IN VITRO DEVELOPMENT OF HOLSTEIN AND JAPANESE BLACK BREEDS EMBRYO

PERKEMBANGAN *IN VITRO* EMBRIO SAPI-SAPI HOLSTEIN DAN JAPANESE BLACK

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ABSTRACT

Two experiments were conducted with 3,457 oocytes, aspirated from the ovaries of slaughtered Holstein and Japanese Black breeds cows to compare the use of superovulated cow serum (SCS) and fetal calf serum (FCS) supplementation into medium for *in vitro* development (experiment I) and the effect of different source of oocytes (experiment II). The endpoint was development to cleavage on day 2 and to blastocyst up to day 9 after insemination. In experiment I, oocytes were matured and cultured *in vitro* (after fertilization) in TCM-199 supplemented with 5% SCS or 5% FCS. The cleavage rate of zygotes cultured in medium supplemented with 5% SCS was higher (P<0,05) than 5% FCS (562/860, 65,3% vs 445/742, 60,0%). The blastocyst production rate was significantly higher (P<0,01) in SCS treatment than FCS (232/860, 27,0% vs 124/742, 16,7%). In experiment II, 956 oocytes collected from Holstein breed and 899 oocytes from Japanese Black breed were matured, fertilized and cultured *in vitro* in medium supplemented with 5% SCS. The mean oocyte number and blastocyst production per ovary were 12,0 (956/80) and 3,2 (252/80) for Holstein breed, 15,0 (899/60) and 3,1 (184/60) for Japanese Black breed. The cleavage rate of zygotes derived from Holstein and Japanese Black breeds ovaries was not significantly different (635/956, 66,4% vs 569/899, 63,3%, respectively). However, the blastocyst production rate was significantly higher (P<0,01 in Holstein than Japanese Black breeds (252/956, 26,4% vs 184/899, 20%). These results indicated that the developmental rate to blastocysts of embryos cultured *in vitro* differ from breed to breed.

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ABSTRAK

Telah dilakukan dua eksperimen dengan memakai 3.457 oosit yang diperoleh melalui aspirasi ovari sapi Holstein dan Japanese Black, untuk mengetahui pengaruh serum sapi superovulasi (SSS) dan Fetal Calf Serum (FCS) yang disuplementasikan ke dalam medium perkembangan *in vitro* (Eksperimen I) dan pengaruh sumber oosit yang berbeda (Eksperimen II). Pengamatan dilakukan terhadap perkembangan sampai tingkat pembelahan pada hari ke-2 dan tingkat blastosis pada hari ke-9 setelah inseminasi. Pada eksperimen I, oosit dimatangkan dan dikultur *in vitro* (setelah dibuahi) dalam medium TCM 199 yang ditambah dengan 5% SSS atau 5% FCS. Rata-rata tingkat pembelahan sigot yang dikultur dalam medium diberi tambahan SSS 5% lebih tinggi ($P<0,05$) dibanding FCS 5% (562/860, 65,3% vs 445/742, 60,0%). Sedangkan rata-rata blastosis yang dihasilkan secara bermakna lebih tinggi ($P<0,01$) dalam SSS dibandingkan dengan FCS (232/860, 27,0% vs 124/742, 16,7%). Dalam eksperimen II, sebanyak 956 oosit yang diambil dari sapi Holstein dan 899 oosit dari Japanese Black dimatangkan, dibuahi dan dikultur secara *in vitro* dalam medium yang diberi tambahan SSS 5%. Rata-rata jumlah oosit dan blastosis yang dihasilkan per ovari dari sapi Holstein adalah 12,0 (956/80) dan 3,2 (252/80); sedangkan dari Japanese Black adalah 15,0 (899/60) dan 3,1 (184/60). Rata-rata tingkat pembelahan zigot yang berasal dari ovari sapi Holstein dan Japanese Black tidak bermakna yaitu masing-masing 635/956, 66,4% dan 569/899, 63,3%. Namun demikian, rata-rata jumlah blastosis yang dihasilkan oleh ovari sapi Holstein secara nyata lebih tinggi ($P<0,01$) dibanding yang dihasilkan oleh ovari Japanese Black (252/956, 26,4% vs 184/899, 20,0%). Hal ini menunjukkan bahwa tingkat perkembangan embrio yang dikultur secara *in vitro* untuk mencapai tingkat blastosis berbeda dari satu jenis dengan yang lainnya.

INTRODUCTION

The developmental capacity of bovine oocytes after *in vitro* fertilization are influenced by factors such as the morphology of cumulus cells attached to oocytes, morphology of the ooplasm, the bull providing the semen and culture conditions (Shioya *et al.*, 1988; Younis and Brackett, 1991; Kroetsch and Stubbings, 1992; Kajihara *et al.*, 1990; Boediono *et al.*, 1994). Normal embryonic development of *in vitro* matured and fertilized bovine oocytes has been achieved. Bovine oocytes matured with cumulus cells led to the production of zygote, and it develop, with subsequent pregnancies after transfer to recipient (Kajihara *et al.*, 1990; Xu *et al.*, 1990).

Nuclear and extranuclear maturation events take place in vivo during follicular growth and ovulation. The changes go hand in hand with follicular maturation and are induced by

characteristic changes in gonadotropic hormone profile. The presence of serum in culture medium was necessary for oocyte maturation, fertilization and culture since sera could provide cumulus oocyte complexes with protein requirements for *in vitro* maturation.

The purpose of the current study was to investigate the pre-implementation development of oocytes collected from Holstein and Japanese Black breeds after mature, fertilize and culture *in vitro* in medium supplemented with superovulated cow serum (SCS).

MATERIALS AND METHODS

Oocyte collection and *in vitro* maturation :

Cow's ovaries were obtained at a local slaughterhouse and were brought to laboratory within 3 h in physiological saline solution [0.9% (w/v) NaCl] supplemented with Penicillin-G (100 IU/ml) and streptomycin sulfate (0.2 mg/ml) at 30°-32°C. Oocytes in follicles 2-5 mm in diameter, were collected by aspiration with an 18-G needle filled on a 5 ml syringe in Modified-PBS. Only oocytes surrounded by cumulus cells over more than one-third of their surface and an evenly granulated cytoplasm were used in this experiment (Fig. 1A). Oocytes were then washed 2-3 times using maturation medium (TCM-199, Earle's salt, Gibco, Grand Island, NY. USA) supplemented with 0.01 mg/ml follicle stimulating hormone (FSH, Denka Pharmaceutical Co., Kawasaki, Japan), 50 μ g/ml gentamycin sulfate (Sigma Chemical Co., St. Louis MO., USA) and 5% superovulated cow serum (SCS. collected on day-7 of estrus) or 5% fetal calf serum (FCS, Sigma Chemical Co., St. Louis MO., USA). The oocytes (100-200 oocytes) were then introduced into the maturation medium (2.5ml) in a polystyrene culture dish (35 mm diameter, Falcon 1008, Becton Dicknson Co. Ltd., Oxnard, CA., USA) covered with mineral oil (E.R. Squibb & Son, Inc., Princeton, NJ, USA) and cultured for 20 to 22 h at 38.5°C under 5% CO_2 in humidified air.

***In vitro* fertilization and culture :**

Frozen-thawed semen from a single bull (Japanese Black breed) and the same lot was used for fertilization *in vitro*. Frozen sperm was thawed in water (30°-35°C), and the spermatozoa were diluted to about 6 ml with 2.5 mM caffein added to Brackett and Oliphant's medium (Caff-BO, Brackett and Oliphant, 1975) without bovine serum albumin and washed twice by centrifugation at 1800 rpm for 5 min each. The resultant sperm pellet was resuspended in Caff-BO supplemented with 0.3% bovine serum albumin (BSA, Sigma Chemical Co., St. Louis MO., USA) and 20 μ g/ml heparin (Shimizu Pharmaceutical, Shimizu, Japan) to a final concentration of 5 x 10^6 spermatozoa per ml. A 100 μ l aliquot of the sperm suspension was covered with mineral oil and pre-incubated for 1 h under the condition described above. Oocytes matured *in vitro* (Gig. 1B) were transferred into sperm microdrops (20 to 25 oocytes per microdrop) for insemination (Fig. 1C).

After 5 h of insemination, oocytes with adherent cumulus cells were washed by repeated pipetting in culture medium and transferred to the culture medium (TCM-199) supplemented with 5 μ g/ml insulin (Wako Pure Chemical Industries, Osaka, Japan), 50 μ g/ml gentamycin sulfate and 5% SCS or 5% FCS for further development. The culture medium (0.5 ml) in a polystyrene dish (4-well multidish; Nuclon, Roskilde, Denmark) was covered with mineral oil (0.5 ml).

The cumulus cells surrounding the embryos were removed by pipetting after 48 h of insemination, but the cumulus cells layer (forming a monolayer at that time) attached to the bottom of culture dish were not disrupted and the embryos were cultured on this layer. The culture medium was replaced with new medium 4 days after insemination.

Experiment I :

During maturation and culture of bovine oocytes fertilized *in vitro*, the medium was supplemented with 5% SCS or 5% FCS. Each treatment was repeated four times, the sample of SCS and FCS remaining constant throughout.

Experiment II :

Oocytes of Holstein and Japanese Black breeds were collected separately. According to the results of experiment I, the maturation and culture medium in this experiment was supplemented with 5% SCS. The examinations were replicated 7 times using 956 Holstein oocytes and 899 Japanese Black oocytes.

Embryo evaluation :

The proportions of embryos that had cleaved (2-, 4- and 8-cell stage) were recorded 48 h after insemination (when oocytes were taken from cumulus cells). Blastocyst development was assessed on day-7, -8 and -9, when counted, blastocysts were removed from the culture dish to avoid double counting. The data were analyzed by Chi-square test. A probability of $P<0.05$ and $P<0.01$ were considered to be statistically significant.

Superovulated cow serum (SCS) collection :

The SCS was collected from superovulated cows treated with a total 20 mg FSH. Prostaglandin was administrated 48 h after FSH injection to induce luteolysis. Blood was collected on day-7 (on the time of embryo collection). Whole blood samples were centrifuged twice (3500 rpm, 10 min at 5°C). The serum obtained was then heat-inactivated (56°C, 30 min), distributed in small tube (2 ml) and kept in deep freezer (-20°C). The frozen serum were thawed before use.

RESULTS

Experiment I :

The cultured oocytes displayed a marked expansion of cumulus cells as characteristic for matured ova (Fig. 1B). Oocytes were evaluated 48 h after insemination, when developed to cleavage (2-, 4-, 8-cell stage, Fig. 2A), and when developed to blastocyst on day-7, -8 and -9 (Fig. 2C, 2D). The development rate of embryos cultured in medium supplemented with different sources of serum to cleavage rate of zygotes cultured in medium supplemented with 5% SCS was higher ($P<0.05$) than 5% FCS (562/860, 65.3% vs 445/742, 60.0%). The blastocyst productions rate was significantly higher ($P<0.01$) in SCS treatment than FCS (232/860, 27.0% vs 124/742, 16.7%.

Experiment II :

Total 956 oocytes collected from Holstein breed and 899 oocytes from Japanese Black breed were used for *in vitro* maturation, fertilization and culture. The development rate of Holstein and Japanese Black breeds *in vitro* produced embryo are shown in Table 2. The mean oocyte number and blastocyst production per ovary were 15.0 (956/80) and 3.2 (252/80) for Holstein breed, 15.0 (899/60) and 3.1 (184/60) for Japanese Black breed. The cleavage rate of zygotes derived from Holstein and Japanese Black breeds ovaries was not significantly different (635/956, 66.4% vs 569/899, 63.3%, respectively). However, the blastocyst production rate was significantly higher ($P<0.01$) in Holstein than Japanese Black breeds (256/956, 26.4% vs 184/899, 20%).

The development *in vitro* of Holstein and Japanese Black breeds embryos on day-2 (when it developed to 2-, 4-, and 8-cell stage), -7, -8, and -9 (when it developed to blastocyst) after insemination are shown in Figure 3.

DISCUSSION

Suzuki *et. al.* (1992) and Shioya *et. al.* (1988) reported that follicular cells, especially the cumulus cells surrounding immature oocytes, play central role in developmental competence in inducing not only nuclear maturation but also cytoplasmic maturation. Mucinate expansion of cumulus cells after *in vitro* maturation is a sign of nuclear and cytoplasmic maturation (Fig. 1B). When cumulus cells are removed, the zona pellucida hardens (Katska *et al.*, 1989) and this occurs under a prolonged culture period of germinal vesicle-stage oocytes. The cumulus cells surrounding the oocytes and the present of serum in medium will protect the zona pellucida against hardeing. Following oocyte maturation in the absence of cumulus cells and/or serum, fertilizability is decreased in proportion to sperm penetration. The nutrients required for oocyte maturation are transported into the ooplasm via

the junction gap between the ooplasm and cumulus cells used for *in vitro* fertilization a high number of the fertilized bovine oocyte cleaved and developed into blastocysts.

Co-culture with somatic cells is necessary for *in vitro* oocyte development to the blastocyst stage following fertilization. Fertilized oocytes have been co-cultured with bovine oviductal epithelial cells (BOEC; Durnford and Stubbings, 1992; Rorie *et al.*, 1992), granulosa cells (Mochizuki *et al.*, 1991), and cumulus cells (Kajihara *et al.*, 1990, Boediono *et al.*, 1994). In the present study, the embryos were co-cultured with cumulus cells for further development. As shown by Suzuki *et. al.* (1992) and Kajihara *et. al.* (1990), the co-culture of *in vitro* fertilized oocytes with cumulus cells provides an appropriate environment for embryonic development, and they obtained pregnancies and offspring by transferring embryos resulting from co-culture with cumulus cells.

In early work hormonal and follicular factors were found to affect maturation of sheep oocytes *in vitro* and their subsequent developmental capacity (Moor and Trounson, 1977). *In vitro* matured oocytes in hormone free medium show a limited developmental competence (Hensleigh and Hunter, 1985). Several researcher used the medium supplemented with fetal calf serum (FCS; Saeki *et al.*, 1990), estrus cow serum (ECS; Schellander *et al.*, 1990), and superovulated cow serum (SCS; Boediono *et al.*, 1994; Suzuki *et al.*, 1992). When maturation and culture medium was supplemented with serum, a high proportion of the fertilized bovine oocytes developed into blastocysts. Suzuki *et. al.* (1982) reported that the medium supplemented with serum of the same species was having a beneficial effect for the survival of the embryo cultured *in vitro*. The results reported here demonstrated that SCS contains substances which increase the cleavage competence and developmental capacity into blastocysts of *in vitro* matured and fertilized bovine oocytes. This conclusion draws support from result of Boediono *et al.* (1994) who used SCS for oocyte maturation, fertilization and culture of bovine oocytes, which developed into blastocysts.

The further development *in vitro* of Holstein and Japanese Black breeds fertilized oocytes to cleavage (2 to 8-cell stage) were not differ significantly. However, the blastocyst production rate was significantly higher in Holstein and Japanese Black breeds. McLaren and Bowman (1973) found that the difference in the start of cleavage in mouse was determined by maternal genotype. In the present study, the maternal genotype had no affect on the cleavage rate of bovine oocyte. However, blastocyst rate produced from Holstein breed was higher than from Japanese Black breed. These results indicated that the developmental rate into blastocysts of embryos cultured *in vitro* differ from breed to breed.

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Table 1. Development of IVF embryos cultured *in vitro* using different sera.

Serum	No.Oocytes assessed	No.of embryo cleaved				Blastocyst (%)
		2-cell	4-cell	8-cell	Total (%)	
SCS	860	42	146	374	562[a] (65.3)	232[c] (27.0)
FCS	742	62	72	311	445[b] (60.0)	124[d] (16.7)

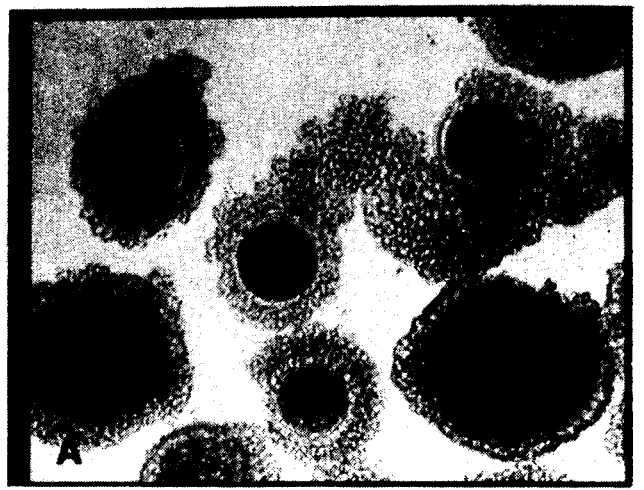
SCS = superovulated cow serum; FCS = fetal calf serum

[a,b] within columns, means with different superscripts are significantly different ([a,b] P< 0.05; [c,d] P< 0.01, X^2-test).

Table 2. Development of Holstein and Japanese Black embryos in culture medium supplemented with superovulated cow serum.

Breed	No.ovary	No.oocytes	Developed to (%)	
			cleavage	blastocyst
Holstein	80	956	635 (66.4)	252 (26.4)[a]
Jpn. Black	60	899	569 (63.3)	184 (20.4)[b]

[a,b] within columns, means with different superscripts are significantly different (P< 0.01, X^2-test).



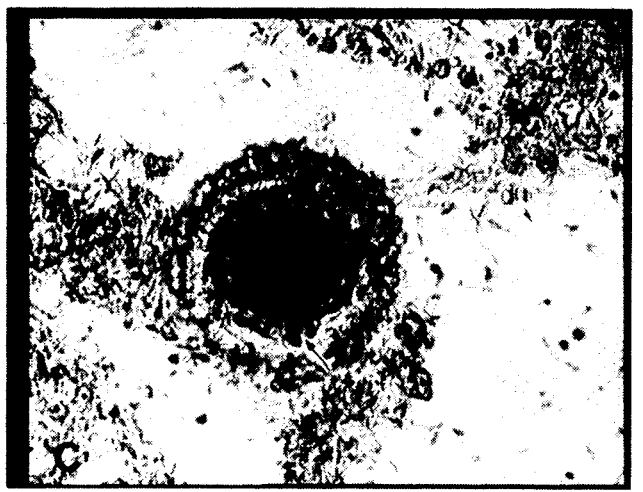


Figure 1. Maturation and Fartilization *in vitro* of Bovine Oocytes
(A) Immature oocytes, oocytes surrounded by cimmulus cells and evenly granulatted cytoplasm
(B) Matured oocytes, oocyrtes with mucinate expansion of cummulus cells after 21 h cultured *in vitro*
(C) *In vitro* fertilization of matured oocytes (with I and II polar body present) by using capacitated sperm (I and II polar body = arrow)

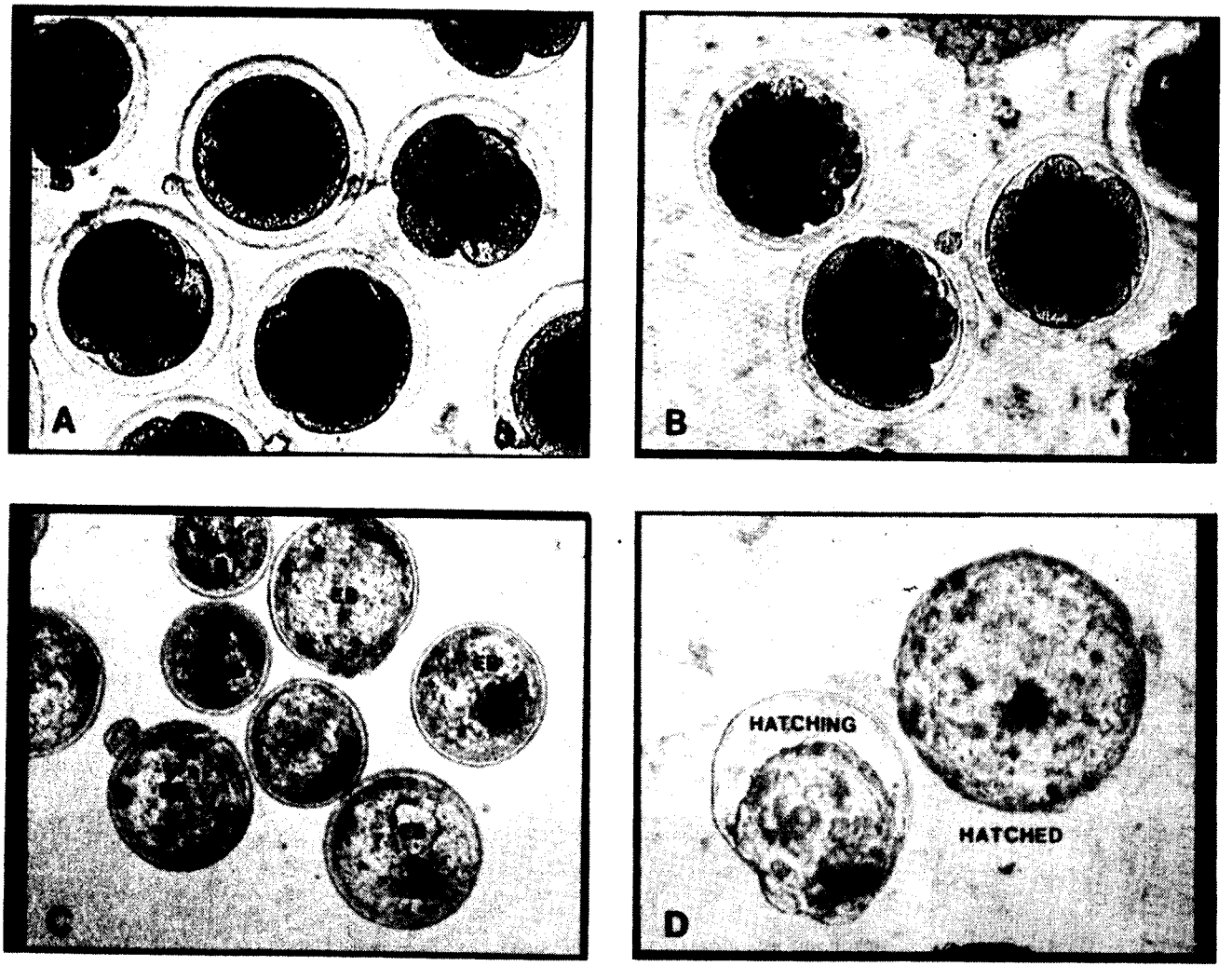

A
B
C
HATCHING
HATCHED
D

Figure 2. Development *in vitro* of Bovine Embryos

(A) On day 2 when it developed to cleavage (2-, 4- and 8-cell stage)

(B) On day 4 when it developed to morula

(C) On day 8 when it developed to blastocyst (B=blastocyst, EB=expanded blastocyst)

(D) On day 9 when it develop to hatching and hatched blastocyst

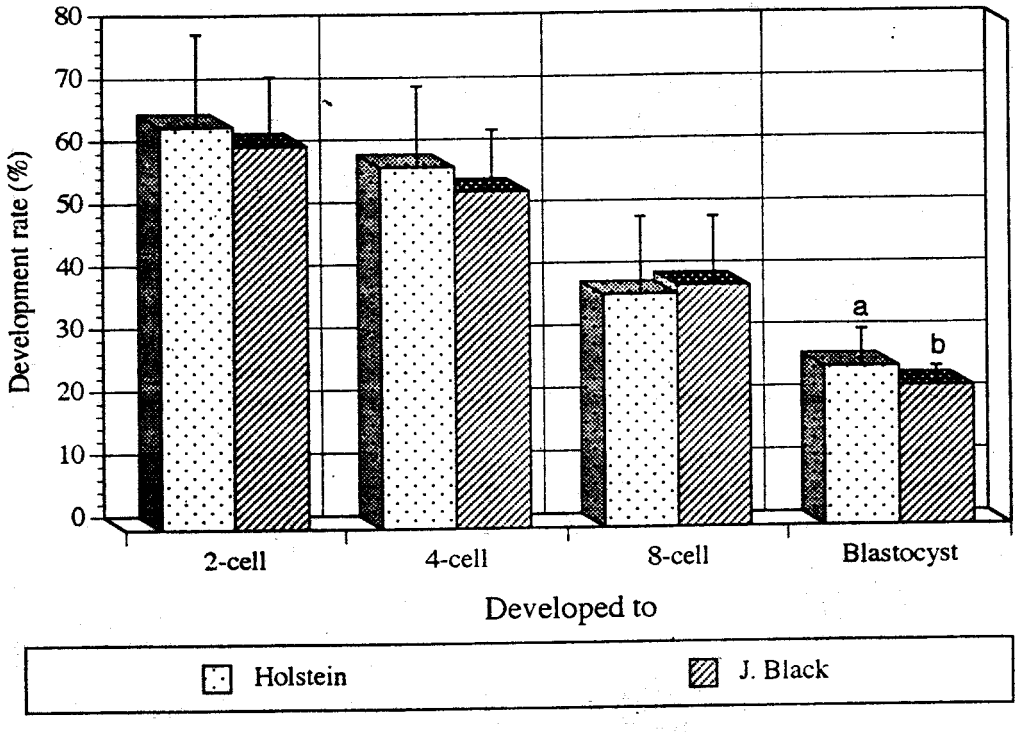


Figure 3. Development rate of Holstein and Japanese Black breeds in vitro produced embryos (a-b, $P<0.01$).

Development rate of Holstein and Japanese Black breeds in vitro produced embryos

Developed to	Holstein (%)	J. Black (%)	Holstein-sd	J.Black-sd
2-cell	64.330	61.110	14.810	11.220
4-cell	57.670	53.670	13.090	9.970
8-cell	37.000	38.330	12.430	11.080
Blastocyst	25.170	22.110	6.030	3.060