— Review —

Evaluation of Bovine Embryos Produced in High Performance Serum-Free Media

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Abstract. This review evaluates the quality of bovine embryos developed from *in vitro*-matured (IVM) and -fertilized (IVF) oocytes cultured in either serum-free or serum-containing media. Bovine embryos cultured in serum-supplemented medium contain numerous cytoplasmic lipid droplets and immature mitochondria compared to those cultured in serum-free medium. The accumulation of cytoplasmic lipids in embryos developed in serum-containing medium may be a result of incorporation of lipoproteins from the serum and may result in impaired function of mitochondria. The improved serum-free media (IVMD101 and IVD101) offer several advantages over culture in serum-containing medium, including increased rates of blastocyst formation and higher cell numbers. Additionally, the survival and hatching rates of embryos produced in serum-free media after post-thaw culture were superior to those of embryos produced in the serum-containing medium, suggesting that the abnormal accumulation of cytoplasmic lipids in embryos may have a negative effect on the sensitivity of embryos to chilling and freezing. These serum-free culture systems have proven to be beneficial for the production of good quality embryos from IVM-IVF bovine oocytes. Furthermore, recent studies have shown a correlation between mitochondrial function (oxygen consumption) and embryo quality. A new method using scanning electrochemical microscopy may be capable of assessing the viability and developmental potential of bovine embryos.

Key words: Embryo culture, Cryotolerance, Oxygen consumption, Ultrastructure, Cow

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Bovine embryos produced by IVM and IVF techniques are able to develop up to the blastocyst stage in various formulations of media and under different culture conditions [1-5]. 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 and chelators of heavy metals. In cultures of mammalian pre-implantation embryos, serum is frequently used as the fixed nitrogen source in cultured media, especially when the embryos are co-cultured with somatic cells [6].

However, serum has a biphasic effect on bovine embryo development, inhibiting the first cleavage division but stimulating blastocyst development [1, 7] and the addition of serum to culture medium can cause morphological deviations of embryos [6, 8-10]. Furthermore, serum is suspected of contributing to the large offspring syndrome in sheep [9, 11, 12]. For these reasons, culture systems using serum-free media are desirable.

Novel Serum-Free Culture Systems for Oocyte Maturation and Embryo Culture

Mammalian oocytes are arrested at the diplotene stage of the first meiotic prophase and these

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Table 1. Composition of media for *in vitro* maturation (IVM) and *in vitro* embryo culture (IVC)

Components	IVMD101	IVD101	HPM199
D-glucose	5.56 mM	2.22 mM	5.56 mM
Sodium pyruvate	0.91 mM	0.27 mM	0.91 mM
Sodium lactate	–	2.48 mM	–
L-cysteine	–	0.05 mM	–
GSH	–	200 μ M	–
Taurine	5 mM	5 mM	5 mM
Selenium	5 nM	5 nM	5 nM
Insulin	5 μ g/ml	–	–
TGF- α	10 ng/ml	–	–
<i>apo</i> -transferrin	10 μ g/ml	10 μ g/ml	–
bFGF	–	10 ng/ml	–
TGF- β_1	–	1 ng/ml	–
TIMP-1	–	0.5 μ g/ml	–
Aprotinin	–	0.5 μ g/ml	–
BSA	1 mg/ml	1 mg/ml	–
HEPES	5 mM	5 mM	5 mM
Gentamycin sulfate	10 μ g/ml	10 μ g/ml	10 μ g/ml

Basal medium (DM199) of IVMD101, IVD101, and HPM199 does not contain glucose, Tween-80 and *para*-aminobenzoic acid from original components of TCM199.

immature oocytes acquire the ability to be fertilized during the process of oocyte maturation. In general, serum-supplemented media have been used for *in vitro* maturation of immature oocytes [13, 14]. In order to formulate a serum-free medium for bovine oocyte maturation, 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 fertilizability [15]. Additionally, the same study showed that TGF- α and/or EGF are beneficial for obtaining matured oocytes of high quality for *in vitro* fertilization.

It is known that many factors are involved in embryonic development *in vitro* [16]. It has been suggested that somatic cells such as oviduct epithelial cells and granulosa cells secrete certain embryotrophic factors [17, 18], and we have investigated the embryotrophic activity of bovine granulosa cell-conditioned medium (BGC-CM) using a serum-free culture system. The rate of embryos developing to the blastocyst stage was significantly higher in BGC-CM compared to fresh control medium [19]. The embryotrophic activity of BGC-CM has been purified and identified as a

tissue inhibitor of metalloproteinase-1 (TIMP-1) [20]. TIMP-1 enhanced the developmental rates from the morula to blastocyst stage and increased cell numbers of embryos at the blastocyst stage. Furthermore, it has been reported that both basic fibroblast growth factor (bFGF) and transforming growth factor- β_1 (TGF- β_1) increased the rate of bovine embryos developing to the blastocyst in a serum-free medium without somatic cell co-culture [21].

In addition, it is known that the gaseous environment [16] and the glucose concentration [22] in culture media are important for the embryonic development *in vitro*. The presence of a high concentration of glucose was found to be harmful for the development of bovine embryos, especially in the early stages [1]. Based on these findings, we performed experiments that found that the combination of low oxygen atmosphere (5% O₂) 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 [15].

On the basis of the aforementioned studies, two serum-free culture media (IVMD101 and IVD101) were developed for *in vitro* maturation (IVM) of oocytes and *in vitro* culture (IVC) of bovine embryos [23]. The basal medium of IVMD101 and IVD101 is prepared from DM199 medium which

Fig. 1. Light micrographs of bovine blastocysts cultured in serum-free medium (IVMD101; a, c) and serum-supplemented medium (HPM199 + CS; b, d). a, b: Normarski differential interference contrast micrographs. c, d: Blastocysts are stained with Sudan Black B. Arrows indicate large sudanophilic lipid droplets. Bar = 50 μ m.

excludes glucose, Tween-80, and *para*-aminobenzoic acid from the original components of TCM199. The formulations of these serum-free media are described in Table 1. 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% CO₂ in air. IVD101 medium is used for embryo culture in the absence of somatic cell co-culture under a low oxygen condition (5% O₂).

Morphological Differences Between Embryos Developed in Serum-Free and Serum-Supplemented Cultures

Conspicuous differences in the morphological features between bovine embryos cultured in serum-free and serum-supplemented media have

been observed [10]. Morulae cultured in serum-free media were more compact and lighter in color than those cultured in serum-containing medium when observed under a light microscope. Blastocysts cultured in serum-supplemented medium were darker and contained numerous cytoplasmic inclusions compared to those cultured in serum-free medium (Fig. 1a, b). Staining by Sudan Black B revealed a large number of sudanophilic particles of various sizes present in bovine embryos cultured in serum-free media, whereas blastocysts cultured in serum-containing medium had many, much larger sudanophilic lipid droplets (LD) present (Fig. 1c, d). Furthermore, quantitative histochemical examinations demonstrated that sudanophilic LD increased significantly in embryos from the morula to the blastocyst stages when cultured in serum-containing medium [24].

Fig. 2. Electron micrographs of bovine morulae cultured in serum-free medium (IVMD101; a, c) and serum-supplemented medium (TCM199 + CS; b, d). a: Many lysosome-like vesicles (Ly) were partially filled with electron-dense materials. Lipid droplets (LD) were present in cytoplasm, but were less abundant than in b. b: Note cells filled with numerous lipid droplets with high electron density. c: Note presence of elongated mitochondria (M) with transverse cristae. d: Note immature mitochondria showing ovoid structures with a hooded appearance and a dark electron-dense matrix. N, nucleus. Bars: a, b = 2 μ m, c, d = 1 μ m.

Our electron microscopic studies showed major differences in the ultrastructural features between the morulae and blastocysts from serum-free and serum-supplemented cultures [10, 24]. The blastomeres of morulae and blastocysts grown in serum-supplemented medium contained large, electron-dense LD, while those cultured in serum-

free medium had fewer LD (Fig. 2a, b). Morulae and blastocysts derived *in vivo* contained less cytoplasmic lipid, which was similar to those developed in serum-free cultures [25]. These results suggest that the presence of serum in the culture medium may be the cause of the abnormal accumulation of cytoplasmic lipids. Similar

findings showed that ovine embryos cultured in human serum-supplemented medium had abundant cytoplasmic LD [6, 9].

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 [26]. Bovine embryos cultured in a serum-supplemented medium showed a different fatty acid composition compared to those cultured in a serum-free medium [27]. The fatty acid composition of embryos grown in calf serum (CS)-containing medium was similar to those of CS by itself, suggesting that the accumulation of long-chain saturated and unsaturated fatty acids in serum-exposed embryos is directly from serum. It has been reported that excessive accumulation of triglycerides occurs in bovine embryos cultured in serum-containing medium [28]. A recent study in our lab showed that bovine embryos developed in serum-free media supplemented with bovine serum plasma lipoproteins contained numerous LD in the cytoplasm (unpublished results). These findings suggest that the source of cytoplasmic lipids in bovine embryos cultured in CS-supplemented medium originates from the lipoproteins of the serum itself.

Conspicuous differences in the ultrastructural features of mitochondria between embryos cultured in serum-free and serum-supplemented medium were observed at the morula stage. There were numerous elongated mitochondria in the morulae developed in IVMD101 and IVD101, while many of the mitochondria of the morulae cultured in HPM199 + CS were spherical or ovoid, indicating an immature form (Fig. 2c, d). The maturation of mitochondria in embryos is associated with increases in metabolism reflected in their oxygen consumption [29] and CO₂ production [30], and it seems to be related to the depletion of stored products (cytoplasmic lipids). Similar mitochondrial changes were found in ovine embryos [31]. We suggest that the presence of serum has adverse effects on the structure of mitochondria by causing the accumulation of cytoplasmic lipids which causes impaired function of mitochondria, since intracellular lipid is normally metabolized by mitochondria.

Physiological Evaluations of Bovine Embryos Cultured in Serum-Free Medium

Serum-free media (IVMD101 and IVD101) showed improvements in the developmental rates and quality of bovine blastocysts from IVM-IVF oocytes [23]. 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 low oxygen condition (37.1%) was significantly higher than in serum-containing medium (TCM199 + 5% calf serum) with BCGC co-cultures (25.1%). The mean cell numbers of 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).

A comparative study of *in vivo* and *in vitro* produced embryos showed remarkable differences in their morphology, metabolism, and embryo quality with regard to cell numbers and frozen embryo viability [4, 25, 32, 33]. Although bovine *in vitro*-produced embryos have yielded live calves after transfer [34, 35], standard cryopreservation methods yielding 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 chilling and cryopreservation than *in vivo*-derived embryos [36–38]. 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 [39, 40].

We examined the tolerance of cryopreservation of embryos developed from IVM-IVF oocytes cultured in either serum-free or serum-containing media [23]. Bovine embryos were frozen using the conventional slow freezing method in a ethylene glycol and cultured on feeder layers of BCGC in TCM199 supplemented with 5% CS in 5% O₂ in air at 38.5 C after thawing [41]. 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 TCM199 + CS medium (48.1%). To further evaluate the correlation between the lipid accumulation and cryotolerance, embryos at different developmental

Table 2. Post-thaw survival of bovine embryos developed in serum-free media (IVMD101 and IVD101) and serum-supplemented medium (HPM199+5% CS)

Embryonic stage at freezing	Culture media	No. of embryos thawed	No. of embryos surviving after 24 h (%)	No. of embryos hatching after 96 h (%)
Morula	IVMD101	49	27 (55.1) ^a	14 (30.6) ^a
	IVD101	45	24 (53.3) ^a	12 (26.7) ^a
	HPM199+5% CS	53	15 (28.3) ^b	3 (5.7) ^b
Early blastocyst	IVMD101	18	18 (88.9) ^a	10 (55.6) ^a
	IVD101	49	43 (87.8) ^a	30 (61.2) ^a
	HPM199+5% CS	31	17 (54.8) ^b	11 (35.5) ^b
Blastocyst	IVMD101	75	71 (94.7) ^a	56 (74.7) ^a
	IVD101	68	58 (85.3) ^{ab}	44 (64.7) ^{ab}
	HPM199+5% CS	64	48 (75.0) ^b	32 (50.0) ^b

a,b: Values with different superscripts with each column differ significantly ($P < 0.05$).

stages grown in either serum-free or serum-containing media were examined after freeze/thaw procedures [24]. 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 the serum-containing medium (Table 2). These results strongly suggest that bovine embryos with high lipid content are more sensitive to cryopreservation procedures. In summary, sensitivity to cryopreservation of bovine embryos developed from IVM-IVF oocytes could be improved by serum-free cultures. The serum-free media described in this paper are useful for the production of high quality bovine embryos for cryopreservation.

It is well known that embryo transfer of *in vitro*-derived bovine embryos often results in the production of bovine calves with abnormalities such as heavier than normal birth weight and a high incidence of dystocia [42–45]. In a recent review, 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 (TCM199 and HPM199 + 5% CS) [46]. The pregnancy rate of recipients receiving embryos from serum-free cultures (39.6%, $n=154$) was slightly higher than those receiving embryos from serum-supplemented cultures (32.8%, $n=67$). Although the calving rate and abortion rate for embryos in serum-free and serum-supplemented

cultures were not 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 from embryos obtained from serum-supplemented cultures were slightly higher than those for embryos obtained from serum-free cultures (male: 37.5 kg vs 34.0 kg; female; 36.1 kg vs 32.1 kg, respectively). However, these mean birth weights of calves from embryos derived from serum-free and serum-supplemented cultures were within the previously reported normal range of birth weights for Japanese Black calves produced from IVM-IVF embryos [47].

Evaluation of Embryo Quality by a Novel Non-invasive Method

Embryo quality is an important determinant of the success of embryo transfer procedures. Several methods for embryo evaluation have been described. Dye exclusion tests [48], measures of enzyme activity [49], glucose uptake [50], and live/dead stains [51] are useful in predicting embryo survival following transfer, but most of these methods are invasive and less useful for evaluation of individual embryos. Currently, morphological evaluation is the noninvasive technique that has been widely used to evaluate embryo quality and has been shown to be useful in predicting

Table 3. Relationship between oxygen consumption and further developmental potential of 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 scanning electrochemical microscopy, the embryos were incubated in IVD101 medium for 4 days in a humidified atmosphere of 5% CO₂ / 5% O₂ / 90% N₂ at 38.5 C.

pregnancy rates for groups of bovine embryos following transfer [52–56]. However, morphological evaluations remain one of the most subjective and quasi-quantitative aspects of embryo transfer, and categorization standards vary among investigators.

It is well known that metabolic processes drastically change during embryonic development because of 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 from a single embryo has been attempted [29, 57–59]. Oxygen consumption is an ubiquitous parameter which can provide valuable information on metabolic mechanisms. Oxygen consumption of mammalian embryos has been studied with various methods such as Cartesian diver [29], spectrophotometrics [60, 61], fluorescence [62–64], and electrochemical techniques [65–68].

Currently, the correlation between oxygen consumption and embryo quality is of great. Our recent study showed conspicuous differences in the ultrastructural features between morulae of high and low quality [69]. The morulae classified as fair and poor quality by morphological classification contained nucleoli with low transcriptional activity, a large number of LD, and immature mitochondria, suggesting that low quality embryos have low metabolic activities, including oxygen consumption. More recently, we succeeded in non-invasively and quantitatively determining oxygen consumption of individual bovine embryos cultured in serum-free media by scanning

electrochemical microscopy (SECM) [70]. SECM is a technique in which the tip of a microelectrode monitors the local distribution of electroactive species (O₂) near the sample surface (embryos). Using this technique, oxygen consumption has been monitored at different developmental stages of single, identical bovine embryos. The morulae with the higher oxygen consumption on day 6 developed into blastocysts of larger sizes and higher oxygen consumption on day 8 after continuous cultivation [70]. The morulae with higher oxygen consumption have a strong potential for further development into expanded and/or hatched blastocysts (Table 3). These results show the potential of the SECM procedure to non-invasively assess the embryo quality and the subsequent developmental potential of embryo. This method may be widely applicable for judging embryo quality in the future.

Conclusions

Serum-free cultures offer several advantages over the cultures using serum-supplemented media. Specifically, the improved serum-free media (IVMD101 and IVD101) are useful for production of higher quality bovine embryos for cryopreservation. Furthermore, it has been shown that there is a correlation between mitochondrial function (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 capable of assessing the quality of bovine embryos and their developmental potential.

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References

1. **Pinyopummintr T, Bavister BD.** In vitro-matured/ in vitro-fertilized bovine oocytes can develop into morulae/blastocysts in chemically defined protein-free culture media. *Biol Reprod* 1991; 45: 736–742.
2. **Voelkel SA, Hu YX.** Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. *Theriogenology* 1992; 37: 1117–1131.
3. **Shamsuddin M, Larsson B.** In vitro development of bovine embryos after fertilization using semen from different donors. *Reprod Dom Anim* 1993; 28: 77–84.
4. **Shamsuddin M, Larsson B, Gustafsson H, Rodriguez-Martinez H.** In vitro development up to hatching of bovine in vitro-matured and fertilized oocytes with or without support from somatic cells. *Theriogenology* 1993; 39: 1067–1079.
5. **Shamsuddin M, Larsson B, Rodriguez-Martinez H.** Culture of bovine IVM/IVF embryos up to blastocyst stage in defined medium using insulin, transferrin and selenium or growth factors. *Reprod Dom Anim* 1993; 28: 209–210.
6. **Gardner DK.** Mammalian embryo culture in the absence of serum or somatic cell support. *Cell Biol Int* 1994; 18: 1163–1178.
7. **Van Langendonck A, Donnay I, Schuurbiens N, Auquier P, Carolan C, Massip A, Dessy F.** Effects of supplementation with fetal calf serum on development of bovine embryos in synthetic oviduct fluid medium. *J Reprod Fertil* 1997; 109: 87–93.
8. **Gardner DK, Lane M, Spitzer A, Batt PA.** Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: amino acids, vitamins, and culturing embryos in groups stimulate development. *Biol Reprod* 1994; 50: 390–400.
9. **Thompson JG, Gardner DK, Pugh PA, McMillan WH, Tervit HR.** Lamb birth weight is affected by culture system utilized during in vitro pre-elongation development of ovine embryos. *Biol Reprod* 1995; 53: 1385–1391.
10. **Abe H, Yamashita S, Itoh T, Satoh T, Hoshi H.** Ultrastructure of bovine embryos developed from in vitro-matured and -fertilized oocytes: comparative morphological evaluation of embryos cultured either in serum-free or in serum-supplemented medium. *Mol Reprod Dev* 1999; 53: 325–335.
11. **Holm P, Walker SK, Seamark RF.** Embryo viability, duration of gestation and birth weight in sheep after transfer of in vitro matured and in vitro fertilized zygotes cultured in vitro or in vivo. *J Reprod Fertil* 1996; 107: 175–181.
12. **Sinclair KD, McKvoy TG, Carolan C, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ, Broadbent PJ.** Conceptus growth and development following in vitro culture of ovine embryos in media supplemented with bovine sera. *Theriogenology* 1998; 49: 218 (abstract).
13. **Leibfried-Rutledge ML, Critser ES, First NL.** Effects of fetal calf serum and bovine serum albumin on in vitro maturation and fertilization of bovine and hamster cumulus-oocyte complexes. *Biol Reprod* 1986; 35: 850–857.
14. **Moor RM, Dai Y, Lee C, Fulka JJ.** Oocyte maturation and embryonic failure. *Hum Reprod Update* 1998; 4: 223–236.
15. **Kobayashi K, Yamashita S, Hoshi H.** Influence of epidermal growth factor and transforming growth factor- α on in vitro maturation of cumulus cell-enclosed bovine oocytes in a defined medium. *J Reprod Fertil* 1994; 100: 439–446.
16. **Bavister BD.** Culture of preimplantation embryos: facts and artifacts. *Hum Reprod Update* 1995; 1: 91–148.
17. **Eyestone WH, First NL.** Co-culture of early cattle embryos to the blastocyst stage with oviductal tissue or in conditioned medium. *J Reprod Fertil* 1989; 85: 715–720.
18. **Gandolfi F.** Autocrine, paracrine and environmental factors influencing embryonic development from zygote to blastocyst. *Theriogenology* 1994; 41: 95–100.
19. **Kobayashi K, Takagi Y, Satoh T, Hoshi H, Oikawa T.** Development of early bovine embryos to the blastocyst stage in serum-free conditioned medium from granulosa cells. *In Vitro Cell Dev Biol* 1992; 28A: 255–259.
20. **Satoh T, Kobayashi K, Yamashita S, Kikuchi M, Sendai Y, Hoshi H.** Tissue inhibitor of metalloproteinases (TIMP-1) produced by granulosa and oviduct cells enhances in vitro development of bovine embryo. *Biol Reprod* 1994; 50: 835–844.

21. **Hoshi H.** In vitro production of bovine preimplantation embryos in a serum-free medium. In: Muramatsu T (ed.), *Gene Expression and Nutrition in Animals: From Cells to Whole Body*. Trivandrum, India: Research Singpost; 1996: 59–70.
22. **Thompson JGE, Simpson AC, Pugh PA, Donnelly PE, Tervit HR.** Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *J Reprod Fertil* 1990; 89: 573–578.
23. **Yamashita S, Abe H, Itoh T, Satoh T, Hoshi H.** A serum-free culture system for efficient in vitro production of bovine blastocysts and their improved viability after freezing and thawing. *Cytotechnology* 1999; 31: 1–9.
24. **Abe H, Yamashita S, Satoh T, Hoshi H.** Accumulation of cytoplasmic lipid droplets in bovine embryos and cryotolerance of embryos developed in different culture systems using serum-free or serum-containing media. *Mol Reprod Dev* 2002; 61: 57–66.
25. **Abe H, Otoi T, Tachikawa S, Yamashita S, Satoh T, Hoshi H.** Fine structure of bovine morulae and blastocysts in vivo and in vitro. *Anat Embryol* 1999; 199: 519–527.
26. **Spector AA.** Fatty acid, glyceride, and phospholipid metabolism. In: Rothblat GH, Cristofalo VJ (eds.), *Growth, Nutrition and Metabolism of Cells in Culture*. New York: Academic Press; 1973: 257–296.
27. **Sata R, Tsujii H, Abe H, Yamashita S, Hoshi H.** Fatty acid composition of bovine embryos cultured in serum-free and serum-containing medium during early embryonic development. *J Reprod Dev* 1999; 45: 97–103.
28. **Ferguson EM, Leese HJ.** Triglyceride content of bovine oocytes and early embryos. *J Reprod Fertil* 1999; 116: 373–378.
29. **Mills RM, Brinster RL.** Oxygen consumption of preimplantation mouse embryos. *Exp Cell Res* 1967; 47: 337–344.
30. **Brinster RL.** Carbon dioxide production from glucose by the preimplantation mouse embryo. *Exp Cell Res* 1967; 47: 271–277.
31. **Dorland M, Gardner DK, Trounson AO.** Serum in synthetic oviduct fluid causes mitochondrial degeneration in ovine embryos. *J Reprod Fertil* 1994; 13: 25 (abstract).
32. **Greve T, Avery B, Callesen H.** Viability of in-vivo and in-vitro produced bovine embryos. *Reprod Dom Anim* 1993; 28: 164–169.
33. **Massip A, Mermillod P, Dinnyes A.** Morphology and biochemistry of in-vitro produced bovine embryos: implications for their cryopreservation. *Hum Reprod* 1995; 10: 3004–3011.
34. **Fukuda Y, Ichikawa M, Naito K, Toyoda Y.** Birth of normal calves resulting from bovine oocytes matured, fertilized, and cultured with cumulus cells in vitro up to the blastocyst stage. *Biol Reprod* 1990; 42: 114–119.
35. **Zhang L, Barry DM, Denniston RS, Bunch TD, Godke RA.** Birth of live calves after transfer of frozen-thawed bovine embryos fertilized in vitro. *Vet Rec* 1993; 132: 247–279.
36. **Leibo SP, Loskutoff NM.** Cryobiology of in vitro-derived bovine embryos. *Theriogenology* 1993; 39: 81–94.
37. **Pollard JW, Leibo SP.** Comparative cryobiology of in vitro and in vivo derived bovine embryos. *Theriogenology* 1993; 39: 287 (abstract).
38. **Hasler JE, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, Neely B, Shuey LS, Stokes JE, Trimmer SA.** Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology* 1995; 43: 141–152.
39. **Leibo SP, Pollard JW, Martino A.** Chilling and freezing sensitivity of “reassembled” in vitro-derived bovine embryos. *Theriogenology* 1995; 43: 265 (abstract).
40. **Ushijima H, Yamakawa H, Nagashima H.** Cryopreservation of bovine pre-morula-stage in vitro matured/in vitro fertilized embryos after delipidation and before use in nucleus transfer. *Biol Reprod* 1999; 60: 535–539.
41. **Takagi M, Otoi T, Boediono A, Saha S, Suzuki T.** Viability of frozen-thawed bovine IVM/IVF embryos in relation to aging using various cryoprotectants. *Theriogenology* 1994; 41: 915–921.
42. **Farin P, Farin C.** Perspectives on large calves following transfer of embryos produced in vitro. *Embryo Transfer Newslett* 1997; 15: 15–19.
43. **Behboodi E, Anderson GB, BonDurant RH, Cargill SL, Kreuscher BR, Medrano JF, Murray JD.** Birth of large calves that developed from in vitro-derived bovine embryos. *Theriogenology* 1995; 44: 227–232.
44. **Kruip TAM, den Daas JHG.** In vitro produced and cloned embryos: effects on pregnancy, parturition and offspring. *Theriogenology* 1997; 47: 43–52.
45. **Schmidt M, Greve T, Avery B, Beckers JF, Sulon J, Hansen HB.** Pregnancies, calves and calf viability after transfer of in vitro produced bovine embryos. *Theriogenology* 1996; 46: 527–539.
46. **Hoshi H.** In vitro production of bovine embryos and the application for embryo transfer. *Theriogenology* 2003; 59: 675–685.
47. **Yazawa S, Aoyagi Y, Konishi M, Takedomi T.** Characterization and cytogenetic analysis of Japanese Black calves produced by nuclear transfer. *Theriogenology* 1997; 48: 641–650.
48. **Kardymowicz O.** A method of vital staining for determining the viability of fertilized sheep ova stored in vitro. *Proc 7th International Congress on Animal Reproduction & AI, Munich* 1972; 1: 503–506.
49. **Schilling E, Smidt D, Sacher B, Petac D, El Kaschab S.** Diagnosis of the viability of early bovine

- embryos by fluorescence microscopy. *Ann Biol Anim Biochem Biophys* 1979; 19: 1625–1629.
50. **Renard JP, Philippon A, Menezo Y.** In vitro uptake of glucose by bovine blastocysts. *J Reprod Fertil* 1980; 58: 161–164.
 51. **Schilling E, Niemann H, Cheng SP, Duepke HH.** DAPI—a further fluorescence test for diagnosing the viability of early cow and rabbit embryos. *Zuchthyg* 1979; 14: 170–172.
 52. **Elsden RP, Nelson LD, Seidel GEJ.** Superovulating cows with follicle stimulating hormone and pregnant mare's serum gonadotrophin. *Theriogenology* 1978; 9: 17–26.
 53. **Lindner GM, Wright RWJ.** Bovine embryo morphology and evaluation. *Theriogenology* 1983; 20: 407–416.
 54. **Schneider HJJ, Castleberry RS, Griffin JL.** Commercial aspects of bovine embryo transfer. *Theriogenology* 1980; 13: 73–85.
 55. **Shea BF.** Evaluating the bovine embryo. *Theriogenology* 1981; 15: 13–42.
 56. **Wright JM.** Nonsurgical embryo transfer in cattle: embryo-recipient interactions. *Theriogenology* 1981; 15: 43–56.
 57. **Wales RG, Whittingham DG, Hardy K, Craft IL.** Metabolism of glucose by human embryos. *J Reprod Fertil* 1987; 79: 289–297.
 58. **Rieger D, Loskutoff NM, Betteridge KJ.** Developmentally related changes in the uptake and metabolism of glucose, glutamine and pyruvate by cattle embryos produced in vitro. *Reprod Fertil Dev* 1992; 4: 547–557.
 59. **Khurana NK, Niemann H.** Energy metabolism in preimplantation bovine embryos derived in vitro or in vivo. *Biol Reprod* 2000; 62: 847–856.
 60. **Magnusson C, Hillensjo T, Hamberger L, Nilsson L.** Oxygen consumption by human oocytes and blastocysts grown in vitro. *Hum Reprod* 1986; 1: 183–184.
 61. **Magnusson C, Hillensjo T, Tsafiriri A, Hultborn R, Ahren K.** Oxygen consumption of maturing rat oocytes. *Biol Reprod* 1977; 17: 9–15.
 62. **Houghton FD, Thompson JG, Kennedy CJ, Leese HJ.** Oxygen consumption and energy metabolism of the early mouse embryo. *Mol Reprod Dev* 1996; 44: 476–485.
 63. **Thompson JG, Partridge RJ, Houghton FD, Cox CI, Leese HJ.** Oxygen uptake and carbohydrate metabolism by in vitro derived bovine embryos. *J Reprod Fertil* 1996; 106: 299–306.
 64. **Donnay I, Leese HJ.** Embryo metabolism during the expansion of the bovine blastocyst. *Mol Reprod Dev* 1999; 53: 171–178.
 65. **Overstrom EW, Duby RT, Dobrinsky J, Roche JF, Boland MP.** Viability and oxidative metabolism of the bovine blastocyst. *Theriogenology* 1992; 37: 269 (abstract).
 66. **Smith PJS, Hammar K, Porterfield DM, Sanger RH, Trimarchi JR.** Self-referencing, non-invasive, ion selective electrode for single cell detection of trans-plasma membrane calcium flux. *Microsc Res Tech* 1999; 46: 398–417.
 67. **Land SC, Porterfield DM, Sanger RH, Smith PJS.** The self-referencing oxygen-selective microelectrode: detection of transmembrane oxygen flux from single cells. *J Exp Biol* 1999; 202: 211–218.
 68. **Trimarchi JR, Liu L, Porterfield DM, Smith PJS, Keefe DL.** Oxidative phosphorylation-dependent and -independent oxygen consumption by individual preimplantation mouse embryos. *Biol Reprod* 2000; 62: 1866–1874.
 69. **Abe H, Matsuzaki S, Hoshi H.** Ultrastructural differences in bovine morulae classified as high and low qualities by morphological evaluation. *Theriogenology* 2002; 57: 1273–1283.
 70. **Shiku H, Shiraishi T, Ohya H, Matsue T, Abe H, Hoshi H, Kobayashi M.** Oxygen consumption of single bovine embryos probed with scanning electrochemical microscopy. *Anal Chem* 2001; 73: 3751–3758.