Indian Journal of Animal Sciences **83** (2): 111–117, February 2013/Review article

Quality control measures for improving *in vitro* embryo production: A review

S D KHARCHE[1] and PUJA GOEL[2]

Central Institute for Research on Goats, Makhdoom, Uttar Pradesh 29122 India

Received: 7 March 2012; Accepted: 18 May 2012

ABSTRACT

Despite considerable progress in determining the requirements of the mammalian embryo, the multi-step process of *in vitro* embryo production remains significantly inferior to the complex and dynamic environment found *in vivo*. Attempts are being made for further improvement of methods for *in vitro* production of embryos. *In vitro* production of embryos has been made possible by improvements in oocyte collection, maturation, fertilization and early embryo culture systems. *In vitro* cell culture technique is still not very efficient due to several limiting factors affecting the outcome of the results. In the present review the practical approaches for the improvement of *in vitro* embryo production will be discussed.

Key words: Embroy culture system, *In vitro* embryo production, Maturation, Zygotes

In vitro culture of mammalian embryos allows examination of the complex interactions and control of development that bring about life from a single cell into a multicellular organism. The customary practice in caprine *in vitro* embryo production (IVP) is to handle oocytes and embryos after acquiring experience and expertise in the handling of single zygote. *In vitro* culture has been made possible by improvements in culture systems and attempts are being made for further improvement (Kharche *et al.* 2010). However, despite considerable progress in determining the requirements of the cell culture, the multi-step process of *in vitro* culture remains significantly inferior to the complex and dynamic environment found *in vivo*. Determination of the requirements and responses of the cell culture for the availability of nutrients is therefore important to promote development and elucidate the conditions and mechanisms that control development. Besides the use of different culture conditions with various media and supplementations some additional factors are also responsible that influence *in vitro* culture system. An additional attribute to quality control in the laboratory encompassing not only choice of culture media but also the continuous monitoring of all possible toxic conditions is required. The ideal *in vitro* culture media is expected to posses certain physiological and chemical properties such as pH, oxygen, carbon dioxide, buffering, osmolarity, viscosity, temperature in such a way that it should support growth and proliferation of cultured animal cells. *In vitro* cell culture technique is still not very efficient due to several limiting factors affecting the outcome of the results (Kharche *et al.* 2006). The review can be divided into two major themes, inherent factors in zygotes and culture techniques. In the following sections, the practical approaches for controlling these factors are discussed.

Present address: [1]Senior Scientist, Physiology Reproduction and Shelter Management Division (kharche62@gmail.com), [2]SRF, Physiology Reproduction and Shelter Management Division (pujabiotech2k@gmail.com).

Inherent factors in zygotes

Oocyte 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, Kharche *et al.* 2011b). 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). Being the key factor in *in vitro* fertilization technology, oocyte quality profoundly affects monospermic fertilization, early embryonic survival, maintenance of pregnancy and even fetal development (Wang *et al*. 2007). Therefore selection criteria of cumulus oocyte complexes (COC's) are extremely important for successful *in vitro* embryo production program (Kharche *et al*. 2008b). Morphology of the cytoplasm and cumulus cell investment surrounding oocyte is the primary criteria for the categorization and selection of oocyte. Oocytes should also have homogenous cytoplasm, absence of cracked zona pellucida and absence of vesicle in ooplasm. An important role of cumulus cells is to provide nutritional support to the developing oocyte (Buccione *et al*. 1990). A subtle coordination between maturation of the oocyte and its

adjacent follicular cells is essential in order to produce an oocyte that is fully competent to undergo fertilization and embryonic development. Cyclical growth and atresia of ovarian follicles result in changes in overall oocyte quality, which is likely to be associated with alterations in the composition of the follicular fluid. During the period of follicle growth, oocyte acquires competence to undergo meiotic maturation by an interaction between oocyte and theca and granulosa cells (Miyano 2003) and accumulates transcripts and proteins that will guide maturation, fertilization, and initiate embryo development (Kruip *et al*. 2000). Hormonal stimulation of donors can be used to increase developmental competence of prepubertal oocyte by enhancing cytoplasmic maturation and increasing the number of follicles available for puncture on ovarian surface but blastocyst production rate may remain inferior to those obtained using *in vivo* matured ones. Aspect that should be taken into account is the genetic information transmitted by spermatozoa to the embryo. The expression of compromised genetic information from spermatozoa can impair embryo quality (Leibfried-Rutledge 1999) and interfere with an *in vitro* embryo development program's success. The quality of semen influences fertilization and embryonic development rates and this have to be controlled when implementing new procedures. Additionally differences of sperm sensitivity, sperm concentration between each male requires that optimum conditions for each male be characterized. Trials should be repeated to get accustomed to the new technique. A repetition of experiment is necessary to evaluate the potential improvements brought by the new procedures. Therefore, it is needed to select the most fertile male for semen collection, selection of excellent grade oocytes and standardization of processing techniques

Culture techniques

Chemical composition of culture medium

Effect of water quality: An area of major concern is the negative impact of water quality on *in vitro* embryo development (Rienhart *et al*. 1988). Water is used in IVF laboratory for purposes such as a solvent for culture media when preparing from powdered formulae or media concentrates, finally rinsing steps in washing up reusable glassware's which will be used for media preparation, storage of media, which demand a high level of purity in particular. Extreme attention is to be paid to chemical constituents and water quality while media formulation.

The presence of metal ions and organics in water can be very harmful to cell culture and can prevent healthy *in vitro* embryo development. The demand of pure water in media preparation and washing materials for mammalian cell culture is well established. IVF Laboratories may be partially attributable to disparate quality of the water used in preparation of culture media. The removal of contaminants in the water is of paramount importance since water constitutes the predominant component in any media formulation. To assist in selection, operation and maintenance of a water purification system, the level of contaminants must be carefully monitored. Conductivity and resistance are used to measure the purity of natural and ultrapure water respectively. Ideally a water purification system situated in the wash up area can serve as a continuous supply of ultrapure water for all purposes. This will also help in the long term to minimize cost of media and other sterile tissue culture reagents.

Removal of impurities is particularly important. Wiemer *et al*. (1998) carried out complete analysis of impurities that can be found in water; this universal solvent provides the medium for most biological and chemical reactions and is more susceptible to contamination than any other common solvents. Three categories of contaminant are generally present: inorganic, organic particles and microorganisms. Water purification processing system includes distillation, deionization, micro porous filtration, reverse osmosis, ultrafiltration, particulate filtration, carbon absorption, activated carbon cartridge filtration and electro deionization. An ultraviolet oxidation system before final filtration through 0.22 µm filters membrane to remove trace particles and prevents reverse bacterial contamination from the environment. This prepared water should be stored in plastic container to prevent leaching of metallic ions from the glass. Thus the Milli-Q synthesis is a water polisher that combines a range of advanced purification techniques designed to remove trace contaminants that may affect molecular biology, biochemical and cell biology research. The obvious alternative to one manufacturing their own water for media production is to purchase commercially prepared media. It will be advisable to buy ultrapure water for the preparation of solutions of reagents and deionized water from a conventional double distilled apparatus may be adequate for glassware rinsing and other general purposes. Thus, it may be concluded that water purity is essential requirement for media preparation and washing procedures and the system should be periodically checked for organic contaminants and endotoxins.

Osmolarity: An important parameter of cell culture medium is the osmotic pressure expressed as osmolarity. Measurement of osmolarity of the culture medium is based on the measurement of the freezing point of the liquid with the principle that freezing point is lowered as the total number of all dissolved particles (ionic and nonionic) is increased. The effect of each component in the medium to the overall osmolarity is additive and dependent upon its dissociation, e.g. 15 mM glucose will increase the osmolarity by 15 mOsm/l whereas 15 mM NaCl will increase the osmolarity by 30 mOsm/l. Osmolality of culture media is one of the key factors affecting the success of *in vitro* culture. The osmolarity of standard culture medium is approximately 270 mOsm/l and is optimal for cell culture (Naglee *et al.* 1969) however cells

can normally tolerate variations up to 10–20 mOsm following normal development. Composition of culture medium directly affects osmolarity of the medium. Osmolarity of culture media is based on the media composition but during media preparation it may deviate because of minor differences in measurements. If the osmolarity deviate more than 10–20 mOsm, new media should be prepared for more acute measurements. The osmolarity of a culture may increase during cell growth as a result of the production of low molecular weight metabolites such as ammonia and lactic acid. Therefore culture media should be replaced at every 24 h with regards that osmolality of culture medium must remain stable during *in vitro* culture process. Osmotic stress can damage DNA; affect DNA replication, DNA transcription and mRNA translation, leading to cellular damage (Burg *et al.* 2007). Evaporation probably affected cellular viability, due to both increase in salt concentration and osmolality. Regardless of the evidence that high osmolality during early embryonic stages decreases the number of apoptotic cells and improves blastocyst formation, the increase in osmolality is highly detrimental for later stages of embryo development (Ngyuen *et al.* 2003, Hwang *et al.* 2008). The oil overlay avoids the water evaporation from cell culture media. However, some studies indicated that use of oil can have disadvantages related to migration of positive lipophilic factors into the oil (Shimada *et al.* 2002), toxic factors from oil to media (Erbach *et al.* 1995) and oil storage (Otsuki *et al.* 2009). *Gasperin et al.* (2010) tested the alternate of oil overlay by presence of water in the central hole of culture plate to maintain osmolality stability during different steps. The maintenance of osmotic equilibrium and *in vitro* culture rate confirm that 4-well plate with water in the central hole can be a feasible alternative to oil for *in vitro* embryo production. In addition, it is also common to use paraffin oil to cover media to prevent evaporation of media that causes changes in component concentration or osmolality in medium. Liu and Foote (1996) showed a drastic reduction in bovine embryo production when medium osmolality was 300 mOsm/kg. In addition, high levels of NaCl impair fertilization, increase polyspermy, decrease blastocyst formation and decrease number of blastomeres (Liu and Foote 1996, Roh *et al.* 2002). Miyoshi *et al.* (1995) showed the effect of osmolarity in RlECM (Rat 1 cell embryo culture medium) containing a reduced concentration (63.8 $mmol^{-1}$) of NaCl on development of rat one-cell embryos and found culture media at 246 mOsm significantly enhanced blastocyst production rate. Naglee *et al.* (1969) cultured rabbit embryos from 2-cell to 4-cell stage embryos in a modified Ham's F-10 defined medium prepared so as to give calculated osmolarities of 230, 250, 270, 290, 310 and 330 mOsm. They found that number of blastocyst, which had enlarged and partially or completely escaped from zona pellucida at 270 mOsm was significantly higher than other groups. Therefore, osmolarity of the medium should remain in consideration prior to proceeding for *in vitro* culture.

Use of defined media: Co-culture of oocytes and embryos with granulose, cumulus or oviductal cells presents a major risk of contamination. This type of co-culture could be avoided by using defined culture media containing the complete nutritional requirement of cells. With successful endeavors, surprisingly little is known about the beneficial effects of serum, although the production of growth factors, modulation of nutrient profile, and protection against culture induced stress may be contenders. But the serum is an undefined biological product and variation in bioactivity results in inconsistencies. Culture systems without the support of biological products i.e. serum, physiological fluids are commonly referred to as defined systems, where synthetic macromolecule supplementation is used (Kharche *et al.* 2010). An approach that is widely employed is to replace serum with a set of hormones, growth factors, attachment proteins and transport proteins that will substitute for serum in the cell culture system being used. Chemically defined embryo culture system aimed to define the role of complex biological products and resolving the problems such as variation in different lots as well as risks of contamination. Pre-implantation stage embryos can develop in different media whose compositions range from simple balanced salt solutions and carbohydrates, e.g. Charles Rosenkrans 1 and 2 (CR1 and CR2), synthetic oviductal fluid (SOF) and potassium simplex optimization medium (KSOM)) to very complex constituents, such as tissue culture medium (TCM)-199, with further supplementation of serum and/or a feeder layer of somatic cells. Defined culture media i.e. media without BSA or FBS improved developmental competence of *in vitro* cultured bovine embryos and delivery of viable calves after embryo transfer (Lim *et al.* 2007). Utilization of KSOM has also resulted *in vitro* embryo development and birth of twin kids following embryo transfer into recipient doe (Kharche *et al.* 2011a). The advantages of completely defining the cell culture environment were recognized early in the development of cell culture. However, several distinct advances were necessary to achieve the goal maintaining functional mammalian cells in totally defined media. The formulations were prepared very precisely with milligram quantities of essential amino acids, vitamins, salts and carbohydrates often based on the analysis of body tissues. The roles of essential amino acids, vitamins, minerals, salts, trace metals and other nutritional components were first demonstrated and later amplified. Once these requirements were met in more complex media, it became possible to examine the role of serum, undefined components of cell culture media. Researchers have done great progress in the development of serum free systems for the growth of mammalian cells *in vitro*. Work from different laboratories and cell culture systems have contributed to our understanding of the role of serum in supporting cell growth and function. When a medium such as Eagle's minimal

essential medium is used serum can remain a source of essential nutrients and co-factors such as vitamins and some of the amino acids. A more complex medium may provide all of these nutrients and still be inadequate to support the growth of many cells in serum free medium. It has been shown that optimization of nutrient concentration and balancing the ratios of concentrations of various nutrients in the culture medium can reduce, or in some cases eliminate, the requirement of serum (Gandhi *et al.* 2000). The optimal nutrient balance and concentrations will depend on the cell type, cell density and culture system employed. Optimization of nutrients is a time consuming task, but is rewarded with a culture system that has been determined to be optimal for the cell type and conditions used at a minimal cost. The optimal set of factors will again be cell-type specific. The addition of growth factors and essential amino acids to culture media have shown evidence that improvements could be made in culturing mammalian cells by eliminating serum. It has been found that use of defined media in embryo culture is capable of supporting the development of goat embryos in the absence of co-culture with somatic cells (Kharche *et al.* 2010). The development of defined embryo culture systems also holds promise for ongoing efforts in reproductive physiology (Kharche *et al.* 2011b). Thus it may be concluded that good development of embryos can be obtained in a completely defined medium.

Environmental factors

Sterilization: The more recent approach to ensure that *in vitro* culture does not possess the risk of contamination is based on the sanitary status of cell, culture conditions and the associated culture media. This approach clearly demonstrated that embryos could be generated *in vitro* without transmitting pathogens, provided that standardized methods of collection and processing of oocytes (Kharche *et al.* 2008a). But contamination may arise during processing as inadvertent introduction of microorganisms during processing, and may lead to stop the further development of cell. Recently, a decrease in implantation rates has been attributed to air contaminants (Cohen *et al*. 1997). Maintenance of contamination free culture system during *in vitro* environment is highly desirable for success of tissue culture laboratory. To minimize the risks of contamination several features has been adopted including use of sterilized or disposable glass wares, use of sterilized (UV exposed) laminar air flow, use of antibiotics, serial washing and clean laboratory environment. *In vitro* embryo production culture system is generally followed by 8–9 days, which is sufficient long period for the growth of pathogens and cause contamination. Environmental microbes associated with an operator, tissue or the laboratory may pose risks during the *in-vitro* culture. The transferable embryos obtained through IVP system must be free of pathogens. Sources of contamination may include agents introduced with culture supplements of biological origin, e.g. serum, supporting granulose and oviductal co-culture cells, or cell lines. In this regard, inadvertent inclusion supporting cells from slaughter house material presents a major risk of contamination, especially by viruses (Booth *et al.* 1995) and inclusion of serum from infected animal into the pool may cross-contaminate, leading to batches of contaminated cell culture. This type of co culture should only be used if the cells can be controlled for the presence of viruses and results known before embryo transfer into recipients. Established cell lines such as buffalo rat liver (BRL) or vero cells are good systems if they can also be controlled before use and are often certified as pathogen free. Culture in simple defined media such as synthetic oviductal fluid (SOF), potassium simplex optimization medium (KSOM), Charles Rosenkrans medium (mCR2aa) without the any cell support would be the ideal solution from the sanitary point of view (Kharche *et al.* 2010). Additionally the media, solutions, sera and additives used must be free from contaminants and living microorganisms. Washing by the gradient centrifugation method appears to be effective for reducing the microbial population of supporting cells like oviductal epithelial cells, fetal fibroblast cells and is harmless for them. Multiple drops washing in sterile medium is recommended for the prevention from chances of contamination.

Application of enzymes to disinfect the *in vitro* cultured cells is possible regardless of cells sensitivity (Stringfellow 1998). Oocytes and embryos are surrounded by the zona pellucida (ZP), a glycoprotein shell, which protects them from physical injury and infection. It is well known that the intact ZP of uterine stage and IVF embryos is an effective barrier against penetration by most pathogens, even though some may adhere firmly to the surface (Stringfellow 1998). Disinfection of embryos through enzymatic treatment must be carried out in strictly controlled conditions to prevent dissolution of the ZP and damage to the embryonic cell membranes which could, in turn affect embryo survival**.** Currently, a trypsin-like protease is the only enzyme that is used routinely for rendering embryos free from viruses when a mechanical washing procedure is not fully effective. Antibiotics are routinely added to media used for the long-term culture of cells and tissues, to avoid contamination. However, antibiotics are biologically active substances that always have the potential to affect cell function. So the quantity of antibiotics in the media is kept at appropriate concentrations to control microorganisms in culture media, without any apparent effect on the development of embryos. Penicillin (100 IU/ml) and streptomycin (100 mg/ml) or gentamicin (50 µg/ml) are the most common antibiotics with no harmful effect on viability and growth of cell (Kharche *et al.* 2008b). Study also suggests that if antibiotics are to be used, gentamicin is the safest antibiotic (Zhou *et al.* 2000). Various media have been formulated to improve cell viability at standard concentration and quality of antibiotics with

successive days of culture. However, use of antibiotics at higher concentrations is unsuitable, as they can compromise the viability of cells. So there is need of maintaining sterile culture condition for appropriate tissue culture. In the event of cell cultures becoming contaminated with bacteria, fungi or mycoplasmas, the best course of action is to discard the culture, check cell culture reagents for contamination. In case of contamination with a spore-forming organism, and where such facilities exist, room fumigation may also be done. Maintenance of sterile culture conditions from all potentially pathogenic microorganisms are difficult until and unless sanitary conditions are provided.

Oxygen tension: The proliferation and scale up of *in vitro* embryo production depends upon the oxygen supply without causing cell damage. *In vitro* culture deviates from *in vivo* conditions in many respects, but one of the critical factors that are generally not considered is the oxygen tension under which oocytes/embryos are cultured. Cultured cells depend on the dissolved oxygen present in the media. High concentration of the oxygen may act as toxin to the cultured cells due to generation of free radicals (Olson and Seidel 2000). Therefore oxygen must be supplied in such a way that it fulfills all the cell requirements and avoids toxic effects on *in vitro* embryo productions. Numerous studies have demonstrated that atmospheric oxygen conditions during culture have detrimental effects on *in vitro* embryo development (Olson and Seidel 2000, Voelkel and Hu 1992). *In vivo*, the embryo is surrounded by oviductal or uterine fluid from which it draws oxygen and metabolic substrates required for continued growth and survival. It actively accumulates organic molecules and ions by specific transport mechanisms, and exchanges nutrients with its environment. Not only the microenvironment is critical *in vivo*, but also the contact with somatic cells, and mimicking this condition was necessary for the successful production of the first *in vitro* blastocysts (Goto *et al.* 1988). Therefore the presence of somatic cells often influences the choice of atmosphere under which embryos are to be culture. Oxygen is an important component of the oviductal and uterine environments and may have important roles in regulating embryonic development, particularly through the regulation of metabolism. In the mammalian oviduct and uterus, the oxygen tension is maintained between 1.5 and 9% oxygen (Fishers and Bavister 1993). Direct measurements of oxygen tension show that it can vary between 2 and 6% in the oviduct and uterus, depending on the species (Fischer and Bavister 1993). In defined embryo culture systems *in vitro*, 5% oxygen was demonstrated to be optimal (Yuan *et al.* 2003). However, when somatic cells are used in co-culture with oocytes or embryos, 20% oxygen is customarily used (Correa *et al.* 2008). Although, nowadays somatic cells are generally no longer used for caprine embryo culture due to undefined nature and chances of contamination. The majority of studies suggested that lower oxygen conditions are encountered in both the oviduct and uterus. Oxygen reduction can be achieved by using special gas mixers. More blastocyst with a higher cell number can be obtained when zygotes are cultured in an oxygen tension lower than that of the atmosphere (Goovaerts *et al.* 2009). The use of a low O_2 concentration was also found beneficial for embryo development *in vitro* to the blastocyst stage in various animal species (Goovaerts *et al.* 2009). Furthermore, a reduced O_2 concentration of 5% has been shown to result in a slightly decreased formation of reactive oxygen species in mouse embryos as compared to 20% O_2 (Goto *et al.* 1993). Ovulated oocytes and early embryos are rich in protective enzymes, particularly superoxide dismutase (Mouatassim *et al.* 1999), and to some extent they are protected against the harmful effects of reactive oxygen species. So mammalian embryos can be cultured successfully using a gas phase containing either atmospheric (~20%) or reduced (5%) O_2 concentration depending upon somatic cells culture used.

Temperature: Maintaining the optimal and constant temperature is basic fundamental necessity for consistency of culture conditions as well as for viability of the cells during *in vitro* embryo production which is usually 38.5°C. The direct effects of temperature on cells are obvious, but the temperature also affects the pH of the media due to increased solubility of CO_2 at lower temperature. A change in the temperature of a solution is reflected by a subsequent change in pH. The pH of a typical medium at room temperature is around 0.2 units lower than at 37°C. The pH of the buffer and the degree of ionization of serum components is also affected by the temperature. A slight decrease in temperature from optimal may slow down the cell growth rate but an increase in temperature is likely to be far more detrimental to the cells. Therefore, it is desirable to maintain the constant temperature during *in vitro* embryo production and even during the processing outside the incubator.

Therefore in extreme cold, blowers or hot air conditioners are very essential as low working temperature can affect cell growth and proliferation even can cause cell damage. There is also necessary need of air conditioning for laboratories in warm climates when many heat generating items are concentrated in small space, incubators may overheat if the ambient temperature is too close to their operating temperature. Handling of culture at high room temperature may affect cell growth and survivability. Elevated temperature can reduce developmental competence of the preimplantation embryo (Rivera and Hansen 2001). Following fertilization, the embryo is very sensitive to heat shock. However, 4–5 days after insemination, the embryo has acquired increased resistance to elevated temperature indicating that embryonic development can be disrupted by a short-term severe or a prolonged mild heat shock and that the effects of heat shock are not artifacts of changes in pH or high oxygen tension (Rivera and Hansen 2001). Thus it may be concluded that the deviation from required temperature

directly affects the *in vitro* embryo development potential.

Maintaining pH of culture medium: One parameter that requires close scrutiny in this endeavor is pH. pH is an important environmental factor that can dramatically affect the growth and survival. The challenge of pH regulation can be particularly because many organisms alter the pH of their environments through their metabolic activities. pH management and control is an important aspect of designing cell culture media in the tissue culture laboratory. pH buffering is deemed desirable because the growth of many cells is restricted to narrow limits of pH. Most cell culture media contain components intended to provide pH buffering. pH is broadly defined as the negative logarithm of the hydrogen ion concentration. The H^+ ion concentration is one of the most important parameters which determines the rates and steady state concentrations in chemical and biochemical reactions. Supplying and maintaining appropriate pH is critical to minimize stress imposed upon cultures cells and to optimize the *in vitro* culture conditions. With regard to *in vitro* culture of cells, it seems logical to consider that culture media composition should reflect as much as possible the natural conditions of cells. Though *in vitro* cultured cells have a limited ability to regulate their internal pH, thus careful attention to external pH of culture media is imperative (Swain 2010). The CO_2/bicarbonate buffering system is used to control pH. It is also notable that, although the pH in the culture medium is an important factor for providing optimal culture conditions, the optimum intracellular pH level is essential for cell survivability or growth (Swain 2010). Ability to protect from pH deviations during *in vitro* culture and processing depend on the media composition and culture conditions. HEPES (N-hydroxyethylpiperazine-N-ethanesulfonate) is a widely used buffer to maintain the pH of the media at approximately 7.2–7.6 thus prevented the deleterious effects of alkaline conditions on the development i.e. pH of the culture media under atmospheric conditions during processing can be maintained by using HEPES modified medium. In recent developments HEPES is avoided for long time *in vitro* culture because several reports have shown that these buffers may be toxic or detrimental to embryo development. Successful *in vitro* culture has been carried out under various conditions with attention to maintain the pH of culture medium. A tank of compressed CO_2 is attached to the incubator and delivers CO_2 at a constant level. Several studies have investigated the effect of CO_2 concentration on *in vitro* culture developmental potential. Culture media buffered with sodium bicarbonate are designed to maintain their pH at 7.4 at 5% CO_2 (Geshi *et al.* 1999). The pH of the medium becomes significantly higher (7.6–7.7) when the CO_2 concentration is reduced from 5 to 2% (Geshi *et al.* 1999) and vice versa. Therefore the composition of media and CO_2 concentration works together in stabilizing the pH of culture medium during *in vitro* culture.

In-vitro embryo production is still not very efficient due to several limiting factors affecting the outcome of each step of the process. Improvement in *in vitro* embryo production requires strong research efforts to develop *in vitro* treatments, more adapted to precise requirements of oocytes and early embryos. The ideal *in vitro* culture conditions posses certain physiological and chemical properties such as pH, oxygen, carbon dioxide, buffering, osmolarity, viscosity, temperature in such a way that it should support growth and proliferation of cultured animal cells. Attempts are being made for further improvement of *in vitro* culture conditions, more adapted to precise requirements of embryos by providing different media with various supplementations and co culture. But the additional attribute to quality control in the laboratory encompasses not only choice of culture media but also the continuous monitoring of all possible toxic conditions.

REFERENCES

Booth P J, Stevens D A, Collins M E and Brownlie J. 1995. Detection of bovine viral diarrhea virus (BVDV) in ovarian and oviductal tissue. *Journal of Reproduction Fertility* **28**: 700–20.

Buccione R, Schroeder A C and Eppig J J. 1990. Interaction between somatic cells and germ cells throughout mammalian oogenesis. *Biology of Reproduction* **43**: 543–47.

Burg M B, Ferraris J D and Dmitrieva N I. 2007. Cellular response to hyperosmotic stresses. *Physiology Review* **87**: 1441–74.

Cohen J, Gilligan A, Esposito W, Schimmel T and Dale B. 1997 Ambient air and its potential effects on conception *in vitro*. *Human Reproduction* **12**: 1742–49.

Comargo L S A, Viana J H M, Sa W F, Ferreira A M, Ramos A A and Vale Filho V R. 2006. Factors influencing *in vitro* embryo production. *Animal Reproduction Science* **3**: 19–26.

Correa G A, Rumpf R, Mundim T C D, Franco M M and Dode M A N. 2008. Oxygen tension during *in vitro* culture of bovine embryos: effect in production and expression of genes related to oxidative stress. *Animal Reproduction Science* **104**: 132–42.

Erbach G T, Bhatnagar P, Baltz J M and Biggers J D. 1995. Zinc is a possible toxic contaminant of silicone oil in microdrop cultures of preimplantation mouse embryos. *Human Reproduction* **10**: 3248–54.

Fischer B and Bavister B D. 1993. Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *Journal of Reproduction and Fertility* **99**: 673–79.

Gandhi A P, Lane M, Gardener D K and Krisher R L. 2000. A single medium supports development of bovine embryos throughout maturation, fertilization and culture. *Human Reproduction* **2**: 395–401.

Gasperin Bernardo G, Barreta Marcos H, Santos Joabel T, Ferreira Rogério, Neves Jairo P, Oliveira Joao F C and Gonçalves Paulo B D. 2010. Oil-free culture system for *in vitro* bovine embryo production. *Italian Journal of Animal Sciences* **9**: 32–37.

Goovaets I G F, Leroy J L M R, Soom A Van, Clercq J B D De, Andries S and Bols P E J. 2009. Effect of cumulus cell coculture and oxygen tension on the *in vitro* developmental competence of bovine zygotes cultured singly. *Theriogenology* **71**: 729–38.

Geshi M, Yonai M, Sakaguchi M and Nagai T. 1999. Improvement of *in vitro* co-culture systems for bovine embryos using a low concentration of carbon dioxide and medium supplemented with

ß-mercaptoethanol. *Theriogenology* **51**: 551–58.

Goto Y, Noda Y, Mori T and Nakano M. 1993. Increased generation of reactive oxygen species in embryos cultured *in vitro*. *Free Radical Biology Medicine* **15**: 69–75.

Hwang I S, Park M R, Moon H J, Shim J H, Kim D H, Yang B C, Ko Y G, Yang B S, Cheong H T and Im G S. 2008. Osmolarity at early culture stage affects development and expression of apoptosis related genes (Bax-alpha and Bcl-xl) in pre-implantation porcine NT embryos. *Molecular Reproduction Development* **75**: 464–71.

Kharche S D, Goel A K, Jindal S K and Sinha N K. 2006. *In vitro* maturation of caprine oocytes in different concentrations of estrous goat serum. *Small Ruminant Research* **64**: 186–89.

Kharche S D, Goel A K, Jindal S K and Sinha N K. 2008a. Birth of a female kid from *in vitro* matured and fertilized caprine oocytes. *Indian Journal of Animal Sciences* **78**: 680–85.

Kharche S D, Goel A K, Jindal S K, Goel Puja and Jha B K. 2011a. Birth of twin kids by transferring i*n vitro* matured and fertilized goat oocytes. *Indian Journal of Animal Sciences* **81**: (Accepted).

Kharche S D, Goel A K, Jindal S K, Sinha N K and Yadav P. 2008b. Effect of somatic cells co-culture on cleavage and development of *in vitro* fertilized embryos. *Indian Journal of Animal Sciences* **78**: 686–92.

Kharche S D, Goel Puja, Jha B K, Goel A K and Jindal S K. 2011b. Factors influencing *in vitro* embryo production efficiency of caprine oocytes: A review. *Indian Journal of Animal Sciences* **81**: 344–61.

Kharche S D, Yadav P, Goel A K, Jindal S K and Sharma M C. 2010. Influence of defined and complex culture media on fertilization and embryonic development of *in vitro* matured caprine oocytes. *Reproduction and Fertility and Development* **22**: 227 (abstract)

Kruip T A M, Bevers M M and Kemp B. 2000. Environment of oocyte and embryo determines health of IVP offspring. *Theriogenology* **53**: 611–18.

Leibfried-Rutledge M L. 1999. Factors determining competence of *in vitro* produced cattle embryos. *Theriogenology* **51**: 473–85.

Lim K T, Jang G, Ko K H, Lee W W, Park H J, Kim J J, Lee S H, Hwang W S, Lee B C and Kang S K. 2007. Improved *in vitro* bovine embryo development and increased efficiency in producing viable calves using defined media. *Theriogenology* **67**: 293–302.

Liu Z and Foote R H. 1996. Sodium chloride, osmolyte, and osmolarity effects on blastocyst formation in bovine embryos produced by *in vitro* fertilization (IVF) and cultured in simple serum-free media. *Journal of Assist. Reproduction and Genetics* **13**: 562–68.

Miyano T. 2003. Bringing up small oocytes to eggs in pigs and cows. *Theriogenology* **59**: 61–72.

Miyoshi K, Abeydeera LR, Okuda K and Niwa K. 1995. Effects of osmolarity and amino acids in a chemically defined medium on development of rat one-cell embryos. *Journal of Reproduction and Fertility* **103**: 27–32.

Mouatassim S, Guerin P and Menezo Y. 1999. Expression of genes encoding antioxidant enzymes in human and mouse oocytes during the final stages of maturation. *Molecular Human Reproduction* **5**: 720–25.

Naglee D L, Maurer R R and Foote R H. 1969. Effect of osmolarity on *in vitro* development of rabbit embryos in a chemically defined medium. *Experimental Cell Research* **58**: 331–33.

Ngyuen V T, Kure Bayashi S, Harayama H, Nagai T and Miyake M. 2003. Stage specific effects of the osmolarity of a culture medium on the development of parthenogenetic diploids in the pig. *Theriogenology* **59**: 719–34.

Olson S E and Seidel G E. 2000. Reduced oxygen tension and EDTA improve bovine zygote development in a chemically defined medium. *Journal of Animal Science* **78 (1)**: 152–57.

Otsuki J, Nagai Y and Chiba K. 2009. Damage of embryo development caused by peroxidized mineral oil and its association with albumin in culture. *Fertility and Sterility* **91**: 1745–49.

Rienhart J S, Bavister B D and Gerrity M. 1988. Quality control in the *in vitro* fertilization laboratory: comparison of bioassay systems for water quality. *Journal of in vitro Fertilization and Embryo Transfer* **5**: 335–36.

Rivera R M and Hansen P J. 2001. Development of cultured bovine embryos after exposure to high temperatures in the physiological range. *Reproduction* **121**: 107–15.

Roh S, Hwang W, Lee B, Lim J and Lee E. 2002. Improved monospermic fertilization and subsequent blastocyst formation of bovine oocytes fertilized *in vitro* in a medium containing NaCl of decreased concentration. *Journal of Veterinary Medicine Sciences* **64**: 667–71.

Schultz R M. 2002. The molecular foundations of the maternal to zygotic transition in the preimplantation embryo. *Human Reproduction Update* **8**: 323–31.

Shimada M, Kawano N and Terada T. 2002. Delay of nuclear maturation and reduction in developmental competence of pig oocytes after mineral oil overlay of *in vitro* maturation media. *Reproduction* **124**: 557–64.

Stringfellow D A and Seidel S. 1998. *Manual of the International Embryo Transfer Society*. 3rd edn. USA: IETS, Savoy, Illinois.

Swain Jason E. 2010. Optimizing the culture environment in the IVF laboratory: impact of pH and buffer capapcity on gamete and embryo quality. *Reproduction and Bio Medicine* **21**: 6–16.

Voelkel S A and Hu Y X. 1992. Effect of gas atmosphere on the development of one cell bovine embryos in two culture systems. *Theriogenology* **37 (5)**:1117–31.

Wang Z G, Xu Z R and Yu S D. 2007. Effect of oocyte collection techniques and maturation media on *in vitro* maturation and subsequent embryo development in Boer goat. *Czech. Journal of Animal Science* **52**: 21–25.

Wiemer K E, Anderson A and Stewart B. 1998. The importance of water quality for media preparation. *Human Reproduction* **13**: 166–72.

Yuan Y Q, Van Soom A, Coopman F O J, Mintiens K, Boerjan M L, Van Zeveren A, De Kruif A and Peelman LJ. 2003. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured *in-vitro*. *Theriogenology* **59**: 1585–96.

Zhou H, McKiernan S H, Ji W and Bavister B D. 2000. Effect of antibiotics on development in vitro of hamster pronucleate ova. *Theriogenology* **54**: 999–1006.