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Can Oocyte Selection, Cleavage and Developmental Rates of *In vitro* Produced Bovine Embryos Assist in the Gender Selection of the Pre-implanted Embryos

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Abstract: Knowing the sex of embryos produced for use in an embryo transfer program can assist farmers in managing their resources more effectively. With an objective of determining the relationship between embryonic development rate and sex ratio of the *in vitro* produced (IVP) bovine embryos, a total of 930 oocytes divided into 2 groups (excellent and good) according to their morphological score, time of first cleavage and the rate of blastocyst formation were employed. The proportion of embryos that cleaved before 36 h post insemination (pi) was significantly higher ($P < 0.05$) in each group compared with slow cleaving (cleaving after 40 h pi) embryos (55.6 and 50.2% Vs 29.3 and 27.8%, for excellent and good, respectively). Moreover, fast cleaving embryos yielded higher ($P < 0.05$) percentages of fast growing embryos (i.e., those that reached blastocyst stage within the fist 7 days of culture: 30.5 Vs 18.7%, for excellent- and good-classified oocytes, respectively). Although there was no significance difference among groups in terms of cleavagetime, oocytes from the excellent group showed a significant increase ($P < 0.05$) in total blastocyst rate compared with those originating from group classified as good (32.6 Vs 20.7, respectively).
There was no significant difference between fast and slow growing embryos with regard to their sex ratio. Both fast and slow growing embryos biopsied either at 8-cell or at > 8-cell stages showed a significant ($P < 0.05$) different sex ratio from the expected 1:1. The overall sex ratio was 77 Vs 23% for male and female respectively, from both developmental groups probably due to the type of sperm used and/or both cultural and manipulation conditions. These results suggest that although there might be some differences in developmental rate between male and female embryos, such phenomena are associated with other factors such as the source of sperm as well as cultural conditions. As such, more studies are needed to clarify this aspect.

Key words: morphology, *in vitro* fertilised, cleavage, blastocyst, sex ratio

Introduction

Gender pre-selection in domestic animals is potentially of great economical importance to the global agricultural efficiency because in most cases, regardless of the breeding methods used, offspring of one sex will have significantly higher value than the other. Remarkable progress have been made in the past decade in improvement and refinement of the different techniques for pre-implantation sex determination in both semen and embryos.
Although the ideal method of controlling the sex ratio would be to separate X- and Y-bearing spermatozoa and to introduce the separated spermatozoa into an ova, efforts to sex bovine semen have so far resulted in numerous promises as well as failures (Seidel *et al.*, 1996). Various methods have been employed including flow fractionation (Gledhill, 1988) and flow cytometry (Johnson, 1988; Van Vliet *et al.*, 1989), but none of these have been generally accepted as successful up to now. The alternative approach to sex selection involves pre-implantation embryo sexing (Booman, 1986). Depending on whether or not a biopsy of embryonic tissue is required, embryo sexing method are classified as either invasive or noninvasive (Betteridge, 1989). Several nonivasive methods for pre-transfer determination of sex have been developed (Williams, 1986; Anderson, 1987; Seidel, 1998), but to date none have the required accuracy. On the other hand, invasive approaches which require a sample of cells to be removed (biopsy) from the embryos for direct genetic analysis, the procedure is by and large affected by its technological demands as to be readily applicable under field conditions (Herr *et al.*, 1990).
As it has become evident in the bovine embryo that sexual dimorphism may occur even before activation of the embryonic genome (Yadav *et al.*, 1993), it is assumed that there may be a relationship between embryonic growth rate and life span. However, there seems to be two schools of thoughts with regard to the rate of embryonic growth in different species. While some researchers believe that male cattle embryos develop faster than the female embryo both *in vivo* (Itoh and Goto, 1986; Avery *et al.*, 1989) and *in vitro* (Marquant-Le Guiene *et al.*, 1992, Xu *et al.*, 1992; Dominko and First 1993; Yadav *et al.*, 1993) during early cleavage stages, others (Berg *et al.*, 1992; Callesen *et al.*, 1992; Thibier and Nibart, 1995) reiterate that the ratio between male and female does not differ from 1:1. Apparently, male and female embryos may commence cleavage at about the same time, but the difference in male new-borns at birth indicates a possible different developmental competence between the two, which may have originated from the embryonic stage. The proper mechanism of this effect is not known, but Tiffin *et al.* (1991) reported that in male cattle embryos, glucose metabolism was twice that of females and increased between the morula and expanded blastocyst stages.
The present study was therefore conducted to first examine whether or not *in vitro*-derived bovine embryos differed on their developmental competence, and second, to exploit the possibilities of finding some criterion to be used as a means of avoiding an invasive approach for bovine embryo sexing.

Materials and Methods

In vitro Maturation (IVM): Ovaries from Holstein cows were collected from a slaughterhouse and transported to the laboratory in Ringer's solution supplemented with penicillin G

(100 IU/ml) and streptomycin sulfate (0.2 μg/ml) at 30-32 °C. Cumulus-oocyte complexes (COCs) were aspirated from follicles of 2 to 6 mm in diameter using an 18-g needle connected to a 5-ml syringe, and then transferred to a petri dish containing modified PBS (Gibco, Grand Island, NY, USA) supplemented with 3% BSA. After aspiration, although it is only those COCs with an intact and surrounded by cumulus cells over more than one-third of their surface that were considered suitable for IVF procedure, the COCs were further classified into two groups: excellent and good, based on the number of cumulus cells layer surrounding an oocyte (as described bellow). The classified COCs were washed three times in maturation medium (TCM-199, Earle's salts; Gibco) supplemented with 5% Day-7 superovulated cow serum (SCS:Matsuoka *et al.*, 1992), 0.01 mg/ml FSH (Denka Pharmaceutical Co., Kawasaki, Japan) and 50 μg/ml gentamycin (Sigma Chemicals, St. Louis, MO, USA). Thereafter, the COCs were separately incubated (according to their respective score) in maturation medium for 21 to 22 h at 38.5° C in humidified 5% CO_2 in air.

In vitro Fertilization (IVF) and Culture (IVC): Frozen-thawed semen from a bull whose reproductive qualities are known (Sumatri *et al.*, 1997), used for *in vitro* fertilisation. Frozen semen was thawed in a water-bath (37°C), then washed 2 times using 2.5 mM caffeine in Brackett and Oliphants's medium (Caff-BO), as previously described (Brackett and Oliphant, 1975), followed by centrifugation at 500*g* for 5 min. The sediment was then suspended in Caff-BO supplemented with 1% BSA (Sigma) and 20 μg/ml heparin (Shimizu Pharmaceutical Co., Shimizu, Japan) to yield a final sperm concentration of 5 × 10^6/ml. A 100 μl aliquot of sperm suspension was covered with mineral oil and then pre-incubated for 1 h at 38.5°C in 5% CO_2 in air. Matured oocytes were transferred into sperm microdrops (20-25 oocytes per drop) for insemination.

Five hours latter, the COCs were washed 3 times and transferred into fresh culture medium (TCM-199 supplemented with 5% SCS), 5 μg/ml insulin (Wako Pure Chemical Industries Ltd, Osaka Japan) and 50 μg/ml gentamycin for further development. At 48 h after fertilization, the cumulus cells surrounding the embryo were partially removed by repeated pipetting, while the cumulus cell layer attached to the bottom of the culture dish was undisturbed and used as a co-culture. The culture medium was replaced with a new after 96 h.

Assessment of oocytes' quality score in relation to cleavage and developmental rate: Aspirated COCs with intact cytoplasm and surrounded by unexpanded cumulus cells over more than one-third of their surface were grouped into two classes (excellent and good), based on their respective layers of cumulus cells. An oocyte was classified as excellent when together with the morphological characteristics describe above, it had > 5 layers of cumulus cells, and as good when cumulus cells were between 3 and 5 layers. The cleavage rate was evaluated at 36 and 40 h post *in vitro* fertilisation, and cleaved embryos from each class present at each time point were recorded and regrouped together in either one or two wells (depending on the number of oocytes) of the 4-well culture dishes. All the cleaved embryos were then cultured for up to 10 d (fertilisation day = 0 d) and their development to blastocyst was noted. Embryos that cleaved within the first 36 h following the IVF were regarded as fast-cleaving, while the ones that cleaved after 40 h post insemination were classified as slow-cleaving. Similarly, embryos that reached the blastocyst stage before or up to Day 7 were classified as fast developing whereas embryos that reached the same stage from day 7 onwards were considered to be slow-developing.

Effects of developmental capacity on sex ratio and survival rate of the embryos biopsied at different cell stages: In order to accomplish the above objectives, two groups of 50 zygotes each were formed according to their developmental rates: fast- and slow-growing embryos. Then each group was further subdivided into two subgroups, depending on the stage at which the embryo was biopsied; i.e., either at 8- and ≥ 8-cell stages, respectively. Twenty-five embryos were employed per each cell stage (subgroup) and their corresponding sex ratio (following the PCR results) as well as their capacity to develop up to blastocyst stage were recorded.

Sex determination by PCR method: Biopsies of 2 to 6 cells were used for the polymerase chain reaction (PCR) assay. The biopsy procedure was carried out by micro-manipulation done in a 100-mm sterile plastic petri dish containing 100-μl micro-drop of PBS supplemented with 0.05% fatty acid free BSA (Sigma). Biopsied embryos were washed in the culture medium before being replaced back for further development, and the isolated cells were transferred in 1-2 μl of PBS buffer to the bottom of 0.5 ml reaction tube (vials), then 8-9 μl of autoclaved double distilled water was added and covered with 10-μl mineral oil. This was followed by denaturation of the sample at 95 °C for 1 min, and the samples were either stored in the deep freezer (-20 °C) until the day sex was intended or directly subjected to the PCR assay.

The PCRs were performed using sequencer TSR-300 (Iwasaki Glass, Co. Ltd., Japan) in 0.5 ml reaction tubes using reagents supplied by Takahara Shuzo Co., Ltd. (Biochemical Group, Otsu, Shiga, Japan). The reaction mixture consisted of 5 μl of 10× buffer (10 mM Tris-HCl, 50 mM NaOH, 0.5 M EDTA 2Na, and 0.0275 g/ml boric acid), 5 μl Primer I (BM_{1-5}), 5 μl Primer II (BM_{2-5}), 2 μl dnT (AGTC) and 0.25 μl Taq Polymerase; all adjusted to a total volume of 50 μl per sample by adding double distilled water and covered with 50 μl mineral oil. Reaction tubes containing the reaction mixture with 10 μl of male and female DNA each were used as positive controls. Forty-five PCR cycles each consisting of three steps: template denaturation, primer annealing, and primer extension at 95, 70, and 50 °C, respectively, were run. After the last cycle, the samples were incubated at 38.5 °C for 5 min, then cooled to room temperature. The PCR products were electrophoretically separated in 3% agarose gels containing ethidium bromide at a concentration of 20 μg/ml were visualised on an UV transilluminator (Iwasaki Glass Co.: 320 nm). Electrophoresis marker consisted of 0.5 μl (100 base pairs DNA ladder), 1 μl of loading dye and 3.55 μl buffer.

Statistical analysis: The data were statistically analysed by Chi-square (χ^2) test, or when some expected values were less or equal to 5, Ficher's exact probability test were used. Differences at a probability (P) value of 0.05 or less were considered significant.

Results

A Total of 930 oocytes divided into 2 groups (Excellent and Good) according to their morphological score were *in vitro*

Table 1: Cleavage rates of *In vitro* fertilised bovine oocytes according to the oocytes' morphological score

Oocytes' Classification	No. of fertilised oocytes	Cleavage (%)		
		Fast[A]	Slow[B]	Total
Excellent	430	239 (55.6)[a]	126 (29.3)[b]	365 (84.9)
Good	500	251 (50.2)[a]	139 (27.8)[b]	390 (78.0)

[A,B]Cleaved within the first 36 and after 40 h post insemination, respectively.
Values with different superscripts within a row are significantly different ($P < 0.05$)

Table 2: Effect of time of cleavage and oocyte score on developmental competence of *in vitro* fertilised bovine embryos

Oocytes' classification	Type of cleavage (No.)	Development to blastocysts (%)		
		Fast	Slow	Total
Excellent	Fast (239)	73 (30.5)[aa'a'']	18 (7.5)[b]	89 (37.2)[a'a''] [119/365 = 32.6%]a'
	Slow (126)	18 (14.3)[ab']	12 (9.5)[b]	30 (23.8)[b']
Good	Fast (251)	47 (18.7)[aa'b'']	15 (5.9)[b]	62 (24.7)[a'b''] [81/390 = 20.7%][b']
	Slow (139)	14 (10.1)[ab']	5 (3.6)[b]	19 (13.7)[b']

Values with different superscripts within a row ([a-b]) and column ([a'-b'] or [a''-b'']) are statistically different. ($P < 0.05$).

Table 3: Sex ratio and survival rate of fast- and slow-growing IVP-bovine embryos biopsied at different cell stages

Growth category	Biopsied at	Survival (%)		Sex		Ratio (M:F)
		No.	(%)	Male (%)	Female (%)	
Fast	8-cell	25	17 (68)	22 (88)[a]	3 (12)[b]	22 : 3
	> 8-cell	25	20 (80)	20 (80)[a]	5 (20)[b]	20 : 5
Slow	8-cell	25	14 (56)	17 (68)[a]	8 (32)[b]	17 : 8
	> 8-cell	25	20 (80)	18 (72)[a]	7 (28)[b]	18 : 7
Total		100	71 (71)	77 (77)[a]	23 (23)[b]	77 : 23

[a,b]Values with different superscript letter within the rows are significantly different ($P < 0.05$).

matured, fertilised, and cultured to determine their developmental competence. The percentage of embryos that cleaved before 36 h post insemination (pi) was significantly higher ($P < 0.05$) within, but not among the two groups (excellent and good) than those that cleaved 40 h pi (Table 1). The fast cleaving oocytes were 55.6 and 50.2% compared with the slow cleaving ones 29.3 and 27.8%, for excellent and good groups, respectively.

In order to study the correlation between developmental competence with the time of cleavage, two more criterions were established. That is, after separating the zygotes according to their cleavage time, cleaved embryos were further classified into two classes depending on the time it took them to reach the blastocyst stage. Fast cleaving embryos had the highest ($P < 0.05$) percentages of fast growing embryos (30.5 Vs 18.7%, for excellent- and good-classified oocytes, respectively). Moreover, in both groups and cleavage times, there were significant difference ($P < 0.05$) between them in terms of growth rate (Table 2). Among the groups, although there were no significant difference among cleavage times, oocytes from the excellent group showed a significant increase ($P < 0.05$) in total blastocyst rate compared with those originating from good oocytes.

For sex determination, fast and slow growing *In vitro* produced blastocysts were employed (Table 3). Both fast and slow growing embryos biopsied either at 8-cell or more than 8-cell stages showed a significant ($P < 0.05$) different sex ratio from the expected 1:1. However, there was no significant difference between fast and slow growing embryos with regard to their sex. The overall sex ratio obtained was 77 Vs 23% for male and female respectively, from both developmental groups. On the other hand, although not significantly different, embryos biopsied during their early developmental stages showed a tendency of low survival rate compared with those that were biopsied at an advanced stage of growth.

Discussion

In the present study, bovine embryos obtained by *in vitro* fertilisation and culture showed variable developmental rates starting from the time of cleavage up to the blastocyst formation. Basing on the previous reports that bovine oocytes starts to cleave at 31 (Hyttell *et al.*, 1988) and between 26-32 h (Xu *et al.*, 1987; Barnes and Eyestone, 1990) post insemination for *in vivo* and *in vitro* respectively, only 2 time points: 36 and 40 h pi were selected so as to avoid exposing the embryos longer time out of the incubator. Fast cleaving embryos yielded a higher rate of blastocyst development than those that cleaved after 40 h pi, coinciding with previously reported results (Plate and King, 1992; Van Soon *et al.*, 1992; Yadav *et al.*, 1993; Miller *et al.*, 1994). Although there were no significant difference between oocyte classification groups on cleavage time, fast and slow cleaving zygotes from the excellent group yielded higher rates of fast growing blastocysts than their corresponding counterparts from the group of oocytes classified as good. These results support the concept that the development *in vitro* of bovine embryos depends, upon other factors, the oocyte morphology and size (Assey *et al.*, 1994; Longergan *et al.*, 1994).

Differences in the growth rate between embryos depend on various factors, including differences in fertilisation time, inherent growth rate of individual embryos, and sex-related growth (Avery *et al.*, 1992). Several reports have indicated that embryonic development is regulated by sex-specific traits or genes leading to altered sex ratios. Fast growth by male embryos has been related to the process of gender sex differentiation (Burgoyne, 1989; Heslop *et al.*, 1989) in some way, and glucose metabolism (Tifffin *et al.*, 1992) or growth stimulating effects of oxygen radicals (Peipo and Bredbacka, 1996) in another.

Despite the fact that sex ratio between fast and slow growing embryos did not differ significantly, the total sex ratio of fast- and slow-developing bovine blastocyst stage embryos in this experiment was significantly skewed towards the males rather than females. Our results are therefore contrary to the previously reported (Tsunoda *et al.*, 1985; Xu *et al.*, 1992; Dominko and First, 1993; Yadav *et al.*, 1993) that male embryos develop faster than female embryos *in vitro*; since both fast- and slow-growing embryos in this study were significantly male in proportion. Consequently, our findings

neither agrees with the standard sex ratio of 1:1 obtained *in vivo*. There are two possible reasons for this. The first reason is that since the semen used for fertilisation came from only one bull, the latter might have contributed heavily towards the higher proportion of male embryos as it has been observed that there is sire differences in the sex ratio of progeny, in cattle (Powell *et al.*, 1975; Leibo and Rall, 1990), and in buffalo (Totey *et al.*, 1996). The second reason is based on the hypothesis that manipulation and culture of bovine embryos result in a preferential loss of female embryos (King *et al.*, 1992). However, the mechanism underlining these two aspects are not totally elucidated, and from the sample population taken in this experiment, it is hard to reach conclusive remarks. Hence, further studies, including a variation in the source of sperm used for *in vitro* fertilisation, ought to be conducted.

In summary, our study demonstrated that developmental competence of the *in vitro* produced bovine embryos is dependent upon the quality of the oocytes used, and the fast cleaving embryos within a pool results to higher rates of fast growing and total blastocyst formation. Secondly, for a proper analysis of the correlation between fast-growing embryos and sex ratio, at least the comparison of the bulls together with the number of sampled population needs to be considered.

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