

The incidence of polyploidy and mixoploidy in early bovine embryos derived from in vitro fertilization

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Summary – The present work describes a cytogenetic study of early bovine embryos (two to sixteen blastomeres) produced in vitro to determine the proportion of embryos carrying chromosome abnormalities. The embryos were produced from follicular oocytes matured in vitro and fertilized by sperm prepared using the 'swim up' method. Slides were prepared according to an 'air drying' method and the chromosomal complement of embryos was studied by Giemsa-staining. Approximately 45% of embryo preparations were suitable for analysis. The results revealed that 23% of cytogenetically analysed embryos were chromosomally abnormal. The abnormalities observed included triploidy (6.9%), tetraploidy (4.0%), mixoploidy (7.9%) and haploidy (4.5%). The results of this study were compared to the results of other studies with several species.

bovine embryo / cytogenetic analysis / in vitro fertilization / mixoploidy / polyploidy

Résumé – **Incidence de la polyplôidie et de la mixoploïdie chez des embryons bovins à un stade précoce après fécondation in vitro.** Dans ce travail on présente une étude cytogénétique des embryons bovins dans leur stade initial (deux à seize blastomères) produits in vitro afin de déterminer la proportion d'embryons possédant des anomalies chromosomiques. Les embryons ont été obtenus à partir d'ovocytes folliculaires mûris in vitro et fécondés par du sperme préparé selon la méthode de migration ascendante. On a fait les préparations cytogénétiques en utilisant la méthode du séchage à l'air. Les compléments chromosomiques ont été étudiés avec la coloration de Giemsa. On a obtenu des résultats analysables sur 45 % des embryons. L'étude cytogénétique a montré la présence d'anomalies chromosomiques dans 23,3 % des embryons. Les anomalies observées étaient : triploïdie (6,9 %), tétraploïdie (4 %), mixoploïdie (7,9 %) et haploïdie (4,5 %). Les résultats de cette étude ont été comparés avec d'autres résultats sur plusieurs espèces.

embryon bovin / analyse cytogénétique / fécondation in vitro / mixoploïdie / polyplôidie

INTRODUCTION

The development of techniques for in vitro maturation, in vitro fertilization of follicular oocytes and in vitro embryo culture (IVM/IVF/IVC) has created a source of gametes and embryos for investigation. Chromosome abnormalities have been reported in embryos of most domestic animals and humans. It has been suggested by King (1990) that about a quarter of the abnormalities (mainly aneuploidy) can be attributed to errors in meiosis and the remaining three-quarters occur around the time of fertilization and early embryonic development (haploidy, polyploidy, mixoploidy). Therefore the fertilization process seems to be a critical time for chromosome abnormalities to develop. Most of the reports about chromosome anomalies in embryos of domestic animals and humans describe numerical aberrations comprising aneuploidy, haploidy, polyploidy (triploidy and tetraploidy) and mixoploidy with a frequency ranging from 5 to 39% according to species (Long and Williams, 1982; Iwasaki et al, 1989a; Iwasaki and Nakahara, 1990a, b; King, 1990, 1991a, b; Murray et al, 1985; Kawarsky et al, 1996) and even 60% (Murray et al, 1986). There have been some reports that in vitro fertilization itself increases the incidence of chromosome anomalies found in embryos (Frazer et al, 1976; Maudlin and Frazer, 1977, 1978; Iwasaki and Nakahara, 1990a). The cytogenetic analysis of bovine embryos cultured in vivo and in vitro has revealed a slightly higher incidence of abnormalities in the latter group (28 and 37.5%, respectively) (Iwasaki and Nakahara, 1990a). Frazer et al (1976) reported an increased incidence of triploid IVF embryos (12.8%) in mice in comparison with those produced in vivo (2.7%). It is normal for polyploid cells to appear in trophoblast during early development and fetal differentiation (Long and Williams, 1982; Murray et al, 1986). However, the study of Iwasaki and Nakahara (1990b) revealed that the occurrence of chromosome aberrations (including haploid and polyploid cells) in isolated inner cell mass (ICM) from bovine blastocysts cultured either in vivo or in vitro was quite high. Moreover the incidence of embryos carrying anomalies in the ICM did not differ significantly from those of entire embryos.

Most chromosome aberrations found in two- to eight-cell bovine embryos were polyploidy (mainly triploidy) caused by polyspermy (Iwasaki et al, 1989a). Polyploid cells have been observed only occasionally in bovine blastocysts, which may suggest that embryos with such cells are eliminated (Iwasaki and Nakahara, 1990a; Kawarsky et al, 1994, 1996).

In the present study early bovine embryos (two to sixteen blastomeres) produced in vitro have been cytogenetically analysed and the incidence of chromosomally unbalanced embryos investigated.

MATERIALS AND METHODS

Collection of ovaries

Ovaries from randomly chosen slaughtered cows were collected at the local slaughterhouse within 1 h (30–40 ovaries) and then transported to the laboratory in saline solution at 30–37 °C. They were then washed twice in a fresh saline solution.

Oocyte collection

Cumulus–oocyte complexes (COCs) were aspirated from visible ovarian follicles (2–5 mm in diameter) using low pressure (~ -0.1 bar) created by a water pump

according to the method developed by Berg and Brem (1991). After 10 min the pellet of COCs was removed from the bottom of the cylinder and transferred to the collecting medium (TCM-199 medium (Sigma, USA) + 20% estrus cow serum (ECS) + 50 µg/mL gentamycin (Polfa, Poland)). Only oocytes surrounded by compact cumulus cells, selected according to the criteria of Leibfried and First (1979) and Madison et al (1992), were used in the present experiments.

Oocyte maturation

The COCs were washed twice in maturation medium (TCM-199 medium + 20% ECS + 10 µg/mL FSH (Sigma, USA) + 1 µg/mL estradiol-17β (Sigma, USA) + 50 µg/mL gentamycin) and transferred in groups of 10 or 25 to droplets of maturation medium (50 µL and 300 µL, respectively) and covered with paraffin oil (Merck, Germany). The oocytes were then incubated in the droplets at 39 °C in humid 5% CO₂ atmosphere for 26 h.

Sperm treatment

Frozen sperm was processed according to the method of Parrish et al (1986) with some modifications. The thawed sperm pellets were layered in a sterile tube under 1 mL of Talp medium and incubated for 1 h at 39 °C. Afterwards the sperm pellet was washed twice and centrifuged. Sperm concentration was adjusted to 1–5 × 10⁶/mL.

In vitro fertilization

After 26 h incubation oocytes were washed and placed into fertilization droplets (10 oocytes in 50 µL droplet) overlaid by paraffin oil (Merck). Before insemination, hypotaurine (4.6 µg/mL), epinephrine (7.7 µg/mL) and freshly prepared heparin (3.4 µg/mL) solutions were added to the droplets. Sperm and oocytes were co-cultured for 20 h at 39 °C in 5% CO₂ in humid air.

Embryo culture

At the end of the co-culture period the oocytes were washed twice and transferred back to the maturation droplets. By that time a granulosa cell monolayer had been formed on the bottom of the culture dish.

Slide preparation

After 2–3 days of embryo culture, colcemid (0.1 µg/mL; Gibco) or vinblastin (0.08 µg/mL) was added to the droplets and embryos were cultured for a further 6–8 h. Chromosome slides were prepared according to the Tarkowski's method (1966). Briefly, embryos were placed in 0.075 M KCl solution for 5–10 min and fixed on slides with a mixture of acetic acid/methanol (1:3) dropped on the top of the embryo. Chromosome slides were dried, kept in fixative solution overnight and stained with 5% Giemsa solution (Sigma) for 10–12 min.

RESULTS

A total of 468 embryos were subjected to chromosomal analysis. Metaphase plates were found in 202 of them (43.2%), whereas the remaining embryos (56.8%) displayed only interphase nuclei in blastomeres (table I). Some 155 (76.7%) of analysed embryos had a normal diploid chromosome set (fig 1). The abnormal complements were as follows: 6.9% of triploid embryos, 4.0% of tetraploid and 7.9% of mixoploid. Haploid embryos with frequency of 4.5% were also noticed (Lechniak, 1995). Most triploid and tetraploid embryos exhibited only one metaphase spread. It was thus impossible to classify these embryos as being pure polyploid or mixoploid. However, in two 6–8 blastomere embryos three triploid sets of chromosomes were present. The majority (56%) of mixoploid embryos were haploid/diploid ($n/2n$) (table II). The full sex chromosome complement (XXYY) was established in the tetraploid line of the $3n/4n$ mosaic embryo (fig 2).

Table I. Polyploidy and mixoploidy in early bovine embryos derived from in vitro fertilization.

Analysed	No of embryos									
	With anomaly		Haploid		Triploid		Tetraploid		Mixoploid	
	No	(%)	No	(%)	No	(%)	No	(%)	No	(%)
202	47	23.3	9	4.5	14	6.9	8	4.1	16	7.9

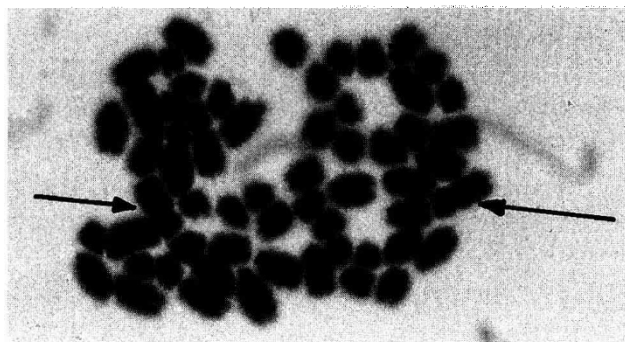


Fig 1. Chromosome set of a normal, diploid bovine IVF embryo ($2n = 60$, XX; Giemsa staining, the arrows indicate X chromosomes).

Table II. Chromosome set arrangements in the mixoploid IVF bovine embryos.

Chromosome arrangement	No of embryos
$n/2n$	9
$n/3n$	1
$2n/3n$	2
$2n/4n$	3
$3n/4n$	1
Total	16

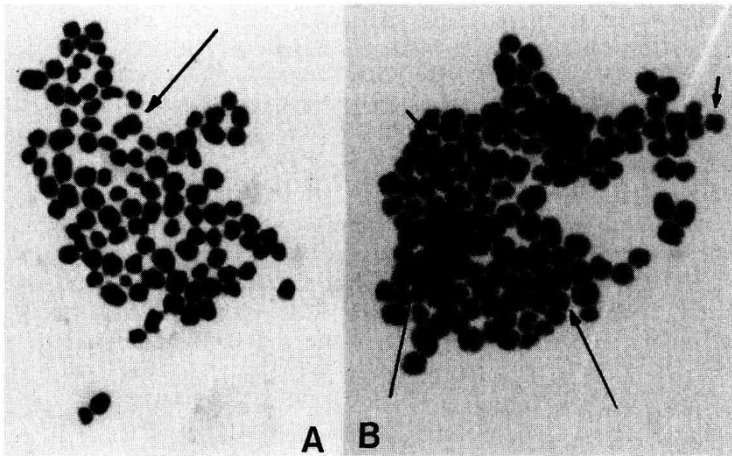


Fig 2. Metaphase plates derived from a mosaic IVF bovine embryo: **A:** triploid set; **B:** tetraploid set (Giemsa staining, the short arrows indicate Y chromosomes, the long arrows indicate X chromosomes).

DISCUSSION

In the present study of early bovine embryos the rate of polyploid and mixoploid embryos reached 23.3%. This finding is in agreement with previous reports concerning this species (13.7%, Iwasaki et al, 1989a; 15.5% King, 1991a; 18–36% Iwasaki and Nakahara, 1990a, b; 36.3–39.2% Kawarsky et al, 1996).

Mixoploidy was the main abnormality observed in the present study (7.9%). This aberration has been reported for bovine IVF embryos with a frequency varying from 0.3% (Iwasaki et al, 1989a), 7.5% (King, 1991a), 12% (Kawarsky et al, 1996) to 32% (Iwasaki and Nakahara, 1990a). Long and Williams (1982) reported that mixoploid cells were located mainly in trophoblast of d10 pig embryos with the frequency of 64% whereas the incidence of unbalanced cells in the ICM was low (5.1%). Only mixoploidy (6%) was found among d3–4 pig embryos by Van der Hoeven et al (1985); this was caused by endoreduplication in early differentiating trophoblast cells. Murray et al (1986) worked with d13 and d14 sheep embryos and older fetuses, and demonstrated that mixoploidy was the main abnormality observed with a frequency of 46–69%. Diploid/polyploid mixoploidy has been observed in the trophoblast of most domestic species during the second week of development and it is considered as a normal feature of trophoblast cells (King, 1990). However, the mixoploidy may be attributed to abnormal embryonic development. The presence of cell lines displaying various multiples of haploid chromosome complements within an embryo may be a result of cell fusions or endoreduplication which is associated with abnormal cell division (King, 1990). Therefore it seems likely that embryos with a lower cell number or those showing signs of degeneration may be a group at high risk of being carriers of chromosome aberrations. The results of experiments carried out on bovine embryos by Kawarsky et al (1996) proved this thesis and showed that the rate of development evidenced by cell number for d5 embryos was slowest for haploid and polyploid embryos, fastest for diploid and mixoploid

and intermediate for aneuploid. The cytogenetic analysis of d7 bovine embryos collected from superovulated cows with either poor morphological quality or lower cell number revealed a high frequency of chromosome unbalanced embryos (mainly mixoploidy), whereas no abnormalities have been noted in morphologically normal embryos (King et al, 1987, 1995). Moreover, among IVF embryos of the same age, developmentally less advanced embryos showed a higher frequency of abnormalities than more advanced ones (King, 1991b). Mixoploidy has mainly been observed in developmentally more advanced stages, therefore the rate of this abnormality found in the present study (7.9%) is considered to be high for early bovine embryos in comparison with 0.3% reported by Iwasaki et al (1989a). No mixoploids have been reported for d4 pig embryos (Underhill et al, 1991). Long and Williams (1980) found only one mixoploid embryo among 89 d2-3 sheep embryos analysed.

In order to examine the presence of mosaicism within an embryo it would be ideal to study the chromosomal complement of the majority of blastomeres. Cytogenetic evaluation of an embryo based on analysis of a single, biopsied blastomere described by Kola and Wilton (1991) is not sufficient as a mosaic embryo may be classified as normal if a blastomere from a diploid line was analysed. The triploidy and tetraploidy observed in the present study were diagnosed mainly on the basis of a single metaphase so it is possible that these embryos may have been mixoploids.

Since the mixoploid cell line is believed to be a part of normal trophoblast development, their presence in the ICM of early embryos should be considered as a sign of abnormal cell division negatively influencing the subsequent development of the fetus.

The rate of polyploid embryos observed in the present study was 10.9% and did not exceed the frequencies already reported for bovine embryos (11.2%, Iwasaki et al, 1989a; 21.3%, Iwasaki and Nakahara, 1990b; 4.9%, King, 1991a; 10%, Kawarsky et al, 1996); 63.6% of polyploid embryos were triploid. It has been reported that triploidy was the main numerical aberration observed in cattle IVF embryos (10%, Iwasaki et al, 1989a; 16.4%, Iwasaki and Nakahara, 1990b; 12.8%, King, 1990; 7.7%, Kawarsky et al, 1996). Triploid cattle embryos have been observed at various developmental stages (two-cell stage until d12-13) (King, 1990), but this abnormality is usually reported to occur at the early stages of embryonic development. In the study of Kawarsky et al (1994) the most common type of chromosome abnormality in d2 bovine embryos was polyploidy, mainly triploidy. However, the recent findings reported by Dortland et al (1993) showed a triploid compact morula that after 24 h of additional culture developed into a morphologically normal blastocyst. The chromosome complement was investigated by measuring DNA content of interphase nuclei.

Triploid embryos may be digynic in origin (when the extra set of chromosomes is of maternal origin) or diandric (when the extra set is of paternal origin). The main source of triploidy has been found to be polyspermy (15.1-36.9%, Iwasaki et al, 1989a; 7.9%, Iwasaki and Nakahara, 1990b) whereas the incidence of diploid spermatozoa is very low (Carothers and Beatty, 1975). The 2-12% incidence of diploid secondary oocytes (King, 1990; Lechniak et al, accepted for publication in *Theriogenology*) should be considered as a possible source of digynic triploid embryos. Taking all the possibilities mentioned above into consideration the frequency of triploid embryos should be much higher than observed. According

to Angell et al (1986) the majority of trippronuclear zygotes do not develop into triploid embryos. At the first mitotic division three possible types of events may occur: 1) triploid daughter cells can be produced; 2) one of the haploid sets may be excluded from the metaphase plate and it may either degenerate or be incorporated into a diploid cell during the next cell division to yield a $2n/3n$ embryo; or 3) three daughter cells may be produced via a tripolar spindle. Usually only 50% or less of trippronuclear zygotes develops into triploid embryos.

It has been shown that triploid embryos can be caused by many factors such as: PMSG dose (Maudlin and Frazer, 1977), delay in fertilization, aging of oocytes (Maudlin and Frazer, 1978; Ho et al, 1994), the IVF system itself (Frazer et al, 1976; Maudlin and Frazer, 1978; Iwasaki and Nakahara, 1990a), sperm motility, and the percentage of morphologically normal spermatozoa (Ho et al, 1994).

Tetraploid embryos were observed in the present study with a frequency of 4.0% which was in agreement with the previously reported data (2.7%, King et al, 1987; 4.9%, Iwasaki and Nakahara, 1990b; 3.0%, King, 1991a). Tetraploid embryos may have been caused by polyandry (trispermic fertilization of a haploid egg), by a combination of polyandry and polygyny, by endoreduplication at the zygote stage or by the inhibition of the first cleavage division of a diploid zygote (King, 1990). Some of the tetraploid bovine embryos (2.7%) produced by electrofusion reached the morula stage, although most of them did not progress beyond the fourth cleavage division (Iwasaki et al, 1989b). In the pig a d11 tetraploid embryo was reported by Moon et al (1975).

CONCLUSIONS

The results of the present study revealed that mixoploidy was found in early, functionally undifferentiated bovine embryos. The rate of polyploid embryos (especially triploid) may be influenced by both polyspermy and the incidence of diploid secondary oocytes. A technical artefact (the mixing of chromosomes originating from different blastomeres during slide preparation) cannot be excluded.

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