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# The effect of sperm–oocyte incubation time on in vitro embryo development using sperm from a tetraparental chimeric bull

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**Abstract**

The present study was designed as 5 × 4 factorial to investigate the effects of using sperm from 5 bulls, and varied sperm–oocyte incubation times (5, 10, 15 and 20 h) on the fertilization, cleavage rates and blastocyst formation on an in vitro bovine embryo production system. The bulls included a tetraparental Chimera, its sires (Japanese Black and Limousin), its maternal grand-sires (Japanese Brown and Holstein). The proportion of polyspermy, 2-pronuclei formation, fertilization, cleavage and development to blastocyst were affected ($p < 0.01$) by the duration of sperm–oocyte incubation, as well as by the interaction between bulls and their corresponding sperm–oocyte incubation time. Blastocyst rate observed after 5 h in oocytes inseminated with Chimera, Japanese Black and Limousin were higher ($p < 0.05$) than those observed at 20 h incubation. The proportion of blastocysts from oocytes inseminated with Japanese Black observed at 10 h of incubation did not differ from that of Chimera, but both were higher ($p < 0.05$) than those observed for the Limousin, Japanese Brown and Holstein sires. The present study showed that there was an effect by the duration of sperm–oocyte incubation on in vitro embryo development. The optimal time of sperm–oocyte incubation for the Chimera was similar to that of its sires (Japanese Black and Limousin) but differed from its maternal grand-sires (Japanese Brown and Holstein). The fertilization rates for the sperm from the Holstein bull increased up to 15 h suggesting that this might be the only bull that would benefit from a long incubation period for insemination. © 1997 Elsevier Science B.V.

*Keywords:* Sperm–oocyte incubation time; Tetraparental; Embryology; In vitro fertilization

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## 1. Introduction

Sperm from individual bulls have been reported to differ in their ability to fertilize matured oocytes in vitro and in the development to the pre-implantation stage (Leibfried-Rutledge et al., 1987, 1989; Shi et al., 1990, 1991). Similar findings were also reported in rams (Fukui et al., 1988). Spermatozoa from a single bull had fertilization and cleavage rates affected with different heparin concentrations and incubation periods (Iritani et al., 1986; Fukui et al., 1990). Also, spermatozoa from different semen lot and straws within the same semen lot from a single bull affected the developmental capacity of embryos (Otoi et al., 1993).

In vitro maturation of bovine oocytes incubated with sperm for 18–20 h after in vitro fertilization has been reported (Long et al., 1993). It has been suggested that the optimal time of sperm oocytes incubation for achieving maximum fertilization rate after IVM–IVF is 24 h (Rehman et al., 1994). In some IVF systems, a significantly higher incidence of polyspermy has been shown in oocytes cultured with sperm for 24 h compared to those of 8 h (Chian et al., 1992).

Tetraparental chimeric cattle were successfully produced by aggregating IVF embryos of F1(Holstein × Japanese Black) and F1(Japanese Brown × Limousin) and culturing in vitro without the zona pellucida (Boediono et al., 1993), and the sperm from this tetraparental chimeric bull used for producing IVF bovine embryos (Sumantri et al., 1997). However, the optimal time of sperm–oocyte incubation required for tetraparental chimeric bull was not determined.

Therefore, the present study was conducted to examine the effect of sperm–oocyte incubation time on in vitro fertilization and later developmental stages of IVF embryos using sperm from tetraparental Chimera, its sires (Japanese Black and Limousin) and its maternal grand sires (Japanese Brown and Holstein).

## 2. Materials and methods

### *2.1. In vitro maturation of oocytes*

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 $\mu$g/ml) at 30 to 32°C. Cumulus–oocyte complexes (COCs) were aspirated from follicles of 2 to 5 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 solution. The aspirated COCs were washed 3 times in maturation medium (TCM-199, Earles salt; Gibco, Grand Island, NY, USA) supplemented with 5% day-7, superovulated cow's serum (SCS; Matsuoka et al., 1992), 0.01 mg/ml FSH (Denka Pharmaeutical, Kawasaki, Japan), and 50 ( $\mu$g/ml) gentamicin (Sigma Chemical, St. Louis, MO, USA). Thereafter, the COCs were incubated in maturation medium for 22 h at 38.5°C in humidified 5% $CO_2$ in air.

### 2.2. *In vitro fertilization*

Frozen–thawed semen from a 17 month-old tetraparental Chimera (CH) was used for in vitro fertilization. Frozen–thawed semen obtained from its sires (Japanese Black/JB and Limousin/L), and from its maternal grand-sires (Japanese Brown/JBr and Holstein/H) were also 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), followed 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 ( $\mu$g/ml) heparin (Shimizu Pharmaceutical, Shimizu, Japan) to yield a sperm concentration of $5 \times 10^6$/ml. A 100 $\mu$l aliquot of the sperm suspension was covered with mineral oil and pre-incubated for 1 h at 38.5°C in humidified 5% $CO_2$ in air. Matured oocytes were transferred into sperm microdrops (20 to 25 oocytes per microdrop) separately for each bull: Chimera (O-CH), Japanese Black (O-JB), Limousin (O-L), Japanese Brown (O-JBr) and Holstein (O-H) followed by incubation for different times (5, 10, 15 and 20 h).

### 2.3. *Experiment 1: Assessing pronuclear formation and fertilization rates*

A total of 761 matured inseminated oocytes from different sperm–oocytes incubation time (5, 10, 15, and 20 h) treatments were washed and cultured for 15, 10, 5, and 0 h, respectively; followed by staining to observe fertilization rate. The cumulus cells surrounding the embryos were removed by several pipettings in PBS (Gibco, Grand Island, NY, USA) supplemented with 5% SCS, before being fixed in carnoy 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 2 or more pronuclei.

### 2.4. *Experiment 2: Assessing cleavage and blastocyst rate*

The inseminated oocytes with cumulus cells from all treatments were washed and transferred separately into culture medium for further development. The culture medium consisted of TCM-199 supplemented with 5% SCS, 5 ( $\mu$g/ml) insulin (Wako Pure Chemicals Osaka, Japan) and 50 ( $\mu$g/ml) gentamicin. At 48 h after fertilization, the cumulus cells surrounding the embryos were removed by several pipettings, 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 one after 96 h.

The cleavage (2-, 4- and 8 cell stage) rate was calculated from the number of total ova inseminated after 48 h of insemination (IVF = Day 0). The blastocyst rate was calculated from the total cleavage that had developed to the blastocyst stage by day 9 after insemination.

### 2.5. *Embryo evaluation*

All experiments were repeated 3 times. Data were analyzed as $5 \times 4$ factorial (5 types of sperm from different bull, and 4 for sperm–oocytes incubation time 5, 10, 15 and 20

h). Mean proportions were subjected to least-square analysis of variance with arcin transformation. Duncan's Multiple Range Test was used for specific comparisons.

## 3. Results

The proportion of polyspermy observed after 5 h of sperm–oocyte incubation was significantly lower ($p < 0.05$) than that observed after 20 h (Fig. 1). The proportion of polyspermy gradually increased when oocytes were incubated with sperm for longer periods with each of the bulls (7.1 vs 22.5%, 8.9 vs 27.1%, 0.0 vs 11.4%, 7.0 vs 17.3% and 4.8 vs 22.5% for O-CH, O-JB, O-L, O-JBr and O-H, respectively). Nonetheless, J. Black had a higher ($p < 0.05$) polyspermy rate after 10 and 15 h of incubation than the other bulls.

The proportion of 2-pronuclei formation observed after 5 h sperm–oocyte incubation for oocytes inseminated with Chimera sperm did not differ from that observed after 20 h (Fig. 2). However, the proportion of 2-pronuclei formation observed at this point for the oocytes inseminated with J. Black and Limousin were higher ($p < 0.05$) than those observed after 15–20 h. In contrast, oocytes inseminated with J. Brown and Holstein had lower formation of 2-pronuclei at 5 h incubation compared to that of 20 h ($p < 0.05$), (74.9 vs. 73.8%; 70.1 vs. 56.8%; 69.5 vs. 51.2%; 39.5 vs. 54.3% and 17.7 vs. 63.8% for O-CH, O-JB, O-L, O-JBr and O-H; respectively).

Oocytes inseminated with J. Black and Limousin sperm retained the same fertilization rate in each time treatment (Fig. 3). However, the fertilization rates observed after 5 h sperm–oocyte incubation in the Chimera, Japanese Brown and Holstein were lower ($p < 0.05$) than those observed after 20 h of incubation (81.9 vs 96.3%, 79.1 vs 83.9%,

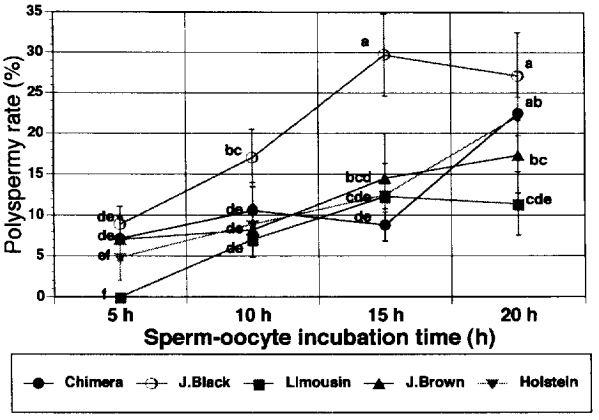


Fig. 1. The effect of sperm–oocyte incubation time on polyspermy rate. Chimera ($n = 159$), J. Black ($n = 144$), Limousin ($n = 158$), J. Brown ($n = 145$) and Holstein ($n = 155$), $n$ (No. of stained oocytes). Each treatment was replicated 3 times. Mean with different superscripts are significantly different (ANOVA, Duncan's multiple range test, a vs. b, c, d, e; b vs. c, d, e, f; c vs. d, e, f; d vs. e, f; e vs. f; $p < 0.05$, and a vs. f; $p < 0.01$).

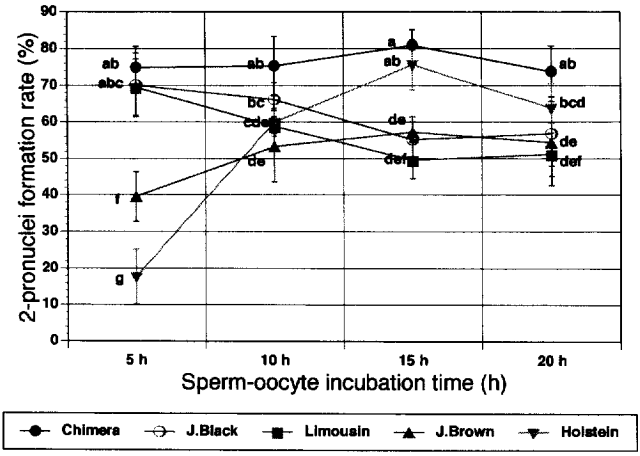


Fig. 2. The effect of sperm–oocyte incubation time on 2-pronuclei formation rate. Chimera ($n = 159$), J. Black ($n = 144$), Limousin ($n = 158$), J. Brown ($n = 145$) and Holstein ($n = 155$), $n$ (No. of stained oocytes). Each treatment was replicated 3 times. Mean with different superscripts are significantly different (ANOVA, Duncan's multiple range test, a vs. b, c, d, e; b vs. c, d, e; c vs. d, e, f, g; d vs. e, f, g; e vs. f, g; f vs. g; $p < 0.05$ and a, b vs. f, g; $p < 0.01$).

67.8 vs 62.9%, 46.6 vs 71.6%, and 20.5 vs 85.9% for O-CH, O-JB, O-L, O-JBr and O-H sperm, respectively).

A total of 4057 matured oocytes were inseminated for producing IVF embryos to study the effect of sperm–oocyte incubation time on cleavage rate. The cleavage rate for oocytes inseminated with Limousin sperm remained the same for each incubation time

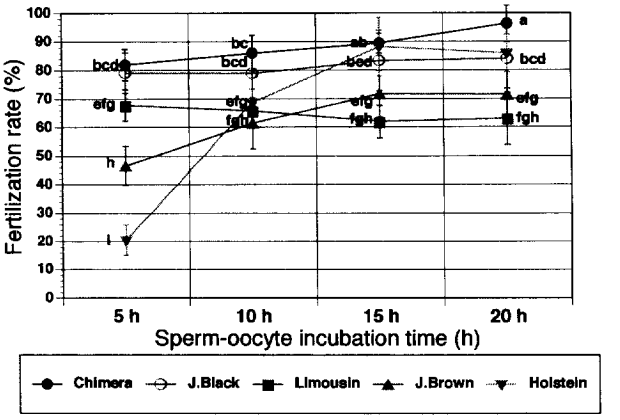


Fig. 3. The effect of sperm–oocyte incubation time on fertilization rate. Chimera ($n = 159$), J. Black ($n = 144$), Limousin ($n = 158$), J. Brown ($n = 145$) and Holstein ($n = 155$), $n$ (No. of stained oocytes). Each treatment was replicated 3 times. Mean with different superscripts are significantly different (ANOVA, Duncan's multiple range test, a vs. b, c, d, e, f, g, h; b vs. c, d, e, f, g, h; c vs. d, e, f, g, h, i; d vs. e, f, g, h, i; e vs. f, g, h, i; f vs. g, h, i; h vs. i; $p < 0.05$; a, b, vs. h, i; $p < 0.01$).

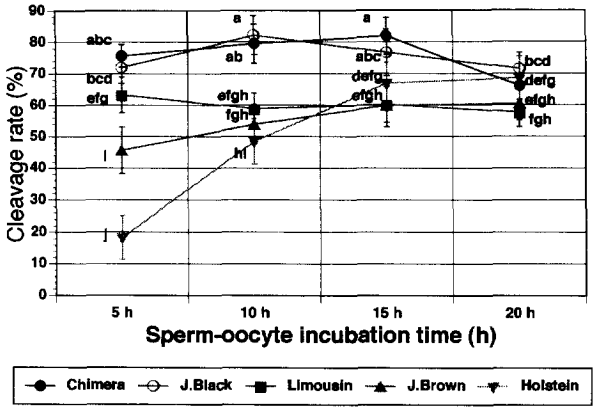


Fig. 4. The effect of sperm–oocyte incubation time on cleavage rate. Chimera ($n = 917$), J. Black ($n = 763$), Limousin ($n = 825$), J. Brown ($n = 771$) and Holstein ($n = 781$), $n$ (No. of inseminated oocytes). Each treatment was replicated 3 times. Mean with different superscripts are significantly different (ANOVA, Duncan's multiple range test, a vs. b, c, d, e, f, g, h, i; b vs. c, d, e, f, g, h, i; c vs. d, e, f, g, i, j; d vs. e, f, g, h, i, j; e vs. f, g, h, i, j; f vs. g, h, i, j; g vs. h, i, j; i vs. j; $p < 0.05$, a, b, vs. j; $p < 0.01$).

(Fig. 4). Only small changes occurred using sperm from J. Black and Chimera. The trends with sperm from J. Brown more greater (45.7, 53.9, 59.9 and 60.5% for 5, 10, 15 and 20 h, respectively). Cleavage rates using Holstein sperm to inseminate oocytes increased from 18.3% at 5 h to 68.6% at 20 h ($p < 0.01$; Fig. 4).

The results of blastocyst formation are summed in Fig. 5. In this case, a total of 2550 cleaved (2-, 4-, and 8-cell) embryos were used. In general terms, as the time of sperm–oocyte incubation increased, the blastocyst rate decreased. Also, the Limousin,

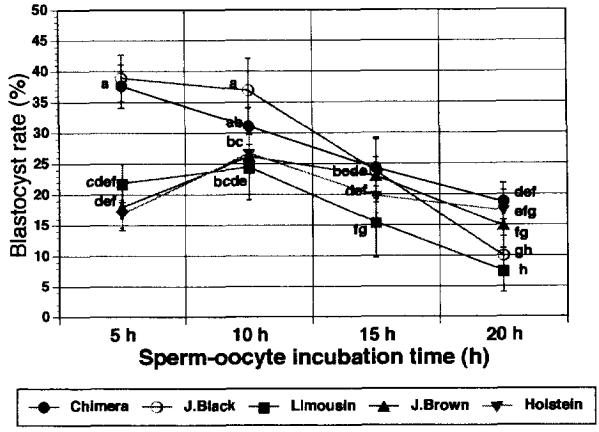


Fig. 5. The effect of sperm–oocyte incubation time on Blastocyst rate. Chimera ($n = 692$), J. Black ($n = 567$), Limousin ($n = 488$), J. Brown ($n = 403$) and Holstein ($n = 400$), $n$ (No. of cleaved 2-, 4- to 8-cell stage). Each treatment was replicated 3 times. Mean with different superscripts are significantly different (ANOVA, Duncan's multiple range test, a vs. b, c, d, e, f, g; b vs. c, d, e, f, g; c vs. d, e, f, g, h; d vs. e, f, g, h; e vs. f, g, h; f vs. g, h; g vs. h; $p < 0.05$, and a, b, vs. h; $p < 0.01$)

Holstein and J. Brown sperm inseminated oocytes showed a lower blastocyst formation than the other bulls (Chimera, and J. Black) for all incubation times.

Blastocyst rate for the Chimera and J. Black sperm inseminated oocytes were similar at 5 h, but different from Limousin, J. Brown and Holstein inseminated oocytes ($p < 0.05$) which did not differ from each other. Subsequent ranges and differences tended to be smaller. At 20 h of incubation, Chimera inseminated oocytes were superior ($p < 0.05$) on blastocyst rate (18.8%) compared to J. Black (10.0%) and Limousin (7.6%) bulls, but similar to J. Brown (17.5%).

## 4. Discussion

The incidence of polyspermy among the bulls used in this study showed a tendency to increase with time of sperm–oocyte incubation but the magnitude of this effect differed from one bull to another. J. Black seemed to be the most susceptible, while the Limousin bull had the lowest rate. The extent of these differences among the bulls in terms of the sperm capacitation times and acrosome reactions has been reported by Parrish et al. (1986).

The fertilization patterns of matured oocytes inseminated with Chimera was similar to its sires (J. Black and Limousin), but different from that of its maternal grand sires (J. Brown and Holstein). The latter two bulls had lower fertilization rates after 5 h of incubation. Increasing the sperm–oocyte incubation time improved the fertility rate. Similar differences among the bulls have been documented by others (Iritani et al., 1986; Leibfried-Rutledge et al., 1987; and Shi et al., 1990); and in sheep (Fukui et al., 1988).

The results in Fig. 5, indicate that the rate of blastocyst formation decreases with increased sperm–oocyte incubation time for each of the 5 bulls. These observations contrast with those reported by Long et al. (1993) and Rehman et al. (1994). They found that the blastocyst rate from IVM–IVF oocytes was not affected by sperm–oocyte incubation period. Similarly, Long et al. (1993) showed that sperm concentration had no effect on monospermic fertilization. These differences with our results may be due to differences between the bulls used in each study. There are two possibilities contributing to the decline in rate of blastocyst formation at 20 h in this experiment: First, the increasing incidence of polyspermy could have lead to an increased rate of mortality in pre-implantation embryos; and second, the bull effect in our case seemed to be dependent on the interrelationship among them (that is, Chimera has its sires, J. Black and Limousin, and its grand sires, J. Brown and Holstein).

The similarity between Chimera and its sire (Japanese Black) in fertilization, cleavage and blastocyst rate might be associated with the significantly higher proportion of the cell in the gonads derived from Japanese Black than from the Limousin which contributed in the formation of these chimeric cattle. Sumantri et al. (1996) reported that 78.6% of microsatellite DNA present in the tetraparental chimeric cattle were uniquely contributed from the Japanese Black and only 21.4% from the Limousin. Detailed analysis of mosaicism in interspecific Chimeras between *Mus musculus* and *Mus caroli* have revealed that cells of the two species could coexist and interact normally in all

tissues (Rossant and Chapman, 1983). These findings indicate that there may be a paternal effect on fertilization, cleavage and blastocyst rate. Shire and Whitten (1980a,b) and Goldbard and Warner (1982) reported similar cases in the mouse embryo, where the cleavage rate and speed of development were dependent upon genetic factors, including maternal and paternal effects. There is also a strong paternal effect on pre-implantation development and blastocyst formation in the human embryo (Janny and Menezo, 1994).

In conclusion, incubation time did affect in vitro embryo development. Although there were inherent differences with the Chimera and its sire (Japanese Black) having the highest rate of blastocyst production, there were no significant differences between 5 and 10 h for the Chimera, J. Black and Limousin. Only the Chimera and Japanese Black decreased at 15 h and 4 of the 5 sires decreased at 20 h.

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