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Chromosomal Abnormalities and Developmental Kinetics in In Vivo-Developed Cattle Embryos at Days 2 to 5 after Ovulation[1]

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ABSTRACT

The frequency of chromosome abnormalities was investigated in cattle embryos (n = 256) derived from superovulated heifers (n = 35) on Days 2, 3, 4, and 5 postovulation (PO). Interphase nuclei (n = 4358) were analyzed for chromosome abnormalities using fluorescent in situ hybridization with chromosome 6- and chromosome 7-specific probes and the developmental rate was described by scoring cell numbers. We found that 93%, 85%, 84%, and 69% of the embryos from Days 2, 3, 4, and 5 PO, respectively, displayed a normal diploid chromosome number in all cells. Of the embryos containing abnormal cells, mixoploidy was significantly more frequent than polyploidy. The percentage of mixoploidy at Days 2, 3, 4, and 5 PO was 5%, 13%, 16%, and 31%, respectively, whereas the percentages of polyploidy were 2%, 2%, 0%, and 0%, respectively. The mean number of cells per embryo was 4.7, 8, 11.5, and 48.3, respectively, at Days 2, 3, 4, and 5 PO. Thus, in vivo-developed embryos were significantly more advanced than the in vitro-produced (IVP) embryos except for Day 2. In conclusion, a significantly lower frequency of chromosomally abnormal embryos, in particular displaying polyploidy early after fertilization, was seen in in vivo versus IVP embryos, and these chromosomal abnormalities may be inherent to the process of IVP in cattle.

conceptus, developmental biology, implantation/early development

INTRODUCTION

Mammalian embryos are often mixoploid, i.e., contain a mixture of diploid and polyploid cells, and cattle are no exception. For example, an early cytogenetic study of trophoblast biopsies from 159 cattle Day 12–18 embryos revealed that about half of the embryos contained polyploid cells [1]. These authors found that a level of 25% polyploid cells was compatible with pregnancy and suggested that mixoploidy up to this level was without biological significance. Other studies using cytogenetic methods have largely confirmed these data and have further shown that chromosome abnormalities occur from 0% and up to 56% in the earlier stages of cattle embryos [2–7]. The large span of this interval is most likely due to the inherent difficulty in obtaining accurate estimates of the frequency of polyploid cells in an embryo using cytogenetic techniques, because only a few metaphase spreads are present and can be analyzed. The use of fluorescence in situ hybridization (FISH) with chromosome-specific probes has provided an opportunity to obtain more accurate estimates of the frequency of polyploid cells, because all or almost all cells of an embryo can be analyzed. Our initial studies using FISH have revealed that 25% of in vivo-developed cattle embryos at Day 7–8 were mixoploid but at a level where the mixoploid embryos contained few and generally less than 10% polyploid cells [8]. Our investigation of Day 7–8 embryos also revealed that the chromosome abnormality frequency in in vitro-produced (IVP) embryos was significantly higher than in such produced in vivo. We have recently extended the data on mixoploidy and polyploidy frequencies in IVP cattle embryos by including Day 2, 3, 4, and 5 after fertilization [9], but comparable data on the frequency of such chromosome deviations in cattle embryos developed in vivo are lacking. We have therefore studied a total of 256 in vivo-developed cattle embryos isolated on Days 2, 3, 4, and 5 after ovulation with respect to the chromosome abnormality frequency. In doing so, we were also able to describe the developmental rate of in vivo embryos by scoring cell numbers during the first cleavage divisions. The results are given below and they are compared to the results that we have previously obtained on cattle IVP embryos in the discussion.

MATERIALS AND METHODS

Embryo Production

To obtain embryos at precisely defined times after ovulation heifers were treated for superovulation using a procedure with a controlled LH surge according to van de Leemput et al. [10]. Holstein-Friesian heifers (n = 13; first session) and cows (n = 22; second session) were selected on the basis of general clinical examination and normal ovarian cyclicity during at least 3 wk as established by the progesterone concentration in peripheral blood samples taken three times a week [11]. The heifers/cows were housed in groups of six animals and were fed silage and concentrate (to a maximum of 1 kg per heifer and 3 kg per cow per day) and supplied water ad libitum. The experiments were carried out in October/November 1999 (first session) and in April/May 2000 (second session). Heifers/cows were assigned at random to groups for the collection of embryos.

Before the experiments estrus was synchronized using an ear implant for 9 days (3 mg norgestomet, Crestar; Intervet International BV, Boxmeer, The Netherlands) accompanied by an i.m. treatment with 3 mg norgestomet and 5 mg estradiol-valerate. Two days before removal of the implants, prostaglandin (PG) (15 mg Prosolvin vet; Intervet International BV, The Netherlands) was administered i.m. to ensure complete regression of the corpus luteum. At Day 8 (estrus is Day 0) of the subsequent cycle all follicles >8 mm were ablated by transvaginal ultrasound-guided punctur-

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ing to prevent eventual suppressive action of dominant follicles on the follicle population to be stimulated. Stimulation for superovulation initiated on Day 10 with sheep FSH (Ovagen ICP, Auckland, New Zealand) administered twice daily during 4 days (first day 3.5 ml, second day 2.5 ml, third day 1.5 ml, fourth day 1.0 ml; in total 17 ml equivalent to 299 IU NIH-FSH-S1). An ear implant (Crestar) was inserted concomitantly with the first administration of FSH to suppress endogenous release of the preovulatory LH surge. Prostaglandin (15 mg Prosolvin) was administered i.m. 48 h after the start of the FSH stimulation. The ear implant was removed 48 h after the PG treatment at the time prematuration was assumed to be completed, and GnRH (1.0 mg Fertagyl in 10 ml saline i.m.; Intervet International BV) was administered to start the in vivo maturation.

Heparinized blood samples were collected daily from the jugular vein from the start of FSH administration until removal of the ear implant. After administration of the GnRH, blood samples were collected every 6 h to establish the time of the LH surge. The dose of GnRH used in this study induces an LH surge ~2 h later that was also confirmed in the present experiment. All animals were inseminated 12–14 h after the GnRH administration with two straws of semen from the same bull (one straw per uterine horn).

The time point of 24 h after the LH surge was taken as the starting time of multiple ovulations. The time periods between expected ovulation time and slaughter were 50 and 46 h (i.e., Day 2, sessions 1 and 2, respectively; aiming at 4-cell embryos), 74 and 70 h (i.e., Day 3, sessions 1 and 2, respectively; aiming at 8-cell embryos), 100 h (i.e., Day 4, session 2; aiming at 16-/32-cell embryos), and 130 h (i.e., Day 5, session 1; aiming at morulas). These time periods were ± 1.5 h.

Embryo Collection

Oviducts, uteri, and ovaries were placed in saline (37°C) and transported immediately to the laboratory in a thermocontainer. The time period between slaughter and flushing ranged from 45 min to 2 h. Oviducts with uterine horns were flushed with PBS (37°C) from the infundibulum toward the uterine horn under gentle massage through a blunt needle (inner diameter 2 mm) [10]. For the Day 2 group, only the oviducts were flushed. The PBS was collected into an embryo recovery filter (Embryo Concentrator; Immuno Systems Inc., Spring Valley, WI) and the oocytes/embryos were collected by rinsing the filter with saline supplemented with 0.005% BSA.

Immunoassays for Progesterone, Estradiol, and LH

During the superovulatory treatment concentrations of progesterone and estradiol-17β in peripheral blood samples were estimated by solid-phase ^{125}I RIA methods (Coat-A-Count TKPG and TKE, respectively; Diagnostic Products Corporation, Los Angeles, CA) with modifications as described by Dieleman and Bevers [11]. The sensitivity was 0.15 nmol L^{-1} and 7.5 pmol L^{-1}, and the interassay coefficient of variation 11% and 8.9%, respectively. Concentrations of LH were estimated by a validated RIA method as described by Dieleman et al. [12]. The sensitivity was 0.4 μg L^{-1} NIH-LH-B4, and the intra- and interassay coefficients of variation were <9%.

Interphase Cytogenetic Analysis by FISH

All embryonic nuclei were spread, fixed, and FISH was performed as described earlier for cattle embryos [9]. Embryo nuclei were scored according to previously established criteria [9], except that embryos containing diploid nuclei and more than two nuclei with signals as 1 + 1, and none of the false-negative types were included in the analysis as haploid mosaics. The different frequencies were compared using likelihood ratio tests in logistic regression models. The logistic regression models were fitted to data using PROC GENMOD in SAS version 6.12 (SAS Institute, Cary, NC). The number of cells per embryo were analyzed by a two-way analysis of variance model after first having transformed the data by the natural logarithm (based on residual plots). This analysis was carried out using PROC GLM in SAS version 6.12. A logistic regressions model with random cow-effect was used in order to take into account the fact that some cows corresponded to several embryos. The SAS-macro GLMM612 was used for this part of the analysis.

RESULTS

Ploidy of Embryos in Relation to the Day of Collection and the Stage of Embryonic Development

A total of 4358 nuclei were scored in 256 embryos collected on Days 2, 3, 4, and 5 postovulation (PO). The percentage of blastomeres lost during fixation and FISH was 4.6%, and the fraction of blastomeres showing false-negative results after FISH was 2.4%.

The results of the interphase chromosome analysis on Days 2, 3, 4, and 5 PO is presented in Table 1. The frequency of entirely diploid embryos was decreasing from an initial 93% on Day 2 to 69% on Day 5. Of the embryos containing chromosomally abnormal cells, mixoploidy was significantly ($P < 0.001$) more frequent than polyploidy. The percentage of mixoploidy on Days 2, 3, 4, and 5 PO was 5%, 13%, 16%, and 31%, respectively, whereas the percentages of true polyploidy, i.e., all nuclei in the embryo were analyzed and were polyploid, were 2%, 2%, 0%, and 0%, respectively. Although no embryos were categorized as polyploid on Day 4 and Day 5, one 13-cell embryo on Day 4 was found in which 12 of the nuclei could be analyzed and were polyploid (triploid), whereas the last nucleus showed a false-negative result. Consequently, this embryo was categorized as mixoploid, although it might have been a true triploid embryo.

The frequency of mixoploidy increased from Day 2 to Day 5. Statistical analysis by comparing each day with any of the other days revealed that the frequency on Day 2 (5%) was significantly lower that on Day 5 (31%) ($P = 0.001$), but that the frequencies on Days 3 and 4 were only marginally different from that on Day 5 ($P = 0.03$ and $P = 0.08$, respectively). A cow effect was observed on those results as the significance slightly increased from Day 2, 3, and 4 to Day 5 ($P = 0.029$, $P = 0.061$, and $P = 0.09$, respectively) if the fact was taken into account that some cows corresponded to several embryos.

The type of chromosome abnormalities observed in embryos that were not entirely diploid is illustrated in Table 2. In the 40 mixoploid embryos, diploidy-triploidy was the most frequent finding (50%), whereas diploid/haploid, diploid-tetraploid, and diploid-triploid-tetraploid/haploid mosaics were observed in 12.5%, 25%, and 12.5% of embryos, respectively. Diploid-triploid mosaics were present throughout the early developmental stages, whereas diploid-tetraploid mosaics first appeared on Days 4 and 5 (with one exception on Day 3). The three polyploid embryos (2-, 6-, and 8-cell) were all triploid. Around 90% of the embryos contained none or less than 10% of haploid/polyploid cells (93% on Day 2, 85% on Day 3, 91% on Day 4, and 92% on Day 5).

Increase in the Number of Cells per Embryo from Days 2 to 5 PO

As expected, the mean number of cells per embryo increased from Day 2 to Day 5. At Day 2, there were 4.7 cells (95% confidence interval [CI], 4.2–5.3), at Day 3 there were 8.0 cells (CI, 7.3–8.8), at Day 4 there were 11.5 cells (CI, 10.2–13.0), and at Day 5 we observed 48.3 cells (CI, 42.7–54.6).

DISCUSSION

The results of the interphase FISH-based chromosome analysis of the 256 in vivo-developed embryos reveal that the mixoploidy frequency increase from 5% on Day 2 up to 31% on Day 5 and that polyploidy is a very rare phenomenon. These data are significant because they fill a gap in providing reliable data on the mixo- and polyploidy frequency of bovine in vivo-developed embryos.

Compared to previous results on IVP cattle embryos [9], these in vivo-developed embryos showed a significantly

TABLE 1. Ploidy of in vivo-developed embryos in relation to the day of collection and embryonic development stages.

Embryonic stage	Day 2[a]				Day 3				Day 4				Day 5				Total			
	Di	Mix	Poly	Total	Di	Mix	Poly	Total	Di	Mix	Poly	Total	Di	Mix	Poly	Total	Di	Mix	Poly	Total
2 cell	6	1	1	8													6	1	1	8
3–4 cell	23	1	0	24	4	1	0	5	2	0	0	2	0	1	0	1	29	3	0	32
5–8 cell	23	0	0	23	51	6	2	59	15	0	0	17	0	0	0	0	89	6	2	97
9–16 cell	1	1	0	2	23	5	0	28	21	5	0	26	0	1	0	1	45	12	0	57
17–40 cell									8	4	0	12	6	2	0	8	14	6	0	20
41–60 cell													19	9	0	28	19	9	0	28
61–80 cell													7	3	0	10	7	3	0	10
81–100 cell													3	0	0	3	3	0	0	3
>100 cell													1	0	0	1	1	0	0	1
Total	53	3	1	57	78	12	2	92	46	9	0	55	36	16	0	52	213	40	3	256

[a] Di, Diploid; Mix, mixoploid; Poly, polyploid.

lower overall frequency of mixoploidy (25% versus 16%, $P = 0.003$) and polyploidy (6% versus 1%, $P = 0.002$). In addition, a significant difference was observed between in vivo and in vitro with less chromosomally abnormal embryos in the in vivo groups on Day 2 ($P < 0.0001$) and on Day 3 ($P = 0.0179$). That the in vitro production procedure increases the polyploidy frequency has, at least as we are aware, not been convincingly documented before for any species. It is conceivable that it is the in vitro maturation of oocytes that has an influence on the frequency of polyploidy. Preliminary studies [13] have shown that a prematuration effect of the oocytes may take place because Day 3 embryos, derived from in vivo-matured oocytes (aspirated from preLH or 24 h-after-LH follicles) followed by in vitro fertilization and culture, had fewer polyploid embryos compared to the control IVP group. The biological mechanism behind this adverse effect of in vitro oocyte maturation remains an enigma, but it has been proposed that lack of proper capacitation may cause an incomplete cortical reaction with consequent polyspermy, whereas overmaturity may result in second polar body retention (diploid oocyte) [14], which after fertilization could result in a triploid embryo or a haploid/diploid mosaic. In this context it is interesting to note that previous ultrastructural studies have demonstrated perturbances in the peripheral migration of the cortical granules during in vitro maturation of cattle oocytes [15].

The group of mixoploid embryos consisted mainly of diploid-triploid embryos (50%), whereas diploid/tetraploid embryos accounted for 25%. Diploid/haploid and diploid/haploid/triploid or diploid/haploid/tetraploid represented each 12.5%. In this study we have included the category haploid mosaics that consists of the embryos that contain more than two nuclei with signals as 1 + 1 and none of the false-negative types as 0 + 0, 0 + 1, 1 + diffuse, or 0 + diffuse. This represents a change of criteria compared to our previous studies, where we used the very conservative approach of accepting only entirely haploids (in which all cells were 1 + 1). The reason is that we have arrived at a frequency of 2.4% false-negative nuclei after having analyzed approximately 20 000 embryonic nuclei and therefore feel confident that the FISH signals of two differentially labeled chromosome-specific probes is an accurate representation of the chromosome complement, at least with respect to polyploidy and haploidy. We have included the haploid-mixoploid embryos in the statistical analysis when comparison is made between data obtained in this study and data from our previous IVP study. The origin of such haploid mosaics is however not resolvable.

It is interesting to note that the diploid/tetraploid mosaics increase in frequency in the latter half of the interval we studied, i.e., on Days 4 and 5. Combined with the fact that the mixoploidy frequency increases during this time it might suggest that the triploid and tetraploid cells arise at different stages: The triploid cells that are present from fertilization and onward and the tetraploid cells being generated at a later stage. This is the first indication that the etiology of polyploid cells may be different in developing embryos. The diploid-triploid mosaics are a likely consequence of fertilization with two spermatozoa. Tripronuclear human zygotes do not all develop into triploid embryos but some form mosaics [16–18]. Furthermore, Han et al. [19] have very elegantly shown that some polypronuclear pig eggs correct ploidy abnormalities and that they may develop as far as to term. Thus, the fact that most of the mixoploid cattle embryos in our study contain triploid cells

could be explained by a higher likelihood of polyspermy by two than by three spermatozoa. The increase in the frequency of tetraploid cells on Days 4 and 5 could be related to the fact that cell cycle regulation changes from a maternal dominance up to the fourth cell cycle to an embryonic dominance after the embryonic genome is activated during the third cell cycle. Cell-cycle checkpoints normally operate to ensure that essential processes such as chromosomal segregation are completed before the cell proceeds to the next phase of the cell cycle, but such checkpoints may not operate effectively during the cleavage divisions that occur before activation of the embryonic genome. The larger increase in the level of mixoploidy in IVP embryos does, however, suggest that there are also factors relating to the culture system that influence the generation of polyploid cells. That we do not find the same increase in the proportion of tetraploid cells in the IVP embryos as we have demonstrated in the in vivo-produced material further points in the same direction. In this context it is interesting to note that it has been demonstrated that even temperature differences of 1°C can disturb normal spindle structure [20] leading to a disorganized movement of chromosomes and formation of mosaics [17]. Such small temperature differences could easily occur during handling of oocytes, zygotes, and embryos.

We have so far not obtained any information about the level of polyploid cells that are tolerated by developing embryos. The fact that the mixoploid embryos were generally found in the fast-developing group on Day 5 in the IVP embryos suggests that developmental competence is not influenced. If up to 25% polyploid cells are compatible with pregnancy as suggested by Hare et al. [1], then 93% of the in vivo embryos from our study would be viable, the 6% mixoploid containing more than 25% polyploid cells would be doubtful, and the 1% polyploid embryos would most likely be eliminated. In this light chromosomal abnormalities seems only to account for a minor percentage of the embryo losses in cattle where loss in the order of 30–40% is normal [21].

The preparation of spreads of nuclei made it possible to count precisely the numbers of nuclei in each embryo. As illustrated in Figure 1, the in vivo-developed embryos contained significantly more cells at Day 3 ($P < 0.001$), Day 4 ($P = 0.018$), and Day 5 ($P < 0.001$) than the IVP embryos studied previously [9]. In other words do the in vivo embryos grow significantly faster than the IVP embryos, at least in our culture system. This is to our knowledge the first documented developmental kinetics of in vivo and in vitro embryos. The influence of the in vitro culture system on the growth rate has been shown by van Soom et al. [22] who compared the developmental rate of IVP embryos derived from two different media: Menezo B2-coculture and TCM199-coculture. It was concluded that the embryos cultured in TCM199 developed slower than those cultured in B2 resulting in average 18 versus 24 cells on Day 5, whereas no difference was detectable on Day 3. Our IVP embryos were cultured using Menezo B2-coculture but their rate of development actually seems to be more similar to the TCM199 group in the cited study.

If in vitro embryo techniques are to be integral parts of modern breeding strategies, they need to mimic the natural conditions as closely as possible. The data presented here will be important reference points for monitoring the differences in development that may be induced by in vitro manipulations of cattle oocytes and early embryos. This point has been illustrated by demonstrating significant dif-

TABLE 2. Different types of mixoploidy in relation to the day of collection and embryonic development stages.

	Day 2[a]				Day 3				Day 4				Day 5				Total			
Embryonic stage	D/H	D/Tr	D/Te	D/Tr/Te	D/H	D/Tr	D/Te	D/Tr/H	D/H	D/Tr	D/Te	D/Tr/Te	D/H	D/Tr	D/Te	D/Tr/Te	D/H	D/Tr	D/Te	D/Tr/Te
2 cell		1																1		
3–4 cell		1				1									1			2	1	
5–8 cell						4	1	1										4	1	1
9–16 cell	1				3	2				2	2	1			1		4	4	3	1
17–40 cell										2	1	1			1	1		2	2	2
41–60 cell														6	2	1		6	2	1
61–80 cell													1	1	1		1	1	1	
81–100 cell																				
>100 cell																				
Total	1	2			3	7	1	1		4	3	2	1	7	6	2	5	20	10	5

[a] D/H, Mosaic embryo of diploid-haploid; D/Tr, mosaic embryo of diploid-triploid; D/Te, mosaic embryo of diploid-tetraploid; D/Tr/Te, mosaic embryo of diploid-triploid-tetraploid; D/Tr/H, mosaic embryo of diploid-triploid-haploid.

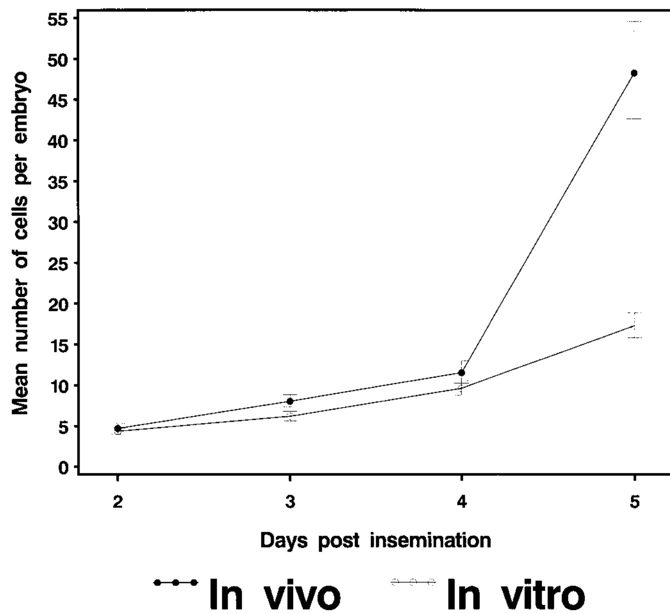

FIG. 1. Developmental kinetics in in vitro- and in vivo-developed bovine embryos on Days 2–5 postinsemination.

ferences between in vivo- and in vitro-produced cattle embryos, not only with respect to the frequency of polyploidy and mixoploidy, but also to the developmental rate. There are, however, still severe deficiencies regarding our understanding of the significance of these differences with respect to the later stages of embryonic development.

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