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Asian Journal of Animal and Veterinary Advances

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OPEN ACCESS Asian Journal of Animal and Veterinary Advances

ISSN 1683-9919
DOI: 10.3923/ajava.2016.737.756

# Review Article
# Factors Influencing *in vitro* Production of Bovine Embryos: A Review

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## Abstract
*In vitro* embryo production (IVP) is currently one of the most important biotechnologies in cattle breeding and husbandry. However, the efficiency of *in vitro* embryo production is still low with only 30-40% of oocytes developing into blastocysts, probably because, the *in vitro* environment cannot mimic *in vivo* environment and results in embryos that have altered morphology and gene expression. Several factors can influence the IVP efficiency and contribute to the existing differences between *in vivo* and *in vitro* produced embryos. There is also evidence showing that IVP can cause some disorders during gestation and in offspring. The aim of this study is to give a brief overview of some factors that influence *in vitro* production of bovine embryos and their ability to develop into blastocyst stage.

Key words: Bovine, *in vitro* fertilization, embryo development, offspring

Received: July 25, 2016 Accepted: September 27, 2016 Published: November 15, 2016

Citation: Ayman H. Abd El-Aziz, Usama E. Mahrous, Sherief Z. Kamel and Ahmed A. Sabek, 2016. Factors influencing *in vitro* production of bovine embryos: A review. Asian J. Anim. Vet. Adv., 11: 737-756.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

## INTRODUCTION

The ability to produce large number of embryos from donors of high genetic merit has considerable potential value in disseminating genetic improvement and shorter the progeny testing and generation interval through the national herd. Some commercial applications of *in vitro* fertilization technology have included efforts to: (1) Upgrade the productive and genetic performance of animals, (2) To overcome infertility of valuable high yielding animals, (3) To produce transgenic and cloned animals, (4) Provide a source of sexed embryos, (5) For twin production in beef cattle and (6) At the molecular level, the technique is used to elucidate events related to maturation, fertilization of oocytes and development of embryos, these events are difficult to study under natural conditions in living animals. In the laboratory, embryos can be routinely produced and developed up to the blastocyst stage using three subsequent techniques: *In vitro* maturation (IVM) of oocytes, followed by sperm capacitation and *in vitro* fertilization (IVF) of matured oocytes and then *in vitro* culture (IVC) of the fertilized oocytes up to the blastocyst stage. *In vitro* embryo development is strongly influenced by events occurring during oocyte maturation, fertilization and the subsequent development of the fertilized oocytes. So, improving the efficiency and identifying the sources of variations between IVF systems or between different laboratories are more important when routinely producing blastocysts from individuals of high genetic merits. Also, the development of specific culture regimes capable of supporting IVM/IVF and IVC to the blastocyst stage is highly desirable. In addition, survival rate of *in vitro* produced embryos after freezing and thawing and following some of the more advanced manipulation is less than for embryos produced *in vivo*, indicating that the techniques used to produce embryos *in vitro* still require considerable improvement.

The following study presents description of the history of embryo transfer (*in vivo* and *in vitro*) and various factors that could affect *in vitro* development of oocytes and their ability to develop into blastocysts in cattle. These factors can be classified into:

## HISTORY OF *in vitro* EMBRYO PRODUCTION

*In vitro* embryo production involves 3 steps: Maturation of oocytes, fertilization with motile, capacitated sperm and then culture to the blastocyst stage. It is still almost unfathomable that it is actually possible to begin with an immature oocyte and end with a developed embryo outside of an animal. Thus, the researchers of today owe a huge debt to the researchers of yesterday, as there were many, many investigations and failed attempts which laid the foundation for the eventual successes found in the 1980s and still enjoyed today.

***In vivo* maturation:** Before exploring *in vitro* maturation, it is prudent to consider the details of maturation *in vivo*. Follicles are stimulated to change from primordial to primary follicles under the influence of as-yet unknown signals. Once they become primary follicles, they develop a zona pellucida and experience abundant hyperplasia of the surrounding granulosa cells as they become a preantral follicle over a period of about 3-4 months[1]. At this point, they are considered gonadotropin-responsive and will continue to grow and develop under the influence of Follicle Stimulating Hormone (FSH) secreted by the anterior pituitary in response to gonadotropin releasing hormone (GnRH) secreted by the hypothalamus. As the preantral follicles respond to FSH, one or more will grow larger than the others and become the dominant follicle.

As the follicle grows (due mostly to increasing antrum size, although the oocyte itself also approximately quadruples in size), it becomes dependent on FSH for its final maturation and development. Just before ovulation the majority of the hormone receptors present on the dominant follicle will change from FSH to Luteinizing Hormone (LH) receptors, which occurs in concert with falling FSH concentrations (due to production of inhibin by the dominant follicle). This assures dominance of the follicle, as other, less developed follicles do not have this changeover in receptors and are still FSH dependent. The final step in *in vivo* maturation occurs in response to the LH surge, which precedes ovulation by approximately 24 h. During this pre-ovulatory maturation (sometimes called oocyte capacitation) the oocyte resumes meiosis to arrest at the metaphase II step. The granulosa cells which are in direct contact with the oocyte (called corona radiata cells) have had very intimate contact with the cytoplasm of the oocyte through zona processes which pass through the zona pellucida and are connected to the oocyte through gap junctions. These gap junctions allow the passage of several cytoplasmic molecules, including cyclic adenosine monophosphate (cAMP). Prior to the LH surge, those processes retreat somewhat from the cytoplasm and after the surge they retract more until they are completely removed from the oocyte[2]. This loss of communication causes, among other things, a decrease in cAMP in the oocyte. This falling level of cAMP within the oocyte is suspected to be a signal for resumption of meiosis[3].

***In vitro* maturation:** The goal of *in vitro* maturation (IVM) is to cause an immature oocyte from a developing follicle to resume meiosis and render itself capable of fertilization, arrested at the second metaphase step of meiosis just like its *in vivo* matured counterpart. Early researchers found that oocyte maturation (as indicated by nuclear changes in chromatin and germinal vesicle breakdown (GVBD)) seemed to proceed spontaneously when oocytes were removed from the follicle[4]. The mechanisms of this spontaneous resumption of meiosis, however were not entirely clear at that time. Another researcher found that culturing murine oocytes in follicular fluid caused a transient suspension of maturation (signaled by a prevention of GVBD) and further, the addition of a cAMP elevating agent ((Bu)2 cAMP) inhibited maturation for at least 21 h (compared to nearly 100% GVBD after 3 h in controls). Thus, it seems that the necessary steps to initiate the maturation process *in vitro* include both removal of the oocyte from the follicular environment and depletion of intracellular cAMP stores[5]. The first report of successful IVM (indicated by polar body extrusion and metaphase plate appearance) of a bovine oocyte[6]. The major medium components (cell culture medium, serum and antibiotics) and incubation conditions (5% $CO_2$ in humidified air) described in this report are, with only slight modifications, still used today. Soon after, similar study was conducted by another group showing that oocytes could start the maturation process *in vitro* (to metaphase I), continue *in vivo* and be successfully fertilized *in vivo* (shown by two pronuclei or evidence of sperm within the ooplasm)[4]. The first report of a blastocyst produced *in vivo* after *in vitro* maturation (in Fetal Bovine Serum (FBS) alone)[7]. Old study performed IVM in a media very similar to Edwards with the addition of pyruvate as well as porcine-origin Follicle Stimulating Hormone (FSH). That study showed that although 39% of *in vitro* matured oocytes appeared to undergo normal fertilization after IVF (compared to 71% of *in vivo* matured and *in vitro* fertilized controls), there were no blastocysts recovered after *in vivo* culture (compared to 37% of controls). This showed that *in vitro* matured oocytes were still quite hampered compared to their *in vivo* matured counterparts and despite being capable of experiencing apparently normal fertilization, further development was severely depressed[8]. A study published one year later from the same researchers showed vastly improved results: The biggest difference in the IVM protocol was the substitution of purified and defined FSH, as well as the addition of purified and defined LH and estradiol. Their results showed improved development over previous study, as 28% of cleaved embryos recovered after *in vivo* culture had proceeded to the morula or blastocyst stage[9]. No control *in vivo* matured group was included buta group of oocytes subjected to IVM in media devoid of hormones resulted in a development rate of 18% which was not significantly different than the hormone-included group. The inclusion of the gonadotropins is intended to mimic the *in vivo* environment and both LH and FSH have shown to improve the health of the oocyte or surrounding cumulus cells[10,11]. Steroid hormones present a bit of an enigma, as the granulosa cells which surround the oocyte have been shown to secrete estrogen, testosterone and progesterone during IVM[12]. Although they may not be added to the media, they are likely present in unmeasured amounts. However, progesterone concentrations declined precipitously in media which was in contact with mineral oil (vs no change in media not in contact with oil). These studies suggest that concentrations of steroids are rather undefined in the media and may be very sensitive to environmental conditions[13].

The results some of the early IVM studies suggest that FBS alone is a media capable of supporting oocyte maturation. However, as the negative effects of serum were discovered, many researchers changed to using FBS only in small concentrations or excluding it altogether from maturation media. Instead, cell culture media such as TCM 199 or DMEM often make up the majority of maturation media[7-9]. In the pursuit of more defined maturation media, several serum free maturation media tested, the common factor among those which produced the highest percentage of blastocysts after IVF and *in vitro* culture (IVC) was glucose supplementation[14]. A further investigation confirmed that moderate (1.5 mM) glucose supplementation in IVM media augments blastocyst development (compared to no supplementation) but revealed that an excess (20 mM) leads to comparatively depressed development after IVF/IVC. This depression in development was seen inspite of the fact that more of the oocytes matured in the high glucose group showed nuclear evidence of progression to metaphase II than those in the moderate glucose media and fertilization between the two groups appeared to be equal[15]. Other additives which have shown benefit when added to maturation media are the gonadotropins (LH and FSH). The IVM has been successfully carried out successfully in defined media without the addition of gonadotropins[14] however, many media used in IVM today include gonadotropins as the eventual development of oocytes matured in their presence following *in vitro* production appears to be higher than those matured without gonadotropins[16].

## FACTORS AFFECTING *in vitro* MATURATION (IVM)

*In vitro* maturation is the most critical step *in vitro* embryo production. There is a constant need to emphasize the fact that effective oocyte maturation is the foundation of embryo production. Identifying these factors will improve the *in vitro* embryo production systems in bovine. These factors include:

**Factors affecting oocytes yield:** The recovery of large number of oocytes with high developmental competence remains an ultimate goal for the mass production of embryos in cattle. At the same time, the origin of the oocyte can play an important role in their IVF and subsequent developmental competence. Oocytes yield and quality can be affected by:

**Effect of follicular size on oocyte competence:** In cattle, the oocyte first acquired competence to develop into blastocysts in *in vitro* system at a follicular size of 2-3 mm. When follicles were pooled according to size, it was shown that large follicles (≥10 mm diameter) contain oocytes with a higher potential to become embryos[17,18]. Some studies described the fate of individual oocytes according to the exact follicular size and it confirmed an increased competence with follicle size, i.e., bovine oocyte complexes (COCs) isolated from ovaries carrying follicles of 2-5 mm in diameter showed lower rates of maturation and blastocyst formation than those from ovaries carrying follicles of >10 mm in diameter[19,20]. This indicate that large follicles (≥6 mm diameter) provide the oocyte with a microenvironment which improves its quality[21]. Dramatic changes in oocyte nuclei, especially nucleoli are known to occur as the bovine follicle grows from 1-20 mm. Such changes may have a crucial effect on the developmental potential of the oocytes. It is known that a very stable form of RNA accumulates in the oocyte and that it is translated during maturation, fertilization and early embryonic development; such RNA accumulation may be influenced by the nature of the follicle growth[22]. However, other reports suggest that follicular size may not the only important criterion, since some bovine oocytes originating from large follicles failed to produce embryos, while some oocytes from medium size follicles already have this capacity[23].

## METHODS OF OOCYTES RETRIEVAL

**Aspiration technique:** Several reports deal with comparison of the different methods used for oocyte recovery. Recovery of bovine oocytes by aspiration of vesicular follicles, using an appropriate pipette or syringe and needle has been the method most commonly employed. The advantage of follicle aspiration is in terms of speed of operation, which may be particularly important in commercial embryo production. One of the difficulties associated with the aspiration approach lies in the fact that oocytes may only be retrieved from 30-60% of the puncture follicles[24]. When comparing between aspiration of follicles and follicular dissection in cattle these study generally supports the view that significantly greater yield of oocytes in the highest-quality grades may be obtained by follicle dissection rather than aspiration[25]. Aspiration of the oocyte can result in greater disruption of surrounding cumulus cells. There is also the possibility that aspiration does not always succeed in retrieving the highest-quality oocytes, this may be due to the cumulus oophorus being firmly attached to the stratum granulosum[26].

**Slicing of ovary:** More recently, slicing procedures have been employed in cattle[27]. Those researchers reported that oocyte yields average 55 animal$^{-1}$. This was a threefold increase on the number than recovered by aspiration. Oocyte recovery by slicing rather than aspiration can resulted in marked increase in blastocyst yield after IVM, IVF and IVC.

**Slicing after aspiration:** The slicing of ovaries after preliminary aspiration of follicles has been dealt in several reports[28]. Those authors concluded that there would be no merit, in terms of oocytes recovered or their quality in combining the two procedures.

**Transvaginal ultrasound-guide oocytes pick up (OPU):** Transvaginal oocyte pick-up (OPU) is an important technique for oocyte retrieval in living previously genetically selected highly valuable donor cow. The success of OPU is measured in part by the recovery rate of oocytes, expressed as a percentage of the number of follicles punctured[29]. This recovery rate in turn is influence by numerous factors such as aspiration vacuum, hormonal pre-treatment of animals, puncture frequency, stage of the estrous cycle and the experience of the operator. Recovery rate declined as the aspiration pressure increased above 50 mmHg. The recovery rate of grade 1 oocytes decreased significantly as the vacuum pressure increased with a corresponding increase in the number of denuded oocytes recovered[30]. Moreover, hormonal pretreatment of donors prior to OPU using gonadotropin significantly increased oocytes recovery rates and blastocyst production[18]. Oocyte competence was increased when the period between p-FSH administration and OPU was

extended[31]. Recently, it is possible to improve the efficiency of OPU and *in vitro* production of embryos by utilization of the growth phase of the first follicular wave before dominant follicle selection in cattle[32].

**Ovary storage: Temperature and time limits:** In the recovery of oocytes for *in vitro* maturation, the time interval between animal slaughter and oocyte recovery from the ovaries and the temperature at which the ovaries should be stored are important considerations. Cattle oocytes were recovered within 1-2 h of animal slaughter; ovaries were usually stored[33] at about 30°C. Another scientist suggested that exposure of COCs to temperature below 35°C during oocyte recovery might significantly decrease both the quantity and quality of bovine embryos produced by *in vitro* methods[34]. Other reports suggested that the time from slaughter to oocyte recovery might extend upto 8 h according to Solano *et al.*[35].

**Oocyte quality:** Proper oocyte selection in the laboratory is crucial for successful embryo production. Presence of an intact complement of cumulus cell layers surrounding the oocyte and a homogenous appearing cytoplasm have been the best indicators of an immature oocyte ability to undergo maturation and embryonic development. Studies have evaluated the impact of cumulus morphology on subsequent development in cattle[36]. When immature oocytes were classified according to the number of layers of cumulus left around the oocytes following aspiration, the thicker the number of layers of cumulus cells, the better were the chances for development[21]. The oocytes that are not of this type have aberrant protein synthesis and complete meiosis at a lower frequency[37,38]. The role of the cumulus cells is to provide nutrients to the oocytes during its growth, to participate in the zona formation and following the LH surge, to synthesis the matrix composed of proteins and hyaluronic acid important in oviductal transport or in sperm trapping[39].

**Culture media:** The culture employed in IVM not only affect the proportion of bovine oocytes that reach metaphase II (M II) and become capable of undergoing *in vitro* fertilization but can also influence subsequent embryonic development[40]. *In vitro* maturation medium can be broadly divided into simple and complex. Simple media are usually bicarbonate-buffered systems containing physiological saline with pyruvate, lactate and glucose and they differ in their ion concentration and in the concentrations of the energy sources. Complex media contains in addition to the basic components of simple media, amino acids, vitamins and purines.

Most IVF laboratories routinely use M-199 as the basic IVM medium in cattle and there have been few reports suggesting that other media may be more appropriate. In one comparison of complex media for IVM, the scientists concluded that under their conditions, F-10 medium is superior to M-199 and B2 media[41]. In another comparison of IVM commercially available complex chemically defined media showed that TCM-199 was superior[42] to RPMI-1640. While, no difference in the rate of embryo development for bovine oocytes matured in TCM-199 or CR2 media[43]. Oocytes matured in medium leading to poor developmental competence have depressed levels of glycolysis that necessary for completion of maturation, the reduced level of glycolysis may reflect reduced activity of the pentose phosphate pathways, which plays an important role in meiotic maturation of bovine oocytes[44].

Dealing with the energy source, the excessive glucose in the media used for oocyte maturation impairs the development of bovine oocytes to the blastocyst stage, possibly due to the increase of Reactive Oxygen Species (ROS) and the decreased in the intracelluar glutathion content of bovine oocytes[15]. Addition of β-mercaptoethanol to TCM-199 medium increased intracellular glutathion levels of bovine oocytes cultured individually and can improve maturation rate leading to the blastocyst stage throughout *in vitro* production[45].

## BOVINE SERUM AND OTHER PROTEIN SOURCES

**Bovine Serum Albumin (BSA):** The FCS generally superior to BSA as a protein supplement in IVM medium[46]. It has been recognized that BSA was probably contaminated with some low molecular weight compound[47]. For example, four lots of fraction V BSA were tested for their ability to support two-cell hamster embryos in culture. Results showed that such preparations can produce highly variable effects on cultured embryos and cells ranging from highly inhibitory to highly stimulatory[48]. Data on the amino acid content of bovine serum albumin is provided by other scientists. They noticed that this constituent could show wide variations; similar variability may presumably be expected with hormones, growth factors, cytokins and vitamins[49].

**Sources of bovine serum:** Bovine serum, in the form of FCS or Oestrous Cow Serum (OCS) has been employed as the main protein source in bovine IVM studies. The OCS had a significant and marked effect, compared with FCS, on the percentage of secondary oocytes that were fertilized and which cleaved during culture[50]. In another study suggested that pro-oestrous serum may be more effective than OCS[51], it

was found, on analysis, it contains high levels of LH and prolactin. In another reported reveled that super ovulated cow serum (SCS) was superior to FCS for oocyte maturation and fertilization and embryo development *in vitro*[52]. Fetuin, a major glycoprotein constituent of FCS, can prevent hardening of the zona pellucida (ZP) during IVM by preventing the action of proteolytic enzymes originating from precociously released cortical granules[53].

**Serum substitutes:** Several commercial products are available as serum substitutes for use in *in vitro* cell culture. the ultroser G (compounds: Growth factors, adhesive factors, mineral trace elements, hormones, binding proteins and vitamins) successfully in cattle IVM without hormone supplementation at a concentration[21] of 1-4%. A study employed polyvinyl-pyrrolidone (PVP) at a 0.3% concentration as a substitutes, in the absence of hormones (Estradiol/LH/FSH) in the IVM medium, there was no yield of blastocyst[54]. However, PVP could effectively replaced serum in the absence of hormones[55].

## HORMONE SUPPLEMENTATION OF THE IVM MEDIUM

**Gonadotropins:** Currently, most IVM protocols do employ Luteinizing Hormone (LH) or Follicle Stimulating Hormone (FSH) or a combination of them. However, the effect of the gonadotropins and their relative importance on *in vitro* maturation and subsequent fertilization and early development is still controversial[10,56]. The use of highly purified LH preparations of bovine origin at a certain level in their IVM medium significantly increased embryo yield after IVF/IVC. Evidence was found that LH may alter calcium distribution within the ooplasm and that the gonadotropin promotes increased glycolysis, combined with increased mitochondiral glucose oxidation, within cumulus-cell-enclosed bovine oocytes. It was also evident that LH exposure resulted in increased glutamine metabolism within the oocyte. In contrary, other reports showed no enhancement of development following addition of LH to maturation medium[57]. Reports revealed that mRNA of the LH receptors was detected exclusively in thecal cells. Absence of LH receptors in oocytes confirmed the previous results[58]. At the same time, much of study suggested that FSH has a beneficial effect and that the presence of this gonadotropin in the *in vitro* maturation medium enhances expansion of the cumulus cells surrounding the oocyte, which in terms enhances sperm capacitation and the fertilization process[59]. Additionally, FSH or eCG supplementation to the IVM medium significantly increased cleavage rate and development of buffalo embryos upto the blastocyst stage when compared with negative control medium[60]. It is concluded that cAMP dependent protein kinase activity regulating by cumulus cells following FSH stimulation plays a role in the complex mechanism of chromatin condensation and MPF activation leading to meiotic resumption in bovine oocytes[61]. Moreover, *in vitro* maturation of bovine Cumulus Oocyte Complex (COCs) in serum free medium supplemented with bovine growth hormone (bGH) accelerated the progression of meiosis, induced cumulus expansion and enhanced the cleavage rate and number of blastocyst following IVF and IVC[62,63]. Growth hormone can influence oocyte maturation by affecting the kinetics of the first polar body extrusion[64]. Also, it causes a better cytoplasmic maturation in terms of proper distribution of cell organelles or the formation of the male decondensation factor[65]. However, GH treatment during IVM had no marked influence on the resumption of meiosis but significantly delayed its completion in a dose related manner[66].

**Steroids:** Maturation of oocytes in the presence of estradiol and FSH reduced the percentage of oocytes undergoing Germinal Vesicle Break down (GVBD), while estradiol alone had no effect. Rostenedione reduced the percentage of oocytes showing GVBD when added alone or with FSH. The presence of estradiol in the culture medium of *in vitro* matured human oocytes had no effect on the progression of meiosis but improved fertilization and cleavage rate suggesting that estradiol supports cytoplasmic maturational changes necessary for *in vitro* fertilization and early post fertilization development[58]. However, maturation of bovine oocytes in the presence of high concentrations of estradiol had a negative effect on spindle formation and first polar body extrusion[67] and may alter protein uptake and incorporation[68]. Estradiol could be added at a concentration[26] of 1 $\mu g\ mL^{-1}$, which is about the concentration in the follicular fluid of preovulatory follicles shortly after the LH peak.

**Growth factors:** The effect of growth factors on oocyte *in vitro* maturation has been examined in cattle by several investigators. The presence of Epidermal Growth Factor (EGF) during IVM stimulated cumulus expansion and significantly increased the proportion of oocyte attaining M II[69]. While, adding EGF to cattle IVM without any evident effect on oocyte maturation[70]. The addition of insulin like growth factor-I (IGF-I)[71] or transforming growth factor-α or β or IGF-2 to the IVM medium significantly improved the quality of oocyte[25]. Stimulating activity of EGF is dependent on the cyclic AMP pathway and probably transduced by proteinase-k cytokines pathway[72].

**Effect of cytokins:** Cytokines are small regulatory peptides or glycoproteins, with molecular weight ranging from 6000-60,000, which are synthesized and secreted by activated immune and mesenchymal cells. Cytokines are believed to act generally in a paracrine or autocrine manner. It is possible that cytokines originating from the oocyte have a role in preparing the maternal immune or endocrine system for subsequent events in *in vitro* fertilization and early pregnancy[73].

**Effect of follicular fluid:** Follicular Fluid (FF) is a serum transudate modified by follicular metabolic activities, contains specific constituents such as steroids and glycoproteins synthesized by the cells of the follicle wall. The supplementation of the IVM medium by bovine follicular fluid (bFF) at the 10-20% level favoured subsequent embryonic development in cattle[21]. Also, the evidence of favourable effect from including bFF in IVM medium at 20-30% level was recorded[74]. The implication in these various reports is that certain factors in bFF may favourably influence oocyte quality. However, the bFF from small follicles could inhibit meiosis in cattle oocytes[75]. Follicular fluid from small and medium size follicles at estrus through mid-diestrus had more GVBD inhibition activity than at early proestrus[76]. Importantly, the high concentration of bFF (10-20%) in maturation medium suppressed both resumption of meiosis, fertilization rates and embryo development[77].

**Effect of maturation time:** In the study on the timing of nuclear events during IVM, the Germinal Vesicle (GV) was evident from 0-6.6 h, GVBD occurred at 6.6-8.0 h, chromatin condensation at 8-10.3 h, metaphase I at 10.3-15.4 h, anaphase I at 15.4-16.6 h, telophase I at 16.6-18.0 h and metaphase II at 18.0-24.0 h[78]. The IVM culture period required for GVBD and abstriction of the first polar body was found to be related to the thickness and compactness of the COCs[79]. The bovine oocytes achieved developmental competency within 14 h of commencing IVM; suggested that early fertilization could lead to significantly higher yields of blastocyst[80]. Under routine IVM systems, maturation time usually 22-24 h in cattle[55].

**Simulated Physiological Oocyte Maturation (SPOM):** A novel *in vitro* maturation system that substantially improves embryo yield and pregnancy outcomes as in the study of Gilchrist and other scientists[81]. They reached to a new approach and a novel system to IVM known as Simulated Physiological Oocyte Maturation (SPOM), mimicing some characteristics of oocyte maturation *in vivo* and substantially improving oocyte developmental outcomes. Adaption of SPOM for clinical application should have significant implications for infertility management and bring important benefits to patients. Bovine or mouse cumulus-oocyte complexes (COCs) were treated with cAMP modulators for the first 1-2 h *in vitro* (pre-IVM), increasing COC cAMP levels ~100 fold. To maintain oocyte cAMP levels and prevent precocious oocyte maturation, COCs were treated during IVM with an oocyte-specific phosphodiesterase inhibitor and simultaneously induced to mature with FSH. Using SPOM, the pre-IVM and IVM treatments synergized to increase bovine COC gap-junctional communication and slow meiotic progression (both $p<0.05$ versus control), extending the normal IVM interval by 6 h in bovine and 4 h in mouse. The FSH was required to complete maturation and this required epidermal growth factor signalling. These effects on COC had profound consequences for oocyte developmental potential. In serum-free conditions, SPOM increased bovine blastocyst yield (69 versus 27%) and improved blastocyst quality (184 versus 132 blastomeres; both $p<0.05$ versus standard IVM). In mice, SPOM increased (all $p<0.05$) blastocyst rate (86 versus 55%; SPOM versus control), implantation rate (53 versus 28%), fetal yield (26 versus 8%) and fetal weight (0.9 versus 0.5 g) to levels matching those of *in vivo* matured oocytes (conventional IVF).

***In vivo* fertilization:** *In vivo* fertilization in the bovine takes place in the reproductive tract of the female when male and female gametes meet and combine. Normally, sperm is deposited just outside the cervix (natural service) or just inside the cervix (artificial insemination). Spermatozoa unite with oocytes in the oviduct, the tube connecting ovary to uterus. According to this relatively long journey through the uterus serves to select for the fastest swimming sperm, which are often those with the longest flagella[82]. However, it is not simply a matter of sperm meeting egg-much like the oocyte, sperm must undergo capacitation in order to successfully fertilize. This became clear when scientists proved in a rabbit model that sperm needed time in the female tract to acquire the ability to successfully fertilize an oocyte[83].

Capacitation in sperm means that it is prepared to undergo the acrosome reaction when it meets the zona pellucida. It involves stripping many of the seminal plasma proteins from the head of the sperm which were attached during ejaculation. Much of what is known about sperm capacitation comes from studies of sperm *in vitro*. *In vitro* studies have shown that capacitation[84] takes 4-6 h but these time points are not as well defined *in vivo* due to many factors specific to the uterine environment[85]. The aforementioned acrosome reaction involves fusion of the plasma and acrosomal membranes of the sperm head, which allows

proteolytic enzymes to leave the sperm head to help break down a small area of the zona pellucid to assist with sperm entry into the perivitelline space. The entry of a single sperm initiates a cascade of events inside the oocyte leading to the zona block which should prevent any other sperm from penetrating. This is important because an oocyte which is fertilized by more than one sperm (a phenomenon called polyspermy) is non-viable.

***In vitro* fertilization:** *In vitro* fertilization (IVF) showed itself as an equal if not greater challenge than IVM, likely owing to the fact that conditions needed to be optimized for two gametes instead of one. The foundational study can again be found reviewed in Wright and Bondioli[86]. After completing the aforementioned *in vivo* capacitation study, Chang[87] went on to carry out one of the first studies to demonstrate successful capacitation which involved flushing sperm from the uterus and oviducts of a rabbit bred 18 h previously to accomplish a low but meaningful rate of IVF (1959). This system of capacitation, although successful was not practical. The development of high ionic strength system of incubation which induced spermatic capacitation *in vitro* resulted in sperm capable of successfully fertilizing rabbit ova[88]. Just a few years later, the birth of the first calf was described as a result of *in vitro* fertilization[89]. The oocytes were matured *in vivo*, collected from the oviduct of donor heifers, coincubated with approximately 1 million freshly collected sperm (which had previously been incubated in a high ionic strength solution for 3 h) in 4 mL fertilization media and the resulting embryos were transferred surgically to the recipient cows who had a natural estrus at the same time as the donor when the embryo was at the 4 cell stage. One live birth (Virgil) resulted. This method, although preliminarily successful, was difficult to replicate and appeared to only study for certain bulls[84].

Further investigations into the composition of oviductal fluid (which induces capacitation *in vivo*) showed very high levels of glycosaminoglycans (GAGs) and a new focus for elucidating the induction of capacitation was found. The GAG heparin sulfate significantly improved fertilization, as well as that this improvement was reduced by the presence of glucose in the media[90]. Heparin binds to seminal plasma proteins present on the sperm head, eventually causing the number of these proteins to decrease. However, changes induced by capacitation do not stop at the surface-indeed, the discovery of heparin as a capacitation agent has aided in the understanding of intracellular changes in the sperm due to capacitation. In the intracellular environment of the sperm, heparin induces alkalinization, increases the amount of cAMP present and increases calcium concentrations both in the acrosome and the intracellular environment of the sperm head[84,91]. Glycolysis induced by the presence of glucose acidifies the intracellular environment (thus antagonizing the heparin-induced alkalinization), hence the reduction in capacitation in media including glucose. Since, its discovery, heparin has become the de facto capacitation agent in bovine IVF.

**Factors affecting *in vitro* fertilization (IVF):** Fertilization is a complex process, which results in the union of two gametes, the restoration of the somatic chromosome number and the start of the development of a new individual. Successful cattle IVF requires appropriate preparation of both sperm and oocyte, as well as culture conditions that are favorable to the metabolic activity of the male and female gametes[92]. The first report of successful IVM and IVF of cattle oocytes was in Japan[93] but the birth of calves was not reported until the study of Hanada *et al.*[94].

**Preparing sperm for fertilization:** Fertilization of the bovine oocyte involves a sequence of events in which the sperm: (1) Is motile (to reach the oocyte and move through the Zona Pellucida (ZP)), (2) Has the ability to undergo capacitation and express the Acrosome Reaction (AR), (3) Has 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 and (4) Able to fuse with the oolemma and be incorporated into the oocyte.

It is clearly important to have highly motile bull sperm available for IVF. This may be achieved by applying various procedures for isolating motile samples. There are also a number of chemical agents which may be employed to stimulate motility and AR of bull sperm and to maintain motility.

## FACTORS AFFECTING SPERM MOTILITY AND CAPACITATION

**Effect of bull as a source of variability in IVF:** Considerable variability was exists among bulls in the ability of their sperm to become capacitated. The High Ionic Strength (HIS) medium employed to capacitate bull sperm, from five different bulls, they recorded that *in vitro* fertilization rates varying from 14-46%, they illustrated that individual variation as one of the most important factors affecting sperm preparation by the HIS method[95]. Another researchers using other capacitation

medium recorded the same finding[96]. Moreover, the outcome of IVF examined when sperm from high and low fertility bulls were employed, the yield of IVF embryos for the high fertility bulls was double than that recorded for those in the low fertility group[97]. Individual bull variability may be related to the stage of season, age of animal, ejaculate sperm quality[92]. In this respect, seminal plasma may have been a source of variation in the sperm used in IVF as it contains: (1) Decapacitation factors and variation in the level of such factors may affect ejaculated sperm[98]. (2) Synthetic activity in oocytes is induced by sperm penetration[99] and (3) Sperm may differ in the time taken for them to capacitate and this may affect subsequent embryonic development of the oocyte after IVF. Moreover, Taft *et al.*[100] have recorded evidence that sperm from subfertile bulls may undergo the Acrosome Reaction (AR) and die prematurely. The use of pooled semen is a well-accepted method of minimizing male variability in cattle IVF study[101]. In contrary, Miller and Hunter[102] failed to find evidence of significant variation among 29 AI bulls in their capability to achieve IVF. This result was confirmed by another scientist who suggested that there was no predictive relationship between bull field fertility and *in vitro* embryo cleavage or developmental rates[103].

**Use of fresh or frozen semen:** Various studies have employed both fresh and frozen bull semen in their cattle IVF studies[104]. Those researchers concluded that fresh semen requires a longer capacitation period than frozen semen. Meanwhile, fresh sperm gave better penetration rates than frozen thawed sperm[105]. Frozen-thawed bull semen is likely to deteriorate more rapidly than fresh ones[26]. One problem in using fresh bull sperm, they have at least passed through an initial screening before freezing.

**Methods of sperm separation:** There have been many reports characterizing Percoll density gradient, swim-up, sephadex, glass wool and other sperm separation procedures for bovine spermatozoa. The recovery rate of motile spermatozoa was higher for sperm separated by Percoll rather the swim-up method. However, swim-up procedure resulted in more ova being penetrated than did by using Percoll method. Increasing number of sperm concentrations during IVF could eliminate this problem[106]. this adverse effect of Percoll is not due to Percoll particle *per se* butmay be ascribed to the effect of unbound PVP in the Percoll. For that reason, the presence of PVP stopped bull sperm motility[107].

**Artificial induction of capacitation and Acrosome Reaction (AR):** Capacitation is a process involving the sperm in a complex series of biochemical and physiological reactions. It is believed that the initial step of capacitation involve the removal and alteration of components derived from the seminiferous tubules, epididymis, vas deferens and seminal plasma, this would permit exposure of receptors sites, allowing sperm to interact specifically with oocyte receptors. Sperm capacitation can be achieved by different methods such as:

**Fertilization medium:** Treatment of semen with a medium of High Ionic Strength (HIS) like Brackett and Oliphant (BO) medium (osmolarity 360-390 mOsm) is described for capacitation of fresh bovine semen[89] and frozen bovine semen[108]. Another report demonstrated that the use of the HIS method as probably being limited to certain bulls; they did not regard the procedure as suitable for general application[109]. Additionally, many authors used TALP-medium for *in vitro* capacitation of bovine spermatozoa[110]. In this respect, a group of scientists observed that a significantly higher proportion of bovine oocytes developed to blastocyst stage after insemination with spermatozoa prepared by swim-up in Fert-TALP supplemented with heparin than by centrifugation in mBO supplemented with 10 mM caffeine-sodium benzoate[111].

**Use of heparin and caffeine:** Studies support the view that capacitation of bull sperm by heparin probably reflects the *in vivo* mechanism[112]. Heparin dosage and incubation period for sperm capacitation are important factors affecting bovine IVF and subsequent embryo development[113]. Heparin induces changes in the calmodulin (CaM)-binding properties of sperm proteins and induces a reduction in $Ca^{2+}$ concentrations during capacitation[114]. The capacitation of bovine sperm with heparin requires extracellular calcium, the maximal kinetics of heparin-induced capacitation occurs when extracellular calcium exceeds 10 μMl[91]. Changes in calcium triggre subsequent increase in cAMP, pH and tyrosine are known to be essential for capacitation[106]. Also, in another study reported a synergistic effect of 20 μg mL$^{-1}$ heparin and 10 nM caffeine in their capacitation treatment of frozen-thawed bull sperm[115]. It was evident that the optimum dose of the agent[116] was 100 μg mL$^{-1}$. Other reports suggested that the fertilization rate may be rapidly improved by adding heparin to the IVF medium at values varied between[117] 0.5-5.0 μg mL$^{-1}$. Preincubation period of 15 min was found to be satisfactory.

**Follicular fluid:** Various procedures have been examined for capacitating frozen-thawed bull sperm and the subsequent use of such sperm in IVF. Using frozen-thawed bull sperm preincubated in media containing bFF reported a sperm penetration[118] rate of 56%. It is clear that bFF contains many compounds (Glucose Amino Glycains (GAGs)) capable of capacitating bull sperm; for that reason, it has been used in bull sperm capacitation medium[119].

**Use of calcium ionophore (A23187):** Various authors have shown the importance of an influx of extra-cellular $Ca^{2+}$ into the sperm in the capacitation process. The calcium ionophore A23187 has been employed to achieve this influx[120]. The ionophore treatment resulted in hyperactivation and a functional AR in bovine sperm, enabling them to penetrate zona-free hamster oocytes[121]. Other studies compared treatment of bull sperm with A23187 and heparin, it was suggested that the simplicity of using the ionophore and the higher yield of embryos as the advantage of using that agent[122]. There are some evidences that the $Ca^{2+}$ influx is the result of calcium entering a non-mitochondrial compartment as a consequence of the equilibration of the ion across the mitochondrail and plasma membrane of the sperm and increase the respiratory activity of the sperm[123]. Although, the ionophore-induced AR is believed by some to be similar to the normal *in vivo* reaction in capacitated sperm. While, another report concluded that the ionophore-induced reaction is not the same as the natural event[124].

**Effect of glucose in fertilization medium:** In cattle, it is reported that glucose inhibits the role of heparin for inducing sperm capacitation[125]. On the other hand, for cattle oocytes inseminated in chemically defined medium, glucose is required for stimulating *in vitro* fertilization of bovine oocyte.

***In vivo* embryo culture and early embryo development:** In its natural environment, the developing embryo stays in the oviduct until 4-7 days post fertilization. The embryo is often at the morula or blastocyst stage by this point. Much of the study done in *in vitro* culture methods has shown that *in vivo* cultured control embryos hold advantages over their counterparts *in vitro*[126-128].

The developing embryo (both *in vivo* and *in vitro*) goes through several recognizable stages of growth. A one-celled structure where male and female pronuclei have fused is called a zygote[129]. In the first few mitotic divisions, growth is only in cell number, as the original cytoplasm of the oocyte is divided among the daughter cells. Each blastomere possesses the quality of totipotency, meaning that it could be induced to become any fetal or adult cell type. After the fourth cell cycle (when the embryo is made up of 8 cells), the embryo undergoes the "Maternal to embryonic transition" which means that the embryo is charged with the task of making its own mRNA instead of using the stockpiled maternal mRNA present in the oocyte. The embryonic genome is highly methylated (and thus unavailable for transcription) in the gamete, so it must be both de and re-methylated reviewed in Zhao *et al.*[130] and Kepkova *et al.*[131]. Not surprisingly, this process is a critical obstacle which is difficult for embryos to overcome. After the blastomeres become too numerous to count (16 cell stage, at the earliest), the embryo is called a morula. Further, this is usually the time at which the cells become differentiated into cells which make up the inner cell mass and those of the trophectoderm. Thus, although they are still pluripotent, they are totipotent no longer. The inner cell mass gives rise to the embryo proper as well as other extra embryonic membranes and the trophectoderm becomes the chorion or the fetal contribution to the placenta. The final developmental milestone is signaled when the embryo develops a fluid-filled cavity called a blastocoel, around which primitive endoderm will enclose the yolk sac. It is at this, the blastocyst stage, when many *in vivo* or *in vitro* produced embryos in commercial production are evaluated and transferred to appropriately timed recipients.

***In vitro* embryo culture:** In the *in vitro* production of embryos, two of the three pieces of the puzzle were in place.

Obviously, study continued to improve methods of maturation and fertilization but embryo culture stood as the final great frontier to *in vitro* embryo production. The benefits offered by IVC are easy to see, such as the ability to observe development in real time, as well as the ability to non-surgically transfer embryos at a later stage of development. The latter is absolutely essential for the widespread commercial application of *in vitro* embryo production. The early study in this area involving bovine embryos is reviewed in Wright and Bondioli[86]. Although each investigation provided some foundational frame study for the investigators who came later, there were a few discoveries which led to large leaps in our understanding of embryo culture and they will be the focus of this discourse.

The first several attempts at bovine IVC used a variety of media. a saline with egg white medium was used (which was first used by Hammond[132] successfully in murine embryo culture)[133]. Dowling's study focused on superovulated *in vivo* matured and fertilized embryos collected at varying stages post fertilization. One donor whose embryos were collected 4 days post insemination gave 148-cell embryos.

All of them divided once in 24 h of culture in the egg-saline medium. Other trial attempted culture of *in vivo* produced embryos at varying stages of development in bovine serum. Less than 1 division per embryo after culture were observed[134] for 72 h. Also, attempted to culture *in vivo* produced embryos in follicular fluid but observed no further development. In the same experiment, 3 of 4 embryos cultured in bovine serum at the 4 cell stage divided once but embryos cultured at other stages did not continue to develop[135].

Although the media choices of the initial attempts at *in vitro* culture were logical as they supplied protein, nutrients and growth factors, embryo culture in them was relatively unrewarding. Researchers set out to find exactly what type of fluid embryos were cultured in *in vivo*. The first scientists elucidated the ionic and protein contents of the oviductal fluid of sheep. Appropriately, the resulting media was called Synthetic Oviductal Fluid (SOF) and forms the basis for many ruminants *in vitro* production protocols of today[136]. The SOF was used for the first time in bovine embryo culture[137]. The *in vivo* produced embryos that started at the eight cell stage proceeded to the morula stage in 30% of embryos, which represented only the second time that a morula had been produced in culture. In the same study, oxygen tension was varied to better approximate the conditions of the oviduct (borrowing a page from murine embryo technology[138]. When $O_2$ levels were reduced to 5% (from the 21% found in the atmosphere) with 5% carbon dioxide and the balance nitrogen, development of 8 cell *in vivo* produced embryos proceeded appropriately and the world saw for the first time a bovine blastocyst as the result of *in vitro* culture. The lower oxygen tension is thought to reduce the load of reactive oxygen species present in the media[139].

Despite early success[140], low oxygen tension was not immediately adopted by all researchers of the time. However, after this relatively slow adoption, the large majority of embryo projects performed today have adopted the lower oxygen tension. In doing this, they have experienced similar successes in development. Early successes in bovine embryo development were often seen either from 1-2 cell embryos to the 8 cell stage or from the eight cell stage to morulae/blastocyst. Thus, the term "8 cell block" was coined to describe the insufficiency of culture systems of the day to support development through the fourth cell cycle. Interestingly, this "Block" occurs at the same point as Embryonic Genome Activation (EGA) or the shift in the embryo from the embryo's dependence on maternal mRNA to embryonic. The same pattern is seen in murine embryo development, where a similar "Block" is commonly encountered at the two cell stage, which again is the point of EGA for that species[140]. It is unclear if these two phenomena are related but the pattern certainly raises suspicion of a correlation. The application of low oxygen tension in bovine embryo culture was the first step in solving the 8-cell block[137].

Although he was not the first to successfully bypass it (possibly owing to the omission of amino acids from the culture media[141], his study laid the foundation for others to come after him. The embryos in the third and fourth cell cycle were most affected by *in vitro* culture conditions and would not proceed in development *in vivo* after *in vitro* culture for 24 h, even though embryos cultured for short periods *in vitro* at other points in development could proceed to the blastocyst stage after *in vivo* culture. Vital staining showed that this cessation of development was not due to embryo death but a suspension in embryo development. In addition to low oxygen tension, somatic cell co-culture has also been shown to overcome this "Block[142]".

## CELLULAR CO-CULTURE

In a page borrowed from murine embryo culture[143], investigators discovered that somatic cell co-culture was beneficial to bovine IVC and helped to overcome the aforementioned "8 cell block." One of the first bovine experiments to use this approach was carried out by Camous *et al.*[144]. *In vivo* produced embryos were cultured in media including serum with and without trophoblastic vesicles. Trophoblastic vesicles are made from *in vivo* produced day 14 bovine embryos which have had their inner cell mass removed. They have been shown to persist in *in vitro* culture for at least 5 days and to maintain corpora lutea *in vivo*[145]. The results showed that the previously identified block to development was overcome in both treatment groups butto a greater extent in the co-culture group (46 vs 18% development to morula). These results, while encouraging, presented difficulties in replication as they depend on a constant fresh supply of bovine blastocysts because the trophoblast could only be maintained in culture for around 5 days. Another co-culture system had previously been reported to improve development of *in vivo* produced mouse embryos[143]. This study used whole-organ co-culture of the fallopian tube with embryos and noted the ability of embryos to bypass the 2 cell block (analogous to the 8 cell block in the bovine). This makes sense, because if the cultured oviductal cells behave similarly *in vitro* as *in vivo*, they may be expected to approximate the *in vivo* environment by producing growth factors and removing wastes. culturing *in vivo* produced ovine embryos on an oviductal epithelial monolayer significantly improved development to the

blastocyst[146]. Thus, another investigators chose to focus on a co-culture of oviductal cells in bovine embryo culture[142]. This was a seminal paper for two reasons: It was the first to show the beneficial effect of oviductal co-culture (or oviductal conditioned media) on bovine embryo development and it was the first to demonstrate a fully *in vitro* system of embryo production which resulted in the birth of 5 normal, healthy calves. With these promising results, somatic cell co-culture was soon adopted by many groups studying embryonic development of a wide variety of species. The benefits seen included faster cleavage, higher blastocyst rates, increased cell numbers, better hatching rates, increased resultant pregnancies and most importantly, increased live births[147]. However, co-culture was not a one-size fits all approach. A few drawbacks were quickly identified, including the differing media needs of cells and embryos, the possibility of viral transfection from cells to embryos and the inherent struggles of reproducibility that come from using undefined biological substrates. One additional difficulty of co-culture is the often necessary inclusion of serum in the culture media, which has been previously established as a source of both positive and negative effects on the embryo.

A type of co-culture which has yet to show negative effects on embryo development is the common practice of group embryo culture. Although they are not somatic cells, they are still able to take from and contribute to the culture environment in a way that has over and over again been shown to be helpful to embryos. The origin of ruminant group culture may well be a holdover from *in vitro* murine embryo study[132], where multiple embryos develop together *in vivo*. As well, this tendency may have come as researchers realized that less development resulted from individual culture[141]. In any case, group culture often stands as the standard to which individual culture is compared.

## SERUM ADDED TO CULTURE MEDIA

Serum was found to have a beneficial effect on embryo development almost as soon as *in vitro* production methods were developed. At that time, due to the high level of interest in human IVF and close ties between researchers in both human and animal fields, the additive of choice for researchers in some parts of the world (especially Australia) was human serum[148]. Researchers in other parts of the world used bovine serum. This provided an ample source of protein, growth factors and other hormones. Owing to its biological origins, the exact composition of both human and ruminant source serum, however, differs from batch to batch and thus it remains an undefined medium. One study compared embryos cultured in SOF with those cultured in SOF+10% Fetal Bovine Serum (FBS) showed that the development to blastocyst was faster and approximately doubled by the addition of serum (16 vs. 30% oocytes developed to blastocyst)[149]. This effect was maintained even when the SOF was supplemented with glucose (0.67 mM) (15% of oocytes developing to blastocysts) or the FBS was de-salted (components less than 5 kDa removed) (29% of oocytes developing to blastocysts) suggesting both that the increased development was due to components of serum which were over 5 kDa in mass and not simply the added glucose. A similar increase in development with SOF supplemented with serum (34.4 vs 47.5% of cleaved embryos to blastocyst)[150]. This study also showed an interesting effect of serum on sex ratio. Whereas the blastocysts formed from embryos cultured in SOF tended to be around half (51%) males, those embryos cultured in SOF with serum had a statistically significant increase in males (57%). The same researchers showed in a different study that male embryos cultured in serum tend to develop faster but the sex ratio is evened by day 10 in culture[151].

However, this increased development came at a cost, as investigators quickly saw a trend in heavier birth weights, termed Large Offspring Syndrome (LOS). The human serum-cultured ovine offspring in a study also showed other perturbations, such as longer gestation (which could not account for the increase in birth weight) and increased deaths in the perinatal period. These differences persisted in ruminant embryo culture even after the serum source was changed to fetal bovine serum. The mechanism of these changes is not fully understood but is thought to be the result of impaired methylation of the embryonic genome[152].

One gene which has been hypothesized to be involved in large offspring syndrome is insulin-like growth factor, an imprinted gene[153]. The DNA methylation is an epigenesis change which is often involved in down regulating transcription. However, in sheep feti cultured in serum as embryos (and experiencing large offspring syndrome), a complete loss of methylation was found in the Insulin-like growth factor 2 receptor (IGF2R) and these same feti had a 30-60% reduction in IGF2R in tissues[154]. The researchers purported that the marked differences in body weight seen may be attributable to differences in IGF2 experienced during other times in gestation or to differences in IGF2 binding protein concentrations. These changes are seen both in the preimplantation embryo[155] as well as in the fetus[156]. Interesting study done in murine embryos cultured with either bovine serum or Bovine Serum Albumin (BSA) suggests that pups who were cultured in serum as embryos continued to show differences up to nearly 2 years of age. The serum pups

showed higher rates of pneumonia, larger organs (heart and liver) and differences in motor skills which persisted[155]. This study may have been confounded by the unusually poor development in the FBS compared to the BSA groups (43 vs 95%) as well as the lowered survival to parturition (23 vs 44%). It is also important to note that this was a murine model; however, such long term studies have not been carried out in ruminants. Soon after seeing these unfortunate outcomes of serum supplementation, investigators turned to other avenues which would lead to equivalent development without serum. One early study showed that healthy (measured by total cell numbers) blastocysts could be produced in serum-free conditions[141]. However, they found several things necessary in the absence of serum: amino acids must be present in the media, the media must be changed frequently to lessen the concentration of ammonia and the embryos must be cultured in groups. Additionally, many formulations of serum replacer are available today, which, while somewhat undefined (as the exact formulation is proprietary), should be a more consistent formula than FBS. Oocytes and embryos which were matured and cultured in serum substitute in human *in vitro* embryo production outperformed both in morphologic assessments and pregnancy rates their serum-containing counterparts[156].

## MODERN MEDIA CHOICES

For all of the aforementioned reasons, modern embryo culture is trending toward cell-free, serum-free conditions. This results in more defined protocols which are potentially easier to replicate in different laboratories around the world. However, finding the right constituents of media which successfully support embryos through the 8 cell block and help them to form healthy blastocysts has proven a considerable task. Media additives for group culture which have shown some promise include defined amino acids[141], transforming growth factor β and basic fibroblast growth factor[157] and polyvinyl alcohol, insulin, transferrin, selenium, myo-inositol and epidermal growth factor[158].

## ASSESSMENT OF *in vitro* PRODUCED EMBRYO QUALITY AND HEALTH

There are several ways to objectively measure the health and quality of *in vitro* produced embryos. The most obvious measure is to transfer them to synchronized recipients and assess pregnancy and calving rate compared to *in vivo* produced embryos transferred to another group. Indeed, although this might serve as the "Gold standard" test of embryo health, it is often not feasible or practical due to using oocytes of unknown genetic quality or financial constraints of research. Thus, less cumbersome methods of embryo quality assessment have necessarily arisen. There is a validated method of evaluating *in vivo* produced embryos which was developed by members of the International Embryo Transfer Society. These guidelines concern percentage of cellular material which forms the embryonic cell mass as well as the stage of development compared to the expected stage. They serve to inform decisions about transferring, freezing and discarding *in vivo* produced embryos[159].

The IETS grading system allows assessment of live embryos intended for transfer. However, since many research projects have blastocyst development (rather than pregnancy establishment) as their endpoint, researchers can use more invasive methods of measuring embryo health which are not concerned with embryo survival. Application of cell counting techniques to *in vitro* produced bovine embryos for the first time[122] that having been previously applied to the pig[160]. The results validated this method as they showed that higher morphologically graded embryos had higher total cell counts. The cell counting method was advantageous because it removed the subjective element of embryo grading. As well, embryo grading has been shown to be a poorer predictor of pregnancy establishment in *in vitro* (vs *in vivo*) produced embryos[161]. At around the same time, other study applied the immunosurgery techniques to the bovine embryo which had been developed previously in mouse embryos[162,163]. This technique involved selectively permeabilizing the nuclei of the trophectoderm layer of the embryo to allow fluorescent stains to penetrate, which should leave the Inner Cell Mass (ICM) nuclei intact and only penetrable by vital stains. As a result, differences between *in vivo* and *in vitro* cultured embryos were illuminated, as *in vivo* embryos had higher total cell as well as ICM numbers than their *in vitro* cultured counterparts in each developmental stage examined. Another study compared the timing of blastocyst development to total and ICM cell number and found that later developing blastocysts tended to have fewer total ICM cells, a trend which was true of both *in vitro* and *in vivo* produced embryos. As this method became more popular and more data was gathered, *in vivo* produced embryos consistently showed that 30-40% of their total nuclei were made up by inner cell mass cells and this number became a benchmark by which *in vitro* produced embryos were measured[164].

## IMPORTANCE OF *in vitro* PRODUCTION TO THE CATTLE INDUSTRY

According to the latest IETS statistics[165], 1,877,400 oocytes were collected from 128,444 donors largely obtained from ultrasound-guided ovum pickup with a much smaller proportion received from the abattoir. This resulted in 457,455 embryos. Over 350,000 of these embryos originated from South America, mostly Brazil. The total number of embryos collected worldwide in 2012 was 1,143,119. The fact that *in vitro* produced embryos now account for nearly 1 of every 2 embryos produced worldwide should fuel investigators everywhere to develop improved methods of embryo production which are suitable both for the lab as well as for use in clinical practice.

## CONCLUSION

In conclusion, *in vitro* production of bovine embryos is an efficient tool to produce animals of higher genetic merit and its use has increased, mainly in developing countries in South America and Asia. Several factors can influence the success of *in vitro* embryo production and increase or decrease the embryo yield and gestational viability. Differences between donors, sperm and breeds should be taken into account in regard to *in vitro* embryo production. Single breeds adapted to the environment will certainly overcome the effects of heat on oocyte quality and embryo yield. *In vitro* culture environment is another factor that greatly influences embryo production. There are different culture systems and culture media available for *in vitro* fertilized bovine embryos and embryo yield generally varies among them. The efficiency of culture systems may also vary among labs, making data comparisons difficult. Therefore, it may be advisable to test what culture system or culture medium will provide better results for a given lab.

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