Journal of Reproduction and Development, Vol. 63, No 4, 2017

—SRD Young Investigator Award 2016—

Selection of viable *in vitro*-fertilized bovine embryos using time-lapse monitoring in microwell culture dishes

Satoshi SUGIMURA[1)], Tomonori AKAI[2)] and Kei IMAI[3)]

[1)]*Department of Biological Production, Institute of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan*
[2)]*Dai Nippon Printing Co., Ltd., Kashiwa, Chiba 277-0871, Japan*
[3)]*Department of Sustainable Agriculture, Rakuno Gakuen University, Hokkaido 069-8501, Japan*

Abstract. Conventionally, *in vitro*-fertilized (IVF) bovine embryos for transfer are morphologically evaluated at day 7–8 of embryo culture. This method is, however, subjective and results in unreliable selection. We previously described a novel selection system for IVF bovine blastocysts for transfer that traces the development of individual embryos with time-lapse monitoring in our specially developed microwell culture dishes (LinKID micro25). The system can noninvasively identify prognostic factors that reflect viability after transfer. By assessing a combination of identified prognostic factors —timing of the first cleavage; number of blastomeres at the end of the first cleavage; and number of blastomeres at the onset of lag-phase, which results in temporary developmental arrest during the fourth or fifth cell cycle— the pregnancy rate was improved over using conventional morphological evaluation. Time-lapse monitoring with LinKID micro25 could facilitate objective and reliable selection of healthy IVF bovine embryos. Here, we review the novel bovine embryo selection system that allows for prediction of viability after transfer.

Key words: Bovine embryo, Culture dish, Pregnancy, Selection, Time-lapse monitoring

(**J. Reprod. Dev. 63:** 353–357, 2017)

It has long been a practice of embryo transfer practitioners to identify bovine *in vitro*-fertilized (IVF) embryos that are likely to establish a pregnancy and develop to term. Conventionally, the criteria for selecting the bovine embryos for transfer are based on morphological quality and the developmental stage at the time of transfer [1]. However, this approach is widely considered subjective and inadequate because of the significant variation between practitioner classification codes [2]. Furthermore, the pregnancy outcomes of blastocysts estimated by evaluators to be morphologically good to excellent (code 1) remain low (Fig. 1). Therefore, novel criteria allowing objective and reliable selection of embryos for transfer are required to advance bovine *in vitro* fertilization technology.

Aside from morphological quality, embryonic cell number of the inner cell mass (ICM) and trophectoderm (TE) [3], apoptosis incidence [4], hatching competence [5], chromosomal abnormalities [6], and expression of specific genes [7] have been widely accepted for determining embryo quality. Histological, cytogenetic, and epigenetic analyses, however, make transferring the embryo to a recipient more difficult and may also make practical embryo selection more cumbersome and complicated. Noninvasive criteria that could predict not only blastocyst qualities but also viability may, therefore, lead to novel methods for selecting bovine embryos for transfer.

Following ovum pickup (OPU), through which a limited number of oocytes is collected, the culture systems for *in vitro* oocyte maturation (IVM), *in vitro* fertilization, and embryo culture (IVC) individually appear to be practical [8]. Previously, we developed a microwell culture dish (LinKID micro25) based on the well system [9], which allows the tracking of individual embryos with time-lapse monitoring [10]. Time-lapse monitoring is an effective method for continuous imaging

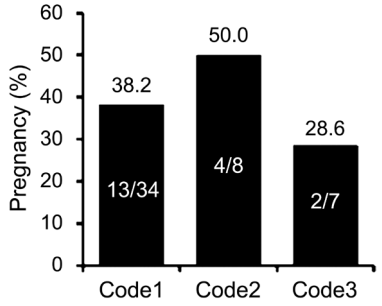

Fig. 1. Effect of conventional morphological code on percentage of pregnancy success at day 30 post-transfer. An *in vitro*-derived blastocyst, which was selected by conventional morphological code at day 7, was transferred to each synchronized recipient. Code 1: irregularities should be relatively minor and at least 85% of the cellular material should be an intact, viable embryonic mass; code 2: at least 50% of the cellular material should be an intact, viable embryonic mass; and code 3: at least 25% of the cellular material should be an intact, viable embryonic mass.

Received: March 28, 2017
Accepted: May 10, 2017
Published online in J-STAGE: May 26, 2017

Correspondence: S Sugimura (e-mail: satoshis@cc.tuat.ac.jp)

of the development of each individual embryo *in vitro*, allowing analysis of the morphokinetics, blastomere number, symmetry of cell division, and the extent of cytoplasmic fragmentation, which have been used to select the best embryos for transfer in human assisted reproduction technology [11–13]. In cattle, these criteria are possible predictors for developmental competence to blastocyst stage [14, 15], but are rarely used to select embryos for transfer.

We identified various prognostic factors that allow for predictions of viability after embryo transfer using time-lapse monitoring with LinKID micro25. The present review describes the specially developed microwell culture dish and prognostic factors for embryo transfer in cattle.

Microwell Culture Dish for Time-lapse Monitoring

Identifying and tracking the developmental progress of each embryo individually may lead to the identification of viability markers that can be used for the assessment of embryo quality and pregnancy success after transfer. However, there are problems with the isolation of embryos; for instance, if a single embryo culture is associated with a low embryo density, embryo development is handicapped, as indicated by the low developmental competence to the blastocyst stage, low cell numbers, and decreased production of interferon (IFN)-tau [16–18]. Aside from the low density, culturing embryos in a small total volume in droplets may lead to the accumulation of toxic substances, such as ammonium [19, 20] and oxygen-derived radicals [21], which may be harmful for embryos or have been suggested to lead to subsequent developmental anomalies [22]. Medium change could theoretically compensate for toxin accumulation, but this manipulation may have a harmful effect on embryo development [23] and would also lead to the elimination of positive-acting autocrine/paracrine factors.

We developed a novel microwell group culture dish (LinKID micro25, Dai Nippon Printing, Tokyo, Japan), which is compatible with time-lapse monitoring (Fig. 2A). Using this culture dish, individual culture without detrimental effects on embryo development, as well as time-lapse monitoring with high visibility, became possible [10, 24].

Design and fabrication

The microwell culture dish has 25 microwells and a circular wall in the center of the 35-mm culture dish. Each well is 270 μm in diameter and 150 μm in depth; the wells are arranged in 5 columns and 5 rows. Each well is separated by 150 μm. The bottom of each well slopes down toward the center of the well (slope angle, 7 degrees). The circular wall, which is 7 mm in diameter and 1.5 mm in height, is used to form a single 125-μl microdrop of culture medium covered with oil. The microwell culture dish is fabricated by the conventional injection molding method. Polystyrene was chosen as the material for the microwell culture dish owing to its non-toxicity for cell culture [10].

Advantageous effect

Embryos cultured in LinKID micro25 can be fixed in alternative microwells, which provide good visibility during time-lapse monitoring. No negative side effects from the culture dish have been observed in *in vitro* development or cell numbers at the blastocyst stage. Furthermore, blastocysts derived from LinKID micro25 have a low incidence of apoptosis and a higher pregnancy rate than those cultured in conventional droplets. This may be due to the accumulation of suitable autocrine and/or paracrine factors in the microwells [10].

In conventional group culture, the effects of positive-acting autocrine/paracrine factors and negative-acting toxic by-products of embryo metabolism depend on the droplet size [22], surface-to-volume ratio [22], distance between cultured embryos [25], quality of neighboring embryos [26], and embryo density (the number of embryos relative to medium volume) [18, 27, 28]. Embryo density has been considered a particularly critical factor, with some studies indicating that embryo density affects embryo development, the number of cells in the ICM and TE [29], IFN-tau secretion [17], and gene expression [28]. We examined the effect of embryo density (the number of embryos per volume of medium) on the rate of *in vitro* development and gene expression in bovine embryos cultured in conventional droplets or LinKID micro25. The microwell cultures did not show the same effects of embryo density on the *in vitro* development rate or gene expression that were observed in conventional droplet cultures. These findings indicate that blastocyst formation and transcription may not be affected by embryo density in LinKID micro25 as they are in conventional droplet culture [24].

Prognostic Factors Identified by Time-lapse Monitoring

We have established a novel system for selecting bovine embryos for transfer using time-lapse monitoring with LinKID micro25 [10, 30]. This system was successful in identifying prognostic factors: (i) timing of the first cleavage, (ii) blastomere number at the end of the first cleavage, and (iii) blastomere number at the onset of the lag-phase (Figs. 2B and 2C). These factors reflected viability after transfer (Table 1). Selection of blastocysts with multiple predictors improved the prediction of viable embryos compared with the conventional selection system (Table 2).

Timing of first cleavage

The mean timing of the first cleavage in successful pregnancies (26.5 ± 1.7 h) was earlier than in failed pregnancies (28.0 ± 2.1 h) (Fig. 3A). Furthermore, we observed that slowly cleaving embryos (> 27.0 h) had lower viability than fast cleaving embryos (≤ 27.0 h) after transfer (Fig. 3B). Previous studies with human cells also indicated that slowly cleaving embryos have a lower likelihood of pregnancy than rapidly cleaving embryos [31]. The reasons why slowly cleaving embryos have inferior viability and lead to lower pregnancy and implantation rates are unknown; however, chromosomal aberrations may play a role and likely reflect the quality of the spermatozoa and oocytes [32]. We confirmed that slowly cleaving embryos had a higher incidence of abnormal chromosomes and were identified as mixoploid more often than rapidly cleaving embryos [30]. As mixoploidy occurs in a relatively large number of *in vitro*-produced embryos, mixoploidy does not seem to critically influence subsequent development. A high frequency of abnormal chromosomes per blastocyst (> 25%) may, however, have serious consequences [33].

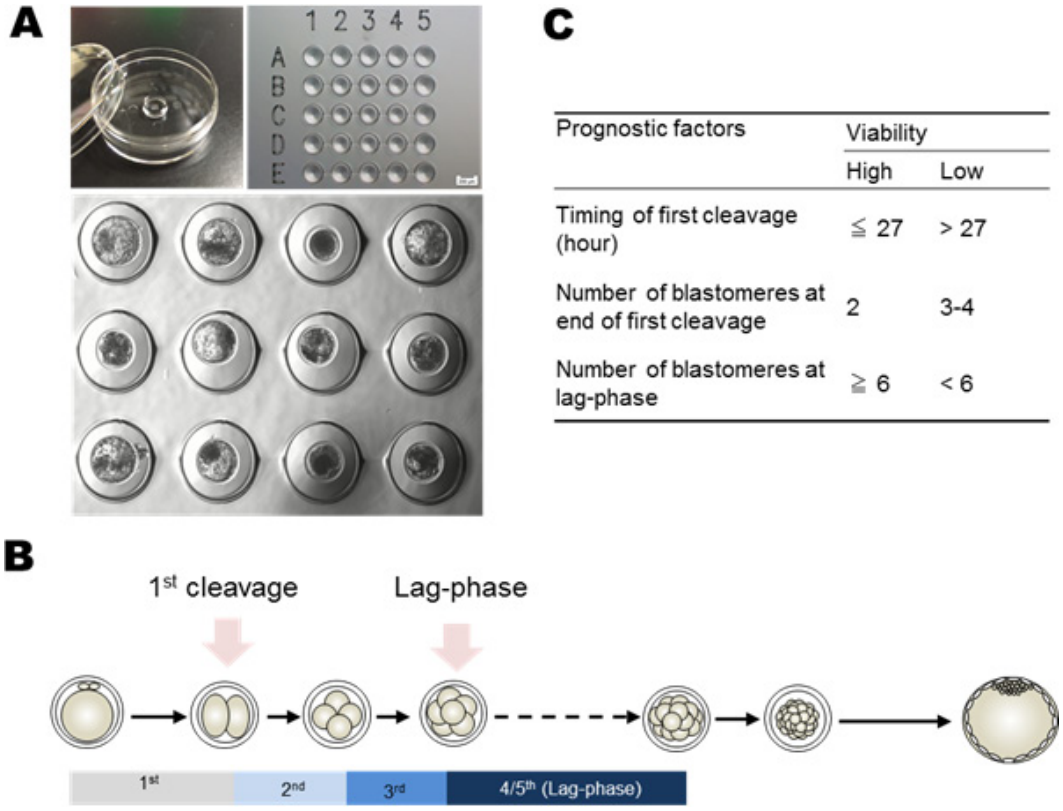

Fig. 2. Overview of the novel selection system. *In vitro* development of bovine IVF embryos in the specially developed microwell culture dish (LinKID micro25) was tracked with time-lapse monitoring (A and B). The timing of the first cleavage, the number of blastomeres at the end of the first cleavage, and the number of blastomeres at lag-phase were examined as prognostic factors (C).

Table 1. Logistic regression analysis of variables reflecting pregnancy status

Potential predictor variables	β [a]	SEM[b]	χ^2	P-value [c]	Odds ratio	95%C.I. [d]
First cleavage: Timing	–15.631	0.159	4.328	0.038	0.718	0.526–0.981
End of first cleavage: No. of blastomeres [e]	–0.331	1.763	5.141	0.023	54.416	1.719–1723.020
Second cell cycle: Duration	3.997	0.596	1.544	0.214	2.097	0.652–6.746
Third cell cycle: Duration	0.741	0.593	1.887	0.17	2.259	0.706–7.224
Cell cycle observed lag-phase	0.815	2.123	0.151	0.697	2.284	0.036–146.306
Lag-phase: Duration	0.188	0.105	3.217	0.073	1.207	0.983–1.484
Lag-phase: No. of blastomeres @4-5	–6.229	2.937	4.498	0.034	0.002	< 0.001–0.624
Lag-phase: No. of blastomeres @6-8	–2.461	2.097	1.378	0.241	0.085	0.001–5.201

[a] Coefficient estimate of multiple regression. [b] Standard error of β. [c] P-value of chi-square (χ^2) statistic. [d] 95% confidence interval.
[e] The number of blastomeres at the end of the first cleavage was categorized as 2 and 3/4 blastomeres.

Number of blastomeres at the end of the first cleavage

We observed that the pregnancy rate following the transfer of blastocysts that had undergone direct cleavage from one cell to 3–4 cells (3–4 blastomeres) was lower than that for blastocysts with normal cleavage morphologies (2 blastomeres) (Fig. 3C). A recent study on human cells indicated that the directly cleaved embryos correlated with impaired implantation and clinical outcomes [34]. Moreover, the study revealed a strong correlation between direct cleavage and multinucleation. Although the correlation in the bovine model is unclear, the direct cleavage morphology may result in subsequent chromosomal abnormalities [35, 36]. This anomalous first cleavage appears to be a product of insufficient oocyte maturation and/or polyspermy fertilization [37, 38]. Hence, improving oocyte maturation *in vitro* should be a key subject for the production of viable bovine IVF embryos [39].

Number of blastomeres at lag-phase

We identified the number of blastomeres at lag-phase, but not

Table 2. Effect of criteria for embryo selection on subsequent pregnancy rate at day 30 and 60 post-transfer

Criteria for embryo selection	No. of transferred blastocysts	No. (%) of pregnancies [c]	
		Day 30	Day 60
Conventional [a]	22	7 (31.8)	7 (31.8)
Identified [b]	27	16 (59.3)	15 (55.5)

[a] Transferred code 1 expanded blastocyst. [b] Transferred blastocyst identified by short duration and 2 blastomeres at first cleavage, and ≥ 6 blastomeres at onset of lag-phase. [c] Data were analyzed by the Fisher's exact test (P = 0.08).

cell cycle observed at lag-phase, as a prognostic factor (Table 1). Time-lapse monitoring showed that a low number of blastomeres at the onset of the lag-phase was related to a low ICM percentage and elevated apoptosis in blastocysts [30]. The lag-phase during the fourth or fifth cell cycle, which corresponds to the 5- to 8-cell stage and to the 9- to 16-cell stage, respectively, is a key step in embryonic gene activation (EGA), which occurs after the maternal-to-zygotic transition [40]. Moreover, the fourth cell cycle may be a conserved step in cell differentiation into ICM or TE cells. Indeed, the first signs of cell polarity in bovine embryos can already be detected at this stage [41]. The parameters of the lag-phase may, therefore, reflect differentiation and/or EGA and may, consequently, be related to the viability of embryos after transfer.

Future Prospects

The risk of pregnancy loss is likely to be reduced with observation of the cleavage pattern 2–3 days after fertilization. Although abnormal chromosomes and/or polyspermy may be potential reasons for the low viability of abnormally cleaved embryos [30], the mechanisms underlying both the abnormal cleavage pattern and low pregnancy rates following transfer are unclear. The data supporting the hypothesis were derived from fixed embryos with Giemsa staining, which makes transferring the embryo to a recipient impossible after the analysis. Moreover, high concentration of lipid droplets on bovine, but not human and mouse, zygotes makes observation of pronuclear with visible spectrum difficult.

Yamagata *et al*. succeeded in developing a less damaging live cell imaging system optimized for preimplantation mouse embryos. It involves microinjection with mRNAs encoding fluorescent proteins and observation using a particular confocal microscope [42]. The live cell imaging allows for long-term observation of molecular dynamics during early embryo development *in vitro*, such as the chromosomal segregation pattern. The live cell imaging system will help to understand why embryos undergoing abnormal cleavage have low developmental competence after transfer.

Time-lapse monitoring with LinKID micro25 could be used for practical selection of healthy IVF bovine embryos as an alternative to conventional morphological assessment at only the blastocyst stage. This system may contribute to the stable availability of dairy cattle and the expansion of beef cattle productivity.

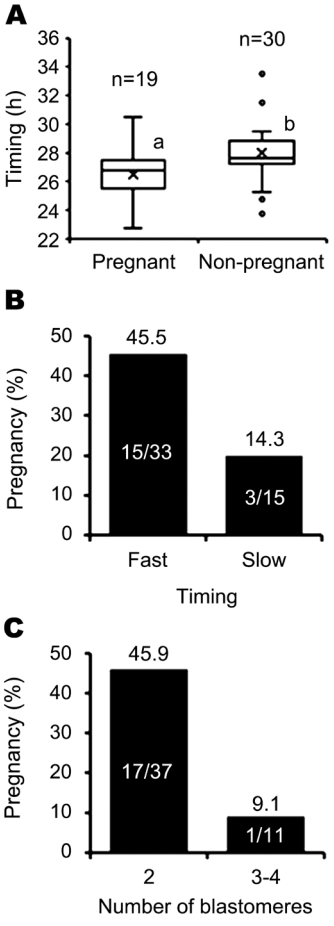

Fig. 3. The relationship between the (A) timing of the first cleavage in embryos resulting in successful or failed pregnancies after transfer, (B) percentage of pregnant cows after transfer of embryos with different timings for the first cleavage, or (C) different numbers of blastomeres after the first cleavage. The boxes indicate the 25th and 75th percentiles, and the middle horizontal line indicates the median. Whiskers indicate the maximum and minimum values within the acceptable range defined by the two quartiles. Circles denote outliers. Data were analyzed with Student's *t*-test. Different letters represent significant differences ([a,b] $P < 0.05$) (A). The percentage of pregnant cows that received embryos with specific first cleavage patterns, based on the timing of cleavage (B) and number of blastomeres (C). Blastocysts were divided into 2 groups, fast and slow, based on the timing of the first cleavage with a cut-off of 27 hpi (B), and 2 or 2–3 blastomeres at the end of the first cleavage (C).

Acknowledgments

The authors thank the members of the National Livestock Breeding Center for the management of the cows.

References

1. **Stringfellow DA, Seidel SM, IETS.** IETS manual. Manual of the International Embryo Transfer Society, 2001.
2. **Farin PW, Britt JH, Shaw DW, Slenning BD.** Agreement among evaluators of bovine embryos produced in vivo or in vitro. *Theriogenology* 1995; **44**: 339–349. [Medline] [CrossRef]
3. **Koo DB, Kang YK, Choi YH, Park JS, Kim HN, Oh KB, Son DS, Park H, Lee KK, Han YM.** Aberrant allocations of inner cell mass and trophectoderm cells in bovine nuclear transfer blastocysts. *Biol Reprod* 2002; **67**: 487–492. [Medline] [CrossRef]
4. **Gjørret JO, Knijn HM, Dieleman SJ, Avery B, Larsson LI, Maddox-Hyttel P.** Chronology of apoptosis in bovine embryos produced in vivo and in vitro. *Biol Reprod* 2003; **69**: 1193–1200. [Medline] [CrossRef]
5. **Massip A, Mulnard J, Vanderzwalmen P, Hanzen C, Ectors F.** The behaviour of cow blastocyst in vitro: cinematographic and morphometric analysis. *J Anat* 1982; **134**: 399–405. [Medline]
6. **Viuff D, Rickords L, Offenberg H, Hyttel P, Avery B, Greve T, Olsaker I, Williams JL, Callesen H, Thomsen PD.** A high proportion of bovine blastocysts produced in vitro are mixoploid. *Biol Reprod* 1999; **60**: 1273–1278. [Medline] [CrossRef]
7. **Mohan M, Hurst AG, Malayer JR.** Global gene expression analysis comparing bovine blastocysts flushed on day 7 or produced in vitro. *Mol Reprod Dev* 2004; **68**: 288–298. [Medline] [CrossRef]
8. **Matoba S, Fair T, Lonergan P.** Maturation, fertilisation and culture of bovine oocytes and embryos in an individually identifiable manner: a tool for studying oocyte developmental competence. *Reprod Fertil Dev* 2010; **22**: 839–851. [Medline] [CrossRef]
9. **Vajta G, Peura TT, Holm P, Páldi A, Greve T, Trounson AO, Callesen H.** New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. *Mol Reprod Dev* 2000; **55**: 256–264. [Medline] [CrossRef]
10. **Sugimura S, Akai T, Somfai T, Hirayama M, Aikawa Y, Ohtake M, Hattori H, Kobayashi S, Hashiyada Y, Konishi K, Imai K.** Time-lapse cinematography-compatible polystyrene-based microwell culture system: a novel tool for tracking the development of individual bovine embryos. *Biol Reprod* 2010; **83**: 970–978. [Medline] [CrossRef]
11. **Wong CC, Loewke KE, Bossert NL, Behr B, De Jonge CJ, Baer TM, Reijo Pera RA.** Non-invasive imaging of human embryos before embryonic genome activation predicts development to the blastocyst stage. *Nat Biotechnol* 2010; **28**: 1115–1121. [Medline] [CrossRef]
12. **Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Andersen AN.** Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. *Hum Reprod* 1997; **12**: 1545–1549. [Medline] [CrossRef]
13. **Shoukir Y, Campana A, Farley T, Sakkas D.** Early cleavage of in-vitro fertilized human embryos to the 2-cell stage: a novel indicator of embryo quality and viability. *Hum Reprod* 1997; **12**: 1531–1536. [Medline] [CrossRef]
14. **Holm P, Booth PJ, Callesen H.** Kinetics of early in vitro development of bovine in vivo- and in vitro-derived zygotes produced and/or cultured in chemically defined or serum-containing media. *Reproduction* 2002; **123**: 553–565. [Medline] [CrossRef]
15. **Lonergan P, Khatir H, Piumi F, Rieger D, Humblot P, Boland MP.** Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *J Reprod Fertil* 1999; **117**: 159–167. [Medline] [CrossRef]
16. **ODoherty EM, Wade MG, Hill JL, Boland MP.** Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. *Theriogenology* 1997; **48**: 161–169. [Medline] [CrossRef]
17. **Larson MA, Kubisch HM.** The effects of group size on development and interferon-tau secretion by in-vitro fertilized and cultured bovine blastocysts. *Hum Reprod* 1999; **14**: 2075–2079. [Medline] [CrossRef]
18. **Khurana NK, Niemann H.** Effects of oocyte quality, oxygen tension, embryo density, cumulus cells and energy substrates on cleavage and morula/blastocyst formation of bovine embryos. *Theriogenology* 2000; **54**: 741–756. [Medline] [CrossRef]
19. **Gardner DK, Lane M.** Amino acids and ammonium regulate mouse embryo development in culture. *Biol Reprod* 1993; **48**: 377–385. [Medline] [CrossRef]
20. **Sinclair KD, Dunne LD, Maxfield EK, Maltin CA, Young LE, Wilmut I, Robinson JJ, Broadbent PJ.** Fetal growth and development following temporary exposure of day 3 ovine embryos to an advanced uterine environment. *Reprod Fertil Dev* 1998; **10**: 263–269. [Medline] [CrossRef]
21. **Johnson MH, Nasr-Esfahani MH.** Radical solutions and cultural problems: could free oxygen radicals be responsible for the impaired development of preimplantation mammalian embryos in vitro? *BioEssays* 1994; **16**: 31–38. [Medline] [CrossRef]
22. **Ferry L, Mermillod P, Massip A, Dessy F.** Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. *Theriogenology* 1994; **42**: 445–453. [Medline] [CrossRef]
23. **Fukui Y, Lee ES, Araki N.** Effect of medium renewal during culture in two different culture systems on development to blastocysts from in vitro produced early bovine embryos. *J Anim Sci* 1996; **74**: 2752–2758. [Medline] [CrossRef]
24. **Sugimura S, Akai T, Hashiyada Y, Aikawa Y, Ohtake M, Matsuda H, Kobayashi S, Kobayashi E, Konishi K, Imai K.** Effect of embryo density on in vitro development and gene expression in bovine in vitro-fertilized embryos cultured in a microwell system. *J Reprod Dev* 2013; **59**: 115–122. [Medline] [CrossRef]
25. **Gopichandran N, Leese HJ.** The effect of paracrine/autocrine interactions on the in vitro culture of bovine preimplantation embryos. *Reproduction* 2006; **131**: 269–277. [Medline] [CrossRef]
26. **Stokes PJ, Abeydeera LR, Leese HJ.** Development of porcine embryos in vivo and in vitro; evidence for embryo cross talk in vitro. *Dev Biol* 2005; **284**: 62–71. [Medline] [CrossRef]
27. **Fujita T, Umeki H, Shimura H, Kugumiya K, Shiga K.** Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. *J Reprod Dev* 2006; **52**: 137–142. [Medline] [CrossRef]
28. **Hoelker M, Rings F, Lund Q, Ghanem N, Phatsara C, Griese J, Schellander K, Tesfaye D.** Effect of the microenvironment and embryo density on developmental characteristics and gene expression profile of bovine preimplantative embryos cultured in vitro. *Reproduction* 2009; **137**: 415–425. [Medline] [CrossRef]
29. **Vutyavanich T, Saeng-Anan U, Sirisukkasem S, Piromlertamorn W.** Effect of embryo density and microdrop volume on the blastocyst development of mouse two-cell embryos. *Fertil Steril* 2011; **95**: 1435–1439. [Medline] [CrossRef]
30. **Sugimura S, Akai T, Hashiyada Y, Somfai T, Inaba Y, Hirayama M, Yamanouchi T, Matsuda H, Kobayashi S, Aikawa Y, Ohtake M, Kobayashi E, Konishi K, Imai K.** Promising system for selecting healthy in vitro-fertilized embryos in cattle. *PLoS ONE* 2012; **7**: e36627. [Medline] [CrossRef]
31. **Fenwick J, Platteau P, Murdoch AP, Herbert M.** Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos in vitro. *Hum Reprod* 2002; **17**: 407–412. [Medline] [CrossRef]
32. **Magli MC, Gianaroli L, Ferraretti AP, Lappi M, Ruberti A, Farfalli V.** Embryo morphology and development are dependent on the chromosomal complement. *Fertil Steril* 2007; **87**: 534–541. [Medline] [CrossRef]
33. **Hare WC, Singh EL, Betteridge KJ, Eaglesome MD, Randall GC, Mitchell D, Bilton RJ, Trounson AO.** Chromosomal analysis of 159 bovine embryos collected 12 to 18 days after estrus. *Can J Genet Cytol* 1980; **22**: 615–626. [Medline] [CrossRef]
34. **Zhan Q, Ye Z, Clarke R, Rosenwaks Z, Zaninovic N.** Direct Unequal Cleavages: Embryo Developmental Competence, Genetic Constitution and Clinical Outcome. *PLoS ONE* 2016; **11**: e0166398. [Medline] [CrossRef]
35. **Somfai T, Inaba Y, Aikawa Y, Ohtake M, Kobayashi S, Konishi K, Imai K.** Relationship between the length of cell cycles, cleavage pattern and developmental competence in bovine embryos generated by in vitro fertilization or parthenogenesis. *J Reprod Dev* 2010; **56**: 200–207. [Medline] [CrossRef]
36. **Sathananthan AH, Kola I, Osborne J, Trounson A, Ng SC, Bongso A, Ratnam SS.** Centrioles in the beginning of human development. *Proc Natl Acad Sci USA* 1991; **88**: 4806–4810. [Medline] [CrossRef]
37. **Wang WH, Abeydeera LR, Prather RS, Day BN.** Morphologic comparison of ovulated and in vitro-matured porcine oocytes, with particular reference to polyspermy after in vitro fertilization. *Mol Reprod Dev* 1998; **49**: 308–316. [Medline] [CrossRef]
38. **Han YM, Wang WH, Abeydeera LR, Petersen AL, Kim JH, Murphy C, Day BN, Prather RS.** Pronuclear location before the first cell division determines ploidy of polyspermic pig embryos. *Biol Reprod* 1999; **61**: 1340–1346. [Medline] [CrossRef]
39. **Sugimura S, Ritter LJ, Sutton-McDowall ML, Mottershead DG, Thompson JG, Gilchrist RB.** Amphiregulin co-operates with bone morphogenetic protein 15 to increase bovine oocyte developmental competence: effects on gap junction-mediated metabolite supply. *Mol Hum Reprod* 2014; **20**: 499–513. [Medline] [CrossRef]
40. **Lequarre AS, Marchandise J, Moreau B, Massip A, Donnay I.** Cell cycle duration at the time of maternal zygotic transition for in vitro produced bovine embryos: effect of oxygen tension and transcription inhibition. *Biol Reprod* 2003; **69**: 1707–1713. [Medline] [CrossRef]
41. **Koyama H, Suzuki H, Yang X, Jiang S, Foote RH.** Analysis of polarity of bovine and rabbit embryos by scanning electron microscopy. *Biol Reprod* 1994; **50**: 163–170. [Medline] [CrossRef]
42. **Yamagata K, Suetsugu R, Wakayama T.** Long-term, six-dimensional live-cell imaging for the mouse preimplantation embryo that does not affect full-term development. *J Reprod Dev* 2009; **55**: 343–350. [Medline] [CrossRef]