

In Vitro Embryo Production in Buffalo: Basic Concepts

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Abstract: *In vitro* embryo production involves a combination of the techniques of *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) of oocytes. The *in vitro* matured oocytes are incubated with suitably processed spermatozoa for an appropriate period of time for carrying out IVF. The presumptive zygotes are then cultured *in vitro* up to the blastocyst stage at which these could either be transferred to synchronized recipients for producing live offspring or cryopreserved for future use. The IVM, IVF and IVC are also integral parts of a number of other reproductive technologies like embryo or somatic cell cloning, production of transgenic animals or production of embryonic stem cells etc., which are expected to bring about a quantum jump in the population of superior genetic merit buffaloes or be useful to mankind in other ways.

Keywords: Buffalo, embryo, fertilization, maturation, culture, oocytes, co-culture.

INTRODUCTION

There is about 174 million buffalo population in the world, and roughly 97% are found in the Asian region. Buffalo is an integral part of livestock agriculture in Asia since many centuries, because they provide draught power, milk, meat and hide to millions of people, particularly small-scale farmers. It is the mainstay of Indian dairy industry since it contributes over 55% of the milk production. However, despite the importance of buffalo to the socioeconomic status, its population has been declining, partly due to poor reproductive performance. The low reproductive efficiency in female buffalo can be attributed to delayed puberty, higher age at calving, long postpartum anoestrus period, long calving interval, lack of overt sign of heat, and low conception rate. In addition, female buffaloes have few primordial follicles and a high rate of follicular atresia. These limiting factors also limit the embryo transfer technology in buffalo. Therefore, the emphasis has now shifted to *in vitro* embryo production (IVEP). IVEP by means of IVF has drawn the interest of innumerable researchers as it can salvage the genetic potential from infertile female and can yield large number of embryos from the ovaries of slaughtered females. A plethora of information is now available on the IVEP from different breeds of buffalo like Murrah [1-4] and Nili-Ravi [5-8] which are the riverine breeds, and in Swamp buffaloes [9-11], but only few laboratories have been able to produce live buffalo calves from *in vitro*-derived buffalo embryos [2, 9]. However, one major problem that has limited the application of this technology is the very low

blastocyst yields around 10 [4, 13] to 20% [12, 14] of the oocytes subjected to IVM, are much lower than the ~30 to 40% observed in cattle [15]. In spite of these limitations, this technology not only offer optimization of high-quality dams, but also allows the preservation and rapid multiplication of genetically superior characters of sire by making embryos available for somatic cell nuclear transfer (cloning), transgenesis, cryopreservation of germplasm through embryo preservation, embryonic stem cells and sexing.

In Vitro Embryo Production

The embryo production is carried out through a combination of techniques of collection of immature oocytes, *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC). However, the significantly contributing factors in the success of IVEP are the quality and number of collected oocytes.

Collection of Oocytes

Oocytes can be obtained from various sources, including live females, slaughterhouse-derived ovaries or *de novo* methods (e.g. the derivation of gametes from haploidisation of somatic cells or the reprogramming of somatic cells to pluripotent stem cells and then re-differentiating these pluripotent cells into gametes [16]). Oocyte recovery and quality vary considerably and depend, more or less, on animal health, the size of the ovary, the number of follicles accessible in the ovary and the methods of retrieval. The slaughterhouse ovaries are the major source of immature oocytes since oocytes can be obtained in numbers large enough for standardization and optimization of various techniques. The methods that

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are generally used for the collection of immature oocytes from slaughtered animal ovaries include: 1) aspiration from surface follicles using 18-20G needle, 2) puncturing of prominent follicle and 3) slicing of ovaries into small pieces. Among these methods, aspiration is mostly used as it is easier and faster to perform and gives high yield of oocytes. Depending on the efficiency of aspiration and the oocyte grading system, the oocyte harvest per ovary can range from 0.46 to 3.0, with an average of 1.5 per ovary [17-19], but the limitation of oocyte collection from slaughtered ovaries is that no information about pedigree of dam is obtained. This limitation is however overcome through ultrasound-guided transvaginal oocyte retrieval, which allows retrieval of oocytes from the best female animal of known pedigree without sacrificing the animal. This technique has widened the application of IVEP by merging assisted reproduction with genetic improvement and allows the selection of young bulls on the basis of the milk production by their half and full siblings rather than their daughters [20].

In Vitro Oocyte Maturation

Oocytes maturation is the process of complex changes in the protein phosphorylation which transform the primary oocyte into a mature secondary oocyte [21]. The fully grown oocyte in the preovulatory follicle resumes meiosis few hours before the rupture of the follicle and ovulation and then progresses from prophase of the first meiotic division to metaphase II. The nucleus and the cytoplasm of the oocyte undergo many changes during maturation, making it receptive to fertilization and competent to support embryonic development. Furthermore, the duration of IVM plays a critical role for incubation of oocytes with sperm during IVF. Improper timing of maturation results in abnormal chromatin, oocyte aging and reduced developmental competence [22]. Although large variations in the timing of oocyte maturation have been reported in buffalo, with the highest proportion of MII oocytes observed between 16 and 24 h [23, 24]. The majority of the workers inseminate buffalo oocytes *in vitro* 24 h after the start of IVM. The different time-scale recorded for oocyte maturation in buffalo may be accounted for by different conditions of IVM and particularly by oocyte quality which is likely to be affected by seasonal variations in this species. It has been recently demonstrated that the duration of IVM affects buffalo oocyte developmental competence, with a progressive decrease of fertilization capability and embryo development as the IVM duration increases from 18 to 30 h [24]. The length of time needed for IVM is a critical

factor to attain the maturation stage (metaphase II). Therefore, the optimal time for IVF in buffalo appears to be at 18 h post-IVM or, in any case, not later than 24 h; in fact, delaying IVF over 24 h resulted in a significant deterioration of oocyte developmental competence. The importance of oocytes aging in this species is also confirmed by activation studies that showed, in contrast to most other species, a deterioration of post-parthenogenetic embryo development at increasing times post-maturation [25].

Beside these factors, the development of a suitable culture system and components of maturation media for IVM of oocytes is a major component of the IVEP procedures. The medium used for IVM of oocytes varies among laboratories. The culture media employed in maturation of oocytes can be broadly divided into simple and complex. Simple media are usually a combination of various salts and bicarbonate buffered systems containing basic physiological saline with low concentration of energy source like pyruvate, lactate and glucose. The main differences between various types of simple media lie in differences in their ion concentration, and in the concentration of the energy sources. The media are generally supplemented with serum or albumin, with trace amounts of antibiotics added. Complex media, on the other hand, contain, in addition to the components of the simple media, amino acids, vitamins, purines and other substances, mainly at levels at which they are found in serum. Fixed nitrogen is present as free amino acids. The most widely used complex media is tissue culture medium 199 (TCM-199), and Ham's F-10 could be also used to mature *in vitro* buffalo oocytes. These media, designed originally for cell culture, are not capable of supporting high levels of oocyte maturation and, therefore, are commonly supplemented with sera.

Our laboratory routinely used TCM-199 supplemented with 10% FBS + 0.81 mM sodium pyruvate + 5% buffalo follicular fluid (buFF) + 50 µg/ml gentamycin sulfate and 5 µg/ml porcine FSH, achieving 82.3% maturation rates for 24 h incubation in a CO₂ incubator (5% CO₂ in air, 90-95% relative humidity) at 38.5°C [26]. In other report, TCM-199 and Ham's F-10 supplementation with fetal calf serum (FCS) which improved the nuclear maturation and fertilization rate compared with buffalo estrus serum (BES) in the absence, but not in the presence of hormones [27]. Upon comparison of EBS, superovulated buffalo serum (SBS), fetal bovine serum (FBS), and steer serum (SS) efficacies for maturation and further development, no differences were detected in terms of cumulus

expansion, nuclear maturation, fertilization, and development to morula or blastocyst stage [28]. Because different sources of serum can support IVM, it would be possible to reduce the IVM costs by replacing FCS or FBS, which are highly expensive, with EBS, SBS, or SS. In addition, Lonergan *et al.* [29] compared synthetic oviduct fluid (SOF) with TCM-199 for the maturation of oocytes but found it to be less effective. Later, Watson *et al.* [30] reported oocyte maturation with amino acid supplemented SOF resulted in blastocysts of a quality comparable to those matured in TCM-199 contained newborn calf serum, and concluded that this formulation of SOF medium could be an effective base medium for cattle oocyte maturation.

***In Vitro* Fertilization**

Mammalian fertilization is a complex event involving many different stages that must be acquired successfully to achieve fertilization. IVF is the most critical step of the IVEP procedures in buffalo, as cleavage rates lower than those obtained in other domestic species have been widely reported [26, 31-33]. Neglia *et al.* [31] reported the lower blastocyst yield in buffalo compared to cattle (26 vs 34 %, respectively), was mainly due to the poor cleavage rate (65% vs. 84%). Successful IVF requires appropriate preparation of sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes. The male gamete influences fertility by affecting fertilization rate and subsequent embryo development. IVF has become a valuable tool for assessing sperm functionality and for studying the success or failure of gamete interaction in most species. To enhance successful fertilization of the oocytes, sperm cells must be motile, have the ability to undergo capacitation and express the acrosome reaction. Sperm must possess the capacity to bind to the zona pellucida and vitelline membrane by acquiring the correct binding proteins during maturation, and exposing these binding sites to the oocyte at the appropriate time. So, spermatozoa are artificially capacitated because they need to undergo biochemical and physiological changes before becoming able to fertilize oocytes. *In vitro* capacitation also induces acrosome reaction that is vital for fertilization. The spermatozoa are treated with appropriate concentration of heparin to induce capacitation and subsequent acrosome reaction. The media used for IVF suggested are BO [34] and TALP, which contain motility enhancing substance like caffeine or theophylline. Caffeine is a cyclic nucleotide phosphodiesterase

inhibitor that has been used as a motility-stimulating agent during IVF. It achieves its action by inhibiting phosphodiesterase, which results in an intracellular accumulation of cAMP that activates respiration and sperm motility of spermatozoa. The bovine serum albumin is also added to fertilization medium for destabilizing cell membrane of oocytes and sperm by removing cholesterol and zinc molecules which eventually enhances capacitation and acrosome reaction. Another report demonstrated that progesterone induces buffalo sperm capacitation *in vitro* and may be considered as an alternative capacitating agent for buffalo IVF [35]. Furthermore, it has been demonstrated that sperm treatment for 2 or 3 h with sodium nitroprusside, a well known generator of nitric oxide *in vitro*, improves the efficiency of buffalo sperm capacitation *in vitro* compared to heparin [36]. The most promising results have been obtained by incubating buffalo sperm with biological fluids, such as BES and the buFF recovered from a pool of dominant follicles [37]. The *in vitro* matured oocytes are incubated with the processed spermatozoa for an appropriate period of 4-6 h which is necessary for inducing vesiculation of the plasma membrane and the outer acrosomal membrane [38] but 16 h sperm-oocyte co-incubation time is required for maximizing the blastocyst yield in buffalo as reported [24].

***In Vitro* Culture**

After the end of sperm-oocyte incubation, IVC is carried out, where presumptive zygotes are cultured *in vitro* in a culture medium at 38.5°C in a CO₂ incubator for up to 9-10 days to get the blastocyst, which could either be transferred to synchronized recipients for producing live offspring or cryopreserved for future use. IVC is perhaps the most important step, not only because of its longer duration of around 10 days compared to that of 24 h for IVM and 6-24 h for IVF but also since the culture conditions and environment during IVC have a profound influence on the outcome of embryo development. Although many authors still prefer the co-culture system with cumulus and oviductal epithelial cells [2, 17, 26] or with established cell lines such as BRL [19] for embryo production in buffalo. It was believed that these cells acted in a protective manner through the reduction or removal of potentially harmful substances or modifying the concentration of the medium constituents to levels more appropriate for embryo development [34, 39] and that they also probably secreted specific embryotrophic factors [40] which were beneficial for the development of embryos *in vitro*. However, the oviductal epithelial

cells, the only source of which is slaughterhouse material, are not only a potential source of introducing pathogens to the embryos, they are also a hazard for a sterile embryo culture laboratory. Moreover co-culture with oviductal epithelial cells introduced an element of variability between different trials since the pathophysiological status of the animal(s) from which oviductal epithelial cells were obtained and the efficacy of these cells in IVC may differ in different animals [4, 13, 26]. Another issue is culture media like complex medium i.e. TCM-199, Ham's F10 etc most commonly used, which were actually designed for the general cell culture work, rather than for culture of embryos, also needs to be avoided since many constituents may be present in this medium at concentrations which are not optimal for supporting embryo development. So, development of an IVC system which obviates the use of oviductal epithelial cells and complex media are an essential requirement for commercial IVEP in buffalo. In this regard, various simple media like Charles Rosenkrans (CR) medium [41], SOF [42], Chatot-Ziomek-Bavister medium [43], hamster embryo culture medium [41], potassium simplex optimized medium [45] and G1.2/G2.2 media [46] were found capable of supporting the development of cattle zygote to the blastocyst stage even in the absence of co-culture with somatic cells [47]. Later on, these media were utilized for buffalo embryo culture and resulted in similar embryo development [11, 19, 26, 33, 48]. In other studies, IVC of buffalo zygote in SOF or CR medium gave a higher yield of embryos than in TCM-199 medium [11, 26]. In order to reduce the accumulation of free radicals, ammonium and other catabolites that may affect embryo development, it has been suggested to partly replenish the medium during culture. However, no significant differences were observed in buffalo embryo development by replacing the IVC medium 2 or 3 times during culture [21]. Hence, it may be due to higher sensitivity of buffalo embryos to fluctuations of temperature and/or pH that normally occur during a culture change even if to limited extents. So it is advisable "do not disturb" buffalo embryos during culture.

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