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Note

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WA King [1,2], A Verini Supplizi [2,3], HEP Diop [2,4], D Bousquet [5]

[1] *University of Guelph, Department of Biomedical Sciences, Ontario Veterinary College, Guelph, ON, N1G 2W1, Canada;*
[2] *Université de Montréal, centre de recherche en reproduction animale, faculté de médecine vétérinaire, CP5000, Saint-Hyacinthe, PQ, J2S 7C6 Canada;*
[3] *Instituto di Produzioni Animali, Facoltà de Medicina Veterinaria, Via San Costanzo 4, 06100 Perugia, Italy;*
[4] *École inter-États des sciences et médecine vétérinaire, département Chirurgie et Reproduction, BP 5077 Dakar, Senegal;*
[5] *BOVITEQ, 1425, Grand Rang Saint-François, Saint-Hyacinthe, PQ, J2S 7A9 Canada;*

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Summary – To determine the chromosomal complement of pre-morula bovine embryos, 30 Holstein heifers were superovulated and inseminated and their embryos were subjected to chromosome analysis on days 2, 3 or 4. Of the 298 embryos/ova recovered, 101 had one or more cells in metaphase and of these, 60 could be karyotyped. Eight of the karyotyped embryos were chromosomally abnormal, including 3 triploids, 1 tetraploid and 4 mixoploids. It was concluded that the abnormalities occurred at or shortly after the time of fertilization.

bovine / embryo chromosome / triploid / tetraploid / mixoploid

Résumé – Analyse chromosomique chez des embryons provenant de vaches superovulées et inséminées artificiellement. *Afin de déterminer la garniture chromosomique d'embryons bovins aux stades de pré-morula, 30 génisses Holstein ont été superovulées et inséminées. Les embryons furent récoltés aux jours 2, 3 ou 4 et soumis à une analyse chromosomique. Sur un total de 298 embryons/ovules récoltés, 101 montraient une ou plusieurs cellules en métaphase et 60 de ceux-ci ont pu être examinés pour leur caryotype. Huit embryons analysés présentaient une garniture chromosomique anormale : 3 triploïdes, 1 tétraploïde et 4 mixoploïdes. Il est conclu que les anomalies se produisent au moment de la fertilisation ou juste après.*

bovin / chromosome d'embryon / triploïde / tétraploïde / mixoploïde

INTRODUCTION

Under intense agricultural management superovulation is used to induce multiple ovulation in cattle to provide genetically valuable embryos for collection and transfer. However, only about 60% of the embryos that are collected from superovulated cattle have normal morphology and are considered suitable for transfer (Schneider Jr, *et al*, 1980; Schiewe *et al*, 1987; Lopez Gatius *et al*, 1988). In humans, chromosomal abnormalities in the embryo or fetus are the most frequent causes of malformations and pregnancy failure (Jacobs *et al*, 1978). Chromosomal analysis of cattle embryos at the morula and blastocyst stages, when embryo transfer is usually performed, has revealed abnormalities that are thought to compromise development (King, 1991). These abnormalities include aneuploidy, mixoploidy and polyploidy (for review see King, 1990). Prior to the morula and blastocyst stages very few observations on the chromosomal constitution of embryos produced by superovulated cattle have been documented (King and Picard, 1985; Murray *et al*, 1985). Hence, little is known of the situation close to the time of fertilization.

The objective of this study was to determine the chromosomal complement of pre-morula stage embryos produced by superovulated cattle. Some of the embryos reported here were included in a preliminary report published in abstract form (Verini Supplizi *et al*, 1988).

MATERIALS AND METHODS

Embryos were produced by superovulated Holstein heifers inseminated once or twice with semen from a highly fertile Holstein bull during the 24 h following onset of behavioural estrus. Superovulation was induced by treatment with follicle-stimulating hormone (FSH-p; Burns-Biotech Laboratory, Oakland, CA, USA) and cloprostenol (Estrumate: ICI Pharms, Mississauga, ON, Canada) as previously described (King *et al*, 1987). Females were checked for signs of behavioural estrus twice daily. The first detection of behavioural estrus was designated day zero.

Embryos were collected by post-mortem retrograde flush of the oviducts on day 2 ($n = 6$), 3 ($n = 23$) and 4 ($n = 1$). Only one oviduct from 11 of the heifers was available for use in this study. For all collections the flushing medium was Dulbecco's phosphate-buffered saline (PBS, pH 7.2) supplemented with 2% fetal calf serum (FCS) and antibiotics (100 iu penicillin, 100 μg streptomycin/ml). Once the embryos were located in the flushing medium they were washed in PBS containing 10% FCS and antibiotics and transferred to Hams F10 containing 20% FCS and antibiotics and colcemide (0.05 μg/ml medium; Sigma, Saint Louis, MO, USA). The embryos were incubated in this medium for 4–8 h and then fixed individually on slides as previously described (King *et al*, 1979). Slides were then stained with Giemsa and examined for cell number and chromosome composition. Fertilization was evaluated after fixation and was considered to have occurred if any of the following were observed: mitotic chromosomes; 2 or more pronuclei/nuclei; or 2 or more blastomeres. Ova that presented meiotic chromosomes or lacked nuclei were considered unfertilized.

RESULTS

Flushing the reproductive tracts of the 30 females yielded a total of 298 embryos/ova. The mean rate of fertilization (percent of total recovery) was 83.2%. In all 101 embryos (33.9%) had one or more cells in metaphase. The karyotype of 60 of these embryos (59.4%) could be determined while 41 had metaphase spreads that were either incomplete or of insufficient quality for analysis. Of the 60 karyotyped embryos, 52 (86.6%) were found to be diploid (60XX or 60XY) and 8 (13.3%) were other than diploid (table I). The abnormal complements included 3 triploids, 1 tetraploid and 4 mixoploids. The triploid and tetraploid embryos were at the 2-cell stage and each embryo presented 2 metaphase spreads. Three of the 4 mixoploid embryos had cleaved to 2-cell stage although 4 nuclei (2 haploid and 2 diploid) were present in each. The fourth mixoploid had not cleaved but contained a haploid and a diploid nucleus (fig 1). The diploid nucleus in this embryo contained 62 chromosomes. In all cases the abnormal embryos were among the least developmentally advanced embryos, estimated on the basis of cell number, within the flush of the donor female from which they originated (table I).

Table I. Stage and within-flush range of development of bovine embryos with abnormal chromosomal complement.

Abnormality	*Karyotype*	*Number of cells*	
		Embryo	*Range in collection*
Triploidy	90XXX	2	1–4
	90XX?*	2	1–4
	90X??*	2	1–4
Tetraploidy	120XXXX	2	1–4
Mixoploidy	30X/60XY	2	1–4
	30Y/60XY	2	1–4
	30Y/60XX	2	1–4
	30X/62XX	1	1–8

* Sex chromosome could not be identified.

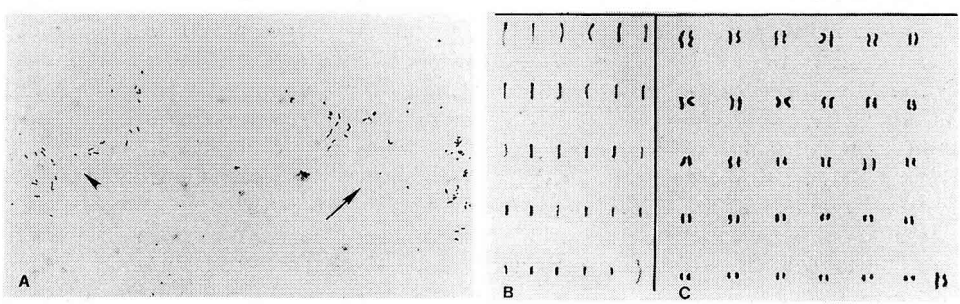

Fig 1. Chromosome preparation from a one cell mixoploid bovine embryo. A: Two separate metaphase spreads of which one is haploid (arrowhead) and one is diploid (arrow). B: Karyotype of the haploid (30X) metaphase. C: Karyotype of the diploid (62XX) metaphase.

DISCUSSION

Chromosome abnormalities have been observed in the embryos of most domestic animals. In sheep and pigs, a frequency of chromosomally abnormal embryos of 10.4 and 6.6%, respectively, has been reported (for review see King, 1990). In cattle the frequency varies from 0 to 36.3% (table II) according to the stage of development and morphological features of the embryo. In the present study of days 2–4 embryos, ranging from 1–8 cells, a frequency of 13.3% was noted while Hare *et al* (1980), reported a frequency 1.9% in elongated blastocysts on days 12–18. The low frequency at the end of the second week of development in the elongated blastocyst stage suggests a loss of abnormal embryos as development progresses. Benevedes-Filho *et al* (1992) reported a frequency of 35.8% in day 7 embryos which exhibited reduced cell number, abnormal morphology and low developmental potential. Similarly, King *et al* (1987) found a higher rate of chromosomally abnormal embryos on day 7 among morphologically abnormal embryos with low cell numbers than among morphologically normal ones. In the present study the abnormal embryos were among the least developmentally advanced within individual donors (table I). If indeed chromosomally abnormal embryos have a slower rate of development and hence a lower cell number, the present observations suggest that development may begin to slow down as early as day 2.

Table II. Summary of cytogenetics studies of *in vivo*-produced bovine embryos.

Stage of development	*Number of embryos*				*Reference*
	Total	*Analyzed*	*Abnormal (%)*		
Zygote to blastocyst	134	34	4	(11.8)	Murray *et al*, 1985
Zygote to blastocyst	548	265	19	(7.1)	Gayerie de Abreu *et al*, 1984
Morula	24	11	4	(36.3)	King and Picard, 1985
Morula/blastocyst	163	39	14*	(35.8)	Benevides-Filho *et al*, 1992
Morula/blastocyst	126	119	16**	(13.4)	King *et al*, 1987
Elongated blastocyst (days 12–18)	198	159	3	(1.9)	Hare *et al*, 1980
Elongated blastocyst (days 12–16)	12	12	0		McFeely and Rajakoski, 1968
Total	1 350	683	71	(10.4)	

* Eleven of the 14 were morphologically abnormal embryos with low cell number; ** all 16 were morphologically abnormal embryos with low cell number.

Chromosome analysis of *in vitro*-produced bovine embryos has shown abnormalities in 12.1% of embryos at the 2-cell stage, 20.0–36.4% at 4- to 16-cell stage and 44.2% at blastocyst (Iwasaki *et al*, 1989). Kawarsky (1994) has noted a frequency of abnormalities of 27.4% on day 2 (1–8 cells) and 32.1% on day 5 (8-cell stage to morula) *in vitro*. As with the *in vivo* studies, both of these *in vitro* studies suggest an accumulation of chromosomally abnormal embryos over the first week of development. Unfortunately the limitation of *in vitro* culture prevents monitoring development beyond the blastocyst stage into the second week of development to

determine if there is an elimination of abnormal embryos as the embryo begins to elongate.

All 8 abnormal embryos were either 1 or 2 cells suggesting that the abnormality occurred at or close to the time of fertilization before completion of the first cell cycle. The 3 triploids and 1 tetraploid were 2-cell embryos. Unfortunately, the exact origin of the extra haploid set(s) of chromosomes could not be determined. In humans, triploid fetuses originate from dispermic fertilization (66%), diploid sperm (24%) or diploid oocytes (10%; Jacobs *et al*, 1978). Tetraploid embryos are less common and mechanisms leading to their production are not well studied. They could, however, arise by combinations of the mechanisms proposed for triploids as well as by failure of cytokinesis at first cleavage or by endoreduplication of the pronuclei. In cattle all of these pathways are possible since polyspermic fertilization, diploid sperm, diploid oocytes and endoreduplication have been reported (King *et al*, 1988; Iwasaki *et al*, 1989; Yadav *et al*, 1991; Kawarsky, 1994). In pigs it has been reported that the incidence of polyploidy arises due to ageing of the oocyte when insemination is delayed (Bomsel-Helmreich, 1961). However, this has not been confirmed in cattle.

All 4 of the mixoploid embryos exhibited haploid nuclei (2 in the 3 two-cell embryos and 1 in the 1-cell embryo). The presence of a Y chromosome in the haploid cells in 2 of the embryos suggests that the oocytes leading to these embryos were fertilized by 2 spermatozoa. The X-chromosome bearing haploid cells may have originated from a spermatozoon, a binucleated oocyte or a polar body. The fate of the haploid cells is not known. King and Picard (1985) and Iwasaki and Hamano (1991) described morula and pre-morula with haploid cells. However, older embryos with such cells have not been reported. It is possible that these nuclei die, become quiescent or are somehow eliminated from the embryo. It is also possible that they eventually fuse with their diploid cohorts as diploid-triploid mixoploids have been reported in blastocysts and elongated blastocysts (Hare *et al*, 1980; King *et al*, 1987).

One aneuploid metaphase was observed in the 1-cell mixoploid (30X/62XX) embryo. This low incidence of aneuploidy (1/60; 1.7%) is consistent with the low incidence of aneuploid spermatozoa (2.8%: Longue and Harvey, 1978) and ova (5.8%: Yadav *et al*, 1991).

It was concluded that roughly 13% of day 2–4 embryos from superovulated cattle that could be cytogenetically analyzed were chromosomally abnormal. The abnormalities most likely arose at or soon after fertilization due to fertilization by a second spermatozoon or failure of polar body extrusion.

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