Chromosomal Analysis in Bovine Embryos Derived from In Vitro Fertilization of Immature Oocytes

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Abstract: *Chromosomal analysis was accomplished in bovine embryos derived from in vitro fertilization (IVF) after insufficient in vitro maturation of oocytes. Bovine oocytes collected from different donors were pooled into two groups; oocytes matured in vitro for 12 hrs were classified as the 12-hr group (insufficient maturation group), and other oocytes matured in vitro for 26 hrs were classified as the 26-hr group. At forty-eight hrs after IVF, most of the embryos (185/223, 83%) in the 26-hr group developed into the 8- or 16-cell stages, while many embryos (125/165, 75.8%) in the 12-hr group were at the 2- or 4-cell stage. The incidence of chromosomally abnormal embryos in the 12-hr group, 66.7% (54/81), was significantly higher than that in the 26-hr group, 27.8% (20/72). These chromosomal anomalies were haploids, polyploids and mixoploids. It was presumed that the increase in these anomalies was caused by sperm penetration with dysfunction of zonae pellucida and/or membrane of the ooplasm of immature oocytes.*

Key words: *Cattle, IVF, Polyspermy, Mosaic, Chromosome*

In vitro fertilization (IVF) techniques in mammals have extended widely from basic academic categories to practical industries or a means of therapy for sterility in some species, e.g. mice, cattle and man. Nevertheless, high incidences of chromosomal anomalies were reported in embryos derived from IVF in mice [1–5], cattle [6–11] and man [12–15]. Various IVF conditions: means of getting gametes, maturation of gametes and other factors, differed among the species. In mice, oocytes ovulated from ovaries stimulated with hormones and spermatozoa recovered from the caudal epididymis are mainly used for IVF. In cattle, follicular oocytes obtained from ovaries without stimulation with hormones are co-cultured with cumulus cells *in vitro*, fertilized with frozen-thawed spermatozoa *in vitro*, and then incubated on the monolayer of cumulus cells into the blastocyst stage for the embryo transfer. In humans, follicular oocytes recovered from ovaries stimulated with hormones, and freshly ejaculated spermatozoa are used for the IVF. It is very important for technical advance to examine what is reflected by these differences in IVF systems among these three species.

Many reports showed high incidences of triploids caused by polyspermy in mouse IVF [1–5, 16], whereas incidences of haploids, polyploids and mixoploids (mosaics) were high in bovine IVF [6–11]. Yoshizawa *et al.* [11] noticed significant increase in haploids and mosaics of chromosomal complements in earlier development stages from 2 cells to 10 cells. Yoshizawa *et al.* [11] presumed that the high incidence of haploids was caused by sperm penetration into immature bovine oocytes because of the presence of a Y chromosome and the statistical result. Iwasaki and Hamano [17] also reported the occurrence of haploids bearing a Y chromosome in bovine IVF. In natural mating also, haploid embryos having a Y chromosome were found in sheep [18]. The presumption of these reports, i.e. haploids which occurred in bovine IVF were derived from the penetration of a spermatozoon into immature oocytes, remains to be confirmed. To certify the presumption, bovine immature oocytes were fertilized with spermatozoa in vitro, and then the incidence of chromosomal anomalies in the resultant embryos was examined in the present study.

Received: September 5, 2001
Accepted: September 25, 2001
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In human IVF, all of the derived zygotes are checked for having two normal pronuclei, and zygotes with one or three pronuclei are abandoned as abnormal embryos. In tripronuclear human embryos derived from IVF also, triploids and mixoploids were found [12, 15]. Especially in man, chromosomal normality is very important in genetic problems. In human subjects, experiments work with human oocytes or embryos are not accomplishable for ethical reasons. Furthermore, as stated above, many problems still remain to be solved in the various species.

The present study aims at solving the problem and establishing the use of mouse or bovine gametes as animal models to estimate chromosomal anomalies in embryos derived from IVF.

Materials and Methods

Bovine oocytes were collected from cow ovaries obtained from different donors in two slaughterhouses in Tochigi prefecture. Oocytes aspirated from 2 to 8 mm follicles were pooled as two groups and they were matured according to the method of Zhu *et al.* [19]. Oocytes cultured *in vitro* with cumulus cells in TCM 199 medium with 5% calf serum for 12 hrs at 38.5°C in air containing 2% CO_2 were classified as the 12-hr group (an insufficient maturation group), and other oocytes matured *in vitro* for 26 hrs were classified as the 26-hr group. The time periods of *in-vitro* culture were determined for insufficient or sufficient maturation of oocytes in the light of the report of Suss *et al.* [20], in which they demonstrated about 22% of the oocytes to be at the metaphase after 10 hrs of *in-vitro* maturation, about 58% at 12 hrs and about 79% at 20 hrs. The IVF was performed according to the method mentioned in our previous report [10]. The conditions for IVF were as follows: co-culture with cumulus cells for maturation of oocytes, a concentration of sperm-suspension of 5–7 × 10^6 cells/ml and the use of frozen-thawed semen of a Japanese black bull, which was treated with caffeine and heparin by the method of Niwa and Ohgoda [21]. After 5 hours of insemination, zygotes were washed in the culture medium and co-cultured again with cumulus cells for 43 hours in the fresh medium under the same conditions as for *in-vitro* maturation. Cleaved embryos were placed in the medium containing vinblastine sulfate 100 ng/ml to accumulate metaphase plates for 10 hours according to our previous report [10]. Chromosome samples were made by Yoshizawa's method [10]. The embryos were washed briefly in a hypotonic solution of 1% sodium citrate, treated in the same solution for 15 minutes, and fixed with a small volume of fixative solution consisting of three parts methanol and one part acetic acid. The fixed embryos were placed on individual glass slides, and immediately re-fixed with the fixative. Dried chromosome samples were stained in 2% Giemsa solution for 10 min. The numbers of nuclei and metaphase plates were counted. On the metaphase plates analyzed numerically, the normality and sex of embryos were examined. The data obtained were analyzed by t-test and χ^2-test.

Table 1. Developmental stages of bovine embryos derived from in vitro fertilization after *in vitro* maturation for different times

	12 hrs	26 hrs
No. of oocytes inseminated	384	292
embryos cleaved	165 (43.0)*	223 (76.4)*
2-cell	65 (39.4)*	14 (6.3)*
4-cell	60 (36.4)*	24 (10.8)*
8-cell	31 (18.8)*	80 (35.9)*
16-cell	9 (6.5) *	105 (47.1)*

*Significant differences between two groups ($P<0.05$).

Results

In the present study, all bovine oocytes were incubated *in vitro* for maturation for an insufficient time of 12 hours or a sufficient time of 26 hours. The total numbers of oocytes inseminated were 384 and 292 in the 12-hr group and the 26-hr group, respectively (Table 1). At forty-eight hours after insemination, percentages of cleaved embryos in total oocytes were 43.0% and 76.4%, respectively. The value for the insufficient group was significantly lower than that for the 26-hr group. Many embryos in the 12-hr group (125/165, 75.8%) were at the 2-cell or 4-cell stage, but most embryos in the 26-hr group (185/223, 83%) developed to more advanced stages, 8-cell or 16-cell stages. The speed of development of embryos differed significantly between the two groups.

As a result of a chromosomal analysis, the proportion of embryos with metaphase plates to the cleaved embryos in the 12- hr group was 58.8% (97/165) (Table 2), which is significantly higher than the 35.0% (78/223) in the 26-hr group. The percentages of embryos analyzed successfully in total embryos with a metaphase were high, 83.5% and 92.3% in both the 12-hr and the 26-hr groups. An especially noticeable rate of embryos with an abnormal chromosome complement, 66.7% (54/81), was seen in the 12-hr group, but a lower rate, 27.8% (20/72), was seen in the 26-hr group. The sig-

Table 2. Incidences of chromosomal anomalies in bovine embryos derived from *in vitro* fertilization after *in vitro* maturation for different times

	12 hrs	26 hrs
No. of cleaved embryos	165	223
embryos with metaphases	97 (58.8)*	78 (35.0)*
analyzed embryos	81 (83.5)	72 (92.3)
normal embryos	27 (33.3)*	52 (72.2)*
anomalous embryos	54 (66.7)*	20 (27.8)*
haploid (n)	13 (16.0)*	4 (5.6)*
polyploid	24 (29.6)*	6 (8.3)*
3n	20 (24.7)*	4 (5.6)*
4n	4 (4.9)	2 (2.8)
mosaic	17 (21.0)	10 (13.9)
n/2n	7 (8.6)	4 (5.6)
n/3n	6 (7.4)*	0 (0)*
2n/3n	4 (4.9)	3 (4.2)
2n/4n	0 (0)	3 (4.2)

* Significant differences between two groups ($P<0.05$).

nificant increase in chromosomal anomalies in the 12-hr group was mainly caused by an increase in haploids and polyploids. The incidences of haploids and polyploids were 16.0% and 29.6%, respectively, in the 12-hr group, and those in the 26-hr group were 5.6% and 8.3%, respectively. Mosaicism, a condition of mixoploidy seen as n/2n (Figs. 1-a, 1-b and 1-c) or 2n/3n etc., did not show a statistically significant difference between the two groups, only 21.0% vs. 13.9%, respectively. No other chromosomal anomalies, aneuploids or structural anomalies, were found in the present study.

The combination of sex chromosomes in Table 3 revealed the origin of the chromosome group. Only the X chromosome originated in an oocyte, and X or Y chromosome in a spermatozoon normally. The number of embryos with a haploid complement of 30 chromosomes containing X or Y was 8 in the 12-hr group. The ratio of X to Y was 6:2 showing no significant statistical difference from the theoretical ratio of 1:1 for sperm penetration into an oocyte. The presence of the Y chromosome indicated that the source of their chromosomes was a spermatozoon. The ratios of XX to XY in embryos with normal diploid chromosomes, 8:9 in the 12-hr group and 17:19 in the 26-hr group, were similar to the theoretical ratio of 1:1 in normal fertilization by single sperm penetration into an oocyte. The ratio of XXX: XXY: XYY in triploid embryos in the 12-hr group, 6:4:3, showed no significant difference statistically from the theoretical ratio, XXX: XXY: XYY=1:2:1, in embryos derived from two-sperm penetration into an oocyte. Various mosaics of combinations of X and Y were observed in

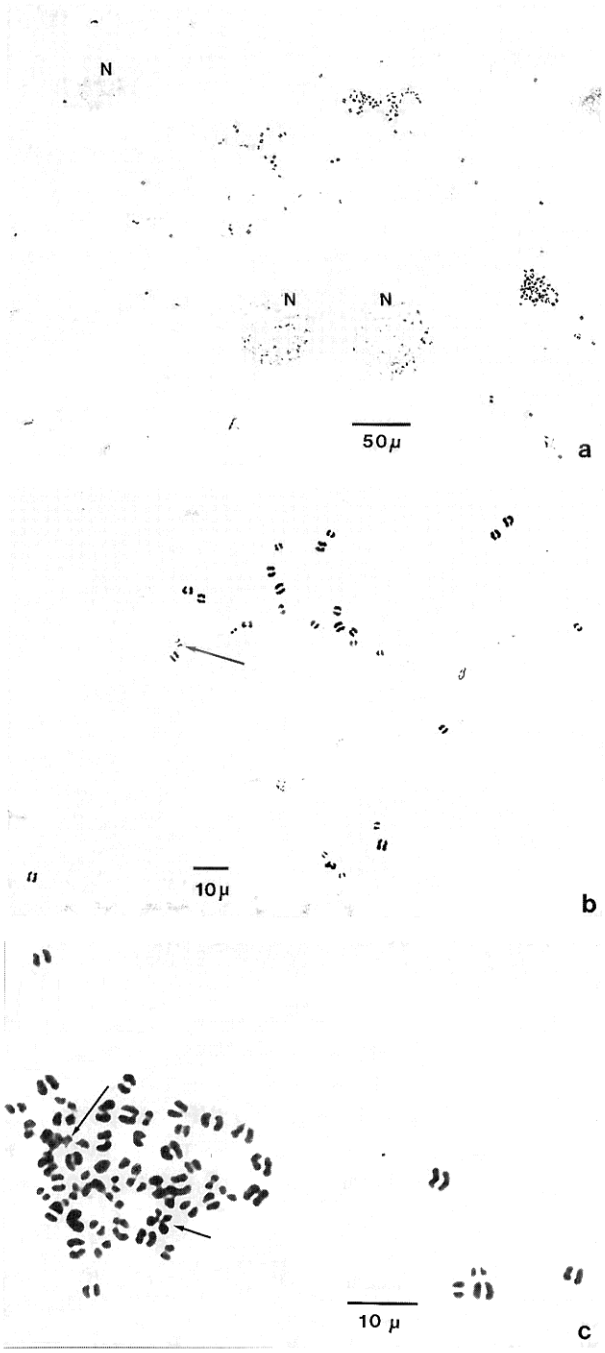

Fig. 1-a. Metaphase spreads from a bovine embryo derived from IVF. There are three metaphase spreads and three nuclei (N). This embryo is mixoploidy (n/2n) of haploid (n) and diploid (2n) chromosome complements.

Fig. 1-b. Highly magnified view of the left metaphase spread (n) in Fig. 1-a. The arrow shows Y chromosome.

Fig. 1-c. Highly magnified figure of the right metaphase spread (2n) in Fig. 1-a. The short and long arrows show X chromosome and Y chromosome, respectively.

Table 3. Sex-chromosome combination in bovine embryos derived from *in vitro* fertilization after *in vitro* maturation for different times

Chromosome complement	No. of embryos analyzed/ No. of embryos prepared		sex-chromosome combination		
	12 hrs	26 hrs		12 hrs	26 hrs
n	8/13	3/ 4	X	6	3
	(61.5)	(75.0)	Y	2	
2n	17/27	38/52	XX	8	17
	(63.0)	(73.1)	XY	9	19
			XX/XY*		2
3n	13/20	1/4	XXX	6	1
	(65.0)	(25.0)	XXY	4	
			XYY	3	
4n	1/ 4	1/2	XXXY	1	
	(25.0)	(50.0)	XXYY		1
n/2n	3/7	2/4	X/XX*	2	2
	(42.9)	(50.0)	X/Y/XX*	1	
n/3n	2/6		Y/XXX*	1	
	(33.3)		X/XXX*	1	
2n/3n	3/4	1/3	XX/XXX*		1
	(75.0)	(33.3)	XY/XXY*	1	
			XY/XYY*	2	

*Mosaic of chromosome complement and/or sex chromosomes.

embryos of tetraploids and mosaics with n/2n, n/3n and 2n/3n in the two groups.

Discussion

In the present study, the embryos in the 12-hr group developed significantly more slowly than those in the 26-hr group. This might be one of reasons why the proportion of embryos with metaphase plates to the cleaved embryos in the 12- hr group was significantly higher than that in the 26-hr group. Nevertheless, because higher incidences of metaphases have been reported in bovine blastocysts, which were derived from IVF of 22-hr matured oocytes, after 10-hr treatment with the same concentration of vinblastine [10], we should conduct finer and more numerous studies by means of fluorescence in situ hybridization if we get a proper probe for the subject.

Although the incidence of haploids increased significantly in immature oocytes in the present study, it was more impressive that the incidence of triploids was extremely high, whereas that of mosaics was high without a statistical difference. In comparison with the incidences reported by other investigators [1, 6–9], the incidence of these anomalies in the 12-h group in the present study was high, although the developmental stage of the bovine embryos examined was earlier than those of other investigators and the IVF conditions differed. It is very interesting and important for us to discover why these chromosomal anomalies occur.

High proportions of mixoploidy were reported in bovine blastocysts produced *in vitro* and recovered from superovulated animals [22], and similar results were obtained from bovine embryos at other developmental stages derived from IVF [23]. Furthermore, the sex-chromosome combination was examined in these mixoploids. Yoshizawa *et al.* [11] found mosaicism of sex chromosomes in cattle.

Triploid (3n) embryos may arise in three cases, showing certain theoretical ratios of sex-chromosome combination of XXX: XXY: XYY as follows. When a diploid oocyte (XX) is fertilized with a normal haploid spermatozoon (X or Y), a digyny triploid occurs and the ratio of their sex-chromosome combination, XXX: XXY: XYY is 1:1:0. When a diploid spermatozoon (XX or YY) is fertilized with a normal haploid oocyte (X), diandry triploids showing a ratio of XXX: XXY: XYY=1:0:1 occur. But the presence of XXY also is possible because it may be considered to occur in spermatozoa bearing XY when the first meiotic division is abnormal and the second meiotic division is normal. When a normal haploid oocyte (X) is fertilized with two normal haploid spermatozoa (X or Y), polyspermic triploids having a ratio of XXX: XXY: XYY= 1:2:1 occur. In the triploids observed in the present study, the ratio of 6:4:3 for XXX: XXY: XYY in the 12-hr group did not show a significant difference from the 1:2:1 theoretical ratio of those in the embryos derived from

dispermy, i.e. a result of penetration of two spermatozoa into an oocyte. However, digynic triploids also may be contained in these XXX. Although there was a high incidence of diploid oocytes matured in vitro in cattle [24], the presence of XXXY and XXYY in the tetraploids (4n) also indicated that the embryo was caused by polyspermy in an oocyte, i.e., polyspermic fertilization. Murray *et al.* [18] suggested that mosaics of n/2n in ovine embryos derived from natural mating might have originated due to polyspermic fertilization because of the presence of the Y chromosome. Murray *et al.* [18] surmised that the second sperm-donated centrosome had organized a second mitotic spindle leading to the production of haploid and diploid cells at the first or second cleavage division. Unlike mice whose centrioles at the first cleavage division were derived from oocytes [25], centrioles of man [26] and cattle [27] were paternally derived from spermatozoa. Furthermore, tripolar spindles were detected in dispermic human embryos. Tripolar spindles altered the first cleavage division to 3 cells [26, 28] and multiple mitotic spindles were observed in polyspermic bovine zygotes [27]. If supernumerary spermatozoa penetrate into a bovine oocyte, the first cleavage division of the resultant zygote will be abnormal because an extra sperm aster works also as a centriole. Kola *et al.* [12] reported that most tripronuclear human zygotes divided into 3 cells directly and they had severely abnormal chromosomal complements, whereas the tripronuclear zygotes which developed into 2 cells were triploids. They indicated that a correlation exists between the pattern of the first cleavage division and the subsequent chromosome complements of these zygotes.

Furthermore, mosaicism of the sex-chromosome combination in other mixoploids observed in the present study showed that the mixoploids were derived from polyspermy in oocytes.

Because of the sex-chromosome combination and the statistical results, we concluded that both polyploidy and mixoploidy resulted from polyspermy in an oocyte. In bovine oocytes incubated insufficiently for maturation in vitro for 12 hours, incidences of chromosomal anomalies, haploids, triploids and mixoploids, increased significantly, and the frequency of polyspermy was much higher than that in the oocytes incubated for 26 hours. Furthermore, some of the diploid embryos derived in the present study may have been caused by two-sperm penetration into an oocyte because half of the oocytes cultured *in vitro* for the insufficient 12 hrs may not have been able to condense their chromosomes [20]. These results suggest that a mechanical or functional disorder of the zonae pellucida and/or the ooplasm membrane of immature bovine oocytes incubated *in vitro* for 12 hr may be the cause of polyspermy.

Acknowledgements

We thank the management and staff of Utsunomiya Slaughterhouse and Otawara Slaughterhouse in Tochigi Prefecture for their help in procuring bovine ovaries, and students of our laboratory for their assistance in the completion of this experiment. This work was supported in part by a Grand-in Aid for Scientific Research (No. 12660251) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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