Embryo Gender Ratio and Developmental Potential after Biopsy of *In Vivo* and *In Vitro* Produced Hanwoo Embryos

Sang-Rae Cho[1,†], Chang-Young Choe[2], Jun-Kyu Son[2], In-Cheol Cho[1], Jae-Gyu Yoo[3], Hyung-Jong Kim[4], Yeong-Gyu Ko[5], Nam-Young Kim[1], Sang-Hyun Han[1], Yong-Sang Park[1] and Moon-Suck Ko[1]

[1]*Subtropical Animal Experimental Station, National Livestock Institute, Rural Development Administration, Jeju, 690-150, Korea*
[2]*Dairy Science Division, National Livestock Institute, Rural Development Administration, Cheonan, 331-801, Korea*
[3]*Animal Biotechnology Division, National Livestock Institute, Rural Development Administration, Suwon 441-706, Korea*
[4]*Planning & Coordination, National Livestock Institute, Rural Development Administration, Suwon 441-706, Korea*
[5]*Division, Animal Genetic Resources Station, National Livestock Institute, Rural Development Administration, Namwon, 590-830, Korea*

ABSTRACT

The present study was to assess the *in vitro* viability and sexing rate of bovine embryos. Blastocysts were harvested on day 7～9 day after insemination(*in vitro* and *in vivo*), and the sex of the embryos was examined using the LAMP method. Embryo cell biopsy was carried out in a 80 μl drop Ca^{2+}, Mg^{2+} free D-PBS and, biopsied embryos viability were evaluated after more 12 h culture in IVMD culture medium. The formation of recovered embryo to expanded and hatching stages had ensued in higher of sexed embryo *in vivo* than *in vitro* (100% vs. 89%, $p<0.05$), and *in vitro*, the rates of degeneration after sexing were significantly ($p<0.05$) higher *in vitro* than *in vivo*(11% vs. 0.0%). The rates of the predicted sex were female 61% vs. 56%, and male 39% vs. 44% *in vivo* and *in vitro*, respectively. The rates of survival following different biopsy methods were seen between punching and bisection method *in vivo* and *in vitro* (100% vs. 89% and 100% vs, 78% respectively). Biopsy method by punching was significantly ($p<0.05$) higher than bisection between produced embryos *in vivo* and *in vitro*. The present data indicate that with microblade after punching for embryo sexing results in high incidence of survivability on development after embryo biopsy. It is also suggested that LAMP-based embryo sexing suitable for field applications.

(Key words : bovine embryos, sexing, biopsied embryos, superovulated)

INTRODUCTION

Gender pre-selection is important to livestock produces because inseminations can be planned to produce a specific gender. In addition to faster genetic progress there are other advantages for the management and efficiency of livestock production.

In the bovine embryo transfer (ET) industry, sexing of preimplantation embryos is an important management tool. It is highly desirable since it provides a means of propagating a desired gender. Various methods have been used to determin the gender of mammalian embryos, including karyotyping at early stages of development(Wintenberger-Torres and Popecscu., 1980), specific antigens detection (Sohal and Allen.,1990), detecting metabolic differences between male and female embryos(White *et al*.,1982) and, DNA analysis methods (Notomi *et al*, 2000). DNA technology in embryo sexing has paved the way the transfer of molecular biology to the field. Polymerase chain reaction (PCR) including nested and primer-extension preamplification-PCR is routinely used in the field of sexing. However, these methods require technical skill and are time consuming. Furthermore, PCR has the risk of false positive because of DNA contamination during the handling of PCR products in duplicate PCR procedures and/or electrophoresis.

Newly introduced PCR techniques(Chen *et al*.,1999), such as nested or primer extension preamplification PCR, are efficient ways to amplify the low DNA template, but non specific amplification of contamination caused by handling two tubes gives rise to PCR failure and relatively long reaction time(Appa and Totey, 1999; Kobayashi *et al*., 1998; Zhang *et al*., 1992).

From the ET industry point of view, embryo sexing needs training and monitoring, whereas from livestock industry point of view, it helps to obtain offspring of the desired gender with savings in the cost and management of recipients. Therefore, a simple, rapid, and precise sexing method needs to be developed bovine ET industry.

† Corresponding author : Phone: +82-64-754-5711, E-mail: E-mail: chosr@korea.kr

Loop-mediated isothermal amplification (LAMP) is novel DNA amplification method that has the ability to synthesize extremely large amount of DNA (Mori *et al*., 2001) and amplifies a target DNA with high specificity, efficiency and rapidity (Notomi *et al*.,2000). The LAMP reaction is carried out under isothermal condition (range, 60~65℃) using DNA polymerase with strand displacement activity and does not need special reagents or electrophoresis to detect the amplified DNA (Mori *et al*., 2001). The aim of this study was to investigate the sex ratio and survival rates of bovine embryos after cell sampling with microblade attached micromanipulator and, to apply an efficient procedure for Hanwoo bovine preimplantation embryo biopsy using LAMP.

MATERIALS AND METHODS

All chemicals and media used in embryo production were purchased from Sigma Chemical Company (St. Louis. MO, USA).

In Vitro Embryo Production

The medium used for oocytes maturation was M199 containing 10% (v/v) FBS (Gibco), 2.5 mM Na pyruvate, 1mM L-glutamine, 25 μg/ml Gentamycin. The medium used for *in vitro* culture (IVC) of embryos was in IVMD medium. For *in vitro* fertilization (IVF), matured oocytes were co-incubated with frozen-thawed semen in modified Brackett and Oliphant medium containing 3 mg/ml fatty acid-free BSA, for 6 h at 39℃ in humidified atmosphere of 5% CO_2 , 5% O_2 in air. After co-incubation, cumulus cells were removed from oocytes, and embryos were cultured for 7 days in IVMD medium(IFP, Japan)

In Vivo Embryo Production

Donor cows were superovulated with follicle stimulating hormone FSH (Folltropin-V, Vetrepham, Canada) twice daily in decreasing dose for 3 days followed by an injection of $PGF_{2\alpha}$ (Pharmacia, Belgium) on the third day. Cow was inseminated 24h after the onset of estrus and embryos were recovered at 7 days after AI. Recovered embryos were cultured in IVMD medium under humidified atmosphere of 5% CO_2, 5% O_2 in air at 39℃.

Embryo Biopsy

For isolation of blastomere, as a simple splitting and biopsy procedure were used in the experiments (Lewis *et al*., 1994). For embryo punching, two micropipettes were prepared from capillaries tube (G-1, NARISHIGE, Japan). One is for the punching with 10 μm inner diameter and the other is for the holding embryos. Briefly, each embryo selected to biopsy was placed in a disposable, Petri dish (100 mm × 20 mm, Corning) with calcium and magnesium free Dulbeco's PBS (Gibco). However, embryos punching were performed in Ham' F-10 medium. The biopsy was carried out using a x 40× inverted microscope and micromanipulator with Bio-cut microblade (FEATHER, Japan) adapted as a cut instrument. Punched morulae embryos were cultured into 50 μl drops of IVMD medium more than 12 h in atmosphere of 5% CO_2, 5% O_2 in air at 39℃, and the development into the hatching blastocysts until embryo sexing, and then the embryos were biopsied with a single vertical movement of microblade. The sampled cells are rinsed three times consecutively in D-PBS containing 0.1% PVA medium, and then deposited in microtube for sex determination. Sexing was performed immediately after biopsy.

LAMP Analysis

The LAMP reaction was performed as previously described(Hirayama *et al*.,2004). The DNA fragments synthesized by the LAMP reaction were detected based on the production of with (+) or without (−) white precipitate of magnesium pyrophosphate, respectively.

The temperature control for the LAMP reaction and the turbidity measurement were performed using a Turbidimeter (Teramecs Co., LTD., Kyoto, Japan) developed for DNA analysis by LAMP(Hirayama *et al*, 2004; Nagamine *et al*., 2002).

Sexing was performed with male-specific and male-female common LAMP reaction. In all experiments, DNA sample extracted from 6 μl biopsied blastomeres with 6 μl extraction solution was divided equally into two DNA tubes; one for male-specific reaction and the other for male-female common reaction. A male specific sequence that was a tandem repeat sequence on the Y chromosome was used for detection of male embryos (Hasler *et al*., 2002). The male-female common reaction was conducted using the 1.715 satellite DNA sequence. The loop primers, which accelerate DNA amplification by LAMP, were also used in each reaction (Nagamine *et al*., 2002). When both of the reactions were positive, the sex of the embryos was judged as male. On the other hand, when only the common reaction was positive, the sex of the embryos was judged as female. No interpretation could be made in the absence of the positive common reaction.

Experimental Design

This study is comprised of three experiments. In Experiment 1, potential of *in vitro* and *in vivo* derived embryo development to hatching blastocysts was investigated after blastomere isolation. In Experiment 2, we investigated the gender ratio after blastomeres isolation. In Experiment 3, the effect of two different blastomere

isolation methods on the survivability of embryos derived from *in vivo* and *in vitro* fertilization/culture condition.

Statistical Analysis

Differences in the developmental rates with respect to the biopsied and control (without biopsy) groups were compared using chi-square test. The statistical significance level was tested at $p<0.05$.

RESULTS

Developmental potential of *in vivo* and *in vitro* produced embryos after blasotmer isolation. The results of Experiment 1 are summarized in Table 1, which shows that the rate of recovered embryos to expanded and hatching blastocyst between *in vivo* and *in vitro* produced embryos. In Table 1, most blastomere isolated embryos developed into expanded and hatching stage in both *in vivo* and *in vitro* produced embryos (100% vs. 88.8%, $p<0.05$). *In vitro* produced embryos were significantly ($p<0.05$) more degenerated than *in vivo* produced embryos (11.2% vs. 0.0%).

Gender ratio after blasotmere isolation with *in vivo* and *in vitro* produced Hanwoo embryos.

The results of Experiment 2 are shown in Table 2. A total of 151 *in vivo* and *in vitro*-derived embryos were subjected to the LAMP-based sexing, derived embryos were subjected to LAMP-based sexing, 21(38.9%) vs. 31(43.7%) and 33(61.1%) vs, 40(56.3%) of them were judged as male and females in Hanwoo embryo respectively. No difference in sexing rate was seen *in vivo* and *in vitro* embryo.

The viability of embryos between different blastomere isolation methods with *in vivo* and *in vitro* produced Hanwoo embryos.

The results of Experiment 3 are shown in Table 3. A total of 138 *in vivo* and *in vitro* derived embryos were subject to blastomere. By using *in vitro* produced embryos, punching methods with had significantly ($p<0.05$) higher survivability rate than bisection method (100% vs. 78.4). However, survivability of *in vivo* produced embryos were not affected by two blastomere isolation methods.

Table 1. Developmental potential of *in vivo* and *in vitro* produced embryos after blasotmere isolation

Embryo source	Embryos used	No. (%) of embryos develop to	
		exBL/hBL*	Degeneration
In vivo	41	41(100.0)[a]	0(0.0)[a]
In vitro	80	71(88.8)[a]	9(11.2)[b]

* exBL: expanded blastocyst, hBL: haching blastocyst.
[ab] Percentage with different superscripts within columns indicate significant difference($p<0.05$).
Replicates 6.

Table 2. Sexing rates according to *in vivo* and *in vitro* embryo production in Hanwoo

Embryo source	Embryos used	No. (%) of sexing of biopsed embryo	
		Female	Male
In vivo	54	33(61.1)[a]	21(38.9)[a]
In vitro	71	40(56.3)[a]	31(43.7)[a]

[ab] Percentage with different superscripts within columns indicate significant difference ($p<0.05$).
Replicates 7.

DISCUSSION

This study presents a simplified and an efficient protocol for bovine embryo sexing method based on LAMP. A gene amplification is performed with the genomic DNA obtained from *in vivo* and *in vitro* produced bovine embryos using male specific sequence and the male-female common sequence as primers. The embryos sex is judged simply and rapidly. The amplification is detected with white turbidity, which is de-

Table 3. The survivability of embryos between different biopsy methods for bovine embryo sexing

Embryo source	Methods	No. of embryos used	No. (%) of embryos survived	No. (%) of embryos degenerated
In vivo	Punching	42	42(100.0)[a]	0(0.0)[a]
	Bisection	18	16(88.9)[a]	2(11.1)[a]
In vitro	Punching	41	41(100.0)[a]	0(0.0)[a]
	Bisection	37	29(78.4)[b]	8(21.6)[b]

Punching : cutting after punching at morula stage, Bisection : splitting at expanded blastocyst stage.
Replicates 7.

rived from magnesium pyrophosphate, by product of the amplification reaction.

The isolation of blastomeres from bovine embryos is essential for embryo sexing by DNA amplification methods. Our LAMP-based embryo sexing analysis was using over 5 blastomeres. Hirayama *et al*(2004) reported that the gender of embryos was determined correctly using 3 to 5 cell samples.

In experimental 1, the results showed that using the biopsy technique for biopsy of produced bovine blastocysts, the efficiency of survivability was similar, and it did not alter developmental potential *in vitro* embryos. as, after biopsy, 100% and 88.8% of the biopsied embryo reached the re-expansion or hatched blastocyst stage between *in vivo* and *in vitro* embryos. However, degeneration rate after biopsy was shown only *in vitro* produced embryos. In these results, Hasler *et al*(2002) reported that a biopsy is harmful to the embryo viability, and reduces the pregnancy rate after embryo transfer, and *in vitro* embryo also exhibited poor survival in IVC following biopsy. These results suggested that the grading and developmental stage of embryos to be biopsied was a critical factor. However, the most likely primary cause for the improvement is that less damage was being done to embryos during the biopsy procedure.

The sex ratio was observed in experiment 2 (Table 2). The assay yielded a definitive result, 51% of embryos had female. These results similar that reported by Shea(2004). When the sex was assigned by LAMP was the error rate was 14%. This cause may be species specific between in donor cows used for bovine embryo biopsy, and the biggest cause of error probably related to contamination of sample (Herr and Reed., 1991). Because PCR is very sensitive, contamination of a biopsy by just a few cells can produce erroneous results. Other factors were related to the visual element of the test. While in most instances there was sufficient product to yield highly visible bands following electrophoresis, on occasion the bands were weak and a Y-sequence was not identified. Once a fetus was identified as male after being sexed as a female embryo, reviewing the gel result often revealed the presence of very weak male band. This problem was minimized by establishing rigid guidelines before assigning sex to an embryo (Shea., 1999). Sex ratio was observed no difference between *in vivo* and *in vitro* embryo in Table 2. In general, the male to female sex ratio in cattle embryos approaches 1:1, with 2~3% more male than females (Jafar and Flint, 1996). Several authors report that IVP bovine embryos deviate from 1.3:1 to 1.9:1 from the normal 1:1 ratio on day 7 (Carvalho *et al*., 1996), However, in two other studies, the sex ration of bovine IVP embryos was approximately 1:1 (Grisart *et al*., 1995), and Bredbacka and Brebacka (1996) and Yadav *et al* (1993) reported that, the rates of male defined embryo were significantly (p<0.05) higher than female defined embryos. These authors also suggested that oxidative stress causes male embryos to develop faster than female embryos as a result of oxidation-induced gene expression. Therefore, LAMP may be a reliable method for embryo sexing (Sohal and Allen, 1990). The rates of female sexing in Holstein IVF embryos, were significantly (p<0.05) lower than male (34.6%, and 65.4%, respectively), and the rates of male defined embryo were significantly (p<0.05) higher than female defined embryo(data is not shown).

In experiment 3 results showed that Table 3, to improvements on embryo micromanipulation techniques led to of embryo bisection technology in commercial embryo transfer programs, and made possible the direct genetic analysis of preimplantation bovine embryos by biopsy (Lopes *et al*., 2001). The relative frequency with which each biopsy procedure was applied depended on a combination of factors and the blade biopsy was the simplest to perform for embryo sexing. However, using the biopsy technique for sexing of *in vivo* and *in vitro* produced bovine embryos, the efficiency was similar. But these methods difference in sex diagnosis affecting the accuracy may be due to the difference of cell number between the biopsy sample and embryo, expressing insufficient or absence of embryonic DNA in the sample. Therefore, the sex of embryo was determined correctly using 5- to 7-cell sample. Hirayama *et al* (2004) reported that high sexing accuracy (75 ~94%) was also obtained with 1- and 2- cell samples many investigators have reported sexing efficiency of >90% and accuracy of about 90% in few or single blastomeres using the PCR technique (Chrenek *et al*., 2001). Therefore, the LAMP-based sexing methods in this study may be a reliable method to use punching method for embryos sexing. In conclusion, this study demonstrates that embryo sexing can be carried out using LAMP, a simple, rapid and effective method in Hanwoo preimplantation embryos, and the Lamp-based sexing method in Hanwoo embryo needs further research on various sex determination factors.

REFERENCES

1. Appa Rao KBC, Totey SM (1999): Cloning and sequencing of buffalo male-specific repetitive DNA: Sexing of *in vitro* developed buffalo embryos using multiplex and nested polymerase chain reaction. Theriogenology 51:785-797.
2. Bredbacka K, Brebacka P (1996): Glucose controls sex-related growth rate differences of bovine embryos produced *in vitro*. J Reprod Fert 106:169-172.
3. Carvalho RV, Del Campo MR, Palasz AT, Plante Y, Mapletoft RJ (1996): Survival rates and sex ratio of

bovine IVF embryos frozen at different developmental stages on day 7. Theriogenology 45:489-498.
4. Chen CM, Hu CL, Wang CH, Hung CM, Wu HK and Choon KB (1999): Gender determination in single bovine blastomere by polymerase chain reaction amplification of sex-specific polymorphic fragments in the amelogenin gene. Mol Reprod Dev 54:209-214.
5. Chrenek P, Boulanger L, Heyman Y, Uhrin P, Laurincik J, Bulla J, Renard JP (2001): Sexing and multiple genotype analysis from a single cell of bovine embryo. Theriogenology 55:1071-1081.
6. Grisart B, Massip A, Collette L, Dessy F (1995): The sex ratio of bovine embryo produced *in vitro* in serum free oviduct cell-conditioned medium is not altered. Theriogenology 43:1097-1106.
7. Hasler JF, Cardey E, Stokes JE, Bredbacka P (2002): Nonelectrophoretic PCR-sexing of bovine embryos in a commercial environment. Theriogenology 58:1457-1469.
8. Herr CM, Reed KC (1991): Micromanipulation of bovine embryos for sex determination. Theriogenology 35:45-54.
9. Hirayama H, Kagiyama S, Moriyasu S, Sawai K, Onoe S, Takahashi Y, Katagiri S, Toen K, Watanabe K, Notomi T, Yamashina H, Matsuzaki S, Minamihashi A (2004). Rapid sexing of bovine preimplantation embryos using loop-mediated isothermal amplification. Theriogenology 62:887-896.
10. Hirayama H, Kagiyama S, Moriyasu S, Hirano T, Sugimoto Y, Kobayashi N, Inaba M. Sawai K, Onoe S, Minamihashi A (2004): Genetic diagnosis of claudin-16 deficiency and sex determination in bovine preimplantation embryos. J Reprod Dev 50(6):613-618.
11. Jafar SI, Flint APF (1996): Sex selection in mammals: A review. Theriogenology 46:191-200.
12. Kobayashi J, Sekimoto A, Uchida H, Wada T, Sasaki K, Sasada H, Umezu M, Sato E (1998): Rapid detection of male specific DNA sequence in bovine embryos using fluorescence *in situ* hybridization. Mol Reprod Dev. 51:390-394.
13. Lopes RF, Forell F, Oliveria AT, Rodrigues JL (2001): Splitting and biopsy for bovine embryo sexing under field condition. Theriogenology 56:1383-1392.
14. Mori Y, Nagamine K, Tomita N, Notomi T (2001): Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun 289:150-4.
15. Nagamine K, Hase T, Notomi T (2002): Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes 16:223-229.
16. Notomi Y, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N (2000): Loop-mediated isothermal amplification of DNA. Nucleic Acids Res 28:63.
17. Shea BF (1999): Determining the sex of bovine embryos using polymerase chain reaction results: a six-year retrospective study. Theriogenology. 43:71-80.
18. Sohal RS, Allen RG (1990): Oxidative stress as causal factors in differentiation and aging: a unifying hypothesis. Exp Gerontology 25:499-522.
19. White KL, Linder GM, Aderson GB, BonDurant RH (1982): Survival after transfer of sexed ouse embryos exposed to H-Y antisera. Theriogenology 18:655-662.
20. Wintenberger-Torres S, Popecscu C (1980): Transfer of cow blastocysts after sexing. Theriogenology 14: 309-318.
21. Yadav BR, King WA, Betteridge KJ (1993): Relationship between the completion of the first cleavage and chromosomal complement, sex and developmental rates of bovine embryos generated *in vitro*. Mol Reprod Dev 36:434-439.
22. Zhang L, Cui X, Schmitt K, Hubert R, Navid W, Arnheim N (1992): Whole genome amplification from a single cell: Implication for genetic analysis. Proc Natl Acad Sci USA 89:5847-5851.

(Received: 7 December 2012/ Accepted: 18 December 2012)