Developmental Rate Differences and Sex of Bovine Preimplantation Embryos Generated In Vitro

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Abstract: *Bovine zygotes produced by in vitro maturation and fertilization (IVM-IVF) were selected according to the time of the first cleavage, and their subsequent developmental ability to the hatched blastocyst-stage and sex were assessed using conventional in vitro culture (IVC) conditions and PCR sexing. Of 669 presumptive zygotes cultured, 124, 227, 88 and 65 cleaving embryos were selected at 22, 26, 30 and 44 h post insemination (hpi), respectively. These embryos were transferred into separate drops of the same medium with cumulus cells and co-cultured for a total of 10 days. Significant correlation was observed between hatching rates and the time of the first cleavage (P< 0.05): 56.5, 40.1, 21.6 and 4.6% of embryos cleaved at 22, 26, 30 and 44 hpi were developed to the hatched blastocyst-stage, respectively. Of 183 hatched blastocysts subjected to PCR for sexing, the sex of 171 embryos was successfully determined and the overall sex ratio was 51.5% (88/171). The sex ratios of early cleaving embryos (22 and 26 hpi) and late cleaving embryos (30 and 44 hpi) were 49.7% (74/149) and 63.6% (14/22), respectively. These ratios were not significantly different from the expected ratio of 1:1 and did not related to the time of the first cleavage. These data suggest that the interval from insemination to the first cleavage of embryos generated in vitro strongly affects their subsequent developmental potential in vitro, but not the sex difference.*

Key words: *First cleavage, Hatching, PCR sexing, Bovine embryo, In vitro culture.*

In early bovine embryos cultured *in vitro*, it is recognized that there is variation in the stage of development reached at any one time, even though the sperm penetration time was accurately controlled using *in vitro* fertilization. Several factors including inherent cleavage rates of the individual embryo, *in vitro* culture (IVC) conditions for embryos, and sex of the embryo, could account for the differences in growth rate between embryos. Recent studies [1–3] have shown that a very early cleavage division of bovine embryos produced by *in vitro* maturation and fertilization (IVM-IVF) is crucial for their subsequent developmental potential *in vitro*. Yadav *et al.* [4] reported that early cleaving embryos categorized according to the interval from insemination to completion of their first cleavage, were more likely to have developed to the 8-cell stage or beyond, contained more cells and were more likely to be male than late cleaving embryos at 5 days after insemination. Since the first report on the observation by Tsunoda *et al.* [5] in mouse, several studies have reported that male embryos generally develop faster than female embryos at the blastocyst-stage using cytogenetic analysis [6, 7] or polymerase chain reaction (PCR) analysis [8] in cattle. However, only a few reports on the relationships between hatching and the sex of embryos have been published [9]. Hatching of embryos is visible sign that they have overcome the various troublesome aspects of developmental process, besides marking the beginning of the process leading to implantation.

In this paper, we observed developmental rate to hatched blastocyst classified according to the time when they completed their first cleavage, and determined the sex of these embryos by PCR sexing.

Materials and Methods

Media: The medium used for IVM and IVC was 25 mM HEPES buffered TCM-199 (Sigma Chemical Co., St. Lois, MO) supplemented with 5% heat-treated (56°C,

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30 min) calf serum, 0.5 mM sodium pyruvate and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The 400-μl drop of medium in plastic dish (Corning, 35 mm treated polystyrene dish, Iwaki Glass, Tokyo) was overlaid with 5–6 ml of mineral oil (Squibb & Sons Inc., Princeton, NJ) and pre-equilibrated overnight at 39°C under a humidified atmosphere of 5% CO_2 in air. BO medium [10] supplemented with 10 μg/ml heparin and 5 mM caffeine was used for sperm washing and IVF. All media were prepared from fresh ultra-pure water (18 megaohm ≦) and sterilized by filtration through a 0.22-μm membrane filter before use.

IVM and IVF of oocytes: Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory in warm (30–32°C) sterile saline (0.85% NaCl) with antibiotics in a thermos flask within 1 h of collection. Follicular contents were aspirated from the ovaries with a 5-ml disposable syringe with a 20-gauge needle. Follicular oocytes were collected and washed in PB1 medium [11] containing 3 mg/ml bovine serum albumin. Only oocytes with compacted cumulus cells and an evenly granulated cytoplasm were selected and cultured in the IVM medium for 24 h. All incubations throughout this study were performed at 39°C under a humidified atmosphere of 5% CO_2 in air. Each droplet contained approximately 40 cumulus-oocytes complexes.

Sperm were capacitated with heparin and caffeine using the methods of Niwa and Ohgoda [12], based on that of Parrish *et al.* [13], with minor modification. Frozen semen from a fertile Japanese black bull was thawed in a 37°C waterbath. Spermatozoa were washed twice by centrifugation and capacitated for a 2-h preincubation in the 100-μl drop of fertilization medium overlaid with mineral oil. After IVM of oocytes for 24 h, 10 to 12 cumulus-enclosed oocytes were transferred into the IVF medium containing capacitated spermatozoa. The time of the first contact between spermatozoa and oocytes is referred to as 0 hpi (h post insemination). Following co-incubation of gametes for 5 hpi, approximately 40 presumptive zygotes (indistinguishable from unfertilized oocytes at this stage) together with attached cumulus cells were transferred into the original culture medium that had been used for IVM.

IVC of embryos: At 22 hpi, oocytes/embryos were manually stripped from their cumulus cells using a finely drawn pipette and the proportion of 2-cell stage embryos was examined at 22, 26, 30 and 44 hpi. Embryos that had developed to the 2-cell stage at each time were removed from the original co-culture medium and transferred into the separate 400-μl drop of the same medium containing of cumulus cell masses that had been previously isolated from 40 cumulus-oocytes complexes. Under these conditions, cumulus cells formed a monolayer on the surface of the culture dish. Care was taken to perform all manipulations of embryos as quickly as possible at an ambient temperature of 30–32°C. Embryos divided into four groups according to the time of the first cleavage were co-cultured with cumulus cells for a total of 10 days. Oocytes which had not developed to the 2-cell stage at 44 hpi were not included in this study, since these oocytes were assumed to be unfertilized or developmentally incompetent.

The developmental stages of embryos in co-culture were evaluated morphologically at 12-h intervals between 164 hpi and 248 hpi. Embryos classified as being at the hatched blastocyst-stage at each time were removed from co-culture medium, washed 3 times in fresh PB1 medium containing 3 mg/ml polyvinylpyrrolidone and directly subjected to PCR for sexing with an extremely small amount (≤ 5 μl) of PB1 medium.

Primers and PCR amplification: Oligonucleotide PCR primers were synthesized by an automatic synthesizer (Applied Biosystems, model 391). Two pairs of primers for bovine male specific (5'-TGGACATTGCCAC AACCATT-3' and 5'-GCTGAATGCACTGAGAGAGA-3') and bovine gender-neutral (5'-GCCCAAGTTGCTAA GCACTC-3' and 5'-GCAGAACTAGACTTCGGAGC-3') were designed from the sequences of p7-1-a and pMF-3, respectively [14].

Conditions for PCR amplification were similar to those described previously [9, 15]. The PCR mixture contained 79.5 μl of distilled water, 10 μl of 10 × PCR buffer (100 mM Tris-HCl (pH 8.9), 15 mM $MgCl_2$, 800 mM KCl, 1% sodium cholate, 1% Triton X-100), 1 μl of 5 mM dNTPs, 0.5 μl of *Tth* DNA polymerase (4 u/μl, Toyobo) and 1 μl of each primer (20 μM/μl). Samples were amplified using a DNA thermal cycler (Perkin Elmer Cetus) for 50 cycles at the following temperatures: denaturation; 94°C, 1 min, annealing; 60°C, 1 min, extension; 72°C, 1 min. In the first cycle, denaturation was at 94°C for 4 min. Ten μl of PCR products were evaluated by electrophoresis in 3% NuSieve 3:1 agarose gel in a Tris-borate-EDTA buffer and staining in ethidium bromide to determine the size of the amplified fragments. DNA bands were photographed under UV light.

Statistical analysis: The chi-square test was used to assess the significance of differences between 2 discrete set data and comparisons of observed sex ratios with the expected ratio of 1:1. The probability of less than 0.05 was considered significant.

Results

As shown in Table 1, out of 669 presumptive zygotes matured and fertilized *in vitro* in seven replicate trials, a total of 504 (75.3%) 2-cell embryos were selected until 44 hpi. The peak for 2-cell embryos selected was at 26 hpi. Most zygotes underwent the first cleavage between 22 and 30 hpi. The remaining 165 uncleaved oocytes at 44 hpi were not evaluated any further.

When developmental rates to the blastocyst-stage (before hatching) were evaluated at 176 hpi, 57.3, 38.8, 25.0 and 6.2% of embryos cleaved at 22, 26, 30 and 44 hpi were developed to the blastocyst-stage, respectively. Significant correlation was observed between developmental rates and the time of the first cleavage ($P<0.05$).

Table 1 also presents the percentages of 2-cell embryos that developed to the hatched blastocyst-stage during observation period. The overall rate of embryos that developed to the hatched blastocyst-stage was 36.3%. However, hatching rates of embryos that underwent the first cleavage at 22, 26, 30 and 44 hpi were 56.5, 40.1, 21.6 and 4.6%, respectively. Statistical differences in the hatching rate could be shown between each group associated with the first cleavage ($P<0.05$). More than 85% of total hatched blastocysts were derived from the 2-cell embryos selected at as early as 26 hpi. Early cleaving embryos (22 and 26 hpi) were more likely to be faster than late cleaving embryos (30 and 44 hpi) in the first occurrence and peak for embryos hatched from the zona pellucida.

Of 183 hatched blastocysts subjected to PCR sexing, the sex of 171 (93.4%) embryos was successfully determined. Twelve embryos not determined the sex may have been due to a technical error in incorrect temperature set at PCR amplification by thermal cycler. However, there was no preferential loss of one time of selection of 2-cell embryos, nor one time of development to the hatched blastocyst-stage.

The number and distribution of 171 sex-determined embryos are presented in Table 2. The overall sex ratio (the number of male embryos/the number of embryos sexed) was 51.5% (88/171), which did not differ significantly from the expected ratio of 1:1. The sex ratios of early cleaving embryos (22 and 26 hpi) and late cleaving embryos (30 and 44 hpi) selected according to the time of the first cleavage were 49.7% (74/149) and 63.6% (14/22), respectively. These ratios were not significantly different from the expected ratio and did not related to the time of the first cleavage. Alternatively, the sex ratios of embryos divided into three groups according to the timing of hatching, were 60.3% (38/63), 46.8% (22/47) and 45.9% (28/61) at the fast (176–200 hpi), intermediate (212 hpi) and slow (224–248 hpi) groups, respectively. These ratios were also not significantly different from the expected ratio of 1:1.

Discussion

The data presented here confirm and extend the concept that a very early cleavage division of bovine embryos produced by IVM-IVF is crucial for their subsequent developmental potential *in vitro* [1–3]. However,

Table 1. Development to the hatched blastocysts by 2-cell embryos selected at various times after insemination of 669 oocytes matured *in vitro*

Time of selection of 2-cell embryos	No. of 2-cell embryos	No. (%) of hatched blastocysts developed at (hpi)							
		176	188	200	212	224	236	248	Total
22 hpi	124	1 (0.8)	17 (13.7)	19 (15.3)	13 (10.5)	12 (9.7)	5 (4.0)	3 (2.4)	70 (56.5)[a]
26	227	1 (0.4)	8 (3.5)	16 (7.0)	31 (13.7)	17 (7.5)	11 (4.8)	7 (3.1)	91 (40.1)[b]
30	88	0 (0.0)	0 (0.0)	4 (4.5)	5 (5.7)	6 (6.8)	3 (3.4)	1 (1.1)	19 (21.6)[c]
44	65	0 (0.0)	0 (0.0)	1 (1.5)	1 (1.5)	0 (0.0)	1 (1.5)	0 (0.0)	3 (4.6)[d]
Total	504	2 (0.4)	25 (5.0)	40 (7.9)	50 (9.9)	35 (6.9)	20 (4.0)	11 (2.2)	183 (36.3)

Percentage of 2-cell embryos developing to hatched blastocysts.
[a–d]Values with different superscripts are statistically different ($P<0.05$).

Table 2. Distribution of 171 sex-determined hatched blastocysts by 2-cell embryos selected at various times after insemination

Time of selection of 2-cell embryos	Sex	No. of hatched blastocysts determined sex at (hpi) 176	188	200	212	224	236	248	Total
22 hpi	Male	1	9	11	4	5	2	1	33
	Female	0	7	8	8	5	3	2	33
	(%)	(100)	(56.3)	(57.9)	(33.3)	(50.0)	(40.0)	(33.3)	(50.0)
26	Male	1	4	9	13	6	4	4	41
	Female	0	3	5	16	10	5	3	42
	(%)	(100)	(57.1)	(64.3)	(44.8)	(37.5)	(44.4)	(57.1)	(49.4)
30	Male			2	4	4	1	1	12
	Female			2	1	2	2	0	7
	(%)			(50.0)	(80.0)	(66.7)	(33.3)	(100)	(63.2)
44	Male			1	1		0		2
	Female			0	0		1		1
	(%)			(100)	(100)		(0.0)		(66.7)
Total	Male	2	13	23	22	15	7	6	88
	Female	0	10	15	25	17	11	5	83
	(%)	(100)	(56.5)	(60.5)	(46.8)	(46.9)	(38.9)	(54.5)	(51.5)

the time chosen for the selection of 2-cell embryos expected to be developmentally competent, were somewhat different between our observation and others [1, 3]. Although the *in vivo* data on these subjects are limited [16–18], the time required for progression of the first cleavage is between 23 and 31 h after fertilization. As the early portion in fertilization events include sperm capacitation, penetration and decondensation of sperm head, and activation and resumption of miosis of oocytes, Barnes and First [19] point out that the duration of the first cell cycle is 20–24 h, when time of the second polar body extrusion is considered to be the start of a true cell cycle. Therefore, we could presume that the most of early cleaving embryos selected at 22 and 26 hpi in this study have resulted from normal fertilization and cleavage. On the other hand, slow cleaving embryos selected 30 and 44 hpi are partly due to inadequate sperm-oocytes interaction under a suboptimal condition of IVF. A large variation has been seen for sperm capacitation and penetration of oocyte obtained when using different bull sperm [20, 21] and for environmental influences such as change of condition of atmosphere and temperature during embryo manipulation [19, 22].

In respect of the normalcy of 2-cell embryos produced by IVM-IVF, Iwasaki *et al.* [23, 24] reported that chromosomal anomalies were observed in 12.1–21.7% of 2-cell embryos obtained at 24–28 hpi and postulated that these anomalies were caused by abnormal fertilization, especially by polyspermy, and abnormal cleavage. We are inclined to consider that a high incidence of chromosomal abnormalities occurs in slow cleaving embryos compared to fast cleaving embryos, as suggested by Yadav *et al.* [4].

The progressive development of early cleaving embryos is also comparable to that observed *in vivo* [25], as most of development to the hatched blastocyst-stage were observed between 188 and 212 hpi. Therefore, the difference in the development to the hatched blastocyst-stage between early and late cleaving embryos in this study may be related to the quality of the embryo itself.

Taken together, these findings suggest that it is important to obtain homologeneous embryos of high quality in order to get a predictable development. As the differences in early development resulted in variation in later development, heterogeneous quality of embryos produced by IVM-IVF may lead to some erroneous conclusions in the studies on the gene expression of early development. The importance of the uniformity of the developmental competence of bovine embryos produced by IVM-IVF-IVC should be emphasized.

In this study, the sex of hatched blastocysts was determined by using PCR. As this technology allows the sex of nearly all embryos to be determined [15, 26], it would be useful for investigating the sexual differences in the early developmental process [8, 9]. Overall sex ratio of 51.5% was not significant from the expected ratio of 1:1 and consistent with our previous results [9]. Several reports also revealed that overall sex ratio of bovine embryos produced both *in vivo* [27, 28] and *in*

vitro [28, 29] did not significantly differ from the expected ratio.

Our observation indicate that the sex ratio of fast-hatching blastocysts (176–200 hpi) was shifted to male (60.3%), although there was no significantly difference from the expected ratio. These data are in agreement with our previous report [9] in respect of timing of hatching. The sex ratios of both early and late cleaving embryos selected according to the time of the first cleavage were not significantly different from the expected ratio, suggesting no correlation between the sex of embryos and the early cleavage division. Xu *et al.* [7] presented evidence that male embryos develop to more advanced stages than do females during the first 8 days after insemination *in vitro* and suggested that sex-related gene expression affects the development of embryos soon after activation of the embryonic genome. However, Yadav *et al.* [4] reported that embryos in the early cleaving at 24–30 hpi were more likely to be male at 5 days after insemination. Several factors could account for the discrepancies, including the time chosen for the selection of 2-cell embryos, the subsequent culture period, successful rate of sex analysis of embryos and the use of one particular bull. On the basis of these results, future experiments would be necessary to determine the sex of embryos that had reached around the 8–16 cell stage soon after activation of the embryonic genome, since we have no direct evidence of the sex of embryos arrested the development before hatching during culture period.

References

1) Plante, L. and King, W. A. (1992): Effect of time to first cleavage on hatching rate of bovine embryos *in vitro*. Theriogenology, 37, 274 (abstract).
2) Van Soom, A., Van Vlaenderen, I., Mahmoudzadeh, A. R., Deluyker, H. and de Kruif, A. (1992): Compaction rate of *in vitro* fertilized bovine embryos related to the interval from insemination to first cleavage. Theriogenology, 38, 905–919.
3) Grisart, B., Massip, A. and Dessy, F. (1994): Cinematographic analysis of bovine embryos development in serum-free oviduct-conditioned medium. J. Reprod. Fert., 101, 257–264.
4) Yadav, B. R., King, W. A. and Betteridge, K. J. (1993): Relationships between the completion of first cleavage and the chromosomal complement, sex, and developmental rates of bovine embryos generated *in vitro*. Mol. Reprod. Dev., 36, 434–439.
5) Tsunoda, Y., Tokunaga, T. and Sugie, T. (1985): Altered sex ratio of live young after transfer of fast- and slow-developing mouse embryos. Gamete Res., 12, 301–304.
6) Avery, B., Madison, V. and Greve, T. (1991): Sex and development in bovine *in-vitro* fertilized embryos. Theriogenology, 35, 953–963.
7) Xu, K. P., Yadav, B. R., King, W. A. and Betteridge, K. J. (1992): Sex-related differences in developmental rates of bovine embryos produced and cultured *in vitro*. Mol. Reprod. Dev., 31, 249–252.
8) Avery, B., Jorgensen, C. B., Madison, V. and Greve, T. (1992): Morphological development and sex of bovine *in vitro*-fertilized embryos. Mol. Reprod. Dev., 32, 265–270.
9) Itagaki, Y., Kimura, N., Yamanaka, M. and Sutou, S. (1993): The sex ratio of bovine hatched blastocysts produced and cultured *in vitro* as determined by the polymerase chain reaction. J. Mamm. Ova Res., 10, 1–10.
10) Brackett, B. G. and Oliphant, G. (1975): Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod., 12, 260–274.
11) Quinn, P., Barros, C. and Whittingham, D. G. (1982): Preservation of hamster oocytes to assay the fertilizing capacity of human spermatozoa. J. Reprod. Fert., 66, 161–168.
12) Niwa, K. and Ohgoda, O. (1988): Synergistic effect of caffeine and heparin on *in-vitro* fertilization of cattle oocytes matured in culture. Theriogenology, 30, 733–741.
13) Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Critser, E. S., Eyestone, W. H. and First, N. L. (1986): Bovine *in vitro* fertilization with frozen-thawed semen. Theriogenology, 25, 591–600.
14) Kudo, T., Sato, S. and Sutou, S. (1993): Sexing of bovine embryos with male-specific repetitive DNA by polymerase chain reaction: cloning and characterization of bovine male-specific repetitive DNA. J. Reprod. Dev., 39, 55–63.
15) Itagaki, Y., Sato, S., Shitanaka, Y., Kudo, T., Yamaguchi, Y. and Sutou, S. (1993): Sexing of bovine embryos with male-specific repetitive DNA by polymerase chain reaction: sexing of bovine embryos and production of calves with predicted sex. J. Reprod. Dev., 39, 65–72.
16) Hamilton, W. J. and Laing, J. A. (1946): Development of the egg of the cow up to the stage of blastocyst formation. J. Anat., 80, 194–204.
17) Thibault, C. (1966): La culture *in vitro* de l'oeuf de vache. Ann. Biol. Anim. Bioch. Biophys., 6, 159–164.
18) Hyttel, P., Greve, T. and Callesn, H. (1988): Ultrastructure of *in-vivo* fertilization in superovulated cattle. J. Reprod. Fert., 82, 1–13.
19) Barnes, F. L. and First, N. L. (1991): Embryonic transcription in *in vitro* cultured bovine embryos. Mol. Reprod. Dev., 29, 117–123.
20) Hillery, F. L., Parrish, J. J. and First, N. L. (1990): Bull specific effect on fertilization and embryo development *in vitro*. Theriogenology, 33, 249 (abstract).
21) Shi, D. S., Lu, K. H. and Gordon, I. (1990): Effect of

bulls on fertilization of bovine oocytes and their subsequent development *in vitro*. Theriogenology, 33, 324 (abstract).
22) Massip, A., Zwijsen, W. and Mulnard, J. (1983): Cinematographic analysis of the cleavage of the cow egg from 2-cell to 16-cell stage. Arch. Biol. (Bruxelles), 94, 99–106.
23) Iwasaki, S., Shioya, Y., Masuda, H., Hanada, A. and Nakahara, T. (1989): Incidence of chromosomal anomalies in early bovine embryos derived from *in vitro* fertilization. Gamete Res., 22, 83–91.
24) Iwasaki, S., Hamano, S., Kuwayama, M., Yamashita, M., Ushijima, H., Nagaoka, S. and Nakahara, T. (1992): Developmental changes in the incidence of chromosome anomalies of bovine embryos fertilized *in vitro*. J. Exp. Zool., 261, 79–85.
25) Betteridge, K. J. and Flechon, J.-E. (1988): The anatomy and physiology of pre-attachment bovine embryos. Theriogenology, 29, 155–187.
26) Herr, C. M., Holt, N. A., Matthaei, K. I. and Reed, K. C. (1990): Sex of progeny from bovine embryos sexed with a rapid Y-chromosome-detection assay. Theriogenology, 33, 247 (abstract).
27) Bondioli, K. R., Ellis, S. B., Pryor, J. H., Williams, M. W. and Harpold, M. M. (1989): The use of male-specific chromosomal DNA fragments to determine the sex of bovine preimplantation embryos. Theriogenology, 31, 95–104.
28) King, W. A., Yadav, B. R., Xu, K. P., Picard, L., Sirard, M.-A., Supplizi, A. V. and Betteridge, K. J. (1991): The sex ratios of bovine embryos produced in *in vivo* and *in vitro*. Theriogenology, 36, 779–788.
29) Iwasaki, S., Shioya, Y., Masuda, H., Hanada, A. and Nakahara, T. (1988): Sex ratio of early embryos fertilized *in vitro* with spermatozoa separated by Percoll. Theriogenology, 30, 1191–1198.

体外作出牛胚の着床前期中の発生速度の差と性

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体外成熟一体外受精によって作出した牛胚を第1卵割時期に従って分類し，これらの2-細胞期胚のその後の脱出胚盤胞までの発生と性について体外培養とPCRによる性判別によって検討した．体外受精に供した669個の胚のうち，媒精後22，26，30および44時間で選んだ卵割胚はそれぞれ124，227，88および65個であった．これらの胚を卵丘細胞層の拡がった同じ培地に別々に移して10日間共培養した．媒精後22，26，30および44時間で選んだ胚の脱出胚盤胞への発生率はそれぞれ56.5，40.1，21.6および4.6%となり，これらの間には統計学的な有意な差がみられた（$P<0.05$）．さらに，早く卵割した胚（媒精後22および26時間）では遅く卵割した胚（媒精後30および44時間）に較べて脱出胚盤胞の最初の出現あるいはピークが早かった．性判別のためにPCRに供した183個の脱出胚盤胞のうち，171個で性を判定することができ，全体での性比は51.5%（88/171）であった．早く卵割した胚および遅く卵割した胚から発生した脱出胚盤胞の性比はそれぞれ49.7%（74/149）および63.6%（14/22）となり，第1卵割時期に関係していなかった．以上の結果から，媒精から第1卵割の終了までの時間は性差についてではなく，胚のその後の発生に大きく影響することが示唆された．

キーワード：第1卵割，発生，PCRによる性判別，牛胚，体外培養．