—Review—

# Factors Affecting Development In Vitro of Bovine and Rat 1-Cell Embryos

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Development of culture systems to support early embryonic development in mammals is quite important not only for basic research to clarify the mechanism controlling embryological development, but also for the application of new technologies to provide transgenic animals. Such development would be achieved by accumulating knowledge on the physiological requirements of embryos in culture. Studies on the culture of mammalian preimplantation embryos have progressed greatly [1–3] since the development of a biological medium containing egg white and egg yolk [4] and a chemically semi-defined medium with bovine serum albumin [5] for mouse embryos, but we have not yet found culture media capable of supporting complete preimplantation development of embryos in most mammalian species.

One of the greatest obstacles to the development of complete culture systems was that embryonic development was blocked *in vitro* at species-specific stages, whereas no such *in vitro* block of embryonic development is usually apparent in primates and rabbits. For example, *in vitro* development of embryos is blocked at the 2-cell stage in hamsters [6, 7], outbred mice [8–11] and rats [12–14], at the 4-cell stage in pigs [15] and at the 8-cell to 16-cell stages in cattle [16, 17] and sheep [18]. Several methods have been employed to overcome the developmental block. Explanted mouse oviduct can maintain the development of mouse 1-cell embryos to the blastocyst stage [19, 20]. Cultured mouse oviducts are also known to support the *in vitro* development of hamster 2-cell embryos [21, 22] and support the development of pig 1-cell embryos [23]. In addition, successful embryonic development has been achieved in co-culture systems with various somatic cells [24–41], oviductal fluid [42, 43] and conditioned media which are prepared by preculturing oviductal tissue [30, 44]. Under these culture conditions, however, it is quite difficult to clarify the embryotoxic or embryotropic factors because various unknown factors which are secreted from somatic cells or included in supplemented fluid are present in the media.

Schini and Bavister [45] have found that phosphate and glucose which are common components in culture media are associated with the *in vitro* block of development of hamster 2-cell embryos and have developed a chemically defined, phosphate/glucose-free medium. This medium, designated as hamster embryo culture medium 1 (HECM-1), supports the early development of hamster embryos *in vitro* beyond the 2-cell and 4-cell stages to the blastocyst stage [45–48]. Although HECM-1 was applied to the culture of 1-cell embryos in other species, such as cattle [49] and rats [50], proportions of embryos developed to the blastocyst stage were very low. We have recently conducted a series of experiments to modify HECM-1 and examined the effects of some chemical and physical factors in the early development of bovine and rat 1-cell embryos. This review aims to present recent information obtained in culturing early mammalian embryos with special reference to the characteristics of culture conditions affecting early development of bovine and rat embryos.

## Effects of Phosphate and Glucose

Detrimental effects of phosphate and glucose on embryonic development have been examined in various mammalian species [51–64]. In hamsters, phosphate inhibits the development of 4-cell embryos to the blastocyst stage [61], and glucose alone does not block the development of 4-cell [62] and 8-cell [63, 64] embryos. However, glucose in the presence of phosphate inhibits the development of 8-cell embryos [63, 64], but not 4-cell embryos [62]. Glucose is detrimental to mouse

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embryos until the morula stage, although glucose supports embryonic development from the morula to the blastocyst stage [51, 52, 54, 55]. In cattle, development of 1-cell embryos to the blastocyst stage is not blocked by phosphate alone [49] but noticeably blocked by glucose alone [35]. We have also demonstrated that the blastocyst formation of bovine embryos in a modified Tyrode's solution (mTLP-PVA, Table 1) is inhibited by glucose regardless of the presence of 1.05 mM phosphate [65]. An adequate concentration (0.35 mM) of phosphate appears to be essential for the development of bovine embryos beyond the 8-cell stage in mTLP-PVA [65]. The addition of glucose at 120 h postinsemination improves development of bovine 1-cell embryos to the blastocyst stage in the absence [66] and presence [65] of phosphate. In rats, in contrast, phosphate at a relatively low concentration (10 $\mu$M) completely blocks the development of 1-cell embryos beyond the 2-cell stage in a chemically defined medium, designated as rat 1-cell embryo culture medium (R1ECM, Table 2), but very low concentrations (0.001–0.01 $\mu$M) of phosphate do not affect the development to the blastocyst stage [67]. Glucose does not affect the development of rat embryos to the morula stage in R1ECM [67], but adequate concentrations (7.5–10.0 mM) of glucose appear to stimulate the development of morulae to the blastocyst stage [67]. Although the supplementation of mR1ECM with 0.4 mM phosphate after 0–64 h of culture reduces the percentage of rat embryos that develop to the blastocyst stage, the addition of 0.4–1.2 mM phosphate after 80 h of culture accelerates blastocyst formation and increases the number of cells in blastocysts [68]. Therefore, phosphate and glucose seem to affect embryonic development in different ways among different species and also different developmental stages of embryos. Phosphate and glucose appear to be important factors in the development of bovine and rat embryos, especially beyond the morula stage.

It is still under debate how phosphate and glucose inhibit early embryonic development. A strong opinion is that inhibition by phosphate and glucose may be due to the "Crabtree effect" [69], in which enhanced glycolysis results in the inhibition of mitochondrial respiration/oxidative phosphorylation in cultured embryos [45, 63, 64, 70]. Oxidizable substrates and intermediates of the tricarboxylic acid cycle maintain embryonic development only in the absence of both phosphate and glucose, and inhibitors of the tricarboxylic acid cycle cause a developmental block in hamster 8-cell embryos [70], but an increased concentration of phosphate cannot maintain early development of rat and hamster embryos [61, 67] even in media which are expected to overcome the Crabtree effect [71]. On the other hand, Brown and Whittingham [55] explain that glucose prevents the expression of specific metabolic genes that are transcribed in response to changes in the availability of different carbon sources in cultured embryos. This phenomenon is known as "glucose repression" or "catabolite repression" [72]. In general, these developmental blocks appear to coincide with the transition from maternal to embryonic gene control, which occurs at characteristic stages in different species [73–78]. Although there are

**Table 1.** Formulae of media used for culture of bovine 1-cell embryos

| Components | Concentration (mM) | |
|---|---|---|
| | mTLP-PVA[a] | BECM[b] |
| NaCl | 110.0 | 89.0 |
| KCl | 3.2 | 3.2 |
| $CaCl_2$ | 2.0 | 2.0 |
| $MgCl_2$ | 0.5 | 0.5 |
| $NaHCO_3$ | 25.0 | 25.0 |
| Sodium lactate | 10.0 | 10.0 |
| Sodium pyruvate | 0.5 | 0.5 |
| Polyvinylalcohol | 1 mg/ml | 1 mg/ml |
| $NaH_2PO_4$ | - | 0.35 |
| BME-AAS[c] | - | 1% (v/v) |
| MEM-NAAS[d] | - | 1% (v/v) |

[a] Kim *et al.* [65]. [b] Lim *et al.* [145]. [c] Basal Medium Eagle's amino acid solution. [d] Minimal Essential Medium non-essential amino acid solution.

**Table 2.** Formulae of media used for culture of rat 1-cell embryos

| Components | Concentration (mM) | | |
|---|---|---|---|
| | mHECM-1[a] | R1ECM[b] | mR1ECM[c] |
| NaCl | 98.0 | 78.8 | 76.7 |
| KCl | 3.2 | 3.2 | 3.2 |
| $CaCl_2$ | 2.0 | 2.0 | 2.0 |
| $MgCl_2$ | 0.5 | 0.5 | 0.5 |
| $NaHCO_3$ | 25.0 | 25.0 | 25.0 |
| Sodium lactate | 10.0 | 10.0 | 10.0 |
| Sodium pyruvate | 0.5 | 0.5 | 0.5 |
| Polyvinylalcohol | 1 mg/ml | 1 mg/ml | 1 mg/ml |
| Glucose | - | 7.5 | 7.5 |
| Glutamine | - | - | 0.1 |
| MEM-AAS[d] | - | - | 2% (v/v) |
| MEM-NAAS[e] | - | - | 1% (v/v) |
| Osmolarity | 276 mOsm | 244 mOsm | 246 mOsm |

[a] Miyoshi *et al.* [67]. [b] Miyoshi *et al.* [88]. [c] Miyoshi *et al.* [152]. [d] Minimal Essential Medium amino acid solution. [e] Minimal Essential Medium non-essential amino acid solution.

various opinions against this relation [3], it is of interest that the development of rat 1-cell embryos to the 4-cell stage is stimulated, even in the presence of phosphate, by the inhibition of embryonic genome activation [78].

## Effects of Osmolarity and NaCl

Optimal osmolarity of media for early embryonic development is species specific and depends on the developmental stage of the embryo. For example, development of rabbit 2-cell embryo is maintained in the 230–339 mOsm range [79]. The ranges of osmolarity that support possible development of hamster 2-cell and 8-cell embryos are 250–325 mOsm [47] and 225–300 mOsm [80], respectively. More limited ranges of osmolarity are required for the development of mouse 1-cell (250–280 mOsm) [81] and 2-cell (272–280 mOsm) embryos [82, 83]. Further reduction in osmolarity to as low as 229 mOsm substantially improves *in vitro* development of mouse embryos [84]. This appears to be due to a reduced NaCl concentration. In pigs [85] and rabbits [86], embryonic development is affected by the NaCl concentration in culture media. Similarly, the NaCl concentration is an important factor in the development of bovine embryos to the blastocyst stage [87]. In contrast, the ability of rat 1-cell embryos to develop beyond the 2-cell stage appears to be dependent on the osmolarity adjusted by adding sorbitol [88]. Sorbitol is known as a small organic effector molecule, organic osmolyte, which exist universally in cells, stabilizes the cell volume by preventing large changes in intracellular ionic strength, and reverses enzyme activities inhibited by salts [89]. A low NaCl concentration or the presence of sorbitol is known to maintain the intracellular glutathione content and microfilament organization in pig oocytes and consequently to increase developmental ability following *in vitro* fertilization [90]. Several amino acids also protect the embryo from high osmolarity (see "Effects of Amino Acids") because amino acids are also known to act as organic osmolytes [89]. The above evidence therefore indicates that culture conditions with a relatively low NaCl concentration or in the presence of organic osmolytes appear to be required for normal embryonic development because intracellular ionic strength is probably associated with enzyme activities regulating embryonic development. In mice [91, 92] and rabbits [93, 94], the activity of $Na^+$-$K^+$-adenosine triphosphatase ($Na^+$-$K^+$-ATPase) is responsible for the blastocyst formation and for expanding of blastocoele. The $Na^+$-$K^+$-ATPase activity is very low in ovulated oocytes and 2-cell embryos [95, 96], but it begins to increase in the late morula stage [97, 98]. Since it is generally accepted that extra- or intra-cellular $Na^+$ is closely related to $Na^+$-$K^+$-ATPase [98–101], the development of embryos from the morula to the blastocyst stage may be dependent on the $Na^+$-$K^+$-ATPase activity of embryos. Low NaCl in the medium is also known to increase the stability of mRNA and protein synthesis in mouse embryos [102, 103], and the presence of organic osmolytes in culture medium containing a higher NaCl concentration increases the relative rate of protein synthesis in mouse 4-cell embryos [102].

## Effects of Amino Acids

Requirements of amino acids for *in vitro* development of mouse [104–107], rabbit [108, 109], hamster [80, 110, 111], pig [58, 112, 113] and sheep [114, 115] embryos have been demonstrated. The presence of amino acids in culture media is beneficial not only for embryo development *in vitro* but also for fetal development following transfer to recipients [104, 107, 116–118].

Supplementation of a chemically defined [65] or semi-defined [119] medium with amino acids noticeably improves development of bovine 1-cell embryos to the morula and blastocyst stages. Amino acids are beneficial for the development of rat 8-cell embryos to the blastocyst stage [117], whereas they are neither beneficial nor detrimental to the development of rat 1-cell embryos to the morula stage in R1ECM [88]. Further, a low concentration (0.1 mM) of glutamine together with 19 amino acids stimulates blastocyst formation of the morulae and hatching of the blastocysts in R1ECM [88]. In hamsters, the presence of 19 amino acids and glutamine in a chemically defined medium appears to be beneficial for development of 2-cell embryos to the blastocyst stage, whereas some amino acids appear to inhibit development of 1-cell embryos [120, 121]. These amino acids also appear to be detrimental for development of embryos after the 8-cell stage and finally it is reported that only 4 amino acids are sufficient for hamster 8-cell embryos to develop to the late blastocyst stage [48]. Mouse embryos are known to undergo a switch in amino acid requirements during the preimplantation period [118]. Further, development of mouse 1-cell embryos in medium supplemented with 20 amino acids is stimulated by transferring them to fresh medium after either 48 or 72 h of culture [106, 118]. This effect is most likely due to removing embryos from an increased concentration of ammonium which is produced by embryos and also generated by the breakdown of amino acids. A similar result has been obtained in sheep

in which replacing the culture medium every 48 h to alleviate ammonium toxicity improved development of 1-cell embryos in the presence of 20 amino acids [114].

There are several ways in which amino acids could contribute to embryonic development. It is possible that some amino acids may be used as substrates for protein synthesis. Exogenous amino acid pool sizes in mouse embryos change between the 8-cell and blastocyst stages [122]. At that time, there is a substantial increase in *de novo* protein synthesis [123–125]. In contrast, the protein content of rat embryos *in vivo* does not increase between the morula and blastocyst stages [123]. The requirement of amino acids for blastocyst formation in rats may therefore be for other metabolic needs, perhaps as substrates for energy production rather than for protein synthesis. The importance of glutamine as an energy source for embryo development has been suggested [51, 53, 58, 110, 126]. On the other hand, the concentrations of amino acids in oocytes, embryos and fluids of the reproductive tract are far in excess of any metabolic requirements [122, 127–130]. These results suggest that amino acids also play other roles in early embryonic development. One of the strong possibilities is, as described in a previous section, that amino acids act as an intracellular osmolyte protecting embryos from high ionic environments [84, 86, 131–133] and consequently affecting intracellular pH [134] in the early embryos which appear to be lack the $Na^+/H^+$ antiporter [135, 136]. Amino acids are also known as chelators of embryo toxins such as heavy metals [104].

## Quality and Viability of *In Vitro* Developed Embryos

Since delayed development, reduced number of cells in blastocysts, and poor viability following transfer have been observed when early embryos were cultured in unsuitable conditions [137–143], the quality of cultured embryos has been judged by comparing them with the morphology and number of cells in embryos developed *in vivo*. Fully expanded bovine blastocysts developed *in vivo* are known to contain about 160 cells [144]. Supplementation of a bovine embryo culture medium (BECM, Table 1) with fetal calf serum increases the mean number of cells in blastocysts obtained 192 h postinsemination from 119 cells to about 150 cells [145]. Chemically defined conditions appears to be still inadequate to obtain bovine embryos of high quality.

An unequivocal test to examine the normality of *in vitro* cultured embryos is the production of fetuses or offspring. Some successful developments of transferred embryos that were developed in chemically defined conditions have been demonstrated. Rabbit morulae and blastocysts [146] and mouse blastocysts [107] developed in chemically defined media from 1-cell embryos can develop into normal fetuses following embryo transfer. In hamsters, 8-cell to morula stage embryos developed in chemically defined conditions from the 2-cell stage [147] or following *in vitro* fertilization [148] can develop into living offspring. Rat morulae and blastocysts cultured in a chemically defined medium, mR1ECM, from the 1-cell stage [88] and after *in vitro* fertilization [149] can develop into full-term fetuses, but it is still unclear if these embryos can develop to term with the same efficiency as *in vivo* developed embryos, although the developmental competence of morulae and blastocysts has been examined following transfer just after collection [150, 151] and after culture from the 8-cell stage [117]. Further comparative studies of *in vivo* and *in vitro* developed embryos are required to determine the developmental competence to term.

## Conclusion

Some factors that regulate *in vitro* developments of bovine and rat 1-cell embryos to the blastocyst stage have recently been identified. Detrimental effects of phosphate and glucose on embryonic development are different in bovine and rat 1-cell embryos, although both compounds are conducive to each embryonic development when they are added to media after an appropriate lag time. Intracellular ionic strength reflected by the NaCl concentration and the osmolarity of the culture medium appears to be the most important factor in the development of bovine and rat embryos. The presence of organic osmolytes such as sorbitol and amino acids may improve embryonic development by rescuing it from detrimental effects of salts. Based on these results, new chemically defined media designated BECM [145] and mR1ECM [152] have been developed for bovine and rat 1-cell embryos, respectively. Relatively high blastocyst production has been achieved in bovine [87] and rat [88] 1-cell embryos by using these media (33 and 90%, respectively). To improve the efficiency and quality of embryos developed *in vitro*, however, further studies to clarify the mechanisms regulating embryonic development are required. BECM and mR1ECM should be useful media for these studies.

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