BIOLOGY OF REPRODUCTION **71,** 1096–1100 (2004)
Published online before print 9 June 2004.
DOI 10.1095/biolreprod.104.030635

Effect of the Post-Fertilization Culture Environment on the Incidence of Chromosome Aberrations in Bovine Blastocysts[1]

Patrick Lonergan,[2,3] Hanne G. Pedersen,[4] Dimitrios Rizos,[3] Torben Greve,[4] Preben D. Thomsen,[5] Trudee Fair,[3] Alex Evans,[3] and Maurice P. Boland[3]

Department of Animal Science and Production,[3] University College Dublin, Lyons Research Farm, Newcastle, County Dublin, Ireland
Department of Clinical Studies, Reproduction[4] and Department of Physiology,[5] Royal Veterinary and Agricultural University, DK-1870 Frederiksberg C, Denmark

ABSTRACT

We have previously shown that the postfertilization embryo culture environment has a significant influence on the quality of the resulting bovine blastocyst measured in terms of its cryotolerance and relative abundance for several developmentally important gene transcripts. Using three different culture conditions known to produce blastocysts of differing quality, the objective of this study was to examine whether the postfertilization culture environment had an effect on the incidence of mixoploidy in bovine blastocysts. Presumptive zygotes, produced by in vitro maturation and fertilization, were cultured in vitro in synthetic oviduct fluid (SOF) medium in the absence or presence of fetal calf serum (FCS), or in vivo in the ewe oviduct. Blastocysts were recovered from the three systems at Day 7 and the incidence of mixoploidy was assessed using fluorescence in situ hybridization with chromosome 6- and chromosome 7-specific probes. A total of 10 025 nuclei were scored in 122 blastocysts. The frequency of normal, diploid, blastocysts was 8.8%, 21.4%, and 34.8% in embryos derived from culture in SOF+FCS, SOF, and the ewe oviduct, respectively, the remainder showing some degree of mixoploidy. The incidence of mixoploidy was apparently not related to the presence of serum; omission of serum from SOF resulted in a reduction in the incidence of mixoploidy (91.2% vs. 78.6%), although this difference was not significant. Culture in vivo, however, resulted in a significant ($P < 0.01$) reduction in the incidence of mixoploidy compared with culture in vitro in the presence of serum (65.2% vs. 91.2%, respectively). Among the mixoploid blastocysts, the majority contained less than 10% polyploid cells, irrespective of culture group (SOF, 69.7%; SOF+FCS, 64.5%; ewe oviduct, 60.0%). More than one type of polyploidy was frequently observed in mixoploid blastocysts. Overall, diploidy-triploidy was the most frequent abnormality, but diploid-tetraploid and diploid-triploid-tetraploid mosaics were also observed. A significantly higher proportion ($P < 0.05$) of blastocysts derived from SOF+FCS had more than one type of abnormality (80.6%, 25/31) compared with those derived from SOF (45.4%, 15/33) or in vivo culture (53.3%, 16/30). In conclusion, the postfertilization culture environment of the developing embryo can affect the incidence and severity of mixoploidy in the resulting blastocyst.

chromosome constitution, developmental biology, early development, embryo, oocyte development, oviduct, preimplantation development

INTRODUCTION

It has previously been demonstrated that the postfertilization embryo culture environment has a significant influence on the quality of the resulting bovine blastocyst measured in terms of its cryotolerance following vitrification [1, 2]. These differences are reflected in modifications of the relative transcript abundance for several developmentally important genes [3]. Furthermore, modification of the media used during in vitro culture can alter the pattern of mRNA expression to more closely mirror that of in vivo embryos with an associated increase in cryotolerance [4]. It was subsequently shown that, depending on the transcript, these changes in mRNA expression pattern can arise after as little as 10 h of culture [5].

Fluorescence in situ hybridization (FISH) analysis, involving hybridization of chromosome-specific DNA probes followed by detection of the bound probes under a fluorescence microscope, has been used recently to study the chromosome constitution of bovine embryos [6–9]. Using such techniques, it has been shown that a high proportion (>70%) of in vitro-derived bovine blastocysts contain polyploid cells, i.e., are mixoploid, compared with those recovered from the uterus following in vivo maturation, fertilization, and development (25%) [6, 10]. This indicates that the conditions of oocyte maturation, fertilization, and early embryo culture in vitro increase the proportion of mixoploid embryos but does not indicate at which stage(s) of the process the changes occur. The same authors subsequently demonstrated that mixoploidy was detectable as early as Day 2 in both in vivo [11] and in vitro [12] embryos and increased from Day 2 to Day 5, with a significantly lower frequency of abnormalities in in vivo-derived embryos.

Using three different culture conditions, known from our previous work to produce blastocysts of differing quality, the objective of this study was to examine whether the postfertilization culture environment in vitro had an effect on the incidence of mixoploidy in bovine blastocysts.

MATERIALS AND METHODS

Blastocyst Production

Immature cumulus oocyte complexes recovered from the ovaries of cattle slaughtered at a local abattoir were submitted to in vitro maturation

[1]Supported by Science Foundation Ireland under Grant 02/IN1/B78 to A.E. and P.L. (the opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the Science Foundation Ireland).
[2]Correspondence. FAX: 353 1 6288421; e-mail: pat.lonergan@ucd.ie

Received: 5 April 2004.
First decision: 18 April 2004.
Accepted: 18 May 2004.

ISSN: 0006-3363. http://www.biolreprod.org

and fertilization using standard techniques [2]. Approximately 20 h after insemination, presumptive zygotes were denuded and randomly allocated to one of three postfertilization culture treatments: 1) in vitro culture in synthetic oviduct fluid (SOF) supplemented with 3 mg/ml BSA, 2) in vitro culture in SOF + 3 mg/ml BSA and 10% fetal calf serum (FCS), 3) in vivo culture in the ewe oviduct. These culture conditions were known from previous work to produce blastocysts of differing quality in terms of cryotolerance [1, 2, 4], ultrastructural morphology [13, 14], and relative mRNA transcript abundance [3–5].

For in vitro culture, zygotes were transferred to 25-μl culture droplets (1 embryo/μl) of SOF supplemented with 3 mg/ml BSA under mineral oil (SOF). Half of the in vitro-cultured embryos received FCS at 48 h postinsemination (SOF+FCS). A third group (ewe oviduct) of zygotes were transferred to the ligated ewe oviduct as previously described [1, 2]. Blastocysts were recovered from the three systems at Day 7. All experimental procedures were licensed by the Department of Health and Children, Ireland, in accordance with the cruelty to animals act (Ireland 1897) and European Community Directive 86/609/EC.

Embryo Fixation and Fluorescence In Situ Hybridization

Immediately following recovery, the blastocysts were spread using the method described previously [6]. Briefly, individual blastocysts were quickly washed in lysis solution (0.01 N HCl, 0.1% Tween 20) and transferred in a small droplet of lysis solution to a Superfrost Plus slide. The blastocyst was constantly observed using an inverted phase-contrast microscope (Nikon, Diaphot). The zona pellucida and the blastomere cytoplasm dissolved gradually and, just before the nuclei dried out, a 3:1 solution of methanol:acetic acid was added dropwise to the slide. The specimens were then fixed in 3:1 methanol:acetic acid at 4°C for 24 h, air dried, incubated at 60°C overnight, and stored at −80°C. FISH was performed according to a previously described method [12]. Briefly, DNA probes specific for chromosome 6 (p33E39) and chromosome 7 (cJAB8) were labeled with biotin (Life Technologies, Tåstrup, Denmark) or digoxigenin (DIG; Boehringer Mannheim, Mannheim, Germany) by nick translation. The embryonic nuclei were treated with pepsin (Sigma Aldrich), fixed in 1% formaldehyde, and DNA was denatured in 70% formamide (VWR International Aps, Albertslund, Denmark) at 71°C, followed by hybridization at 42°C. Hybridization sites were visualized using Cy3-conjugated avidin (Jackson ImmunoResearch, West Grove, PA) and anti-DIG-fluorescein (Boehringer Mannheim). Nuclear DNA was stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich).

Analytical Criteria

Nuclei were scored only if they were intact and nonoverlapping. The specific signals in a given blastomere were considered to reflect a true chromosome constitution if the signals were of similar size, shape, and intensity and were more than the diameter of a single signal apart. A nucleus was considered diploid (Fig. 1) if it was possible to count two red and two green (i.e., 2+2), 2+1, or 2+0 signals and triploid if 3+3, 3+2, 3+1, or 3+0 signals were observed. Nuclei with higher ploidy were classified accordingly. Embryos with more than one type of chromosome complement were considered mixoploid.

Statistical Analysis

The frequency of chromosomal abnormalities among the three groups of blastocysts was compared with chi-square analysis. A P value <0.05 was considered significant.

RESULTS

A total of 10 025 nuclei were scored in 122 blastocysts collected on Day 7 (Table 1). An average of 82.2 (22–165) nuclei per embryo was analyzed. A low number of analyzed nuclei was due, in most cases, to loss of nuclei from the slide during fixation.

Results of the chromosome analysis of the blastocysts from the 3 groups are shown in Table 2. The frequency of normal, diploid blastocysts was 8.8%, 21.4%, and 34.8% in embryos derived from culture in SOF+FCS, SOF, and the ewe oviduct, respectively, the remainder showing some degree of mixoploidy. Omission of serum from SOF resulted in a reduction in the incidence of mixoploidy (91.2%

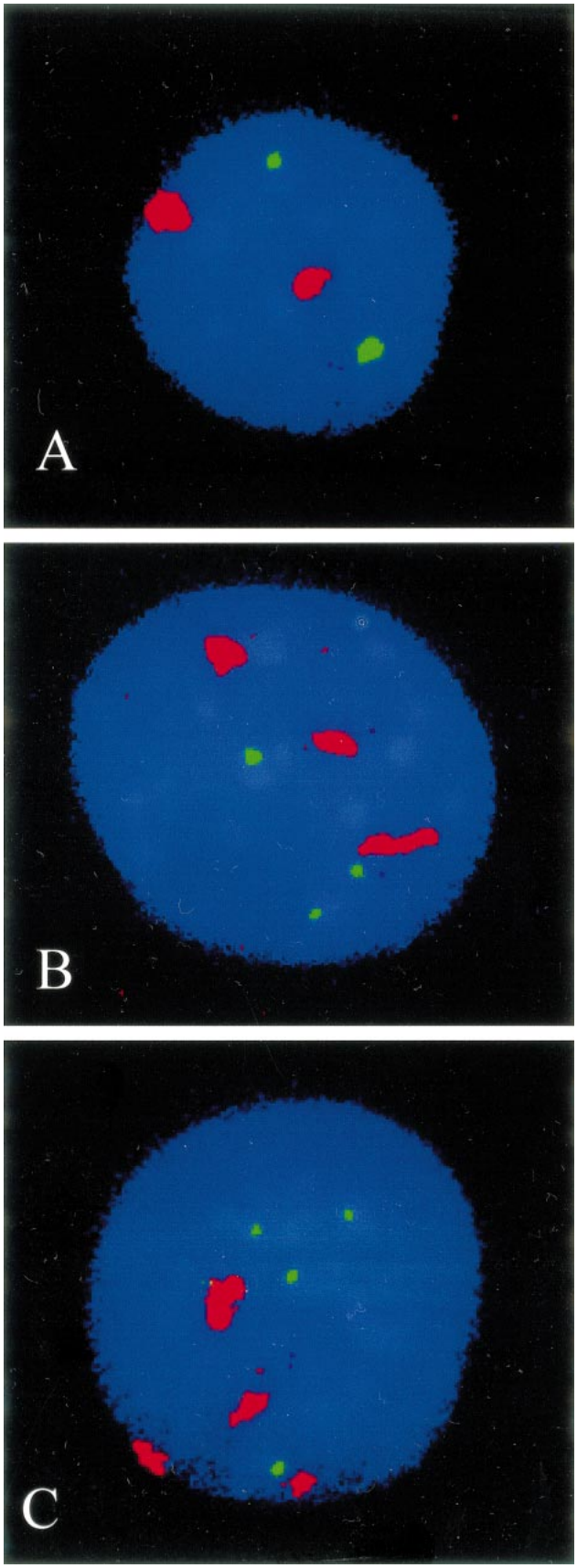

FIG. 1. Representative images of FISH with chromosome 6 (green)- and chromosome 7 (red)-specific DNA probes on extracted nuclei from bovine blastocysts. The nuclei were counterstained with DAPI (blue). **A**) A normal diploid blastomere with two signals from each chromosome. **B**) A triploid blastomere showing three green and three red signals. **C**) A tetraploid blastomere with four signals from each chromosome. Original magnification, ×100.

TABLE 1. Number of nuclei analyzed in bovine blastocysts cultured in vitro in the presence or absence of serum or in vivo in the ewe oviduct.

	Total no. of nuclei examined	No. of nuclei included in analysis*	Mean no. of nuclei examined per embryo (range)
SOF	3262	3192	77.7 (22–162)
SOF + FCS	2988	2917	87.9 (29–163)
Ewe oviduct	3775	3736	82.1 (27–165)
Total	10 025	9845	82.2 (22–165)

* Excluding cases of hybridization error.

TABLE 2. Incidence of mixoploidy in bovine blastocysts cultured in vitro in the presence or absence of serum or in vivo in the ewe oviduct.

	N	No. (%) embryos with chromosomal abnormalities	Mean % of abnormal nuclei per embryo
SOF	42	33 (78.6)[ab]	23.0
SOF + FCS	34	31 (91.2)[a]	18.7
Ewe oviduct	46	30 (65.2)[b]	25.3

[a,b] Values with different superscripts differ significantly ($P < 0.05$).

vs. 78.6%), although this difference was not significant. Culture in vivo, however, resulted in a significant ($P < 0.01$) reduction in the incidence of mixoploidy compared with culture in vitro in the presence of serum (65.2% vs. 91.2%, respectively). Among the mixoploid blastocysts, the majority contained less than 10% polyploid cells, irrespective of culture group (SOF, 69.7%; SOF+FCS, 64.5%; ewe oviduct, 60.0%; Table 3).

More than one type of polyploidy was frequently observed in mixoploid blastocysts (Table 4). Overall, diploidy-triploidy was the most frequent abnormality, but diploid-tetraploid and diploid-triploid-tetraploid mosaics were also observed. A significantly higher proportion ($P < 0.05$) of blastocysts derived from SOF+FCS had more than one type of abnormality (80.6%, 25/31) compared with those derived from SOF in the absence of serum (45.4%, 15/33) or in in vivo culture (53.3%, 16/30).

DISCUSSION

The present study demonstrates that the type of postfertilization culture environment used for the culture of in vitro-derived bovine zygotes can have a significant effect on the incidence of chromosomal abnormalities in the resulting blastocysts. To our knowledge, this is the first time that such an effect has been demonstrated.

FISH with bovine chromosome 6- and 7-specific probes was used to provide estimates of the extent and types of chromosome variability in bovine blastocysts derived from in vitro-produced zygotes cultured under different postfertilization conditions. An average of 82 nuclei were analyzed per blastocyst, and the results revealed an overall mixoploidy rate of approximately 77%. This is entirely consistent with the data of Viuff et al. [6, 10], who used the same probes and the same technique for analysis of blastomere chromosome variability.

Only a small fraction of the total number of cells in a given blastocyst was polyploid. For example, of the mixoploid blastocysts, for the groups SOF, SOF+FCS, and ewe oviduct, respectively, 69.7%, 64.5%, and 60.0% contained less than 10% polyploid cells. Among the three groups, 27.2% (9/33, SOF), 19.3% (6/31, SOF+FCS), and 26.7% (8/30, ewe oviduct) of mixoploid embryos contained more than 25% polyploid cells per blastocyst. This contrasts with the figure of 4% (4/109) reported by Viuff et al. [6] and may reflect differences in the culture conditions employed in the two studies and indicate that the viability of a rather high proportion of embryos is compromised at some step of the experimental procedure.

Inadequate oocyte maturation resulting in lack of polar body extrusion and/or polyspermy may lead to poly- or mixoploidy. In cattle, 21% of blastocysts derived from in vivo-matured oocytes were mixoploid compared with 50% of those from in vitro-matured oocytes [15]. Thus, the level of mixoploid blastocysts from in vivo-matured oocytes was similar to that reported for entirely in vivo-developed blastocysts [6]. This level of mixoploidy in blastocysts from in vivo-matured oocytes is, however, low compared with 50% of those from in vitro-matured oocytes [15]. This would be consistent with several reports indicating that in vivo-matured oocytes have a higher developmental competence than their in vitro-matured counterparts [16–18], even when the two groups have identical developmental histories up to resumption of meiosis (LH surge) [2, 15, 19]. In contrast, other data would suggest that the origin of the oocyte has no effect on the quality of the blastocyst as measured by gene expression [20] and cryotolerance [2], highlighting the importance of the parameters used to assess blastocyst quality. Slimane et al. [8] reported that the incidence of chromosome abnormalities in in vitro-produced bovine two-cell

TABLE 3. Distribution of chromosome abnormalities in bovine blastocysts cultured in vitro in the presence or absence of serum or in vivo in the ewe oviduct.

Nuclei with chromosomal abnormalities (%)	SOF		SOF + FCS		Ewe oviduct	
	N	%	N	%	N	%
1–5	20	60.6	11	35.5	16	53.3
6–10	3	9.1	9	29.0	2	6.7
10–15	—	—	2	6.5	1	3.3
16–20	1	3.0	2	6.5	2	6.7
21–25	—	—	1	3.2	1	3.3
26–30	1	3.0	1	3.2	—	—
31–35	—	—	1	3.2	1	3.3
36–40	—	—	—	—	—	—
41–45	—	—	—	—	1	3.3
46–50	1	3.0	—	—	—	—
>50	7	21.2	4	12.9	6	20.0
Total	33	100	31	100	30	100

TABLE 4. Numbers of different types of abnormalities (triploid, tetraploid, etc.) in bovine blastocysts cultured in vitro in the presence or absence of serum or in vivo in the ewe oviduct.

Number of different abnormalities/blastocyst	SOF		SOF + FCS		Ewe oviduct	
	N	%	N	%	N	%
1	18	54.5[a]	6	19.3[b]	14	46.7[a]
2	9	27.3[a]	16	51.6[b]	10	33.3[ab]
3	4	12.1	7	22.6	6	20.0
4	2	6.1	2	6.4	0	—
Total embryos with abnormalities	33	100	31	100	30	100

[a,b] Values in the same row with different superscripts differ significantly ($P < 0.05$).

embryos varied from 28% to 44%, depending on the probes used. To distinguish the contribution of fertilization and culture to the incidence of abnormalities, Slimane et al. [7] compared in vitro- and in vivo-derived two-cell bovine embryos. The frequency of normal embryos was the same for both groups (~65%), indicating that chromosomal variability occurs as early as the first cleavage and that in vitro fertilization apparently does not increase the frequency of chromosome abnormalities.

Detection of a high incidence of chromosome variability in in vitro-derived blastocysts [6, this study] demonstrates that such abnormalities are not incompatible with blastocyst formation. This raises the question of the impact of such abnormalities on subsequent development. Using blastocysts produced in conditions similar to those used in the present study, Lazzari et al. [21] reported pregnancy rates of 36.9%, 16.7%, 53.8%, and 55.2% following transfer of frozen-thawed embryos produced in SOF+BSA, SOF+serum, the ewe oviduct, or entirely in vivo, respectively. Compared with our data, this suggests that there is an inverse relationship between the level of mixoploid embryos and developmental capacity. This is, however, contradicted by other studies (Hare et al. [22]) that indicate that mixoploidy at low levels, i.e., lower than 25%, seems not to influence the developmental capacity negatively. Actually, if we include all diploid embryos as well as those with less than 25% abnormal nuclei under the term normal, there was no difference between the groups (SOF, 33/42; SOF+FCS, 28/34; ewe oviduct, 38/46).

The incidence of mixoploidy was apparently not related to the presence of serum; omission of serum from the SOF reduced the incidence of mixoploidy (91.2% vs. 78.6%), although not significantly, while culturing the embryos in vivo resulted in a significant reduction in the incidence of mixoploidy. However, the severity of the abnormality was greatest in blastocysts derived from SOF+FCS, as a significantly higher proportion of such blastocysts had more than one type of abnormality (80.6%) compared with those derived from SOF in the absence of serum (45.4%) or in vivo culture (53.3%). This apparent negative effect of serum on developmental processes is consistent with previous studies reporting a negative effect of serum on blastocyst quality measured in terms of ultrastructural morphology [13], cryotolerance [4], and mRNA abundance [3, 4]. It is also well known that serum has an accelerating effect on development [23, 24] and it is possible that the increased rate of development does not leave enough time for normal karyokinesis to occur. In mitosis, and possibly also in meiosis, a spindle-assembly checkpoint ensures accurate chromosome segregation by delaying the onset of anaphase until all chromosomes are correctly attached to the spindle through their kinetochores [25]. It is possible that modification of culture environment resulting in accelerated division perturbs this process.

In conclusion, not only does the postfertilization embryo culture conditions in vitro impact on gene expression in the embryo [3–5, 26], but there is an effect, possibly linked to the speed of development, on the incidence and severity of mixoploidy in the resulting blastocysts.

ACKNOWLEDGMENTS

The authors wish to thank Zaida R. Rasmussen, Bente Synnestvedt, Mary Wade, and Pat Duffy for excellent technical assistance. Dr. Ingrid Olsaker, Norges Veterinærhøgskole, and Dr. John Williams, Roslin Institute, are acknowledged for the generous gift of the chromosome-specific probes p33E39 and cJAB8, respectively.

REFERENCES

1. Enright BP, Lonergan P, Dinnyes A, Fair T, Ward FA, Yang X, Boland MP. Culture of in vitro produced bovine zygotes in vitro vs. in vivo: implications for early embryo development and quality. Theriogenology 2000; 54:659–673.
2. Rizos D, Lonergan P, Ward F, Duffy P, Boland MP. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blastocyst yield and blastocyst quality. Mol Reprod Dev 2002; 61:234–248.
3. Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J, Gutierrez-Adan A. Analysis of differential mRNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. Biol Reprod 2002; 66:589–595.
4. Rizos D, Gutierrez-Adan A, Perez-Garnelo S, De La Fuente J, Boland MP, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 2003; 68:236–243.
5. Lonergan P, Rizos D, Gutierrez-Adan A, Moreira PM, Pintado B, De La Fuente J, Boland MP. Temporal divergence in the pattern of messenger RNA expression in bovine embryos cultured from the zygote to blastocyst stage in vitro or in vivo. Biol Reprod 2003; 69:1424–1431.
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.
7. Slimane W, Heyman Y, Renard J-P. Assessing chromosomal abnormalities by FISH analysis in 2-cell bovine embryos derived from in vitro and in vivo fertilization. Theriogenology 2000; 53:432 (abstract).
8. Slimane W, Heyman Y, Lavergne Y, Humblot P, Renard JP. Assessing chromosomal abnormalities in two-cell bovine in vitro fertilized embryos by using in situ hybridization with three different cloned probes. Biol Reprod 2000; 62:628–635.
9. Slimane-Bureau WC, King WA. Chromosomal abnormalities: a potential quality issue for cloned cattle embryos. Clon Stem Cells 2002; 4:319–329.
10. Viuff D, Palsgaard A, Rickords L, Lawson LG, Greve T, Schmidt M, Avery B, Hyttel P, Thomsen PD. Bovine embryos contain a higher proportion of polyploid cells in the trophectoderm than in the embryonic disc. Mol Reprod Dev 2002; 62:483–488.
11. Viuff D, Hendriksen PJ, Vos PL, Dieleman SJ, Bibby BM, Greve T, Hyttel P, Thomsen PD. Chromosomal abnormalities and developmental kinetics in in vivo-developed cattle embryos at days 2 to 5 after ovulation. Biol Reprod 2001; 65:204–208.
12. Viuff D, Greve T, Avery B, Hyttel P, Brockhoff PB, Thomsen PD. Chromosome aberrations in in vitro-produced bovine embryos at days 2–5 postinsemination. Biol Reprod 2000; 63:1143–1148.

13. Fair T, Lonergan P, Dinnyes A, Cottell D, Hyttel P, Ward FA, Boland MP. Ultrastructure of bovine blastocysts following cryopreservation: effect of method of embryo production on blastocyst quality. Mol Reprod Dev 2001; 58:186–195.
14. Rizos D, Fair T, Boland M, Lonergan P. Developmental, qualitative and ultrastructural differences between ovine and bovine embryos produced in vivo or in vitro. Mol Reprod Dev 2002; 62:320–327.
15. Dieleman SJ, Hendriksen PJ, Viuff D, Thomsen PD, Hyttel P, Knijn HM, Wrenzycki C, Kruip TA, Niemann H, Gadella BM, Bevers MM, Vos PL. Effects of in vivo prematuration and in vivo final maturation on developmental capacity and quality of preimplantation embryos. Theriogenology 2002; 57:5–20.
16. Marquant-Le Guienne B, Gerard M, Solari A, Thibault C. In vitro culture of bovine egg fertilized either in vivo or in vitro. Reprod Nutr Dev 1989; 29:559–568.
17. Greve T, Xu KP, Callesen H, Hyttel P. In vivo development of in vitro fertilized bovine oocytes matured in vivo versus in vitro. J IVF Embryo Transfer 1987; 4:281–285.
18. van de Leemput EE, Vos PLAM, Zeinstra EC, Bevers MM, van der Weijden GC, Dieleman SJ. Improved in vitro embryo development using in vivo matured oocytes from heifers superovulated with a controlled preovulatory LH surge. Theriogenology 1999; 52:335–349.
19. Hendriksen PJM, Vos PLAM, Steenweg WNM, Bevers MM, Dieleman SJ. Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology 2000; 53:11–20.
20. Knijn HM, Wrenzycki C, Hendriksen PJ, Vos PL, Herrmann D, Van Der Weijden GC, Niemann H, Dieleman SJ. Effects of oocyte maturation regimen on the relative abundance of gene transcripts in bovine blastocysts derived in vitro or in vivo. Reproduction 2002; 124:365–375.
21. Lazzari G, Wrenzycki C, Herrmann D, Duchi R, Kruip T, Niemann H, Galli C. Cellular and molecular deviations in bovine in vitro-produced embryos are related to the large offspring syndrome. Biol Reprod 2002; 67:767–775.
22. Hare WCD, Singh EL, Betteridge KJ, Eaglesome MD, Randall GCB, 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.
23. Lonergan P, O'Kearney-Flynn M, Boland MP. Effect of protein supplementation and presence of an antioxidant on the development of bovine zygotes in synthetic oviduct fluid medium under high or low oxygen tension. Theriogenology 1999; 51:1565–1576.
24. Gutierrez-Adan A, Lonergan P, Rizos D, Ward FA, Boland MP, Pintado B, de la Fuente J. Effect of the in vitro culture system on the kinetics of blastocyst development and sex ratio of bovine embryos. Theriogenology 2001; 55:1117–1126.
25. Brunet S, Pahlavan G, Taylor S, Maro B. Functionality of the spindle checkpoint during the first meiotic division of mammalian oocytes. Reproduction 2003; 126:443–450.
26. Lonergan P, Rizos D, Kanka J, Nemcova L, Mbaye AM, Kingston M, Wade M, Duffy P, Boland MP. Temporal sensitivity of bovine embryos to culture environment after fertilization and the implications for blastocyst quality. Reproduction 2003; 126:337–346.