—Review—

Culture Media and their Components and Environmental Factors Affecting Embryo Development In Vitro

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A number of defined and undefined media have been used for *in vitro* embryo culture. For long term culture of embryos, however, more complex media are required [1–8]. They are described as complex media because of the numerous components which are included in their formulation and include vitamins, amino acids, salts, purines, nucleotides, etc. Most of these media are bicarbonate buffered, and must be kept under an atmosphere of 5% CO_2 in order to maintain the proper pH. Hepes or phosphate buffered media offer an advantage in that they do not require a CO_2 controlled gas phase to maintain a relatively constant pH. Development of human embryos occurs in a wide range of culture media with different chemical compositions [9]. Even the complete omission of all proteins and amino acids has no significant effect on human embryo development or viability [10].

Although improvements in embryo culture methods have progressed rapidly and now defined conditions are available in which processes of oocyte maturation, fertilization and embryo development in cattle can progress to the hatched blastocyst stage entirely *in vitro*, embryos produced by *in-vitro* maturation are less competent, especially those arising from defined media. The objective of this article is to review various culture media and components used for *in vitro* culture of preimplantation embryos in a cell free system.

Energy Substrates

Studies on mouse embryos revealed that changes in energy metabolism occur during development. The most pronounced change occurs with glucose utilization. The 2-cell mouse embryo did not metabolize glucose, but utilized pyruvate, unlike the 8-cell embryo, which utilized both substrates [1, 11–13]. Early mouse preimplantation embryos had a low rate of energy metabolism, mainly due to limited activity of certain enzymes of the Kreb's cycle [11], but the advanced-stage embryos such as morula and blastocysts were capable of increased metabolic activity since there was nearly full development of energy producing enzymes. Maximum blastocyst development (>85%) from hamster 8-cell embryos was achieved by culturing for 18 hours in a chemically defined glucose-free and protein free modified Tyrode's medium (TLP-PVA) with amino acids (Phe, Ile, Met and Gln) [12]. Glucose not only inhibited embryo development when used as the sole energy substrate but also inhibited development in the presence of lactate and/or amino acids. The presence of inorganic phosphate in the culture medium was obligatory for glucose inhibition of the development of hamster 8-cell embryos *in vitro* [13].

There are now several studies examining the carbohydrate utilization and requirements for both bovine and ovine embryo development [14–20]. As with most other mammalian species, glucose utilization by bovine embryos generally increases with development [21]. There is sufficient evidence to suggest that D-glucose supplementation of the bovine embryo culture medium is not required until approximately day 3 or 4 of development, at which time the supplementation improves development [16]. Nevertheless, a high glucose concentration is also detrimental to the *in vitro* bovine embryo development [22].

Although not obligatory, development is greatly enhanced in the presence of pyruvate and/or L-lactate

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[15–17, 20] with an optimal pyruvate/D,L-lactate ratio of 1:10 for ovine embryos [20]. There has been little work performed on adjusting the levels of pyruvate and lactate with development in either bovine or ovine embryos [20]. Accumulation of L-lactic acid may be a potential toxin during embryo culture, as Thompson *et al.* [20] have shown that high levels (33 mM Na D,L-lactate) inhibited ovine embryo development *in vitro*.

Serum Supplementation

It has been shown that the addition of biological fluids, such as blood serum, improves fertilization and early cleavage rates in numerous species [23–25]. It is generally believed that the positive effect of serum is due to cyclic adenosine monophosphate, catecholamines, vitamins, putative growth factors, lipids and albumin [25]. Serum brings several classes of lipids, i.e., triglycerides, fatty acids, lecithin, and cholesterol P, generally bound to proteins. These components cannot be added to synthetic media because of their very low solubility except for free cholesterol [26].

The advantage of using fetal calf serum is due to some undefined growth promoting components (such as fetuin) that are absent from the serum of adult animals and that fetal serum lacks components i.e. hormones and immunoglobulins present in adult serum that retard the development of cells *in vitro*. Different types of sera have widely been used in various types of culture media either alone or in combination with growth factors [26–28]. The addition of FCS gave significantly higher rates of fertilization (57–71%) than did ECS (34–52%). The addition of fetal calf serum to the MSOF medium before the 5–8-cell stage accelerated the development of the 9–16-cell stage bovine embryos into morulae [27]. Menezo's B2 medium supplemented with BSA or PVA was capable of maintaining bovine embryonic development to the blastocyst stage, but the yield of transferable embryos was improved by the presence of serum, and without serum or co-culture the development rate was lower [28].

Sera are generally heat-treated at 56°C for 30 min for the removal of complement which has been shown to be toxic to embryos [29]. Wright *et al.* [30] found that premorula bovine embryos developed better in a variety of complete media supplemented with 10% rather than 20% heat treated FCS.

Protein Supplementation

Serum albumin has been the traditional choice of protein supplements. It is an obvious choice as it is the major protein found in reproductive tract fluids. Furthermore, the chelating and binding properties of albumin potentially have beneficial roles, for example, chelating metal cations and preventing non-specific binding of embryos to other surfaces. Bovine serum albumin has been used as a media supplement for its beneficial effect on development and blastocyst hatching, but BSA is not obligatory for normal embryo development [31–33]. The uptake of protein, and in particular albumin, as a means of transporting other molecules into the embryo, is an intriguing possibility that requires further investigation [34]. At this stage, the evidence that protein may constitute a source of metabolic energy is scant, as ATP requirements for the bovine embryo at least, can be fulfilled by the metabolism of carbohydrates [35].

Amino Acid Addition

Since the advent of *in vitro* embryo culture procedures for preimplantation mouse embryos in 1956 [36], amino acids have gone from being considered unimportant to clearly beneficial [37–42]. The addition of essential amino acids during the preimplantation period increases the probability of fetal development [42]. It was suggested that glutamate and aspartate may be involved in signaling among embryonal and uterine tissues near the time of implantation [43]. In addition to their role in protein synthesis, amino acids such as aspartate, glutamine and glycine may be utilized in the synthesis of purines and pyramidines, and some, including glutamine, have been regarded as potential energy sources [44]. Taurine has been shown in several studies [40, 45, 46] to increase both the proportion of 1- or 2-cell embryos that develop to blastocysts *in vitro* and the number of cells that the resultant blastocysts contain. Taurine exerts its beneficial effects exclusively during the first two days post insemination, especially during the 2nd day after fertilization when embryos are at the 2-cell stage [45]. Glycine greatly increases the proportion of preimplantation embryos that develop into blastocysts in medium containing relatively high concentrations of inorganic ions in oviduct fluid [38]. Alanine also improves development, but neither leucine nor taurine is beneficial under such conditions. Glutamine was observed initially to help preimplantation mouse embryos obviate the 2-cell block [37]. This amino acid was subsequently found to protect embryos against the inhibitory effects of a relatively high NaCl concentration under conditions in which a 2-cell block was not detected [47]. L-cysteine increased the number of cells in bovine IVM/IVF blastocysts [48], and ovine oviductal concentrations of amino acids were also

beneficial to hatching rates of bovine embryos produced *in vitro* [49].

A consequence of amino acid addition to embryo culture medium is the accumulation of ammonium ions [50, 51]. The toxicity of ammonium has yet to be determined for bovine embryos but is in the μM range for mouse embryos and produces fetal abnormalities following transfer [50]. Ovine embryos are also sensitive to ammonium accumulation as a result of amino acid metabolism and breakdown [50, 51]. This problem is currently overcome by either of two methods: replacing the medium with fresh medium before accumulation reaches toxic levels, or enzymatically removing the ammonium from the culture system [50].

Water Soluble Vitamins

The expansion of rabbit blastocysts in culture require a group of eleven B vitamins and growth factors present in Ham's F10 medium [52]. These vitamins are required for hatching hamster embryos *in vitro* [53]. No liposoluble vitamins appear to be necessary in the early stages of human embryo development, and only hydrosoluble components added to the medium may be useful [26].

Growth Factors

Various growth factors derived from blood, tissues or produced by cells *in vitro* have been used successfully in mammalian cell culture. Endothelial cell growth supplement, a commercially available extract of bovine neural tissue has been shown to be a superior growth supplement for the proliferation of human umbilical cord venous endothelial cells even in as low a number as 1.25 cells/ cm^2 [54]. Fibroblast growth factor, another commercially available extract of the bovine pituitary gland has been shown to be mitogenic for mesoderm derived cells [55] and may be the compound in bovine fibroblast which allowed for superior bovine embryo development in co culture of bovine embryos with bovine fibroblast [56].

The effect of endothelial cell growth supplement and fibroblast growth factor on early mammalian embryo development *in vitro* has been studied by Larson *et al.* [57]. Their data suggested that the addition of transforming growth factor beta (TGF beta) and basic fibroblast growth factor (bFGF) to serum free cultures of *in vitro* produced, 2-cell bovine embryos produced developmentally competent bovine blastocysts. The effects of TGF beta can be potentiated by bFGF; FGF itself is an effector of protein synthesis and a potent mitogen. A positive interaction between the 2 growth factors resulted in 38.8% of fertilized oocytes maturing beyond the 16-cell stage; of these, 24.6% formed blastocysts. These results support the hypothesis that TGF beta and bFGF act synergistically to promote the development of bovine embryos beyond the "8-cell block" observed *in vitro*.

Whittingham's T6 medium supplemented with insulin or a mixture of growth factors, hormone, and other substances in NU-Serum, which has been used as a serum replacement in media for growth and maintenance of a variety of cell types *in vitro*, had a only a minor influence on promoting embryo development when compared to rate of development *in vivo* [58].

The addition of growth factors to bovine embryo culture medium has revealed that the following enhance development: Epidermal Growth Factor, Transforming Growth Factor-β and Platelet Derived Growth Factor (PDGF) [59–62]. Both Gardner *et al.* [51] and Keefer *et al.* [63] have found that "grouping of ovine and bovine embryos" (respectively) had significant beneficial effects on development, demonstrating auto/paracrine activity. This was further characterized by Thibodeaux *et al.* [62], who found that PDGF was indeed acting as a paracrine growth factor. There is also growing evidence that cytokines play a facilitative role in embryo development [59] and these are likely to interact with growth factors.

Hormones

Estrogen has been implicated in the early embryonic development of rodents, rabbits and pigs. It has been suggested that estrogen is necessary for normal implantation in rodents [64], A specific uptake of estradiol-17β by day-5 pig blastocysts suggested that this uptake was dependent on embryo viability and the presence of specific binding sites for estrogen [65]. Estrogen required for *in vitro* development may be delivered by various sera which are usually added to culture media in order to stimulate development [66]. Sera are known to contain sex steroids apart from a number of other substances [67]. Hormonal supplements (LH, FSH and E2) during *in vitro* maturation of bovine oocytes enhance their subsequent development [68]. Prostaglandin F2α had an accelerating effect on hatching. A high concentration of PGE2 exerted a cytotoxic effect on blastocysts [69]. The production of prostaglandins in blastocysts participated in hatching, as some inhibitors of prostaglandin biosynthesis inhibited hatching of mouse blastocysts [70]. Insulin does not significantly increase glucose uptake by preimplantation mouse blastocysts *in vitro* [71].

Ions

The ions required for successful embryo development include K^+, Ca^{+2}, Na^+, Mg^{+2}, Cl^-, PO_4^{-3} and HCO_3^-. Embryo development occurred over a wide range of K^+ concentrations (0.6 m to 48 mM) and was almost completely inhibited by the absence of K^+ [72]. The absence of Ca^{+2} ions in media has been shown to inhibit cleavage and prevent the compaction of mouse morulae, but normal development has occurred over a wide range (0.4 to 10 mM) of Ca^{+2} concentrations [73, 74]. The complete lack of PO_4^{-3}, Mg^{+2} and SO_4^{-2} in media had little effect on the development of mouse embryos to the blastocyst stage [73]. The role of NaCl is primarily for the osmotic balance of the medium. An important role of HCO_3^- in culture medium is the regulation of pH. Studies by Brinster [75] showed that the removal of HCO_3^- and CO_2 from the culture medium resulted in reduced development. Further studies showed that the mouse embryo fixed CO_2 starting at the 8-cell stage and continued to the blastocyst stage, when overall embryo metabolism is highest [76–78].

An important consideration is the varying composition of oviductal fluid during the estrus cycle and also along the length of the oviduct [79, 80], especially in relation to Ca^{+2} [79]. Furthermore, composition is regulated [80–82] and recently a purinergic receptor has been reported which may be involved in regulating ion transport across oviduct epithelium [81], but little has been done to mimic these changes in ionic composition during *in vitro* culture [82].

Osmolarity and pH

The osmolarity of commonly used media is in the range of 250 to 300 mOms. The use of a variety of media with different osmolarities suggests that the effect of osmolarity on embryo development is limited [66, 83]. Bowen *et al.* [84] determined that bovine embryo development was improved when the osmolarity of SOF medium was 270 mOsm compared to 300 mOsm. Intracellular osmolarity has been suggested as playing an important role during *in vitro* development [85, 45]. The addition of an intracellular osmolyte, such as glycine, has been reported to facilitate and improve bovine embryo development [79]. However, it has yet to be determined if this effect is mediated by reducing the effect of "Na^+-loading," as suggested by the data obtained for the mouse [80, 85].

The pH of commonly used media range from 7.1 to 7.4. Bovine serum albumin has a tendency to lower pH from the physiological range when used at a concentration of 32 mg/ml. Most bicarbonate buffered media are usually equilibrated with 5% CO_2 in air. A major function of CO_2/HCO_3^- in culture media is to act as a buffer. The presence of CO_2 may be necessary as a regulator of intracellular pH, but dependence on CO_2 is not absolute for embryonic development, as ovine embryos will develop to the blastocyst stage when buffered with zwitterionic buffers [87, 88] in a CO_2 free atmosphere. The importance of intracellular pH in a wide variety of cellular mechanisms has been clearly identified (for review see [89]) and internal pH regulation of mouse embryos has recently been reviewed [90]. Nevertheless, the effect of altering the intracellular pH on bovine embryonic development has yet to be fully examined. Like other body fluids, the pH of oviduct and uterine fluid is regulated by the concentration of HCO_3^- and equilibration with CO_2, and varies with the cycle stage [91, 92].

Oxygen

There are a number of reports concerning the effect of the oxygen concentration on bovine and ovine embryo development [93–97]. The evidence demonstrates that a reduction in the concentration from that found in air (approximately 20–21%) to below 10% is associated with increased rates of development and reflects the levels found *in vivo* [95]. High oxygen tension had detrimental effects on bovine embryonic development, probably due to free radicals, including oxygen radicals. Hypotaurine addition exerted beneficial effects on the development of bovine embryos [98].

Oil

The effect of 5 different oils (washed/unwashed) used to cover culture microdrops was analyzed by Borque *et al.* [100]. After chemical washing with oil, the developmental ability of mouse embryos improved significantly (77% vs. 53%; $P<0.01$).

Temperature

Although it is well established that *in vitro* fertilization occurs optimally around 39°C [100, 101], there is surprisingly little information on the optimal developmental temperature for bovine embryos. Wang *et al.* [102] have reported that when *in vitro* development of bovine IVP embryos occurred between 36°C and 40°C, most embryos developed to the blastocyst and hatching

blastocyst stage at 39°C, but there is little information on how temperature changes during development affect developmental capacity [82]. The recent work of Ealy *et al.* [103, 104] has begun to address this question, with particular emphasis on temperature sensitivity and thermotolerance. Their data demonstrate that bovine embryos become more resistant to heat shock as development progresses.

Antibiotics

Antibiotics are added to *in vitro* cultures to prevent bacterial and fungal growth. The most common supplements are penicillin and streptomycin.

Block to Development

With the exception of some inbred and some F1 strains, one cell mouse embryos cease development *in vitro*, and this is termed a "2-cell block" [4, 105]. As a reason for the cell block, a cytoplasmic component(s) has been demonstrated to peak in activity during the transition between G2 and M phases and decline thereafter, and probably plays a role in the regulation of cleavage of the cultured mouse embryos [106]. It has been shown to be a function of the mouse strain [4, 107], media components [1, 108] and culture conditions [108, 109].

The mouse is not the only species which exhibits a block to development *in vitro*. The developmental block in hamster embryos occurs at the 2 and 8 cell stages [110]. Cattle embryos cease development at the 8–16 cell stages [111], porcine and rat embryos at 4-cells [112, 113], the exceptions being embryos of rabbit [67], man, rhesus monkey [114] and some strains of mouse [106].

Various methods have been employed to circumvent the problem. The explanted mouse oviduct [115], cross species preimplantation embryo development *in vivo* [116], and various culture techniques [119, 120] have been used successfully, but understanding of the mechanism of the blocks to development is still minimal. Schini and Bavister [119] reported that the common culture medium components phosphate and glucose block *in vitro* development of hamster 2-cell embryos probably by interfering with efficient production of ATP. Elimination of glucose from their medium resulted in development of 27% embryos beyond the 2-cell stage. Research on some other aspects such as the reduction of the embryo: medium volume [119, 120], addition of transforming growth factor beta & basic fibroblast growth factor to medium [61], and supplementation of bilirubin [121], activin A [122], transferrin [123] and superoxide dismutase and thioredoxin [124], has been carried out with varying levels of success. On the other hand, materials such as lipid peroxides derived from polyunsaturated fatty acids [125], oxygen radicals [126], purines [127], iron [123] and high affinity iron chelator (desferal) [123] are reported to block development of mouse embryos *in vitro*.

Major Problems

Enhanced fetal growth can result from *in vitro* culture of embryos [128–131]. The transfer of cultured sheep embryos resulted in significant increases in mean birth weight, gestation length, incidence of dystocia, and lamb mortality compared to control embryos [129–132]. the birth weight of several lambs was well outside the upper limit of the normal population [131]. The increase was obtained when embryos were cultured from the zygote stage for 3 or 5 days. In the cow, significantly heavier fetuses were obtained following the transfer of cultured IVM/IVF embryos compared with fetuses produced from *in vivo* counterparts [129]. Murine embryos cultured from the 8-cell stage developed into significantly smaller fetuses [132] and human embryos produced by IVF also developed into babies with a reduced mean birth weight compared with that of the general population [134]. Culture of sheep [135, 136] and bovine embryos result in a reduced number of blastocyst cells [137], a skewed ICM/trophoectoderm ratio, precocious cavitation and reduced survivability following transfer when compared to embryos obtained *in vivo* [135–139]. The factors responsible for this variation needs further investigation.

Conclusion

Improved embryo culture techniques have led to a better understanding of the cellular and molecular mechanisms that regulate embryo development during the preimplantation period, but the developmental competence of cultured embryos is compromised irrespective of their source of derivation, whether produced *in vitro* or *in vivo*. Little has been done to mimic the changes in ionic composition during *in vitro* culture [82]. Recently computer generated artificial intelligence has also been utilized for the formulation of embryo culture media [139], but the rate of development of cleaved *in vitro* produced embryos to the blastocyst stage has not changed over the past 5 years (1991–95) in any of the culture systems used.

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