronounced for the oocytes of pre-pubertal females (Ledda *et al.* 1997). Caprine culture media supplemented with gonadotropins (LH and FSH) and estradiol-17 β are reported to improve maturation rates significantly (Keskin-tepe *et al.* 1994, Pawshe *et al.* 1996, Mogas *et al.* 1997a, b, Izquierdo *et al.* 1998, 1999, Kharche *et al.* 2008b, Yadav *et al.* 2010). Researchers are using FSH ranged from 0.1 μ g/ml (Cognie *et al.* 2003) to 10 μ g/ml (Jimenez-Macedo *et al.* 2005, 2007), LH 3 μ g/ml (Ongeri *et al.* 2001) to 100 μ g/ml (Keskin-tepe *et al.* 1994) and estradiol up to 1 μ g/ml (Keefer *et al.* 2002, Jimenez-Macedo *et al.* 2005, 2007, Kharche *et al.* 2008b, Yadav *et al.* 2010) to determine the developmental potential of *in-vitro* produced embryos as Gonadotropins not only supports oocytes maturation but also induce major alteration in the protein profile of oocytes (Schroter and Meinecke 1995). Protein synthesis is essential for the resumption of the first meiotic division in mammalian oocytes. Estradiol may be involved in ooplasmic maturation by stimulating DNA polymerase β and enhancing the synthesis of presumed male nucleus growth factors (Rahman *et al.* 2008). Blastocysts production is significantly increased when oocytes matured in the presence of estradiol-17 β (Pawshe and Totey 2003). However, investigations have shown that

inadequate priming of sheep oocytes with estradiol-17 β exhibit the possibility of embryo cleavage anomalies and thus led to failure of blastocysts formation (Oussaid *et al.* 1999). The addition of hormones is still controversial, as some workers found no significant difference in the number of oocytes reaching metaphase-II or oocytes forming blastocysts in the presence or absence of exogenous hormones (Wahid *et al.* 1992, O-Brian *et al.* 1994). *In-vitro* maturation can be achieved by the supplementation of 20% estrous goat serum. Supplementation of 20% EGS in TCM-199 medium could also be used to achieve maturation following fertilization, embryo culture, and subsequent embryo transfer resulted in successful birth of caprine kid (Kharche *et al.* 2006, Kharche *et al.* 2008a). But addition of hormones like FSH, LH and estradiol may enhance the maturation, fertilization and subsequent embryo development rate of caprine oocytes.

Effect of protein supplementation: Culture media for *in-vitro* oocytes maturation, fertilization and embryo development is always supplemented with some protein sources as almost no maturation (4%) was observed in the medium lacking protein supplement. Selection of protein supplements for IVM plays an important role in *in-vitro* fertilization and subsequent embryo development. 10% Estrus-Goat Serum in maturation medium alone affect the oocytes maturation as it contain unknown substances which are required at the time of *in-vitro* maturation, fertilization and subsequent embryo development. Medium supplemented with FCS support most favorable germinal vesicle breakdown, enhance maturation, fertilization and embryo development rate as compared to media containing goat estrus serum (Pawshe *et al.* 1996). An examination of the total protein content of *in-vitro* derived embryos (Thompson *et al.* 1998) revealed that during early cleavage protein content decreases followed by an increase during compaction and blastulation. In addition to supply of protein and growth factors to the culture media, FCS has a major glycoprotein, fetuin which prevents zona hardening and thereby increases the ability of the oocytes to be fertilized (Downs *et al.* 1988, Pawshe and Totey 2003). Albumin is prevalent protein of the mammalian reproductive tract and it may have a nutritive role during post-compaction embryo development (Thompson 2000). The evidences from the mouse suggest that albumin may have intracellular as well as the additional extracellular supportive roles that it is thought to play (Dunglison and Kaye 1993). Combination of serum and BSA are most commonly used protein source for *in-vitro* culture system. Inclusion of FCS in maturation medium is more effective than BSA alone as FCS induces cumulus expansion (Leibfried-Rutledge *et al.* 1986). Serum is the main component in *in-vitro* embryo production system which is added to the maturation medium at the rate of 10–20% and is known to contain hormones, trace elements, growth factors, amino acids, vitamins, energetic substrates and other

undefined components, some of which may have adverse effects due to the undefined nature, variability in composition and the risk of infection (Bavister 1995). Due to suspicions of detrimental effects of serum on embryonic and fetal development (Young *et al.* 1998), a semi-defined culture medium is preferred by replacing serum with 5–8 mg/ml albumin (fraction V), thus eliminating many potentially harmful components of serum (Bavister 1995). Chemically defined culture system overcomes the problems of complicated culture condition such as variability on embryo production as well as risks of contamination.

Effect of antioxidants: Oxidative stress has been implicated in the etiology of defective embryo development which can alter cell function, minimize cell survival with deleterious effects. *In-vivo* oocytes and embryos seem to be protected against oxidative stress by oxygen scavengers present in follicular and oviductal fluids which eliminate or reduce the effect of Reactive Oxygen Species (Goncalves *et al.* 2006). Reactive Oxygen Species must be continuously inactivated to keep only the small amount necessary to maintain normal growth and development. Glutathione is one of the non-protein sulphhydryl compound, major free thiol which protects the culture from oxidative stress (Meister 1983, Tatemoto *et al.* 2001). Intact cumulus cells are known to produce glutathione (GSH) during IVM and GSH can subsequently be accumulated in the ooplasm of the oocytes (Yoshida 1993). Glutathione content increases during development and maturation of oocytes in the ovary, as the oocyte approaches the time of ovulation (Perreault *et al.* 1988). After fertilization, GSH participates in sperm decondensation in parallel to oocytes activation, and in the transformation of the fertilizing sperm head into male pronucleus (Perreault *et al.* 1988, Yoshida *et al.* 1992, Yoshida *et al.* 1993). GSH also participates in various mechanisms such as amino acid transport, protein synthesis, reduction of disulphide and protection against oxidative damage. It suggests that during *in-vitro* maturation, fertilization and embryo development there is a need of adding antioxidants to protect the oocytes/embryo from oxidative damage. The effect of antioxidant supplementation in various forms has been examined. Cysteamine is a low molecular weight thiol compound that when present during IVM of oocytes and development of embryos, increases the intracytoplasmic oocyte glutathione concentration and improves embryo development rates (De Matos *et al.* 1995, Luvoni *et al.* 1996, De Matos and Furnus 2000). Supplementation of cysteamine (100 μ m) was reported to modify the kinetics of oocyte nuclear maturation and increase blastocyst yield on eighth day post IVF (Cognie *et al.* 2003). Zhou *et al.* (2008) found that the addition of cystine in maturation of goat oocytes increased the GSH level and blastulation only in the presence of cumulus cells (COCs or Denuded Oocytes co-cultured on a cumulus cell monolayer). Addition of cystine alone is enough to improve maturation rate of cumulus oocytes, the

improvement of IVM of denuded/ cumulus less oocytes (DO) requires supplementation of both cystine and cysteamine. Synergic actions between cysteamine, cystine and cumulus cells restore the GSH level and developmental capacity of embryos (Zhou *et al.* 2008). In our previous study we found slightly better *in-vitro* maturation rate in the presence of antioxidant β -mercaptoethanol as compared to base medium (TCM-199 with NCS and 3 mg/ml BSA) but lesser than base medium containing hormones (LH-5 μ g/ml, FSH-5 μ g/ml, Estradiol-5 μ g/ml) (Yadav *et al.* 2010). Based on above discussion, the addition of thiol compound can increase the *in-vitro* maturation rate and subsequently the *in-vitro* embryo development.

Effect of growth factors: The role of growth factors in embryo production has been an active area of research in the recent years. The growth factors present in the culture media or those secreted from oocytes seem to play vital role in acquisition of developmental competence. Several studies have been conducted to investigate the effect of growth factors on the IVM of oocytes. There is some evidence that growth factors secreted by growth supporting cells stimulate mammalian embryo development. Currently, several studies suggest that growth factors in cell-free culture medium improve blastocyst production rate (Byrne *et al.* 2002, Sinclair *et al.* 2003). *In-vitro* studies revealed that insulin and insulin like growth factors are important mediators of follicular development, steroidogenesis, oocyte maturation and subsequent embryo development (Kezele *et al.* 2002, Zaho *et al.* 2002). Insulin-like growth factor 1 (IGF-1) and 2 (IGF-2) increase blastocyst formation rate and cell number (Byrne *et al.* 2002, Sirisathien *et al.* 2003). Furthermore, growth factors can stimulate mitosis (IGF-1), reduce apoptosis (IGF-1 and 2), and stimulate protein synthesis (EGF; Diaz-Cueto and Gorton 2001, Byrne *et al.* 2002, Sirisathien *et al.* 2003), functioning through an autocrine or paracrine mechanism (Diaz-Cueto and Gordon 2001, Hardy and Spanos 2002). A significant positive effect of epidermal growth factor (EGF) on IVM of oocytes was reported in cattle (Park *et al.* 1997), sheep (Guler *et al.* 2000), pigs (Abeydeera *et al.* 1998), buffalo (Nandi *et al.* 2003) and in goats (Yadav *et al.* 2010). Epidermal growth factor (EGF) improves nuclear maturation and cleavage rate (Rieger *et al.* 1998) as well as embryo development (Sirisathien *et al.* 2003). EGF can induce not only nuclear maturation but also cytoplasmic maturation of cumulus enclosed bovine oocytes *in-vitro* (Park *et al.* 1997). EGF significantly influences the steroidogenesis by caprine granulosa cells *in-vitro* and may play important role in the follicular growth and maturation (Behl *et al.* 2001). Supplementation of EGF in maturation medium significantly enhances the *in-vitro* maturation of caprine oocytes. Significant maturation rate could be obtained with the supplementation of EGF on *in-vitro* maturation of goat follicular oocytes with the concentration ranges from 10 ng/ml, 60%, (Yadav *et al.* 2010) to 20 ng/ml 64.5%, (Nagar and

Purohit 2005). EGF influence oocyte maturation and blastocyst production rates in a variety of mammals. Presence of EGF during IVM (1, 10, 100 ng/ml) stimulate cumulus expansion, proportion of oocytes attaining metaphase II, rate of cleavage, as well as blastocyst formation rate (Loneragan 1996). Oocyte maturation with cysteamine and epidermal growth factor (EGF) appears to be efficient in the cytoplasmic maturation of oocytes in goat species (Cognie *et al.* 2004). Goat cumulus cells express EGF receptors (Gall *et al.* 2004) and EGF triggers signaling through the MAPK pathway during IVM in goat CCs (Gall *et al.* 2005). Thus the presence of EGF during oocyte maturation could be beneficial for subsequent embryo development (Yadav *et al.* 2010). EGF stimulates cell function influencing cell differentiation by changing their biochemical activity, cellular growth and regulates their proliferation.

In-vitro fertilization: The expression of compromised genetic information from the spermatozoa can impair embryo quality (Leibfried-Rutledge 1999) and interfere with an *in-vitro* embryo development program's success. Sperm binding to the zona pellucida is the first specific physical interaction between gametes and is thus a key regulatory event in fertilization. IVF encompasses a series of events that results in fusion of male and female gametes, specifically (a) contact with and penetration of the cellular investments of an oocyte by a spermatozoon, (b) penetration of zona pellucida, (c) fusion of the spermatozoon and oocyte external membranes, (d) pronuclei fusion (syngamy) and alignment of their respective chromosomes on the first cleavage spindle. These events generally occur between 18–24 hours at 38.5° in 5% CO₂ in humidified air. IVF process requires appropriate preparation (maturation) of both spermatozoa and oocytes, as well as the culture conditions that are favorable for the metabolic activity of the male and female gametes. *In-vitro* fertilization (IVF) has helped in the understanding regarding fertilization and sperm function, at both the capacitation and acrosome reaction level. Influence of culture media, sperm capacitating agent, sperm concentration, sperm oocytes co-incubation time has been observed by various researchers that affect IVF.

Semen preparation: The most important aspects of the sperm preparation are capacitation of spermatozoa, culture conditions, temperature and pH of the culture medium used (Crozet 1991). Sperm capacitation is generally defined as the physiological and biochemical changes that confer the mammalian sperm capability to fertilize oocyte (Kharche *et al.* 2009). Sperm capacitation is a prerequisite for fertilization, as the response to an oocyte signal can be initiated only in capacitated sperm—the signal transducing mechanisms appeared to be primed during capacitation (Kharche *et al.* 2009). Capacitation is also accompanied by an increase in the membrane fluidity and remodeling of the sperm surface, protein phosphorylation, an increase in internal Ca²⁺, pH and membrane hyperpolarization (Storey

1995). Certain seminal plasma proteins bind to sperm at ejaculation and act as a decapacitation factor (Oliphant *et al.* 1985). Capacitation involves the modification at the plasma membrane level, such as protein and phospholipid transfer and antigen redistribution and changes in intracellular calcium and other ion concentrations (Baldi *et al.* 1996). Capacitation also involves enzymatic removal of a substrate or coating, probably a polysaccharides on the sperm cell head that is acquired in the seminal plasma. Through the membrane alterations, the motile properties of the spermatozoon also change, lead to hyperactivity of the spermatozoa which later allow the spermatozoon to go through the acrosome reaction. The successful fertilization is the union of one and only one sperm nucleus with the female pro nucleus within the activated oocyte. Although polyspermy also occurs and this continues to be a major difficulty, which is affected by numerous factors including culture media, sperm concentration, co-incubation time, source of spermatozoa, age of oocyte donor. *In-vitro* penetration rates are variable, depending upon buck used but standardization is still necessary for IVF protocol by establishing perfect *in-vitro* fertilization media, capacitating agent, sperm count, motility of sperms, co-incubation time of gametes.

Effect of capacitating agents: Capacitating agent provides the functional maturation of spermatozoa. It is well documented that various chemical substances, such as Calcium ionophore (Goto *et al.* 1989, Jiang *et al.* 1992); caffeine (Hanada 1985); Heparin (Parrish *et al.* 1988, Parrish *et al.* 1989, Younis *et al.* 1991, Totey *et al.* 1993, Palomo *et al.* 1995); BSA (Chauhan and Anand 1991, Ling *et al.* 1992) and 20% estrus sheep serum (Smedt *et al.* 1992) can capacitate spermatozoa. Further efforts have to be made to standardize the semen capacitating process to improve the quality and feasibility of *in-vitro* produced embryos. Presently heparin is commonly used capacitating agent which is one of the glycosaminoglycan known to capacitate the bull spermatozoa *in-vitro* (Lu *et al.* 1987). Sperm capacitation, fertilization, cleavage and embryo development rate can be improved by the addition of heparin in *in-vitro* fertilization media (Izquierdo *et al.* 1998, Katska *et al.* 2004, Mogas *et al.* 1997b, Teotia *et al.* 2001, Rho *et al.* 2001, Palomo *et al.* 1999, Cox *et al.* 1994, Younis *et al.* 1991). Heparin apparently binds to sperm and plays a role in the sperm uptake of calcium (Parish *et al.* 1989). Although, the use of heparin in the fertilization medium has been reported to improve embryo yield, several studies have also reported reduced developmental competence and pregnancy rate, perhaps due to a high rate of polyspermy (Cognie *et al.* 2004, Katska *et al.* 2004, Poulin *et al.* 1996, Cognie 1999). Caffeine is also known to enhance and prolong sperm motility in mammals and acts synergistically with heparin in the capacitation of cattle spermatozoa (Niwa and Oghoda 1988). *In-vitro* incubation and washing of spermatozoa may results in spontaneous capacitation. Head to head agglutination of

spermatozoa is considered to be the criteria of sperm capacitation. A positive correlation between cholesterol depletion from the sperm plasma membrane and improved capacitation of the sperm has been observed (Davis 1982, Go and Wolf 1985). Components that act as cholesterol receptors such as albumin are present in the female tract. Thus, albumin is one of the prominent proteins supporting *in-vitro* capacitation by accepting cholesterol (Davis *et al.* 1980, Go and Wolf 1985, Benoff *et al.* 1993). Therefore, bovine serum albumin (BSA) is believed to be essential for capacitation (Bhattacharya 1992, Visconti *et al.* 1995) and it has been widely used in medium for the capacitation of sperm and the acrosome reaction (Andrews and Bavister 1989, Yoshida *et al.* 1993). However different capacitating agent has been investigated, there is further need to investigate different concentrations of capacitating agent as well as durations of capacitation for better *in-vitro* fertilization and embryo development.

Effect of fertilization media: The media employed for IVF must be capable of providing sperm motility, capacitation, the union of 2 gametes, and the start of embryo development. Media employed for sperm capacitation and fertilization include Tyrodes Albumin Lactate Pyruvate (TALP) medium (Cox *et al.* 1994, Mogas *et al.* 1997b, Wang *et al.* 2002, Katska *et al.* 2002, 2004, Kharche *et al.* 2008b, Kharche *et al.* 2010 and Yadav *et al.* 2010); modified Defined Medium (Younis *et al.* 1991, De Smedt *et al.* 1992, Ling *et al.* 1992, Younis *et al.* 1992, Crozet *et al.* 1993, Cognie *et al.* 1995, Crozet *et al.* 1995); TCM-199 (Slavik and Fulka 1992); Brackett and Oliphant (BO) (Crozet *et al.* 1995, Onger *et al.* 2001), and SOF (Rho *et al.* 2001). However, the needs and the metabolic activity of the male and female gametes are not the same (First and Parrish 1987), it is necessary to use one medium for sperm preparation and another for oocyte insemination. In goats, Keskinetepe *et al.* (1994) have reported high fertilization and cleavage rates using modified defined medium for sperm capacitation and TALP medium for oocyte fertilization. Incubation of sperm selected by swim-up in TALP-IVF with heparin (50 µg/ml) for 45 min resulted in excellent fertilization rates (Palomo *et al.* 1999, Katska *et al.* 2002, Katska *et al.* 2004). Similarly, high penetration and fertilization rates were obtained with modified defined medium (mDM) supplemented with heparin for capacitation and TALP supplemented with hypotaurine for fertilization. Today the basic medium for semen treatment and fertilization consists of Tyrode's solution, containing additional bicarbonate, pyruvate and lactate (sperm TALP) in which the spermatozoa are washed, then separated into a population of mobile spermatozoa by swim-up method (Parrish *et al.* 1986, Kharche *et al.* 2008b) or Percoll gradient separation method (Pawsh *et al.* 1994). In our previous studies we also compared the influence of culture media on *in-vitro* fertilization of goat oocytes and found that the utilization of mTALP as a fertilization medium have significantly increased

the cleavage rate of *in-vitro* fertilized oocytes as compared to BO and mSOF medium (Kharche *et al.* 2008c). Younis *et al.* 1991 compared mTALP₂ modified defined medium (Brackett and Oliphant 1975) and mH-199 for fertilization and cleavage of goat oocytes and reported higher cleavage rate in BO medium (60%) than SOF medium (40%). A higher percentage of cleavage rates (52–60%) in BO Medium were also reported by Numabe *et al.* (2001) and Bormann *et al.* (2003). In addition to media formulation, fertilization rates were also affected by culture conditions during capacitation and fertilization (Fukui *et al.* 1988, Morris *et al.* 2003). Combination of the mDM medium for the capacitation of spermatozoa and the TALP medium for fertilization is the protocol which produces the best results for fertilization and cleavage rate (Izquierdo *et al.* 1998).

Effect of protein supplementation: Protein is an essential component of the media used for *in-vitro* Capacitation and fertilization of spermatozoa. Addition of 20% of estrus goat serum or fatty acid free bovine serum albumin (4 µg/ml) has been recorded to be used successfully in the Capacitation and Fertilization media (Kharche *et al.* 2009). Although the medium supplemented with serum gives better cleavage rate than that of media supplemented with fatty acid free BSA (Kharche *et al.* 2009). Higher cleavage rate in a medium supplemented with 20% EGS may be due to the fact that EGS is likely to provide physiological active substances such as energy substrates, amino acids, vitamins and hormones. Therefore, several investigators have also used 20% estrous sheep serum in the fertilization medium for IVF in goats (De Smedt *et al.* 1992, Crozet *et al.* 1995, Martino *et al.* 1995b) and sheep *in-vitro* matured oocytes (Bondioli and Wright 1980, Guler *et al.* 2000). The addition of serum to the capacitation medium supported cholesterol efflux, thought to be a key event in capacitation (Huneau *et al.* 1994). Capacitation of goat sperm can be obtained when heat-inactivated estrus sheep or goat serum was present in the medium and also results in high cleavage rates (De Smedt *et al.* 1992, Katska *et al.* 2004, Crozet *et al.* 1995).

Effect of sperm concentration: It is known that the number of spermatozoa at the site of fertilization *in-vivo* (in oviduct) is much lower than the number of spermatozoon used for *in-vitro* fertilization process. This may be one of the main reasons of *in-vitro* polyspermy and the result may be improved by reducing sperm concentration (Coy *et al.* 1993). It is not known that polyspermy occurs due to inadequate conditions for the maturation of Oocytes or fertilization process or both. Gil *et al.* (2004) had used 4 times higher sperm concentration for cumulus-intact oocytes compared to denude ones to achieve a comparable penetration rate between both groups of oocytes. This study confirms our result that cumulus cells are able to prevent some spermatozoa from reaching the oocyte. The highly hydrated and visco-elastic matrices in the extra cellular space during cumulus expansion may act as general mechanical barriers

to sperm penetration of the oocyte. Moreover, spermatozoa possess a hyaluronidase activity which is necessary to penetrate the HA rich matrices. It might be possible that only motile spermatozoa are able to bind the extra cellular matrix of the oocyte as they try to invade and higher sperm concentration is required to fertilize loosely cumulus enclosed oocytes.

In-vitro embryo culture: Maintaining embryo viability during *in-vitro* culture is a key to the application of *in-vitro* embryo production. *In-vitro* embryo production, the culture system may deeply influence embryonic development (Lonergan *et al.* 1999). The early embryogenesis is a complex process characterized by the use of maternal proteins and transcripts to support the development of the embryo until its genome activation (embryonic genome activation), leading to the synthesis of new transcripts and proteins at the right amount and stage of development (Memili and First 1999). Physiology and biochemistry of zygote from the initial stage of preimplantation period to blastocysts is different along with the morphological differences. Although the mammalian embryo has great plasticity, which allows it to survive *in-vitro*, it usually shows low quality and viability under *in-vitro* environments (Lane 2001). Recent advances in the field of biotechnology and allied science for the production of goat embryos has led to development of several media, additives and culture conditions to optimize *in-vitro* embryo development (Kharche *et al.* 2008b). The inadequacy of culture conditions leads either to a developmental block or to a loss of viability in a morphologically normal embryo. With the growing move in *in-vitro* embryo production and transfer with biotechnological tools, there is need to increase embryo viability. The blastocyst stage of embryos show more viable nature having more chances of survival and are able to produce live offspring with higher success of birth-rate as two blastocysts are sufficient for embryo transfer. Some supplements have been added in the culture media in order to provide the embryos nutritional and other metabolic requirements. Some factors responsible for *in-vitro* embryo development are discussed below.

Effect of culture system: *In-vitro* produced embryos display morphological, biochemical and functional deviations when compared with those derived *in-vivo*. Researchers are continuously trying to overcome this gap by developing different culture systems. Major developmentally important events take place during development of embryos from post fertilization to the blastocysts stage. So there is need to developing culture system which can fulfill the requirement of embryo in early and later stages of development. *In-vitro* produced embryos are generally grown in 3 different types of culture conditions i.e. (i) organ culture, in which the embryos are grown within oviducts, (ii) co-culture with somatic cells, (iii) culture in defined media. Pre-implantation stage embryos can be developed in different media whose compositions range from simple balanced salt solutions and

carbohydrates to complex constituents, such as tissue culture medium (TCM) -199, with further supplementation of serum and/or a feeder layer of somatic cells (Krisher *et al.* 1999, Summers and Biggers 2003) but selection of medium for *in-vitro* embryo development markedly effected embryo developmental potency. However co-culture with somatic cells in complex tissue culture media is commonly used method in caprine *in-vitro* embryo production system. It is well established that embryo metabolism, cleavage, and pregnancy rates are affected by the media composition, which may lead to a diminished embryonic and fetal developmental capacity (Eckert *et al.* 1998). It has been suggested that cultured sheep and cattle embryos require contact with somatic cells or exposure to mitogenic factors secreted by the cells (Gandolfi and Moor 1987). However other workers (Tervit *et al.* 1972, Tervit and Rowson 1974, Walker *et al.* 1986, McLaughlin *et al.* 1989) have reported that sheep and cattle embryos develop readily *in-vitro* without somatic cell support if cultured in defined SOF medium under the gas atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Use of undefined culture media makes it very difficult to examine the key epigenetic factors regulating embryo development. Use of defined media is generally recommended for oocyte/embryos culture as it negates the possible effects of unknown components, minimizes risk of contamination with pathogens, reduces variations among different lots of media and prevents variation among different batches of cultures by the use of biological fluids and co-culture system. The advantages of defined media are: First, to understand fully the requirements for embryo development, which are essential to know precise composition of medium in which embryos are grown, Secondly, supplementation with undefined components, such as serum or serum proteins, is a source of undesirable experimental variation. Finally, the use of a defined medium eliminates the potential introduction of viruses and bacteria, and their metabolites, which are common contaminants of undefined components. Defined culture media have been designed to more exactly fit embryo requirements without the need for co-culture. Simple media like Charles Rosenkranes (CR) medium, synthetic oviductal fluid (SOF) and potassium simplex optimized medium (KSOM) were found capable of supporting the development of cattle zygotes to the blastocyst stage even in the absence of co-culture with somatic cells (Merton and Mullart 1999, Westberg *et al.* 2002). Recently it has been found that use of defined media in embryo culture was found capable of supporting the development of goat embryos in the absence of co-culture with somatic cells (Kharche *et al.* 2010) and bovine embryos to blastocyst (Merton and Mullart 1999, Westberg *et al.* 2002). The composition of SOF was established from the composition of bovine oviductal fluid (Tervit *et al.* 1972). SOF compositions have also been successfully used in small ruminant species. Liu Fengjun *et al.* (2005) compared the culture medium (SOF, mCRIaa,

CRIaa) for *in-vitro* culture of parthenogenetic goat embryos and found that SOF gives better cleavage rate (62.79%) than mCRIaa (13.64%) and CRIaa (53.53%). The rate of morphologically normal blastocysts and their survivability after freezing was higher when cleaved embryos were cultured in mSOF than in complex co-culture system (Nandi *et al.* 2003). Liu *et al.* 1995 observe higher percentage 61% blastocyst formation rate in KSOM medium. We also observed higher (31.4%) cleavage rate for embryo development in KSOM medium as compared to TCM and SOF medium. Glutamine has been reported to have an important role in embryo metabolism and blastocyst formation (Rieger *et al.* 1991); glutamine gives an advantage to KSOM medium. The use of chemically defined culture media has multiple benefits (Bavister 1995). Good development of embryos can be obtained in a completely defined medium (Kharche *et al.* 2010) as comparative study with complex culture shows that the embryonic development up to the morula stage was higher in defined medium (potassium simplex optimized medium) compared to complex tissue culture medium (TCM-199). The development of defined embryo culture systems also holds promise for ongoing efforts in reproductive physiology.

Effect of protein supplementation: Protein supplementation of culture medium is beneficial for embryo development *in-vitro* (Carolan *et al.* 1995). Serum and serum albumin are two commonly used protein sources in media for the *in-vitro* culture of mammalian embryos. Serum is a physiological fluid formed by the clotting of blood (Maurer 1992). It is thought to induce chemical alterations with possible detrimental implication for the culturing of embryos (Maurer 1992). Some medium formulations incorporate serum albumin as a protein source to minimize potential negative effects of serum (Bavister 1995). Albumin not only serves as a low affinity, high capacity reservoir for certain beneficial components (i.e. steroids, vitamins, fatty acids and cholesterol), but also scavenges ions and small molecules (Maurer 1992). The early mammalian pre-implantation embryos develop in the oviduct which secretes various proteins and growth factors (Watson *et al.* 1994). BSA improves embryo development, blastocyst formation and hatching rates *in-vitro*. It may also have a nutritional role to play by supplementing amino acids after hydrolysis (Biggers *et al.* 1997) thereby maintaining the intracellular amino acid pools (Thompson *et al.* 1998). BSA may also provide yet undefined embryotrophic (e.g. citrate, steroids) compounds (Gray *et al.* 1992, Bavister 1995, Biggers *et al.* 1993). The purity of the BSA preparation could determine the developmental outcomes, as reported for hamster zygotes (Miyake *et al.* 1991, Juetten and Bavister 2005). Even higher grade albumin preparations retain small undefined molecules after purification (Bavister 1995, Biggers *et al.* 1997), which possibly account for the variation in the embryotrophic effect observed in different batches of BSA (Wright *et al.* 1976a,b,

Lindner *et al.* 1979, McKiernan and Bavister 1992). Similarly cattle embryos grown with different batches of BSA have displayed different levels of developmentally important gene transcripts (Wrenzycki *et al.* 2001). Secretions of the female reproductive tract have several amino acids that can be used as energy substrate for the embryo (Bavister 1995). It was the pioneering work of Bavister colleagues (1990) that first showed the benefits of amino acids during *in-vitro* development. It has been shown that *in-vitro* fertilized bovine embryos can develop in protein-free culture systems (Pinyopummintr and Bavister 1991, Keskinetepe *et al.* 1995, Holm *et al.* 1999). The use of amino acids in serum-free culture media improves embryo development (Pinyopummintr and Bavister 1996, Lee *et al.* 2004), probably through an antioxidant action (Liu and Foote 1995) and controlling pH and osmolarity (Gardner 1998). Amino acids can also reduce the stresses and cell fragmentation caused by *in-vitro* embryo culture (Donnay *et al.* 1999). Amino acid metabolism releases ammonium ions into the culture medium, which is harmful to embryos. Because of this side effect, the culture medium needs to be replaced by a new one after two or three days of embryo culture (Thompson 2000). Chemically defined embryo culture system overcomes the problems of complicated culture condition.

Effect of somatic cell co-culture: In recent years use of somatic cell co-culture plays important role in *in-vitro* development of embryos to blastocyst stage. Complex media, such as TCM-199 supplemented with serum and co-culture, have been used in practical co-culture system. In caprine, the effect of oviductal epithelial cells co-culture system significantly enhanced the morula and blastocysts yield (Katska *et al.* 2004, Kharche *et al.* 2008b, Pawar *et al.* 2009, Singh *et al.* 2010). During culture some of the oviductal epithelial cells formed vesicle like structures in the first two days resembling blastocysts or signet ring shape continuing active rotatory movement of fertilized oocytes caused by cilia while other oviductal fluid fragments and isolated cells become attached to the culture plate, formed confluent monolayers after 2–3 days and supports embryo development by providing nutritive support. Surprisingly little is known about the mechanisms underlying the beneficial effects of co-culture. The enhancement of somatic cell in *in-vitro* embryo production system may be due to the factors that stimulate embryo development, removal of inhibitory substances present in culture medium (Bavister *et al.* 1992) and reduction of oxygen concentration (Volkel and Hu 1992, Goto *et al.* 1994). Somatic cells may contribute to embryo development by removing harmful substances such as heavy metals, modulation of nutrient profile, protection against culture induced stress as well as secreting embryotrophic factors. Goat embryos may have an *in-vitro* block at the early morula stage, in which an oviduct co-culture system may facilitate development as also evidenced in sheep and cattle

(Eyestone *et al.* 1987, Gandolfi and Moor 1987).

Effect of sequential medium: Due to changes in the embryo requirements during growth, the use of culture media with formulations more similar to the secretions found at different sites of reproductive tract during pre-implantation seems to be promising and has led to the concept that media components and physical conditions should be altered during culture to achieve improved development. This concept has subsequently been termed as sequential media systems (Gardner and Lane 1997). Physiologically sequential media are formulated to reflect the carbohydrate levels of the reproductive tract and reduce the cellular stress on the embryos (Lane and Gardner 2003) on the basis of the temporal sensitivity of embryos. More recently, sequential media have been proposed to fit the evolution of embryo requirements during early development (Gardner *et al.* 2000). For, example, glucose seems to be toxic at early cleaving embryos, whereas morula need some glucose in the medium for further development (Donnay *et al.* 2002, Gardner *et al.* 2000). Sequential medium improves development of embryos and offers the options to transfer 2–3 embryos into the mothers womb instead of large number of embryos. Currently there are different sequential media available for embryos culturing such as G1.2/G2.2 (Gardner 1994) and M1/M2 (Zollner *et al.* 2004). Culturing bovine embryos in KSOM medium until day 3 post fertilization with the addition of 1 mg/ml BSA increased the blastocysts rate and the cell number (Nedambale *et al.* 2006). Garcia *et al.* (2007) compared *in-vitro* embryonic development of bovine oocytes cultured in G1.3/G2.3 sequential culture media and CR1aa medium. Their study shows that sequential medium supports higher maturation rate as compared with conventionally used CR1 medium and suggested that the requirements of embryos during developmental stages keeps changing. Thus better blastocyst development can be obtained by sequential culture system which could provide better nutritional environment to the embryos at specific stage of early and late embryo developmental stages. Garcia *et al.* (2007) studied development and quality of sheep embryos cultured in commercial G1.3/G2.3 sequential media and found that ovine *in-vitro* blastocyst development can be achieved in sequential media G1.3/G2.3, although some modifications in the system are required to improve the quality of subsequent embryo development. In fact, a study of Xu *et al.* (2004) has demonstrated higher quality for G1.2/G2.2–cultured mouse embryos compared to those cultured in KSOM or in CZB medium. As said above, early cleavage stage embryo has a low metabolic activity with a limited ability to utilize glucose, it generates energy from low levels of oxidation of pyruvate/lactate and non-essential amino acids (Barnett and Bavister 1996). Conversely, the post-compaction embryo has a high metabolic activity, uses glucose as the preferred nutrient and requires nonessential and essential amino acids for cell proliferation and differentiation as well as specific vitamins

to maintain oxidation (Bavister 1995). These sequential media would mimic the change in environment experienced by the developing embryos *in-vivo*, enabling them to biochemical and morphological changes such as maternal zygotic transition, compaction, blastocoel formation and expansion (Swain *et al.* 2001). The development of stage-specific media based on the environment of the female reproductive tract may facilitate successful embryogenesis *in-vitro*. So it is necessary to optimize/formulate sequential media for caprine embryos to improve blastocyst quality.

Various researchers studied the effects of culture system and various supplements individually or in combination in an attempt to reach the nutritional requirements of embryo which can lead to positive response in *in-vitro* embryo development. In spite of efforts which has been carried out to enhance in reproductive potential of caprine species, there is still a need to improve culture conditions to obtain efficient *in-vitro* embryo development potential. Additionally, research on goat can be demonstrated as research model to improve culture conditions during pre-implantation stages, post implantation embryo development and fetal growth as the goat is commonly used for slaughter. It is therefore important to understand the role of each component in culture system to identify possible sources of cellular stress to the embryo that will ultimately affect the function and viability of the foetus. Some supplements have been used in the culture media in an attempt to reach the embryos nutritional requirements, but unfortunately media for embryo development is still not optimized and may cause molecular and phenotypic alterations in embryos. Subsequently, there is justified scrutiny on the culture system and the media used to sustain the caprine embryo *in-vitro*.

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