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Fertility of sperm from a tetraparental Chimeric bull

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Abstract

The purpose of the present study was to examine the ability of a tetraparental Chimera in producing IVF embryos. Cumulus oocytes complexes (COCs) were matured in vitro for 22 h. Frozen-thawed sperm of a Chimera (CH), as well as Japanese Black (JB), Limousin (L), Japanese Brown (JBr), Holstein (H) bulls were used for IVF.

The chromosome preparations were made from peripheral lymphocytes. Based on chromosome analysis the Chimera had apparently normal chromosomes (29 acrocentric pairs, one large sub metacentric X chromosome and one small sub metacentric Y chromosome).

The proportion of acrosome reacted spermatozoa after 1 h of incubation was higher ($P < 0.01$) with the Chimera (CH) than with the Holstein and in Japanese Brown bulls, but did not differ from Japanese Black and Limousin bull sperm (79.0%, 71.2%, 72.5%, 57.8% and 57.0% for CH, JB, L, JBr and H sperm, respectively).

Fertilization rates observed after 5 h of sperm-oocyte incubation with Chimera (O-CH) sperm were higher ($P < 0.05$) than with Japanese Brown (O-JBr) and ($P < 0.01$) than with Holstein (O-H) sperm, but did not differ from Japanese Black (O-JB) and Limousin (O-L) sperm (36/44, 81.8%; 28/35, 80.0%; 25/36, 69.4%; 19/43, 44.2% and 6/33, 18.2% for O-CH, O-JB, O-L, O-JBr and O-H, respectively).

The cleavage rates of IVM oocytes inseminated with Chimera sperm were also higher ($P < 0.001$) than in Holstein, ($P < 0.01$) Japanese Brown and ($P < 0.05$) Limousin, but did not differ from Japanese Black sperm (181/239, 75.7%; 123/171, 71.9%; 108/186, 58.1%; 80/196, 40.8% and 30/186, 16.1% for O-CH, O-JB, O-L, O-JBr and O-H, respectively). The blastocyst rates of IVM oocytes inseminated with sperm were higher ($P < 0.05$) than in Limousin, Japanese Brown and Holstein, but did not differ from Japanese Black (69/181, 38.1%; 48/123, 39.0%; 27/108, 25.0%; 7/30, 23.3% and 16/80, 17.8% for O-CH, O-JB, O-L, O.-JBr and O-H, respectively).

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These findings suggested the sperm from this tetraparental Chimeric bull were able to be used for producing bovine IVF embryos.

Keywords: Bovine; Chimera; Acrosome reaction; Fertility; Cleavage; Blastocyst

1. Introduction

Chimeras have become important in embryological and developmental biological studies (McLaren, 1976). They are produced in mice by aggregating the embryos without zona pellucida (Mintz, 1971). In cattle, Chimeras are produced by aggregating bisected embryos within the zona pellucida (Brem et al., 1984), and by micro-injection of the inner cell mass (ICM) into the blastocyst (Picard et al., 1990; Summers et al., 1983). Tetraparental chimeric cattle were successfully produced by aggregating bovine IVF embryos of F_1 (Holstein × Japanese Black) and F_1 (Japanese Brown × Limousin) and culturing, in vitro without the zona pellucida (Boediono et al., 1993).

Sperm from individual bulls and rams differ in their ability to fertilize matured oocytes in vitro and support embryo development to the preimplantation stage (Iritani et al., 1986; Fukui et al., 1988; Leibfried-Rutledge et al., 1987). Reproduction of interspecific chimeric mice between *M. caroli* and *M. musculus* have been reported (Rossant and Frels, 1980) and between sheep and goat (MacLaren et al., 1993). However, the ability of tetraparental bovine chimera sperm to fertilize matured oocytes and support further embryo development in vitro has not been reported.

The present study was conducted to examine the ability of sperm from a tetraparental Chimera to fertilize matured oocytes and support cleavage and blastocyst development of IVF embryos.

2. Materials and methods

2.1. In vitro maturation oocytes

Ovaries from Holstein cows were collected from the slaughterhouse and transported in Ringer's solution supplemented with penicillin-G (100 IU ml^{-1}) and streptomycin sulfate (0.2 $\mu g\ ml^{-1}$) at 30 to 32°C. Oocytes from follicles, 2 to 5 mm in diameter were aspirated with an 18-G needle washed with modified-PBS (PBS + supplemented with 3% BSA) solution. The aspirated oocytes were washed three times in maturation medium (TCM-199, Earle's salt; Gibco, Grand Island, NY USA) supplemented with 5% Day 7 superovulated cow's serum (SCS; Matsuoka et al., 1992), 0.01 mg ml^{-1} follicle stimulating hormone (FSH; Denka Pharmaceutical Co., Kawasaki, Japan), and 50 μg ml^{-1} gentamicin (Sigma chemical Co., St. Louis, MO, USA). Oocytes surrounded by cumulus cells were incubated in maturation medium for 22 h at 38.5°C in 5% CO_2 in air.

2.2. In vitro fertilization

Frozen-thawed semen from a 17-month-old tetraparental Chimera were used for in vitro fertilization. Frozen-thawed semen obtained from its sires (Japanese Black and Limousin), and from its maternal grand-sires (Japanese Brown and Holstein) were used for comparison. Frozen semen was thawed in a water bath (37°C), washed twice using 2.5 mM caffeine in Brackett and Oliphant's medium (Caff-BO), as previously described by (Brackett and Oliphant, 1975) by centrifugation at 500 *g* for 5 min. Then the semen was resuspended in caff-BO supplemented with 1% bovine serum albumin (BSA, Sigma) and 20 μg ml^{-1} Heparin (Shimizu Pharmaceutical Co. Ltd, Shimizu, Japan) to yield a final sperm concentration of 5×10^6 ml^{-1}. A 100 μl aliquot of sperm suspension was covered with mineral oil and was preincubated for 1 h at 38.5°C in 5% CO_2 in air. Matured oocytes were transferred into sperm microdrops (20 to 25 oocytes per microdrop) for insemination.

2.3. Experiment I: Chromosome analysis

Chromosome preparations were made from peripheral lymphocytes. Heparinized blood (1 ml) was cultured for 72 h in CO_2 incubator at 38.5°C in 4 ml TCM-199 medium containing 10% Fetal Calf Serum, 100 μl PHA-M (Difco Lab, Detroit, MI, USA), and 10 μl gentamicin. Colcemid was added to the culture 2 h before harvesting. Harvested cells were processed by treating with a hypotonic solution (0.075 M KCl) for 20 min at 38.5°C and by fixation in Carnoy's solution (methanol:acetic acid, 3:1). Two drops of fixed cell suspension were dropped onto a wet glass slide. The air dried chromosome spreads were stained in 2% Giemsa solution.

2.4. Experiment II: Assessing acrosome reaction

After 1 h pre-incubation, a 300 μl aliquot of sperm suspension was fixed by the addition of 300 μl of 10% formaldehyde solution for 15 min. Naphthol yellow S plus aniline blue (NA) staining was performed according to procedures previously described (Bryan and Akruk, 1977) with the modification proposed by (Christensen et al., 1994). Briefly, slides were placed in 2.8 mmol l^{-1} solution of naphtol yellow S (Wako Pure Chemical Industries Ltd, Osaka, Japan) in 1% aqueous acetic acid for 15 min. After rinsing, blotting and drying, slides were placed for 10 min in an aqueous solution of 5.6 mmol l^{-1} naphtol yellow S and 1.25 mmol l^{-1} aniline blue (Wako Pure Chemical Industries Ltd, Osaka, Japan), whose pH was adjusted to 3.5 with acetic acid. The slides were examined at 1000 magnification without a filter (Nikon, Industries Ltd, Tokyo, Japan).

2.5. Experiment III: Assessing pronuclear formation and fertilization rates

About 10 to 20% of inseminated ova were stained to observe the fertilization rate. At 15 h after insemination, the cumulus cells surrounding the embryos were removed by repeated pipetting in PBS (Gibco, Grand Island, NY, USA), supplemented with 5%

SCS, before being fixed in Carnoy's solution (ethanol:acetic acid, 3:1) for 72 h and stained in 1% aceto-orcein to examine the formation of pronuclei. The fertilization rate was calculated as the percentage of stained ova that had two or more pronuclei.

2.6. Experiment IV: Assessing cleavage and blastocyst rate

At 5 h after insemination, the oocytes with cumulus cells were washed and transferred into culture medium for further development. The culture medium consisted of TCM-199 supplemented with 5% SCS, 5 μg ml^{-1} insulin (Wako Pure Chemical Industries. Ltd. Osaka, Japan) and 50 μg ml^{-1} gentamicin. At 48 h after insemination, the cumulus cells surrounding the embryos were removed by repeated pipetting, while the cumulus cell layer attached to the bottom of the culture dish was used as a co-culture. The culture medium was replaced with new medium every 96 h.

The cleavage (2-, 4- and 8-cell) rate was calculated from the number of total ova inseminated after 48 h of insemination (Day 0). The blastocyst rate was calculated from the total cleaved that had developed to the blastocyst stage. The blastocyst rate on Days 7, 8 and 9 was calculated separately from the total blastocyst developed until Day 9 after insemination.

2.7. Embryo evaluation

All experiments were repeated three times, except for the acrosome reactions which were repeated six times. Mean proportions were subjected to least-square analyses of variance with arcsin transformation. Duncan's Multiple Range Test was used for comparison.

3. Results

Based on these chromosome analysis (Fig. 1), this Chimera had apparently normal chromosomes (29 acrocentric pairs, one large sub metacentric X chromosome and one small sub metacentric Y chromosome). Semen was collected from the Chimera bull at 17 months of age (Lot No KA-94) and at 29 months (Lot No KA-95) with no differences between Lot No KA-94 and Lot No KA-95 in relation to motility (Table 1). Neither were there differences in relation to the proportion of acrosome reaction (79.0% vs. 76.2%), fertilization rate (81.8% vs. 74.2%), cleavage rate (75.7% vs. 78.8%) and blastocyst rate (38.1% vs. 32.3%) (Table 2). Therefore, for comparisons with its sire (Japanese Black/JB and Limousin/L) and the maternal grand-sires (Japanese Brown/JBr and Holstein/H), Lot No KA-94 was used for insemination of IVM oocytes.

The percentage of acrosomes reacted after 1 h of capacitation obtained in Chimera sperm was higher ($P < 0.01$) than Japanese Brown and Holstein, but did not differ from Japanese Black and Limousin sperm. The results are shown in Fig. 2.

A total of 191 inseminated IVM oocytes were stained for observation of pronucleus formation (Fig. 3). No significant difference was observed between the 1-pronucleus

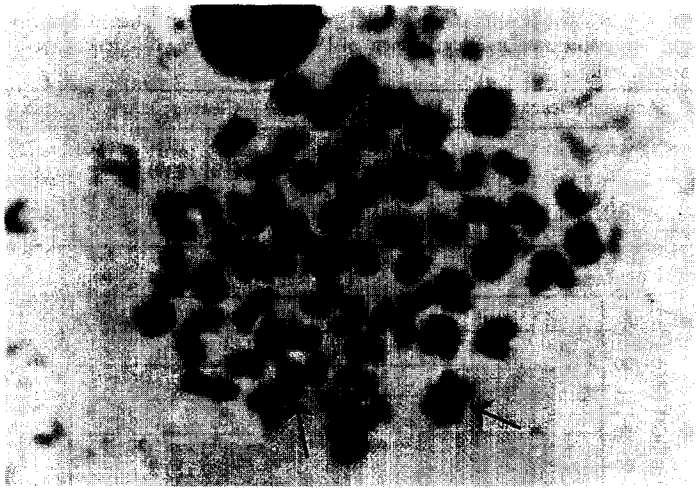

Fig. 1. Chromosome of Chimera bull after giemsa stained (60/XY); one large submetacentric X chromosome and one small submetacentric Y chromosome (arrow).

formation of IVM oocytes inseminated with Chimera and the other sperm used. The 2-pronuclei (Fig. 4) were obtained more frequently in the Chimera groups ($P < 0.05$) compared with Japanese Brown and Holstein groups ($P < 0.01$), but did not differ with the Japanese Black and Limousin groups (33/44, 75.0%; 25/35, 71.4%; 25/36, 69.4%; 17/43, 39.5% and 5/33, 15.2% for O-CH, O-JB, O-JBr and O-H sperm, respectively). Polyspermy occurred in IVM oocytes inseminated with Chimera, Japanese Black, Japanese Brown and Holstein sperm, but did not occur with Limousin sperm (3/44, 6.8%; 3/35, 8.6%; 0/36, 0.0%; 2/43, 4.7%; 1/33, 3.0% and for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively). The fertilization rates of IVM oocytes inseminated with Chimera were higher ($P < 0.05$) than in Japanese Brown and ($P < 0.01$) in Holstein, but did not differ from Japanese Black and Limousin sperm (36/44, 81.8%; 28/35, 80.0%; 25/36, 69.4%, 19/43, 44.2% and 6/33, 18.2% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively; Fig. 3).

A total of 978 inseminated IVM oocytes were cultured for observation of development up to the 2-cell stage. IVM oocytes inseminated with Chimera did not differ from the other sperm used (Fig. 5). Development to the 4-cell stage of IVM oocytes

Table 1
Evaluation of semen from tetraparental Chimeric bull

Fresh semen					Frozen-thawed semen
Lot no. of sperm	Volume (ml)	pH	No. of sperm (10^6 ml^{-1})	Motility (+ + +, %)	Motility (+ + +, %)
KA-94	3.0	7.0	13.6	80	40
KA-95	6.5	6.2	10.2	80	30

Table 2
Acrosome reacted, fertilization rate and development of IVF embryos using differences lot nos of sperm from tetraparental Chimeric bull

Lot no of sperm	Acrosome reacted (%)	Fertilization rate (%)	Cleavage rate (%)	Blastocyst rate (%)
KA-94	79.0 ± 3.5[a]	81.8 ± 15.2[a] (36/44)	75.7 ± 1.6[a] (181/239)	38.1 ± 5.9[a] (69/181)
KA-95	76.2 ± 8.0[a]	74.2 ± 15.1[a] (30/42)	78.8 ± 1.3[a] (231/293)	31.2 ± 5.9[a] (72/231)

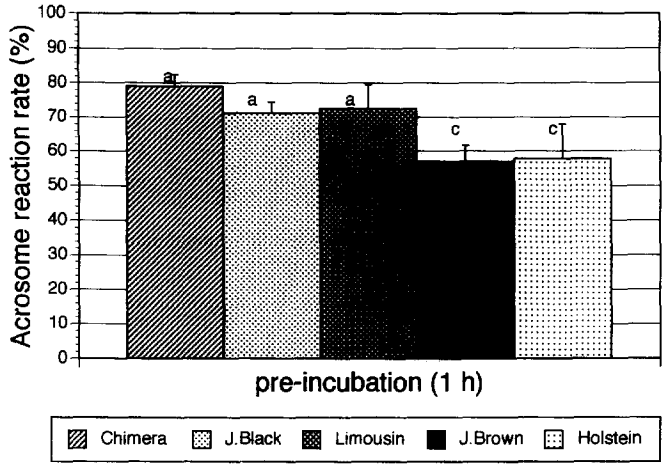

Fig. 2. Acrosome reaction of sperm from Chimera, Japanese Black, Limousin, Japanese Brown and Holstein were assessed after 1 h pre-incubation. Within columns, mean with different superscripts are significantly different (ANOVA, Duncan's test, a–c, $P < 0.01$).

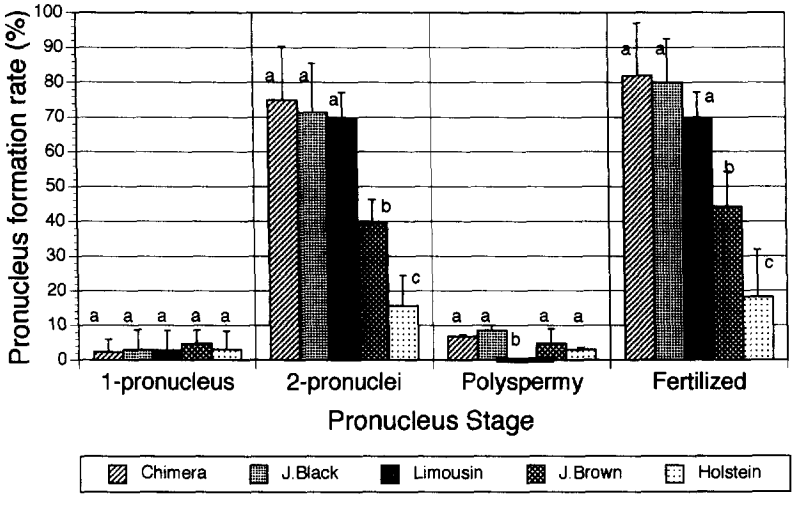

Fig. 3. Pronucleus stage of IVF embryos using Chimera, Japanese Black, Limousin, Japanese Brown and Holstein sperm. Within columns, mean with different superscripts are significantly different (ANOVA, Duncan's test, a–b, $P < 0.05$; a–c, $P < 0.01$).

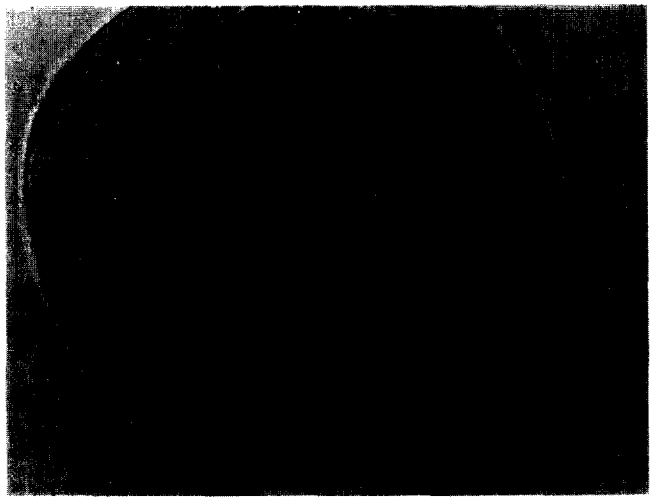

Fig. 4. Two-pronuclei formation of IVM oocytes was observed after fertilization with sperm from Chimera bull.

inseminated with Chimera did not differ from Japanese Black, Limousin and Japanese Brown, but were higher ($P < 0.05$) than in Holstein sperm (62/239, 25.9%; 51/171, 29.8%; 53/186, 28.5%; 37/196, 18.9% and 18/186, 9.7% for O-CH, O-JB, O-L, O-JBr and O-H, respectively). Development to the 8-cell stage of IVM oocytes

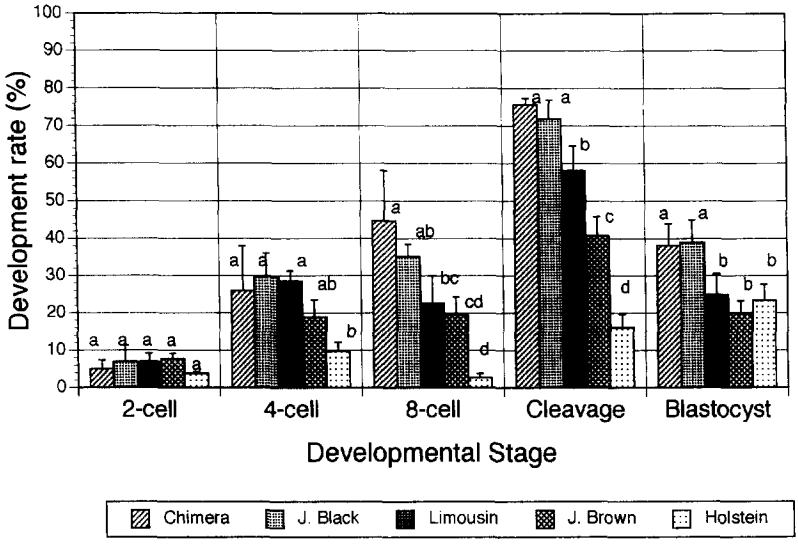

Fig. 5. Development of IVF embryos using Chimera, Japanese Black, Limousin, Japanese Brown, Holstein sperm. Within columns, mean with different superscripts are significantly different (ANOVA, Duncan's-test, a–b, $P < 0.05$; a–c, $P < 0.01$ and a–d, $P < 0.001$).

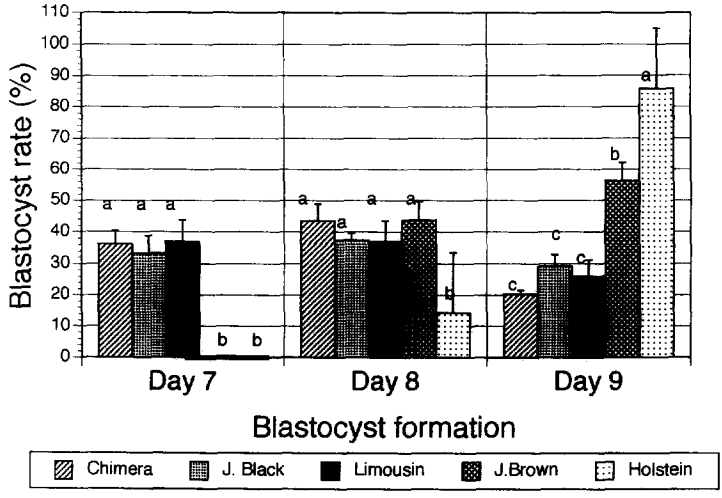

Fig. 6. Blastocyst formation of IVF embryos on Days 7, 8 and 9 after post insemination by using Chimera, Japanese Black, Limousin, Japanese Brown and Holstein sperm. Within columns, mean with different superscripts are significantly different (ANOVA, Duncan's test a–b; $P < 0.05$, a–c; $P < 0.01$).

inseminated with Chimera did not differ from Japanese Black, but were higher ($P < 0.05$) than in Limousin, ($P < 0.01$) in Japanese Brown and ($P < 0.001$) in Holstein sperm (107/239, 44.8%, 60/171, 35.1%; 42/186, 22.6%; 28/196, 14.3% and 5/186, 2.7% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively). The cleavage rates of IVM oocytes inseminated with Chimera sperm did not differ from Japanese Black, but were higher ($P < 0.05$) than with Limousin, ($P < 0.01$) Japanese Brown and ($P < 0.001$) Holstein sperm (181/239, 75.7%; 123/171, 71.9%; 108/186, 58.1%; 80/196, 40.8% and 30/186, 16.1% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively). The blastocyst rates of IVM oocytes inseminated with Chimera sperm did not differ with Japanese Black, but were higher ($P < 0.05$) than Limousin, Holstein and Japanese Brown sperm (69/181, 38.1%; 48/123, 39.0%; 27/108, 25.0%; 7/30, 23.3% and 16/80, 20.0% for O-CH, O-JB, O-L, O-H and O-JBr sperm, respectively).

The proportions of blastocyst formation on Day 7 of IVM oocytes inseminated with Chimera sperm did not differ between Japanese Black and Limousin sperm, but were higher ($P < 0.05$) than in Japanese Brown and Holstein sperm (25/69, 36.2%; 16/48, 33.3%; 10/27, 37.0%; 0/16, 0% and 0/7, 0% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively; Fig. 6). The proportions of blastocyst formation on Day 8 of IVM oocytes inseminated with Chimera sperm did not differ from Japanese Black, Limousin and Japanese Brown, but were higher ($P < 0.05$) than in Holstein sperm (30/69, 43.5%; 18/48, 37.5%; 10/27, 37.0%; 7/16, 43.8% and 1/7, 14.3% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively). The proportions of blastocyst formation on Day 9 of IVM oocytes inseminated with Chimera sperm did not differ from Japanese Black and Limousin, but were lower ($P < 0.05$) than Japanese Brown and ($P < 0.01$) Holstein sperm (14/69, 20.3%; 14/48, 29.2%; 7/27, 25.9.%, 9/16, 56.3% and 6/7, 85.7% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively).

4. Discussion

McLaren (1976) reported that Chimeras made by aggregating embryos with out regard to the sex, should result in 50% of Chimeras being an XX/XY type. The phenotypic sex of XX/XY individuals will show varying degrees of dominance of the male component with the variation seeming to reflect the relative representation of XY and XX cells in the gonadal primordial. Bongso et al. (1981) reported that sterility and infertility in chimerism cattle (60 XX/60 XY) was caused by testicular hypoplasia. However, our Chimera was normal (60 XY); motility (+ + +) of frozen-thawed sperm was 30–40%, and 32 to 38% of cleavage embryos could reach blastocyst (Fig. 7).

The high fertilization rate after 5 h of sperm-oocyte incubation in the Chimera, Japanese Black and Limousin group were attributed to a high occurrence of acrosome reaction at 1 h after capacitation. The first line of evidence of sperm penetration rate increased until 5 h and the proportion of polyspermy was 4 and 7% at 4 and 5 h after insemination (Saeki et al., 1991). As described by Parrish et al. (1986), differences among bulls in fertilization rates are due to variation in the time for capacitation and the acrosome reaction. The lower fertilization rates observed in Japanese Brown and Holstein may be due to the short sperm-oocyte incubation time of 5 h which may not be sufficient for sperm penetration. The numbers of blastocyst produced on Days 7, 8 and 9 of IVM oocytes inseminated with Chimera were similar to that produced with its sires (Japanese Black and Limousin sperm). These findings indicate there may be a paternal effect in blastocyst production on these days. Shire and Whitten (1980a); Shire and Whitten (1980b) and Goldbard and Warner (1982) reported similar cases in the mouse embryo, where the cleavage rate and speed of development are dependent upon genetic factors, including maternal and paternal effects. In the human embryo, there is a strong

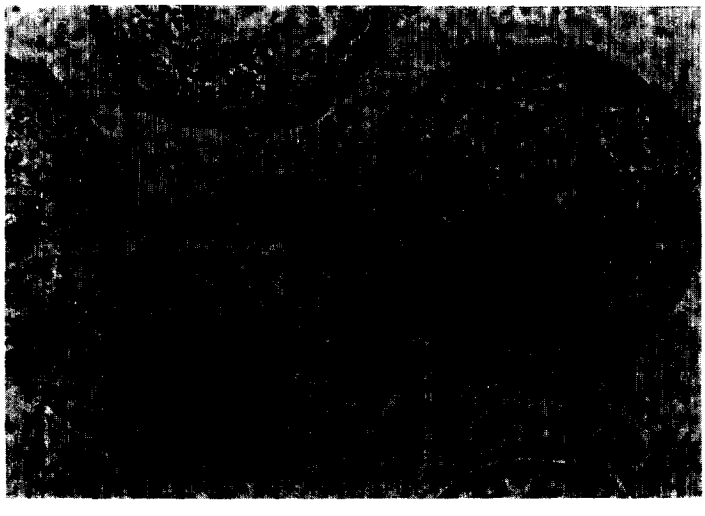

Fig. 7. Blastocyst formation on Day 8 of IVM oocytes was observed after fertilization with sperm from Chimera bull.

paternal effect on preimplantation development and blastocyst formation (Janny and Menezo, 1994).

The cleavage and blastocyst rates of IVF-zygotes inseminated with the Chimera were similar to its sire (Japanese Black), but differed from another sire (Limousin), and from its maternal grand-sires (Japanese Brown and Holstein). Detailed analysis of mosaicism in interspecific Chimeras between *Mus musculus* and *Mus caroli* revealed that cells of the two could coexist and interact normally in all tissues (Rossant and Chapman, 1983). This Chimera originated from aggregated F_1 (Holstein × J. Black) and F_1 (J. Brown × Limousin) (Boediono et al., 1993). Therefore, the high blastocyst production seen when IVM oocytes were inseminated with Chimera sperm might be due to a heterosis effect. These result suggest that this Chimera sperm can be used for producing bovine IVF embryos.

In conclusion, this sperm from this Chimera had the ability to fertilize in vitro matured oocytes and which then developed as blastocysts.

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