Sex Ratio of Different Developmental Stages in Vitro Produced Bovine Embryos

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Abstract The objective of this study was to determine whether the bovine male embryos develop faster in vitro than female embryos. The sex of embryos were determined with PCR(Polymerase chain reaction) technique. On day 5(in vitro insemination= day 0), the embryos were divided by developmental stages into 3 groups: 16 cells, more than 16 cells and morula. In 3 groups, the percentages of males were respectively 32%, 53% and 63%. On day 7(in vitro insemination= day 0), the embryos were classified by developmental stages into 4 groups: morulae, early blastocysts, blastocysts and full-expanding blastocysts. After sexing, the percentages of males were 28%, 38%, 60% and 70%, respectively. The study demonstrated that IVF bovine male embryos develop faster in vitro than female embryos.

Key words bovine, embryo, in vitro development, male, female

A general review of the methods in sexual control of animals, currently the separation of X- or Y-chromosome-bearing spermatozoa[1] and sex determination of preimplantion embryos[2] are being explored two ways. Howerer, more recently, some data showed that male embryos develop at a faster rate in vivo and in vitro than female embryos. Avery *et al*[3] found that the percentages of males in bovine from the 3 developmental groups were 78%, 27% and 11% respectively. Also similar studies on mouse[2,4] and bovine[5] embryos have been reported. However, Berg *et al*[6] indicated that no differences in the sex ratio between fast- and slow-developing embryos could be found by transferred to recipients. For exploring a simple, noninvasive method to embryo sexing, the objective in this study is to reveal the effect of sex on bovine embryonic growth rate in vitro.

1 Materials and Methods

1.1 In vitro embryo production

1.1.1 Media preparation Oocytes were aspirated and washed using TCM-Hepes buffer supplemented with 2% heat-treated cow serum (c.s) and 250 IU/mL heparin

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(aspirated medium). Oocytes were cultured in a TCM-Bicarbonate medium supplemented with 16 IU/mL Suigonan-opl (hCG) and 15% c. s (IVM medium). Spermatozoa were capacitated in CR1-Bicarbonate medium supplemented 30 mg·L^{-1} heparin (IVF medium). Embryos were cultured in CR1-Bicarbonate medium supplemented with 2% c. s (IVC medium).

1.1.2 Culture of oocytes Bovine ovaries were obtained at a local slaughterhouse. Oocyte were aspirated and washed twice in aspirated medium and once in IVM medium, then oocytes were cultured in IVM medium (30~40 oocytes in 500μL drops) under sterile paraffin oil at 39℃ in 5% CO_2 and 95% N_2 for 22~24 h.

1.1.3 In vitro fertilization Frozen semem which divided from a single bull was thawed in water at 35~37℃ for 1 min, and then washed twice by centrifugation at 700 g for 10 min. After maturation culture, matured oocytes moved into IVF medium with treated spermatozoa and cultured under sterile paraffin oil at 39℃ in 5% CO_2, 95% N_2 and 100% humidity for 22~24 h.

1.1.4 Embryo culture After in vitro fertilization, presumptive zygotes were denuded from cumulus cells by vortex mixing. Naked zygotes were cultured with IVC medium under paraffin oil at 39℃ in 5% CO_2 and 95% N_2 for 4 and 6 d.

1.2 Embryo classification

On day 5 (in vitro insemination= day 0), the embryos were morphologically evaluated and divided by developmental stages into 3 groups: 16 cells, more than 16 cells and morula. On day 7 (in vitro insemination = day 0), the embryos were divided into 4 groups: morula, early bastocysts, blastocysts and full-expanding blastocysts.

1.3 Preparation of embryos for PCR-amplification

Embryos were washed twice in pure PBS and once in 1× PCR buffer. Embryos were spliced into 2 parts (A and B samples) under an inverted Olympus microscope, individually, into separate vials containing 10μL of 1× PCR buffer. At that time, 1μL proteinase K was added(10 g·L^{-1}). After centrifuging briefly, each vial was covered with 30μL of light paraffin oil on the reaction mixtures to prevent evaparation. The embryos were kept at 56℃ for 1 hour for digestion. Prior to PCR amplification, each vial was incubated at a temperature of 98℃ for 15 min to denature the proteinase K.

1.4 Polymerase chain reaction

The PCR reaction were carried out in a total 25μL reaction buffer. Amplification were performed in 10 mmol·L^{-1} Tris-HCl(pH8.4), 50 mmol·L^{-1} KCl, 1.5 mmol·L^{-1} $MgCl_2$, 0.01% gelatine, 5 mmol·L^{-1} of each dNTP(dATP, dCTP, dGTP and dTTP) and 0.25 IU of Tag DNA polymerase. BRY.4a-specific primes were added in a volume of 10 pmol to A samples, and 10 pmol of BRY.4a-specific and 2.5 pmol of bovine satellite 1.709 specific primers were added to B samples. A negative control, male and female coutrols (male and female genomic DNA), were included to detect any

contaminations and to check the validity of the reagents. Tab. 1 shows the details concerning sequences and products of both the Y-specific and bovine satellite primers used in the PCR sexing assay.

Tab. 1　Details concerning the primers used in the PCR sexing assay

Template-sequence	Nucleotide position of the primers	Primer sequences	PCR product length (basepairs)
BRY. 4a (Redd et al, 1989)	1844-1872 2284-2312	5′-CAAGACCATACA-TATGTCATTATAGACAG-3 3′-CACAAAAAA-CAAAATTTATGTACTTCATGT-5′	469
satellite 1. 709 (Skowronski et al, 1984)	2749-2770 2974-2994	5′-GCAGTGCATAAATATCAAAAGG-3′3′-CAAGGGATGTTGGAGGACTAG-5′	246

Amplifications were carried out in a Perkin-Elmer Thermal Cycler. All samples were denatured at 94℃ for 2. 3 min, followed by 50 cycles consisting of denaturation at 94℃ for 30 s, annealing at 61℃ for 1 min, and extension at 72℃ for 1 min. After the last cycle, the samples were incubated for further 3 min at 72℃ to assure complete extension and then cooled to 35℃ for 10 s. The PCR products of all samples were then either analyzed immediately by agarose gel electrophoresis or stored in refrigerator until analyzed.

1. 5　Analysis of PCR products

Seven microliters of each sample were added to 1μL of loading buffer containing glycerol, bromplenol blue, xylene cyanol and EDTA before loading on the gel. Electrophoresis was carried out in 2% agarose gel with 1× TBE buffer at 100 volts for 30 min, stained with ethidium bromide and visualized under ultraviolet light. The embryo was judged to be female if only a 246 basepair product from bovine satellite 1. 709 was visible in the sample. If a 469 basepair from BRY. 4a or a 246 and a 469 basepair were visible in the sample, the embryo was judged as a male(Fig.).

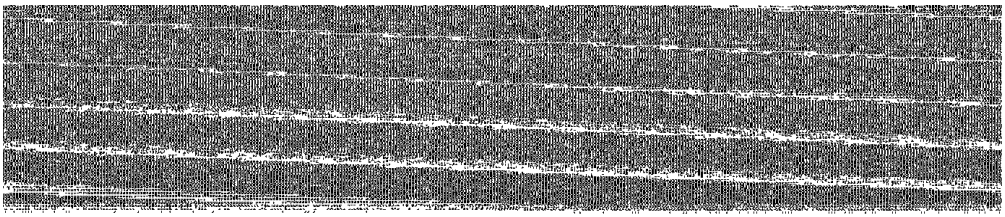

Fig.　Agarose gel electrophoresis after PCR.

(M) PstI digestedλ. 1-7 are embryo samples using the BRY. 4a primers. B, ♀ and ♂ are samples with respectively no, female and male bovine genomic DNA. The samples resolved on the agarose gel below are aliquots of the same samples as above amplified with an addititon of the satellite 1. 709 primerpair. Sample 1, 3 and 5 were assigned males, 2, 6 and 7 were assigned females, while sample 4 was not assigned.

2 Statistical Analysis

To determine if there are differences in the developmental rate between male and female embryos, Chi-square analysis was used.

3 Results

On day 5 after insemination, a total of 110 embryos was submitted for sexing and 105 embryos had their sex determined (Tab. 2). Five embryos (1 16 cells, 2 more than 16 cells, 2 morula) either did not show amplification or were lost during the procedure. When embryos were collected from morulae developmental stage, approximately 63% of fast-developing embryos were males($P < 0.05$). When embryos were collected from 16 cell developmental stage, approximately 68% of slow-developing embryos were females ($P < 0.05$). The overall male percentage was 50% and it was no different from the expected 1∶1 sex ratio($P > 0.05$).

Data in Tab. 3 indicated that male embryos developed at a significantly faster rate than female embryos on day 7($P < 0.05$). When embryos were collected from fast-developing stage, approximately 70% of embryos were males ($P < 0.05$). When embryos were collected from slow-developing stage, approximately 72% of embryos were females ($P < 0.05$). A total of 91 embryos was submitted for sexing and 87 embryos had their sex determined. Four embryos (3 early blastocysts, 1 blastocyst) either did not show amplification or were lost during the procedure. A total of 87 embryos was analyzed and displayed 1∶1 sex ratio($P > 0.05$).

Tab. 2 Sex ratio of bovine IVF embryos by stages of development on day 5

Stages of development	No. of embryos	Males (n)	Females (n)	Sex ratio	Males (%)
16 cells	31	10	21	1∶2.1	32
More than 16 cells	36	19	17	1.1∶1	53
Morula	38	24	14	1.7∶1	63
Total	105	53	52	1∶1	50

Tab. 3 Sex ratio of IVF bovine embryos by stages of development on day 7

Stages of development	No. of embryos	Males (n)	Females (n)	Sex ratio	Males (%)
Morula	18	5	13	1∶2.6	28
Early blastocysts	21	8	13	1∶1.6	38
Blastocysts	25	15	10	1.5∶1	60
Full-expanding blastocysts	23	16	7	2.3∶1	70
Total	87	44	43	1∶1	51

4 Discussion

Earlier findings have demonstrated that the rate of embryonic development and sex ratio of embryos have a clear relationship[2,3,4], and IVF bovine embryos have a higher oocurrence of male among more advanced embryo stages and a higher oocurrence of female among less advanced embryo stages[7]. Itoh and Goto[5] reported that the percentages of males in bovine from the 3 developmental groups (fast development, intermediate development and slow development) were 77%, 40% and 24%, respectively. Seller and Perkins-Cole[4] found that the percentages of males in mouse in the 3 groups (fast development, intermediate development and slow development) were 94%, 45% and 25%, respectively. In this study, 105 and 87 embryos fertilized from a bull sperm could be classified into different developmental stages on day 5 and day 7, respectively, and the results showed that male embryos developed more rapidly than female embryos did. On day 5, the percentages of males from the 3 developmental groups were 63%, 53% and 32%, respectively. And from the 4 developmental groups on day 7, the percentages of males were 70%, 60%, 38% and 28%, respectively. The present results were consistent with the previous findings[3,4,5].

Although reports in several mammals including mouse[2,4] and bovine[3,5] have indicated that the development of male embryos occurs more rapidly than female embryos. Contrary to the results, no differences in embryo development between male and female embryos would be detected. Berg et al[6] found that IVF embryos transferred to recipients (on day 7 after insemination) and taken to term did not show differences in the sex ratios between fast and slow developing embryos. For this reason, it is likely that embryos may have already been degenerate at the time of transfer (slow development), before embryos were classified into different development stages. In addition, the sex of fetus aborted during pregnancy may be another reason.

The faster development of male embryos is not fully understood. It could be argued that male embryos develop faster than female embryos because the redused DNA content would allow faster completion of DNA replication. However, the findings of Burgoyne[8] did not support this. A genetic influence by the Y chromosome on in vitro metabolism may be important. The increased growth in male embryos may be a consequence of differential gene expression caused by the Y-chromosome. In addition, early embryonic development is a very complex process, in vitro-produced embryos can be affected by a range of different factors such as inorganic ions, buffers, gas composition, amino acids, growth factors, vitamins and macromolecules[9].

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体外生产的牛胚胎在不同发育期的性比率

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摘 要 本研究旨在证实牛体外培养的雄性胚胎生长发育是否快于雌性胚胎。胚胎的性别鉴定采用多聚酶链式反应 (PCR)技术。在体外受精的第 5天,胚胎根据发育期划分为三个组: 16细胞期,多于 16细胞期和桑椹期。在 3个组中,雄性胚胎所占百分率分别是 32%, 53%和 63%。在体外受精的第 7天,胚胎根据发育期划分为 4个组:桑椹期、早期囊胚期、囊胚期和扩张囊胚期。在性别鉴定后, 4个组中雄性胚胎所占百分率分别是 28%, 38%, 60%和 70%。结果表明:牛早期胚胎在体外培养条件下,雄性发育快于雌性。

关键词 牛,胚胎,体外发育,雄性,雌性

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