—Brief Note—

Incidence of Chromosomal Abnormalities in Early-stage River Buffalo Embryos Derived from In Vitro Fertilization

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Abstract: *The present study focused on determining the incidence of chromosomal abnormalities in river buffalo embryos produced in vitro to reveal the causes of the low conception rate of transferred embryos. River buffalo oocytes were collected from 2- to 8-mm ovarian follicles of slaughtered river buffaloes in the Philippines and India, matured in vitro, and inseminated with frozen-thawed river buffalo spermatozoa at a final concentration of 1 × 10^6 sperm/ml. At 36–40 h of in vitro culture, 2- to 8-cell stage embryos were treated with vinblastine sulphate for 6 to 10 h, and prepared as chromosomal samples. The rate of chromosomally normal embryos (diploidy 2n = 50) was 52.2% (48/92), while the incidence of chromosomal abnormalities reached 47.8% (44/92) in the analyzed embryos. Polyploidy was the most frequent abnormality (22 embryos; 23.9%), while the incidences of mixoploid (11 embryos, 12%) and haploid embryos (11 embryos, 12%) were the same. The polyploidy and mixoploidy observed in the present study may be the result of polyspermy. The early-stage river buffalo embryos derived from in vitro production displayed a high incidence of chromosomal abnormalities that might affect their subsequent development.*

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Introduction

Birth of river buffalo calves from *in vitro*-derived vitrified embryos was first reported by Huhana-Duran *et al.* [1]. Furthermore, they achieved full-term delivery of *in vitro*-produed and vitrified river buffalo embryos gestated swamp buffalo recipients [2]. However, although they resourcefully transferred excellent frozen-thawed embryos to recipient females, the full-term delivery success rates were not as high as expected. It would appear that failure to implant or early abortion was caused primarily by developmental failure of the embryos [3], although recovery rates of frozen-thawed embryos were not as high as those of fresh embryos. In the Philippines, the co-authors of the present study have routinely achieved success rates of *in vitro* fertilization, cleavage and embryonic development to the blastocyst stage in the river buffalo of 70 to 90%, 60 to 70% and 20 to 30%, respectively. It is worth noting that there have been many reports of the high incidence of chromosomal abnormalities in mammalian embryos produced *in vitro* by many investigators in mice [4, 5], pigs [6–8], cattle [9–16], and humans [17–19]. It is

important to transfer cytogenetically normal embryos, because it is well known that severe cases of chromosomal abnormalities result in abortion [17–19]. Ulloa *et al.* [8, 16] proposed a method for selecting cytogenetically normal blastocysts for successful embryo transfer. Using this method, a combination of distinguishing 5- to 8-cell stage Day 2 embryos and selecting the resultant Grade A blastocysts, the incidence of chromosomal abnormalities was reduced in *in vitro* production (IVP) of pig embryos [8] and cattle embryos [9]. However, there have been no reports about chromosomal abnormalities in buffalo embryos produced *in vitro*. Therefore, the present study focused on determining the incidence of chromosomal abnormalities in river buffalo embryos produced *in vitro*.

Materials and Methods

The method of IVP of river buffalo embryos was performed according to the report of Duran *et al.* [1]. Oocytes were collected from 2- to 8-mm ovarian follicles of slaughtered river buffaloes at slaughterhouses in the Philippines and India. The river buffalo oocytes were matured *in vitro* in Medium 199 (Earle's salts with 25 mM HEPES; Gibco BRL) supplemented with 10% FBS and antibiotics (100 IU penicillin/ml and 100 μg streptomycin/ml) for 23–24 h in 5% CO_2 at 39ºC. The *in vitro*-matured oocytes were inseminated with frozen-thawed river buffalo spermatozoa at a final concentration of 1×10^6 sperm/ml in modified Brackett & Oliphant medium containing 5 mM caffeine, 2 units heparin/ml, and 5 mg/ml BSA (Fraction V, Wako Pure Chemical Ind., Osaka, Japan). After 12–18 h of insemination, the presumptive zygotes were transferred into maturation droplets containing cumulus cells and cultured *in vitro*. At 36–40 h of *in vitro* culture, 2- to 8-cell stage embryos were treated with a concentration of 100 ng/ml of vinblastine sulphate for 6 to 10 h [11]; then, they were prepared as chromosome samples according to the method of Yoshizawa *et al.* [11].

The total numbers of cells with nuclei, analyzable metaphases (complement and non-overlapping), and chromosomes were examined under a phase contrast microscope (Olympus, Tokyo, Japan) at a magnification of 600× (40 × 15) or 900× (60 × 15). Embryos having one or more analyzable metaphases were judged to be recognizable embryos. The embryos that had two sets of chromosomes (2n = 50) in all countable metaphases were labelled normal diploids, and those in which all analyzable metaphases (nuclei) contained one set of chromosomes (n = 25) were labelled haploids. Embryos containing more than two sets of chromosomes in all countable nuclei were considered to be polyploids (3n, 4n, and 5n), and those with mixtures of diploid cells and haploid (n/2n), triploid (2n/3n), or tetraploid (2n/4n) cells were labelled mixoploids.

Table 1. Incidence of chromosomal abnormalities in river buffalo embryos derived from *in vitro* fertilization

Embryos examined	206
with metaphase	105 (51.0)
with analyzable metaphase	92 (44.7)
with 2n	48 (52.2)
with abnormal metaphases	44 (47.8)
polyploidy	22 (23.9)
mixoploidy	11 (12.0)
haploidy	11 (12.0)

Results

The method of IVP of river buffalo embryos used in the present study has routinely achieved success rates of *in vitro* fertilization, cleavage and embryonic development to the blastocyst stage, 70 to 90%, 60 to 70% and 20 to 30%, respectively. The morphological features of river buffalo IVP embryos are similar to those of bovine IVP embryos. Of a total of 206 examined embryos, 105 embryos displayed metaphases (51.0%, Table 1), and 87.6% (92/105) of these (44.7% of total examined embryos 92/206) could be successfully analyzed for chromosomes (Table 1). The rate of chromosomally normal embryos (diploidy 2n = 50) was 52.2% (48/92), while the incidence of chromosomal abnormalities was 47.8% (44/92). Polyploidy was the most frequent abnormality (22 embryos; 23.9%), while the incidences of mixoploid (11 embryos, 12%) and haploid embryos (11 embryos, 12%) were the same. Among 11 mixoploid embryos, the most frequent abnormality was haploid/diploid (6 embryos) (Fig. 1), and other mixoploidies consisted of 2n/3n (4 embryos) and 2n/4n (1 embryo). Triploidy, seen in 14 embryos, was observed most frequently among polyploid embryos; other polyploidies consisted of 4n in 6 embryos and 5n in 2 embryos.

Discussion

In the present study, the incidence of chromosomal abnormalities observed in 2- to 8-cell stage embryos (47.8%) was higher than some reports of incidences in

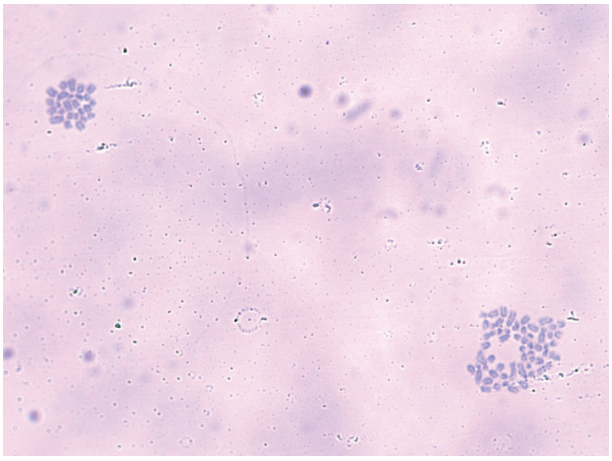

Fig. 1. An example of mixoploidy showing haploidy (n) and diploidy (2n) in an IVF river buffalo embryo. The upper left metaphase has 25 chromosomes (haploidy), and the other has normal 50 chromosomes (diploidy).

Day 2 bovine embryos produced by IVP (22.6% [16], 26.8% [13], and 36.3% [10]), but lower than that of another report (60.6%) [12]. In Day 2 bovine embryos, Ulloa *et al.* [16] reported that the incidence of chromosomal abnormalities was significantly lower in the 5- to 8-cell stage embryos than in the other stage embryos. Furthermore, they found a low incidence of chromosomal abnormalities in morphologically best-quality bovine *in vitro* fertilization (IVF) embryos. They concluded that good-quality bovine embryos, as judged by a low incidence of chromosomal abnormalities, can be selected on Day 2, and the selection of Grade A blastocysts increases the number of chromosomally normal embryos.

The high incidences of polyploidy (23.9%) and mixoploidy (12%) observed in the present study might reflect the high final sperm concentration. In the present study, the final sperm concentration, 1×10^6 sperm/ml, was higher than that used in bovine IVF ($5–7 \times 10^5$ sperm/ml) by Ulloa *et al.* [16]. The final sperm concentration affects the incidence of polyspermy in IVF [4], and it has been suggested that the high incidences of polyploidy and mixoploidy during IVF in mice [4, 5], swine [6, 8], and cattle [9–12] are caused by polyspermy. Therefore, the polyploidy and mixoploidy observed in the present study may also have been the result of polyspermy, although King *et al.* [20] noted that mixoploid embryos could also arise through fusion of blastomeres.

In the present study, a high incidence of haploidy (12%), which accounted for about half of all abnormalities, was similar to the high incidence of haploidy in bovine IVF embryos at Day 2 (10.1% [16]) and the high incidence of haploidy and haploid cells in mixoploid bovine embryos at the 5- to 10-cell stage reported by Yoshizawa *et al.* [12]. The haploidy derived from IVF was probably caused by gynogenetic or androgenic development in IVF zygotes, as described by Iwasaki and Hamano [21], and the haploid cells in the mixoploid embryos probably resulted from abnormal cleavage division in polyspermic zygotes, as proposed by Navara *et al.* [22] and Yoshizawa *et al.* [12].

Conclusion

Early-stage river buffalo embryos derived from IVP displayed a high incidence of chromosomal abnormalities that might affect their subsequent development. However, it may be possible to reduce chromosomal abnormalities such as triploidy by decreasing the final sperm concentration in the IVF procedure, and selection of adequately developed embryos at an early stage (for example, Day 2). Selecting morphologically excellent embryos at the blastocyst stage (for example, Grade A) may also help to exclude embryos with chromosomal abnormalities in IVF buffalo embryos. Further research to classify chromosomal abnormalities in buffalo embryos in detail at the various cleavage stages is needed to clarify the relations between chromosomal abnormality and development stages of buffalo embryos.

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