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—Review—

In Vitro Culture and Evaluation of Embryos for Production of High Quality Bovine Embryos

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Recent advances in technologies associated with assisted reproduction in domestic animals have been remarkable. In cattle, immature oocytes can be developed to the blastocyst stage by *in vitro*-production (IVP) techniques in various formulations of media and different culture conditions [1–5], but many technical problems still remain. For example, the cryotolerance and rates of pregnancy of embryos produced by IVP techniques were not high [6, 7]. Comparative studies demonstrated that the quality of bovine embryos produced by IVP systems was lower than that of *in vivo*-derived embryos [8, 9]. Embryo quality is an important determinant of the success of practical embryo transfer. Accurate evaluation of embryo quality prior to the embryo transfer contributes to improvement in rates of pregnancy. Therefore, successful assisted reproduction in animals requires culture techniques for production of high quality embryos and an accurate method for assessment of embryo quality. This review introduces *in vitro* culture systems for production of high quality bovine embryos and a novel method, with scanning electrochemical microscopy, to assess the embryo quality.

Culture Systems for Embryo Production

Bovine immature oocytes can be developed to the blastocyst stage by an IVP technique. Bovine embryos are routinely cultured in serum-supplemented media with or without somatic cell co-cultures, because serum contains beneficial substances for embryonic development such as growth factors, hormones, anti-oxidative compounds, and chelators of heavy metals [10]. But the biological activities of sera vary from lot to lot and there are potential risks of virus or mycoplasma infections. Furthermore, serum has a biphasic effect on bovine embryo development, inhibiting the first cleavage division but stimulating blastocyst development [1, 11]. Some studies have shown that the addition of serum to culture medium can cause morphological abnormalities in embryos [10, 12–14] and affect gene expression in bovine matured oocytes [15] and in pre-implantation embryos [16]. Serum is also suspected of contributing to the large offspring syndrome in domestic animal species [13, 17, 18]. Obviously, serum is a key factor in the culture environment affecting embryo quality, but because of the negative effects of serum, culture systems with serum-free medium, which is capable of efficient production of high quality bovine embryos, are desirable.

In order to formulate a serum-free medium for bovine embryo production, we conducted investigations to determine the factors required for oocyte maturation and subsequent embryonic development. Transforming growth factor-α (TGF-α) and epidermal growth factor (EGF), as well as luteinizing hormone (LH) and follicle stimulating hormone (FSH) have been shown to enhance cumulus expansion and oocyte fertility [19]. Our study also showed that TGF-α and/or EGF are beneficial for obtaining matured oocytes of high quality for IVF. It is known that certain factors secreted by

somatic cells, such as oviduct epithelial cells and granulosa cells, are involved in embryonic development *in vitro* [20–22]. We investigated the embryotrophic factors in bovine granulosa cell-conditioned medium (BGC-CM) by means of a serum-free culture system. The embryotrophic activity of BGC-CM has been purified and identified as a tissue inhibitor of metalloproteinase-1 (TIMP-1) [23]. TIMP-1 enhanced the developmental rates from the morula to blastocyst stage and increased the numbers of cells in embryos at the blastocyst stage. Furthermore, it has been shown that both basic fibroblast growth factor (bFGF) and transforming growth factor-β_1 (TGF-β_1) increased the rate of bovine embryos developed to the blastocyst stage in a serum-free medium without somatic cell co-culture [24]. It has also been shown that the combination of a low oxygen atmosphere (5% O_2) and low glucose medium (2.22 mM) improved the development of 1–2 cell embryos to blastocysts in a defined medium without somatic cell co-cultures [25].

On the basis of the abovementioned studies, two serum-free media (IVMD101 and IVD101) have been developed for bovine oocyte maturation and embryo culture [26]. The formulations of these serum-free media have been described in other papers [27, 28]. IVMD101 medium is used for both *in vitro* oocyte maturation and embryo culture in the presence of cumulus/granulosa cells under an atmosphere of 5% CO_2 in air. IVD101 medium is used for embryo culture in the absence of somatic cell co-culture under a low oxygen condition (5% O_2).

Physiological Evaluations of Bovine Embryos

These serum-free media showed improvement in the developmental rates and quality of bovine blastocysts produced by IVP systems [26]. The proportion of embryos developing to the blastocyst stage in IVMD101 medium with bovine cumulus/granulosa cells (BCGC) co-cultures (36.5%) and IVD101 medium without BCGC co-cultures under a low oxygen condition (37.1%) was significantly higher than in serum-containing medium (TCM-199 + 5% calf serum; CS) with BCGC co-cultures (25.1%). The mean numbers of cells in Day 7 blastocysts developed in IVMD101 (179.5 cells) and IVD101 (177.1 cells) media were greater than that in the serum-supplemented medium (145.7 cells). The survival rates of blastocysts produced in IVMD101 medium (73.3%) and IVD101 medium (60.0%) based on hatching after 72 h of post-thaw culture were superior to that of blastocysts produced in TCM-199 + CS medium (48.1%).

It is well known that embryo transfer of *in vitro*-derived bovine embryos often results in the production of bovine calves with abnormalities (large offspring syndrome), such as heavier than normal birth weight and a high incidence of dystocia [7, 29–31]. Bovine embryos exposed to a variety of unusual environments prior to the blastocyst have resulted in the development of unusually large offspring. The large offspring syndrome has been started in bovine and ovine embryos after exposure to unusual environments both *in vivo* and *in vitro*. Four different situations have been identified that result in the syndrome: *in vitro* embryo culture, asynchronous embryo transfer into an advanced uterine environment, nuclear transfer, and maternal exposure to an excessively high urea diet [29–32]. Neither the environmental factors inducing the large offspring syndrome nor the mechanisms of perturbation occurring in the early embryo and manifesting themselves in the fetus have been identified. Although the nature of the perturbing environmental factors has not yet been identified, serum-supplemented culture conditions are suspected of contributing to the large offspring syndrome in sheep [13, 17, 18]. Many components of serum such as growth factors, free radicals, ammonia, and progesterone may be candidate factors perturbing environments [33]. Therefore, the normality of calves derived from embryos produced in serum-free media is of great interest. Pregnancy, parturition and calf mortality rates of Japanese Black calves were compared after transfer of embryos developed in either serum-free media (IVMD101 and IVD101) or serum-supplemented media (TCM-199 and HPM199 + 5% CS) [28]. The calving rate of recipients receiving embryos from serum-free cultures (39.6%) was slightly heavier than those receiving embryos from serum-supplemented cultures (32.8%). Although the calving rate and abortion rate for embryos in serum-free and serum-supplemented cultures were not very different (85.2% vs 86.4%; 14.8% vs 13.6%, respectively), calf mortality for embryos from serum-supplemented cultures was greater than that for embryos from serum-free cultures (13.6% vs 4.9%). Average birth weights of calves for embryos from serum-supplemented cultures were slightly higher than those for embryos from serum-free cultures (male: 37.5 kg vs 34.0 kg; female: 36.1 kg vs 32.1 kg, respectively). Although these mean birth weights of calves were within the previously reported normal range of birth weight for Japanese Black calves produced from *in vitro* produced embryos [37], the birth weights of calves from embryos derived in serum-

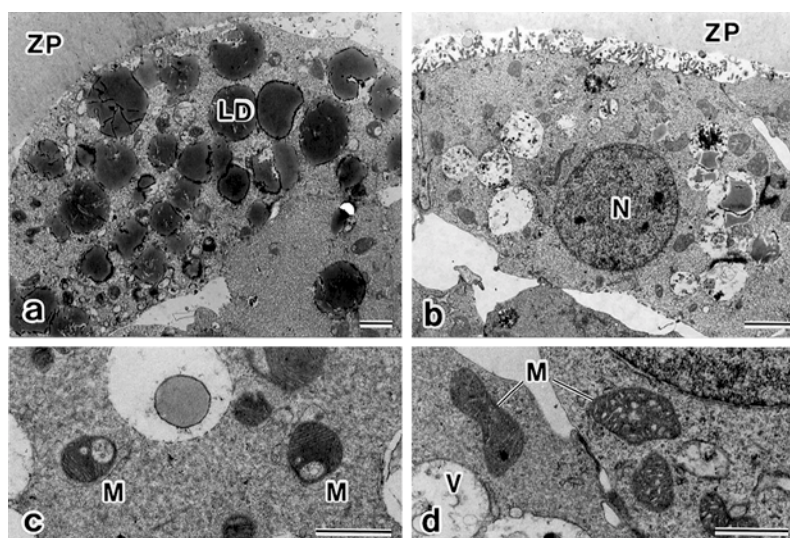

Fig. 1. Electron micrographs of bovine morulae cultured in serum-supplemented medium (TCM-199 + CS; a, c) and serum-free medium (IVMD101; b, d). a: Note cells filled with numerous lipid droplets (LD) of high electron density. b: Lipid droplets were present in cytoplasm, but were not abundant. c: Note immature mitochondria (M) showing ovoid structures with a hooded appearance and a dark electron-dense matrix. d: Note presence of elongated mitochondria with transverse cristae. N, nucleus; V, vesicles; ZP, zona pellucida. Bars: a, b = 2 μm, c, d = 1 μm.

supplemented culture (18–74 kg) were more variable than those from embryos developed in serum-free cultures (23–45 kg).

The serum-free media described in this paper are useful for the production of high quality bovine embryos for transfer. Comparative studies of bovine embryos cultured in different culture systems with serum-free or serum-containing media are examined in more detail below.

Morphological Evaluations of Bovine Embryos

Conspicuous differences in the morphological features of bovine embryos cultured in serum-free and serum-supplemented media have been observed [14]. Morulae and blastocysts cultured in serum-supplemented medium (TCM-199 + 5% CS) were darker and contained numerous cytoplasmic inclusions compared to those cultured in serum-free media (IVMD101 and IVD101). Histochemical examinations revealed that bovine embryos cultured in serum-containing medium had more, much larger lipid droplets in the cytoplasm than those cultured in serum-free media. Cytoplasmic lipid droplets increased substantially in embryos from the morula to the blastocyst stages when cultured in serum-containing medium [38].

Electron microscopic examinations showed major differences in the ultrastructural features of the morulae and blastocysts from serum-free and serum-supplemented cultures [14]. The blastomeres of morulae and blastocysts grown in serum-supplemented medium contained many large, electron-dense lipid droplets, whereas those cultured in serum-free media had fewer (Fig. 1a, b). Morulae and blastocysts derived *in vivo* contained less cytoplasmic lipid, similar to those developed in serum-free cultures. These findings strongly suggest that the presence of serum in the culture medium may be the cause of the abnormal accumulation of cytoplasmic lipids in bovine embryos. Similar findings showed that ovine embryos cultured in human serum-supplemented medium had abundant cytoplasmic lipid droplets [10, 13].

It is interesting to note that a conspicuous difference in the maturation of mitochondria has been observed between embryos cultured in serum-free and serum-supplemented media [14, 38]. There were numerous elongated mitochondria in the morulae developed in serum-free media, whereas many of the mitochondria of the morulae cultured in serum-containing medium were

spherical or ovoid, indicative of an immature form (Fig. 1c, d). Changes in mitochondrial ultrastructure and development were observed in bovine embryos from morula to early blastocyst stages in other studies [39–42]. It seems that the presence of serum had adverse effects on the structure and development of mitochondria. Similar mitochondrial changes were found in ovine embryos cultured in synthetic oviduct fluid (SOF) medium supplemented with human serum [43].

Cryotolerance and Lipid Accumulation

It is of great interest to clarify the correlation between the lipid accumulation and cryotolerance. Comparative studies of *in vivo* and *in vitro* produced embryos showed substantial differences in their morphology, metabolism, and overall embryo quality as assessed by the numbers of cells and frozen embryo viability [3, 8, 9, 38]. Although bovine blastocysts produced by an IVP technique have yielded live calves after transfer [44, 45], standard cryopreservation methods which yield high survival rates for *in vivo*-derived bovine embryos, usually result in low survival rates for *in vitro*-developed embryos, suggesting that *in vitro*-produced bovine embryos are more sensitive to cryopreservation than *in vivo*-derived embryos [6, 46, 47]. The displacement of intracellular lipids by centrifugation significantly improved the survival of *in vitro* developed embryos, suggesting that those lipids were partially responsible for the sensitivity to chilling and freezing [48, 49]. To evaluate the correlation between the lipid accumulation in bovine embryos and the cryotolerance of embryos, we examined the tolerance of cryopreservation by embryos at different developmental stages grown in either serum-free or serum-containing media [38]. The post-thaw viability and hatching rates of bovine embryos developed in serum-free media from the morula to the blastocyst stages was significantly higher than those developed in serum-containing medium, suggesting that bovine embryos with high lipid content are more sensitive to cryopreservation procedures. In summary, sensitivity of bovine embryos produced by an IVP technique to cryopreservation could be improved by using serum-free cultures. Therefore, serum-free media are beneficial for the production of high quality, cryogenically preserved bovine embryos.

The mechanism of cytoplasmic lipid accumulation in bovine embryos cultured in serum-containing medium is still unclear. It is well known that cells in cultures can readily take up fatty acids, phospholipids, and triglycerides from serum-supplemented medium and most of the lipids are derived from triglycerides contained in serum lipoproteins [50]. Bovine embryos cultured in a serum-supplemented medium showed a different fatty acid composition to those cultured in a serum-free medium [51]. The fatty acid composition of embryos grown in calf serum-containing medium was similar to those of calf serum, suggesting that the accumulation of long-chain saturated and unsaturated fatty acids in serum-exposed embryos is from the serum itself. It has been reported that excessive accumulation of triglycerides occurs in bovine embryos cultured in serum-containing medium [52]. Furthermore, a recent study by us showed that bovine embryos developed in serum-free media supplemented with bovine serum plasma lipoproteins contained numerous lipid droplets in the cytoplasm (unpublished results). These findings suggest that the source of cytoplasmic lipids in bovine embryos cultured in serum-supplemented medium is in the lipoproteins of the serum itself.

Novel Technique for Quality Evaluation of Embryos

The quality of embryos prior to embryo transfer affects the pregnancy rates. Several methods for embryo evaluation have been developed. Dye exclusion tests [53], measurement of enzyme activity [54], glucose uptake [55], and live-dead stains [56] are useful in predicting embryo survival after transfer, but most of these methods are harmful and therefore not useful for the evaluation of individual embryos. In the past, morphological evaluation has been widely used to evaluate embryo quality because it is non-invasive and useful in predicting pregnancy rates of groups of bovine embryos after transfer [57–61]. Based on morphological evaluations, embryos of good quality yielded high pregnancy rates and poor embryos resulted in low success rates. Nevertheless, in some cases, embryos of low quality produced a pregnancy, whereas many good embryos as judged by morphological observation failed to result in pregnancy. Furthermore, morphological evaluations remain one of the most subjective and quantitative aspects of embryo transfer because categorization standards vary among investigators.

A recent study by us showed conspicuous differences in the ultrastructural features of morulae of high and low quality [62]. The morulae classified as low quality (fair and poor) by morphological classification contained nucleoli with low transcriptional activity, a large number

of lipid droplets, and immature mitochondria, suggesting that low quality embryos have low metabolic activities, including oxygen consumption. In embryos, the maturation of mitochondria is associated with increases in metabolism reflected in their oxygen consumption [63] and CO_2 production [64], and it appears to be related to the depletion of stored products (cytoplasmic lipids) so that mitochondrial function (oxygen consumption) may be an important factor in embryo quality.

It is well known that metabolic processes drastically change during embryonic development, as indicated by genome activation and large increases in protein synthesis. Radioisotope labeling techniques have been widely used to quantify glucose, pyruvate, lactate, carbon dioxide and amino acids in metabolic pathways, and detection of metabolites originating in a single embryo have been attempted [64–67]. Oxygen consumption is an ubiquitous parameter also used to gain valuable information on metabolic mechanisms. Oxygen consumption of mammalian embryos has been studied with various methods such as Cartesian diver [63], spectrophotometrics [68–70], fluorescence [71–73], and electrochemical techniques [74–78].

Recently, we succeeded in non-invasively and quantitatively determining oxygen consumption of individual bovine embryos cultured in the serum-free medium (IVD101) by scanning electrochemical microscopy (SECM) [79]. SECM is a technique in which the tip of a microelectrode is used to scan and monitor the local distribution of electro-active species (O_2) near the sample surface (embryo). With this technique, oxygen consumption of single, identical bovine embryos at different developmental stages has been monitored. The morulae with the higher oxygen consumption on day 6 developed into blastocysts of larger sizes and higher oxygen consumption on day 8 [79]. The morulae with higher oxygen consumption have a strong potential for further development into expanded and/or hatched blastocysts, thereby increasing the possibility that the SECM procedure could be used to non-invasively assess the embryo quality and the subsequent developmental potential of embryos.

Although SECM may be useful for judging bovine embryo quality, the SECM measuring procedure requires quite a bit of skill, such as gently holding a single embryo with a micropipette positioned with a micromanipulator and aligning the microelectrode very close to the embryo surface. Recently, we designed a new SECM measuring procedure which can be easily used by a non-electrochemist. This new SECM measuring system includes a measuring instrument on an inverted optical microscope stage, a potentiostat (Hokuto Denko Co., Tokyo, Japan), and a notebook computer as controller and analyzer (Fig. 2a). The measuring instruments are covered with a plastic chamber and humidified 5% CO_2 and 95% air gas can be maintained. The motor driven XYZ-stage is located on the microscope stage and this stage and the potentiostat are controlled by a computer. The oxygen consumption rate of embryos is calculated by newly designed software. To easily handle many embryos in a short time, a plate with cone-shaped microwells was designed (Fig. 2b). This plate is designed to amplify oxygen concentration changes due to respiration by the embryo. Using this modified procedure, oxygen consumption has been monitored at various developmental stages of single, identical bovine embryos cultured in IVD101 medium (Table 1). Oxygen consumption rates (*F*) of the single embryos were low

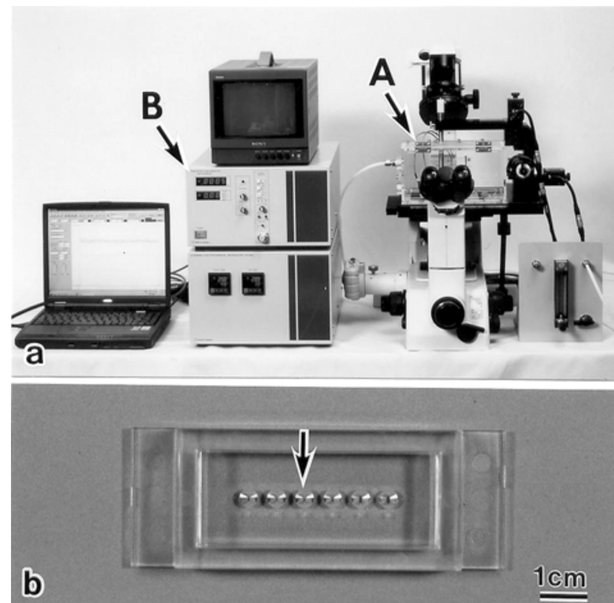

Fig. 2. A new scanning electrochemical microscopy (SECM) system (a) and a plate for measuring respiratory activity of bovine embryos (b). a: SECM includes a measuring instrument on the inverted optical microscope stage (A), a potentiostat (B), notebook computer, and monitor. b: The plate has six cone-shaped microwells (arrow). Individual embryos are transferred into a microwell filled with culture medium. The embryo sinks down to the bottom of the well, remaining at the lowest point. The oxygen concentration profiles are measured according to the method by Shiku et al. (unpublished results).

Table 1. Oxygen consumption rates of the bovine embryos cultured in IVD101 medium at various stages of development

Embryonic stage	No. of embryos measured	Oxygen consumption rate ($F \times 10^{14}$ / mol s^{-1})
2 cell	15	0.46 ± 0.05^a
4 cell	17	0.45 ± 0.03^a
8 cell	18	0.46 ± 0.02^a
Morula	48	1.03 ± 0.05^b
Blastocyst	55	1.86 ± 0.07^c

Values with different superscripts in each column differ significantly ($P<0.05$).

Table 2. Relationship between the oxygen consumption and the further developmental potential of the bovine morulae cultured in IVD101 medium

Oxygen consumption rate ($F \times 10^{14}$ / mol s^{-1})	No. of embryos* measured	No. of embryos developed to blastocyst after 96 h (%)	No. of blastocysts expanded or hatched after 96 h (%)
$F \geq 1.0$	56	50 (89.3)	35 (62.5)
$0.8 \leq F < 1.0$	44	34 (77.3)	20 (50.0)
$F < 0.8$	107	49 (45.8)	28 (26.2)

*: Bovine embryos at the morula stage were selected at day 6 after in vitro fertilization. After measurement by a modified scanning electrochemical microscopy, the embryos were incubated in IVD101 medium for 4 days in a humidified atmosphere of 5% CO_2/5% O_2/90% N_2 at 38.5°C.

from 2-cell to 8-cell stages ($0.48–0.55 \times 10^{-14}$ mol s^{-1}). An increase in the oxygen consumption rate was found at the morula (1.03×10^{-14} mol s^{-1}) stage and blastocysts showed an even higher oxygen consumption rate (1.86×10^{-14} mol s^{-1}). The morulae with the highest oxygen consumption have the strongest potential for further development into expanded and/or hatched blastocysts (Table 2). Furthermore, we found that the oxygen consumption rates of bovine embryos (morulae and blastocysts) produced in serum-free medium (IVD101) were higher than those of embryos cultured in serum-supplemented medium (HPM199 + CS), indicating a correlation between respiratory activity and development of mitochondria (unpublished results). These results demonstrate that the novel SECM procedures are very useful for non-invasively and easily evaluating the oxygen consumption of bovine embryos. This SECM system may have a wide application for judging embryo quality and may be an ideal method for noninvasive quality evaluation of embryos in the future.

Conclusions

With serum-free culture for IVF and IVM bovine embryos can offer several advantages over the cultures with serum-supplemented media. The improved serum-free media (IVMD101 and IVD101) are especially useful for the production of higher quality bovine embryos for cryopreservation. Furthermore, it has been demonstrated that there is a correlation between oxygen consumption and embryo quality. Scanning electrochemical microscopy can be non-invasively applied to measure oxygen consumption of single bovine embryos. This novel method may be useful to assess the quality of bovine embryos and their developmental potential. The combination of novel culture systems and evaluation techniques for bovine embryos may contribute to improvements in the rates of pregnancy with bovine embryos produced by IVP technique.

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