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Maturation, Embryonic Development, and Subsequent Embryo Survival after Freezing and Thawing Following a Single or Group Culture Condition of Japanese Black Cattle Oocytes using Modified Synthetic Oviduct Medium

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mSOF 배양액을 이용한 미성숙 난자의 단독 및 그룹 배양 조건을 적용한 일본 흑우 수정란의 성숙, 배발달 및 동결 융해 후 생존성

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SUMMARY

본 연구는 mSOF(modified synthetic oviduct fluid medium) 배양액을 이용하여 100 μl와 10 μl 배양 소적에서 일본 흑우의 수정란 생산 효율을 개선하기 위하여 수행하였다. 난구세포가 부착된 미성숙 난자는 각각 단독 배양 조건(S; 10 μl 소적) 및 그룹 배양 조건 (G; 100 μl 소적)에서 실시하였고 배양액은 TCM-199의 기본 배지에 10% FCS, 0.02 IU/ml FSH와 1 μg/ml estradiol-17β를 첨가하여 사용하였다. 배반포 단계로 발육한 수정란은 1.5 M ethylene glycol로 직접 이식법에 의한 동결 방법으로 동결을 실시하였고, 세포수는 융해 후 생존 수정란에 대해 조사하였다. 체외 배양 시간이 16~17시간 배양 조건에서 난자의 성숙율은 그룹 배양 조건(27.1±16.8%)보다는 단독 배양 조건(57.1±15.0%)에서 성숙율이 높았다($p<0.05$). 그러나 체외 배양 시간이 18~19 시간과 20~21시간 배양시는 유사한 성숙율을 보였다. 난자의 체외 배양율은 체외 배양 시간의 증가에 의해 성숙도가 86.3±9.9%로 증가하였다. 접합체(zygote)의 분할율은 단독이나 그룹 배양 조건에서도 유사한 결과를 얻었다. 배반포 발달율은 배양 7~8일째에 조사한 결과 단독 배양 방법보다는 그룹 배양 방법에서 발달율이 높았으나, 분할된 접합체를 기준으로 한 경우 배반포 발달율(S; 21.4±10.6%, G; 39.0±13.1%)에서는 유의적인 차이가 없었다. 단독 배양과 그룹 배양에서 6.5~8일 사이에 배반포로 발달된 수정란의 세포수 조사에서는 유사한 결과를 얻었다. 동결 융해 후 24시간 배양 후 배반포 생존율(S; 24.2%, G; 30.2%), 부화율(S; 20.9%, G; 12.7%) 및 생존 수정란수(S; 45.2%, G; 42.8%)에서도 배양 조건에 따른 유의적인 차는 없었다. 결론적으로 mSOF 배양액을 이용하는 경우 미성숙 난자의 체외 성숙 유도 배양 시 단독이나 그룹 배양 시 배반포 발달율에서 그룹간에 유의적인 차가 인정되었다($p<0.01$).

(Key words : single or group culture, mSOF medium, Japanese black cattle)

INTRODUCTION

Improvements in the culture conditions for bovine embryo are of immense practical importance both for bio-technological studies and for the embryo transfer industry (Gordon, 1991). Advances in *in vitro* culture (IVC) systems for bovine embryo production over the years have led to increase blastocyst yields, however little progress has been made for the understanding of the precise factors involved in early embryonic development. Many previous investigations were focused to explore the effects of ovum size and culture media on embryonic survival (Carolan *et al.*, 1995; Palma *et al.*, 1992; Takahashi and First, 1992; Keefer *et al.*, 1991). However, because of group culture, the physiological behavior of specific developing ovum and effect of media on its survivability were largely difficult to understand.

Development of *in vitro* embryo production (IVP) system using individual oocytes (Hajdu *et al.*, 1994) has offered many

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advantages to study embryo metabolism, nuclear cloning and ovum pick up studies. Several studies have, however, reported significantly reduced embryonic development when oocytes/embryos were cultured individually compared to in groups (Carolan *et al.*, 1995; Palma *et al.*, 1992; Keefer *et al.*, 1991). Many studies using individual IVP system suggested that limiting factor to an IVP is the quality of media (Gardner *et al.*, 1994; Takahashi and First, 1992). These studies explained that defined media for embryo production have many advantages to standardize IVC systems for the consistency among laboratories, to eliminate the use of additional animal products in embryo production and to facilitate studies in which the exact requirements of embryo development may be examined.

One such defined medium was modified synthetic oviduct fluid (mSOF), developed by the biochemical analysis of sheep oviduct fluid and subsequently modified (Hailing *et al.*, 2005; Takahashi and First, 1992). The mSOF was a commonly used medium for embryo culture (Gardner *et al.*, 1994) and has been shown suitable for IVC of bovine embryos (Yoshioka *et al.*, 1997). However, the scientific information on production and survival rate of Japanese black cattle embryos cultured in single and group culture system using mSOF is limited. Thus, this study compared the single and group culture of Japanese black cattle embryo in mSOF on their blastocyst production, viability after frozen-thawed rate and cell number's changes in culture and after frozen-thawing.

MATERIALS AND METHODS

1. *In Vitro* Maturation and Fertilization

Cumulus-oocyte complexes (COCs) were aspirated from 2 to 6 mm antral follicles in the ovaries of Japanese black cattle collected from the slaughter house with in 5 h of post-slaughtering time. Oocytes were washed three times with HEPES-buffered Tyrode's medium (TALP-HEPES) supplemented with 3 mg/ml BSA (Fraction V, Sigma Chemical Co., St. Louis, MO), 0.2 mM Sodium pyruvate and 25 μg/ml of gentamicin sulfate (Sigma). Only oocytes with an intact, unexpanded cumulus and evenly granulated cytoplasm (Leibfried and First, 1979) were cultured for 22 to 23 h with TCM-199 supplemented with 10% heat-treated fetal calf serum (FCS, Gibco Laboratories, Grand Island, NY), 0.5 μg/ml of FSH (NIH-oFSH), 5 μg/ml LH (NIH-LH), 1 μg/ml of estradiol-17β (Sigma), 0.2 mM sodium pyruvate and 25 μg/ml of gentamicin sulfate (Sirard *et al.*, 1988). Maturation culture media as well as all other culture media were maintained at 39℃ in humidified air with 5% CO_2. *In vitro* fertilization was performed using frozen semen. The frozen semen was thawed and diluted with modified Braket and Oliphant (BO) isotonic medium (Braket and Oliphant, 1975) without BSA but with 1.7 μg/ml phenol-red and 25 μg/ml of gentamicin sulfate. The sperm cells were then washed twice by centrifugation at 500 × g for 5 min. The COCs were co-incubated for about 22±0.5 h with spermatozoa (5×10^6 cells/ml) in BO medium supplemented with 2.5 mM theophylline and 3 mg/ml fatty-acids free BSA (Sigma) at 39 ℃ in humidified air with 5% O_2, 90% N_2 and 5% CO_2, and high humidity. The oocytes were matured in group condition of 100 μl drop (20 oocytes/drop) or single condition of 10 μl drop (1 oocyte/drop).

2. *In Vitro* Culture

The medium used for IVC was mSOF developed by Takahashi and First (1992), containing 3 mg/ml BSA Sigma fraction V, cat # A6003; essential amino acids, Sigma BME cat # B6766 and non-essential amino acids, Sigma MEM cat # M7145 and with 3 mM Na pyruvate. At 20 to 21 h after insemination, presumptive zygotes were denuded of surrounding cumulus cells by vortexing for 2 min in 2 ml phosphate buffer saline (PBS) and subsequently washed twice in PBS and twice in mSOF before being transferred to the culture droplets. Presumptive zygotes were transferred to droplets of mSOF either in group condition of 100 μl drop (20 oocytes/drop) or single condition of 10 μl drop (1 oocyte/drop) according to *in vitro* maturation and cultured at 39℃ in an atmosphere of 5% O_2, 90% N_2 and 5% CO_2 for 7～8 days.

3. Embryo Freezing

Dulbecco's phosphate buffered saline supplemented with 10 % calf serum was used as the basic medium for cryo-preservation. Embryos were frozen using a direct-transfer freezing method (Dochi *et al.*, 1998). The embryos were exposed to 8.5 % (1.5 M) ethylene glycol by a three-step addition (2.83, 5.66 and 8.5 %) with a 10 min equilibration at each concentration. Embryos were loaded into the center of 0.25 ml straws separated by air bubbles from the other two columns of freezing medium. The straws were then transferred to the ethanol freezer pre-cooled to −5℃ for 1 min before seeding manually with a pair of super-cooled forceps. Five minutes after seeding, the straws were cooled to −30℃ at a rate of 0.3℃ per minute and then plunged into liquid nitrogen.

Thawing was performed in air at room temperature (18~20℃) and embryos were washed in Dulbecco's phosphate buffered saline supplemented with 10% calf serum with 10 min equilibration.

After embryos were cultured for 24 h, the survival rate, live cell numbers, dead cell numbers and M phase (division phase) cell numbers were examined by Giemsa staining method.

4. Statistical Analysis

Differences in means were tested for statistical significance by student's *t*-test and Fisher's PLSD and were considered significant at $p<0.05$, $p<0.01$. The data are presented as means± SEM.

RESULTS AND DISCUSSION

Maturation rate of bovine oocytes by culture conditions were significantly higher ($p<0.05$) with single culture compared with group culture at 16 to 17 h of incubation (Table 1). However, similar maturation rate was observed between single and group cultured oocytes at 18 to 19 and 20 to 21 h of incubation. The maturation rates of oocytes were increased with their increasing incubation time (Table 1). Present results have indicated that bovine oocytes could be cultured for 20 to 21 h with single and group culture methods for their better maturation using mSOF. It could be inferred from these results that drop volume/size has no effect on the maturation rate of bovine oocytes however; incubation time has shown significant effect on the elementary development of bovine oocytes in *in vitro*. Similar results have been reported previously by Long *et al.* (1994).

Table 1. *In vitro* maturation rates of Japanese black cattle oocytes by a single or group culture condition in modified synthetic oviduct fluid medium

Culture condition	Maturation time (h)		
	16~17	18~19	20~21
Single[1]	57.1±15.0^{a}	67.7±24.3	88.9± 6.60
Group[2]	27.1±16.8^{b}	64.9±20.6	84.3±13.9
Total	45.9±21.3	66.3±20.2	86.3± 9.9

^{a,b} Means (±SEM) within the same column with different superscripts differ significantly ($p<0.05$).

[1] The oocytes were matured (% of MⅡ) in the drop size of 10 μl (single; 1 oocyte/drop).

[2] The oocytes were matured (% of MⅡ) in the drop size of 100 μl (group; 20 oocytes/drop).

Blastocyst development rate was higher ($p<0.01$) with group culture compared with single culture of embryos at both 7 and 8 days of embryonic development. But, percent blastocyst yield per zygote cleaved was not ($p<0.01$) different between single and group culture methods (Table 2). Results from this experiment clearly indicate the benefits of culturing oocytes in group compared with single culture using mSOF. Higher zygote cleavage rate and blastocyst development in group culture than single culture could be attributed to the synergistic effects because of some unknown growth factors. Further in group culture the biophysical and biochemical environment is probably more favorable for the development of bovine embryos compared with single culture method. Present results confirm earlier findings (Sirard *et al.*, 1988; Leibfried and First, 1979), which demonstrated that group culture was superior to single oocyte culture. Further, in the present study (Yadav *et al.*, 2000), compact cumulus, homogeneous or slightly granulated ooplasmas (COCs) were used to exclude oocytes with obvious morphological signs of atresia. Our observations indicate that morphological selection skill of oocytes is more important for embryonic development than culture drop size. In this study, we used follicles > 2mm in diameter because the relationship between developmental compatibility of an oocyte to its morphology has been reported to influence its maturation under any culture condition (Jewgenow *et al.*, 1999;

Table 2. Cleavage and blastocyst development rates by a single or group culture condition in modified synthetic oviduct fluid medium

Culture condition	Number of zygote	Cleavage rate (%)	Blastocyst (BL) development rate (%)			% of BL/zygote cleaved
			Day 7	Day 8	Total	
Single	530	83.5±8.1	11.4±9.5^{a}	7.0±6.5^{a}	18.4±10.7^{a}	21.4±10.6
Group	568	86.4±9.0	19.8±9.3^{b}	14.1±7.6^{b}	34.2±13.3^{b}	39.0±13.1

^{a,b} Means (±SEM) within the same column followed by different superscripts differ ($p<0.01$).

Blobdin and Sirard, 1995).

Cell numbers of developed embryos were similar when cultured using either single or group system from 6.5 to 8 days of embryonic development (Table 3). Development of frozen-thawed embryos to the blastocyst stage, their numbers of hatching and live embryos were not significantly different between single and group culturing methods at 24 h of incubation (Table 4). Mean live, dead and M phase (division phase) cells numbers were noticed similar in frozen-thawed embryos culture either using single or group methods (Table 5). Previously (Makarevich *et al.*, 2005), it was reported that with addition of epidermal growth factor (EGF) and bovine follicular growth factor (bFGF) to the maturation drop, blastocyst yields for single oocytes were increased (Catherine *et al.*, 1996; Lim and Hansel, 1996; Lonergan *et al.*, 1996; Lee *et al.*, 1995). However, in our culture system EGF and bFGF were not added. It is evident from the results presented here that oocytes cultured singly are not lacking some vital support which allows for their development to the blastocyst stage, suggesting that pre-implantation embryos are capable of promoting their own development in mSOF culture medium. In studies involving mouse (Sirard *et al.*, 1988) or sheep (Gardner *et al.*, 1994) embryos, no difference was noticed on blastocyst development between group and single culture systems. A similar trend in blastocyst cell number and viability rate was noted in our study.

Table 4. Survival rate after freezing and thawing of Japanese black cattle embryos derived from a single or group culture condition

Culture condition	Number of embryos	Embryonic developmental stages (%)		
		Blastocyst	Hatched	Live embryos
Single	62	15 (24.2)	13 (20.9)	28 (45.2)
Group	126	38 (30.2)	16 (12.7)	54 (42.8)

CONCLUSION

This study was aimed to improve the efficiency of bovine (Japanese black cattle) embryo production using a modified synthetic oviduct fluid medium (mSOF) in a single (1 oocyte/drop) or group (20 oocytes/drop) culture condition. The cumulus-intact oocytes were cultured either singly (small drop size) or group (large drop size) in a maturation medium (TCM-199 containing 10% fetal calf serum, 0.02 IU/ml follicular stimu-

Table 3. Cell numbers of developed live embryo by a single or group culture condition of Japanese black cattle embryos

Culture condition	Embryonic culture time (days)			
	6.5	7.0	7.5	8.0
Single	82.0±24.4 (13)	168.0±20.5 (11)	118.7±67.1 (13)	103.0±26.7 (12)
Group	141.9±51.9 (16)	180.6±32.2 (19)	123.9±38.9 (33)	114.4±53.1 (19)

Table 5. Mean number of live cells, dead cells and M phase cells of frozen- thawed embryos (n=10 at each time point) by days of culture

Culture condition	Cell condition	Embryo culture time (days)					Overall mean (%)
		6.0	6.5	7.0	7.5	8.0	
Single	Live	65.3	88.0	66.4	61.0	56.0	67.3 (84.3)
	Dead	12.3	20.0	14.8	12.5	3	12.5 (15.7)
	M[1]	1	2	–	–	1	–
Group	Live	101.0	80.2	75.3	100.3	35	78.4 (84.7)
	Dead	20.5	15.9	15.7	14.6	4	14.1 (15.3)
	M	2	1.8	1.2	1.4	1	–

[1] : M phase cell ; division phase cell.

lating hormone and 1 μg/ml estradiol-17β). The blastocyst stage embryos were frozen by direct-transfer freezing method with 1.5 M ethylene glycol and their developmental stages and cell numbers were examined after thawing. Maturation rate of bovine oocytes by culture condition was significantly higher with single culture compared with group culture at 16 to 17 h of incubation ($p<0.05$). However, similar maturation rates were observed between single and group cultured oocytes at 18 to 19 and 20 to 21 h of incubation. The maturation rates of oocytes were increased with their increasing incubation time. Mean zygote cleavage rates were noticed similar between single and group cultured methods. Blastocyst development rate was higher with group culture compared with single culture of embryos at both 7 and 8 days of embryonic development ($p<0.01$). Percent blastocyst yield per zygote cleaved was not different between single and group culture methods. Cell numbers of developed embryos were similar when cultured using either single or group system from 6.5 to 8 days of embryonic development. Development of frozen-thawed embryos to blastocyst, their numbers of hatching and live embryos were not significantly different between single and group culturing methods at 24 h of incubation. In conclusion, blastocyst development rate was higher ($p<0.01$) with group culture compared with single culture of embryos at both 7 and 8 days of embryonic development in Japanese black cattle.

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