韓國受精卵移植學會誌(1994) 第 9 卷 第 1 號
Korean J. Emb. Trans. (1994) Vol.9, No.1 pp.53~63

Culture of Bovine Embryos Derived from IVM / IVF into Blastocysts in Defined Simple Media with Different Concentrations of CO_2 and O_2

B. K. Yang, J. B. Kim, H. T. Cheong and C. I. Kim
College of Animal Agriculture, Kangwon National University

CO_2와 O_2의 농도가 소 체외수정란의 체외발육에 미치는 효과

양부근 · 김종복 · 정희태 · 김정익
강원대학교 축산대학

적 요

소 체외수정란의 체외 발육에 CO_2와 O_2의 농도가 미치는 영향에 대하여 두가지 배양액과 서로 다른 Co-culture 체계를 이용하여 검토하였다. 체외 수정후 40~44 시간에 회수한 2~8 세포기 수정란을 여러가지 gas조건하의 다른 배양조건에서 무작위로 옮겨 실험을 수행하였다.

5% CO_2와 5% O_2, 10% O_2 및 20% O_2 조건에서 배양을 실시한 결과, 상실배기 이상 발육된 체외 발육성적은 각각 22.1%, 15.0% 및 12.1%였다($p<0.05$). 한편 배양액에 따른 체외 배양성적은 KSOM배양액 (19.1%)의 경우가 Menezo's B2 배양액(13.7%)에서 배양한 경우보다 더 높은 성적을 얻었다($p>0.05$, Table 1).

2~8세포기 수정란을 5% CO_2 또는 10% CO_2 와 5%, 10% 및 20%의 O_2 조건 하에서 체외 배양을 실시한 경우 각각 15% 와 8%의 체외 발육 성적을 얻었고($P>0.05$), O_2 조건의 경우 10% O_2(17%)와 20% O_2(20%)의 배양조건이 이 5% O_2 (26%)보다 낮은 성적을 나타냈다($P<0.05$), (Table 2).

체외 수정 후 얻은 2~8세포기 수정란을 단순 배양액 과 두개의 다른 공동 배양체계를 이용하여 5% CO_2와 5%, 10% 및 20% O_2의 배양 조건하에서 체외 배양을 실시한 결과 상실배와 배반포기 까지 발육된 체외 발육 성적은 배양액과 공동배양 체계에 따른 차이는 인정되지 않았다($p>0.05$). 그러나 O_2의 농도은 소 체외 수정란의 체외 발육성적에 영향을 미치는 것으로 나타났다(Table 3).

본 실험은 CO_2와 O_2의 농도가 소 체외수정란의 체외 배양할때 배양체계에 따라 다른 영향을 미치는 것으로 나타났다.

Introduction

The incidence of fertilization is high in most mammals around 90 to 100% *in vivo*, but pregnancy fail has showed up to 60 percent during early development depending on species(Overstrom, 1987). The embryonic mortality is the effect of endocrine, hereditary, nutritional and environmental components. The development into blastocysts of embryos derived from IVM /IVF may be also same conditions as *in vivo*. The important aspects of mammalian embryo developmessnt *in vitro* has includes the culture media

for supporting embryo development, such as complex and simple defined media with or without co-culture system containing BSA or serum, and gaseous environment during *in vitro* culture. A lot of studies have attemped to establish the optimal culture condition for supporting the development of bovine embryos derived from IVM /IVF, there has not been a optimal culture condition yet(Bavister *et al.,* 1992). The attempts to improve the culture environment by co-culture system with BOEC and BRL cell may be increased recently the development into blastocysts of bovine IVM /IVF embryos (Ellington *et al.,* 1990; Voelkel and Hu, 1992).

The contribution made by somatic cell co-cultured to the in vitro development of embryo may showed the production of stimulating factor or removal of inhibitory compounds in culture medium to support the embryo development (Bavister *et al.,* 1992). Recent data showing that bovine embryos produced from IVM /IVF with BRL cell co-culture has got a similar results of that of BOEC co-culture. (Voelkel and Hu, 1992). The gaseous environment for mammalian embryo culture is an important factor in their developmental capacity. A common gas environment for mammalian embryo culture has been 5% CO_2 and humidified air(Wright and Bondioli, 1981; Biggers, 1987).

The O_2 is important for energy producing metabolism(Fridhandler, 1961), oxygen consumption is a physiological parameters of blastocyst differentitation such as trans-trophectodermal Na^+ transport and protein synthesis activity (Overstrom, 1987). Rabbit blastocysts have higher oxygen consumption value than mouse blastocysts of comparable age. While the oxygen consumption of rabbit blastocysts decreases 30 to 40 percent after day 5, blastocoelic fluid continues to accumulate at ever-increasing rates(Borland *et al.,* 1977). The oxygen consumption of pig blastocysts is higher than that of rabbit blastocysts(Overstrom *et al,* 1984). The CO_2 is important metabolic regulators of embryos development(Brinster, 1971; Carney and Bavister, 1987), and is required to maintain the pH of bicarbonate buffered media and is readily incorporated into protein and nucleic acids by the mouse embryo(Graves and Biggers, 1970). A reduced oxygen level, between 5 to 7%, has been reported increaseing for embryo development than the 20% on the mouse(Quinn and Harlow, 1978; Umaoka *et al.,* 1992), sheep(Tompson *et al.,* 1990), hamster(McKiernam and Bavister, 1990), rabbit(Li and Foote, 1993), swine (Wright, 1977), cattle(Tompson *et al.,* 1990; Tervit *et al.,* 1992) and goat(Batt *et al.,* 1991).

The benifit conferred by a reduced oxygen level may be attributed to a decrease in the formation of superoxide radicals, which have been associated with developmental arrest in the mouse(Noda *et al.,* 1991; Legge and Sellens, 1991) Voekel and Hu(1992) reported that co-cultured IVM /IVF bovine embryos with different cell type, such as buffalo rat liver cells or bovine oviduct epithelial cells, was favor to different concentration of oxygen. The purpose of this study was to compare the effect of media and co-culture system under different level of corbon dioxide and oxygen on the development during culture into morulae and blastocysts of 2- to 8-cell bovine embryos produced by IVM /IVF.

Meterials and Methods

1. Experimental design

Three separate experiments were carried out each using a completely randomized design to compare the gas environment, complex and sim-

ple media, and two different cell types of co-culture system with different concentration of carbon dioxide and oxygen. Experiment 1 was replicated three times using a total of 691 two to eight cell embryos derived from IVM/IVF in two different culture medium with 5% CO_2 and different levels of oxygen(5%, 10% and 20%).

Experiment 2 was replicated three times using a total of 744 embryos, collected as above. The gas phase of *in vitro* culture was choosen 5% or 10% CO_2, and different concentrations of oxygen in Bigger's KSOM as a simple defined medium. Experiment 3 was replicated four times using a total of 371 embryos, collected as above. The gas phase of *in vitro* culture was used 5% CO_2 and 5%, 10% and 20% oxygen and culture system in Experiment 3 were used in KSOM with bovine oviduct epithelial cell(BOEC) and buffalo rat liver(BRL) monolayer.

2. Oocytes, oocyte maturation and *in vitro* fertilization

Oocytes collection, maturation and *in vitro* fertilization of bovine oocytes was performed according to Yang *et al.* (1993), as described previously. Briefly mentioned that, bovine ovaries were obtained from a local slaughter house and were transported to the laboratory in Dulbecco's phosphate buffered saline containing 0.1% polyvinyl alcohol(PBS-PVA) within 2 hours.

Good-quality oocytes with compact cumulus cell were selected and transferred 100 ul drop of TCM 199 containing 25 mM Hepes(Gibco, Grand Island, NY) and 10% fetal bovine serum (FBS;Gibco) with 0.5 μg/ml FSH, 5μg/ml LH and 1 ug/ml E2.Fifteen good oocytes per drop were matured for 20 to 22 hours with 5% CO_2 and 95% humidified air at 39°C. After maturation, oocytes surrounded by expanded cumulus cell were washed twice in maturation and in IVF medium each, and 10 oocytes were introduced into each 50μl fertilization drop. Fertilization medium was made in Brackett and Oliphant medium(BO, 1975) supplemented with 20mg/ml of fatty acid free BSA(sigma chemical Co., St. louis, MO) and 20μg/ml of heparin (Sigma). Frozen semen from the same bull was used in all experiment.

After thawing, spermatozoa were added to BO medium containing 10mM of caffeine and washed twice by centrifugation at 350×g for 10 minutes. Insemination was performed by placing the 50μl of sperm suspention into each fertilization drop. The final concentrations in the fertilization drop were 5 mM caffine, 10μg/ml heparin and 1.25×10^6 motile sperm/ml. After 40~44 hours of culture, 2 to 8 cell embryos were freed of cumulus cells by repeated pipetting and were allotted randomly to experimental culture media. Embryos were cultured for 5 to 6 days in experimental gasous environment at 39°C. The culture medium was changed every 2 days.

3. Bovine oviduct epithelial cell monolayer preparation

Oviduct cell monolayer was made according to Ellington *et al.* (1989), as reported previously. Briefly, bovine oviducts from local slaughterhouse were transported in plastic bag on ice within 2~3 hours and trimmed free of connective tissue surrounding the oviduct and washed in PBS-PVA. The BOEC were freed by flushing and trasferred to 15 ml centrifuge tube and then washed twice with PBS-PVA and Ham's F 10 supplemented with 10% FBS by centrifugation. They were trasferred to 4-well dishes for culture with same medium for 5 to 7 days. At 24 hours before use, they were changed fresh experimental medium.

4. Buffalo rat liver cell monolayer preparation

The BRL cells were obtained from American

Type Culture Collection(Rockville, MD). Frozen cells were thawed at 37°C in a water bath and transferred into a 15 ml centrifuge tube with 5 ml low glucose Defined Modified Eagle's Medium(DMEM, Gibco) containing 5% FBS. BRL cells were washed by centrifugation at 250×g for 5 minutes. Media was removed and the cells were resuspended in 5 ml DMEM and transferred to a 60 mm tissue culture dish(Corning, NY) and incubated in an atmosphere of 5% CO_2:95% air at 39°C for 1~2 days. After 1~2 days of culture, BRL cells were detached from the culture dishes using 0.25% trypsin with 1 mM EDTA(Gibco).

Released cells were counted with a hematocytometer before making BRL monolayer. The BRL co-culture wells were prepared by introducing 5×10 cells in four-well plates(Nunk, Denmark) with 0.5 ml of DMEM medium 2 days prior to use. DMEM was removed and culture medium was added to the co-culture wells 24 hours before use. The culture medium was replaced every 2 days. The remaining cell suspension was used to make another BRL monolayer dish and maintained in 5% CO_2;95% humidified air at 39°C. The medium in culture dish was changed every 3 days.

5. Embryo culture

The culture dishes were placed within modular incubator chambers purged with the apporpriate gas mixture and with a water-filled dish to supply humidify. The chamber was regassed every 24 hours for 1~2 min with the appropriate gas, sealed and placed at 39°C.

6. Embryo evaluation

After 7~8 days of culture, each embryo was evaluated for appearance and stage of development under light microscope.

7. Statistical analysis

Each batch of oocytes were distributed across all treatments with batchs replicated three or more times in each experiment. Replicates were considered random and treatments as fixed in the analysis of variance of percentage data, using a general mixed linear models procedure (SAS, Institute, Cary, NC). Tukey's honest significant difference was used to test for statistical differences among means.

Results

1. Experiment 1:Effects of media under 5% CO_2 with various O_2 levels

Two-to 8 cell stage embryos collected 40 to 44 hours after *in vitro* fertilization were cultured in KSOM and B2 media under 5% CO_2, and 5% 10% and 20% O_2 environment. The proportion of embryos developing into morulae plus blastocysts and blastocysts in 5% O_2 was higher than those of 10% and 20% O_2 levels($P<0.05$) and were not difference in morulae stage among groups($P>0.05$) And also the development rate according to media were similar to the results of the various concentrations of O_2($P>0.05$)(Table 1).

2. Experiment 2:Effects of different concentration of CO_2 and O_2 in defined simple culture media

Embryonic development in different concentration of CO_2 and O_2 in KSOM containing BSA is summarized in Table 2. There were no significant difference between 5% and 10% CO_2 group in the development into morulae plus blastocysts($P>0.05$), but the development into blastocysts in 5% CO_2(15%) was better than 10% CO_2(6%, $P<0.05$) and the other hand blastosyst development in 5%(15%) and 20%

Table 1. Culture of IVM/ IVF bovine embryos in Bigger's KSOM vs Menezo's B2 medium with 5% CO_2 and different concentrations of O_2

Conc. of O_2%	Media	No. of ICM /IVF embryos	% of embryos developed to:			Morulae plus blastocysts (%)	Blastocyst cell number (Mean S.E.)
			2~8 cells	Morulae	Blastocysts		
5	KSOM	111	74[a]	10[a]	16[a]	26[a]	77.3[a]± 4.4
	B2	121	80[a]	11[a]	9[ab]	20[a]	75.5[a]± 4.3
10	KSOM	109	82[a]	11[a]	7[ab]	18[a]	80.9[a]± 5.4
	B2	120	89[a]	9[a]	2[b]	11[a]	94.5[a]±10.5
20	KSOM	115	86[a]	6[a]	8[ab]	14[a]	62.6[b]± 4.0
	B2	115	88[a]	8[a]	4[b]	12[a]	58.0[b]± 1.9

ab : Means with different superscripts within treatment groups and stage of embryo development are different, P<0.05.

Table 2. Developmental capacity of IVM/ IVF bovine embryos in KSOM medium at 5% and 10% CO_2 and 5%, 10% and 20% O_2

CO_2 (%)	O_2 (%)	No. of ICM /IVF embryos	% of embryos developed to:			Morulae plus blastocysts (%)	Blastocyst cell number (Mean S.E.)
			2~8 cells	Morulae	Blastocysts		
5	5	119	65[a]	16a	19[a]	35[a]	67.7[a]±2.8
5	10	125	84[a]	7[a]	8[b]	16[a]	82.1[b]±5.8
5	20	126	75[a]	9[a]	17[a]	25[a]	62.5[a]±2.6
10	5	123	83[a]	7[a]	10[a]	17[a]	65.8[a]±3.6
10	10	126	81[a]	12[a]	7[a]	19[a]	74.4[a]±4.6
10	20	125	85[a]	9[a]	6[b]	15[a]	76.4[a]±6.8

ab : Means with different superscripts within treatment groups and stage of embryo development are different, P<0.05.

O_2(12%) was significantly different from 10% O_2(7%, P<0.05).

3. Experiment 3: Effects of two different co-culture system under 5% CO_2 with 5%, 10% and 20% O_2 levels

When 2- to 8-cell embryos were cultured in 5% CO_2 with 5%, 10% and 20% O_2 using two different co-culture system in KSOM and CZB media. The development of embryos reaching to morulae plus blastocysts and blastocysts only were not different according to media and co-culture system(P>0.05). The higher proportion of embryos co-cultured with BOEC or BRL in 5% CO_2 and 10% O_2 reached morulae plus blastocysts stage than 5% CO_2 with 5% O_2 (P<0.05).

Discussion

The major objectives of this study were to determine the optimal gas environment of bovine embryos produced by IVM and IVF oocytes and to improve the development beyond morulae and blastocysts. The oxygen tension within the female reproductive tract is 5.2~5.9% in rat

(Yochim and Mitchell, 1968), 7.0~11.2% in rhesus monkey and rabbit(Maas *et al.*, 1976;Mastroianni and Jones, 1965).

The environmental difference between *in vitro* and *in vivo* for embryo development is a higher oxygen levels in the *in vitro* than in the oviducts. Reducing the oxygen concentration for mammalian embryos culture *in vitro*, 5% to 10%, results in a higher proportion of blastocysts in bovine(Fukui *et al.*, 1991;Nakao and Nakatsuji, 1990;Thompson *et al.*, 1990).

The beneficial effects by a reduced oxygen concentration may be attributed to a decrease in the formation of superoxide radicals which have been associated with developmental block of embryos in mouse(Noda *et al.*, 1991; Legge and Sellens, 1991). Oxygen radical have been implicated the ovulation(Miyazaki *et al.*, 1991), luteolysis(Sawada and Carlson, 1991), and sperm capacitation(Bize *et al.*, 1991) and may be important factors in the regulation of growth and development by stimulating intracellular calcium release and increasing the expression of growth-related proto-oncogenes(Sohal and Allen, 1990; Cerutti, 1991).

Nakao and Nakatsuji reproted that reduced oxygen gas pressure from 20% to 5% improved the development of the embryos into blastocysts in bovine(14% vs 8%). And also Fukui *et al.* (1991) reported that when oocytes inseminated *in vitro* were cultured in TCM 199 with 10% FBS, 5% oxygen was superior to 20% for development of bovine embryos into blastocysts(12.9% VS 2.5%), and simple media as synthetic oviduct fluid media was better than that of complex media as TCM 199 for development under low oxygen levels. In our study(Experiment 1) show that overall means of the development into blastocysts in 5% oxygen level was higher than those of 10% and 20% oxygen($P<0.05$) and KSOM was superior to B2 for both blastocysts and morulae plus blastocysts($P<0.05$). Our results are consistant with these reports. Regarding of these results, we can infer that 5% oxygen concentration is better than 10% and 20% oxygen levels regardless of media for developing of bovine embryos *in vitro*. In mammalian embryo culture *in vitro*, the common concentration of CO_2 is 5%, there was no a lot of information on the CO_2 requirements of preimplation embryos developingx *in vitro* and the role that CO_2 plays in the regulation of early development(Brinster,1971). McLinans(1972) reported that CO_2 has been shown to be a potent regulator of glycolysis and amino acid, pyrimidine systhesis and participates in many other metabolic activitis. CO_2 is an important regulator of intracellular pH(Carney and Bavister, 1987). In a study with rabbits, Hallden *et al.* (1992) reported that 10% CO_2 at atmosphere experts a beneficial effect than 5% CO_2 on the development of zygotes into expanding and hatching rabbit blastocysts *in vitro*. Carney and Bavister (1987) reported that a high proportion of hamster 8-cell embryos developing to blastocysts was oberserved upon raising the concentration of CO_2 in the gas phase from 5% to 10% CO_2, suggesting the action of CO_2 as a weak acid in regulating intracellar pH.

In Experiment 2, when 2- to 8-cell embryos derived from IVM/IVF oocytes were cultured in KSOM with 0.1% BSA under various concentration of CO_2 and O_2, the overall means of 5% CO_2 yielded better results than 10% CO_2(24.5% vs 17.9%), showing both for developing into morulae plus blastocysts and blastocysts alone, but there were no significantly difference in all developing stage. Wang *et al.* (1992) reported that when bovine embryos derived from IVM/IVF oocytes were cultured TCM 199 containing 25 mM and 50 mM $NaHCO_3$ with 10% estrus cow serum under 5% and 10% CO_2 in air,

5% CO_2 was better results than that of 10% CO_2, but there was no significantly difference. Our results is consistant with that reported by Wang *et al.*(1993) but is disaccordance with studies Carney and Bavister, and Hallden *et al.* The reason for disaccordance in results from that of Carney and Bavister, and Hallden *et al.* is not known, but it may be due to different species.

In Experiment 3, when 2- to 8-cell bovine embryos were co-cultured in KSOM containg 0.1% BSA with bovine oviduct epithelial cells without oil overlay, 10% oxygen level for developing morulae and blastocysts were better than that of 5% O_2. This finding is consistant with that reported by Fukui *et al.* (1991) who used 0.5 ml of TCM 199 co-cultured with bovine oviduct cells without oil overlay to support development of bovine embryos produced by IVM/IVF.

In contrast, Voelkel and Hu(1992) reported that when bovine embryos were co-cultured in microdrops of TCM 199 medium with 10% FCS and oviduct cells covered with oil, 5% oxygen in the gas atmosphere yielded better results than that of 20% oxygen. The reason for the disaccordance between these experiments is not known, but it is may be due to difference from the IVF method and culture media and the presence or absence of oil overlay. When embryos were co-cultured with buffalo rat liver cells, 10% O_2 level for reaching morulae and blastocysts was better than that of 5% O_2($P<0.05$).

In our results indicated that the effects of oxygen concentration in BRL cell co-culture is similar trends of BOEC co-culture for developing into motulae and blastocysts. This finding is consistant with studies of Voekel and Hu(1992) reported that bovine embryos were co-cultured with buffalo rat liver cells 20% oxygen was more efficacious than the 5% oxygen for supporting embryos development. The different oxygen levels are required for presence or absence of feeder cells in culture of bovine embryos between Experiment 1 and 3 is not clear. Feeder cells may be need more oxygen tension for the development of bovine embryos produced by IVM/IVF oocytes to morulae and blastocysts. The oxygen tension within in the female reproductive tract is approximatrly 5% to 7% in various species. The toxic effect of O_2 is not seen at 5% O_2 concentration, suggesting that atmospheric O_2 concentration are toxic to preimplantation mouse embryos(Pabon *et al.*, 1989)

This investigations indicates that oviduct cells required lower than 5% CO_2 in air. Voekel and Hu reported that BRL cell may be adapted to higher oxygen concentration in which they were established and maintained than those of oviduct cells, but growth curves of BRL cells in 5% or 20% oxygen levels are not different. In addition, Experiment 1 and 3 has used different volume of medium and with or without an oil overlay. This were addtional factors which varied between two experiment whether or not the potential effects of these factors for developing embryos is not clear, but the optimal gas phase for culturing embryos *in vitro* may be altered by the culture system.

The developmental rate in morulae plus blastocysts co-cultured with BRL cell has get similar results of those of co-cultured with BOEC co-culture. This finding is consistant with study of Voekel and Hu, they used the co-culture of bovine embryos with BRL cell resulted in development rates equivalant to those of embryos co-cultured with bovine oviduct epithelial cells. Buffalo rat liver cells have been shown to produce a polypeptide fraction with multiplication-stimulating activity(MSA), non suppressible insulin-like activity, and sulfation fac-

Table 3. Effect of gas phase on development of IVM/ IVF bovine embryos co-cultured with BOEC or BRL cells

CO_2 (%)	O_2 (%)	Media	Cell type	No. of ICM /IVF embryos	% of embryos developed to: 2~8 cells	Morulae	Blastocysts	Morulae plus blastocysts (%)	Blastocyst cell number (Mean S.E.)
5	5	KSOM	BOEC	77	63[a]	27[a]	10[a]	37[a]	94.0[b]±5.3
5	5	KSOM	BRL	73	71[a]	9[a]	20[a]	29[a]	86.8[a]±4.1
5	10	KSOM	BOEC	75	55[a]	24[a]	21[a]	45[a]	89.5[a]±4.3
5	10	KSOM	BRL	70	63[a]	16[a]	22[a]	37[a]	90.6[a]±5.1

[ab]Means with different superscripts within treatment groups and stage of embryo development are different, $P<0.05$.

tor activity, which had chemical properties similar to the MSA of calf serum, requiring for animal cell multiplication, that is the sulfation factor activity of human serum(Dulak and Temin, 1973). These factor containing buffalo rat liver cells may be affect cell attachment, survival and mitosis (Temin *et al.*, 1972).

Buffalo rat liver cells has an abandant source of growth factor such as insulin like growth factor, showing display 93% homology with the functionally related human insulin-like growth factor II(Moses *et al.*, 1980;Marquardt and Todaro, 1981). Buffalo rat liver cells also produce differentiation inhibiting activity(DIA), which is attributable to an acid-stable, non-dialysable activity and is not reproduced by some growth factor such as IGF II and TGF known to be produced by BRL cells(Smith and Hooper, 1987).

And also it is likely that LIF and DIA are the same molecular based on a number of biological and biochemical similarities. Both substances inhibit differentiation of embryonic stem cells and stimulate growth in a variety of cells(Gough *et al.*, 1989). LIF, an excellent pleiotropic growth factor, is produced by endometrial glands and receptors have been found on the 4 day-old mouse embryos and has a physiolosical role in the development of the embryos(Fry *et al.*, 1992). In conclusion, the optimal gas concentration for culturing of bovine embryos beyond morulae stages is different depending on presence or absence of the use of co-culture system. Our results indicated that buffalo rat liver cells co-culture can used to support the development of bovine IVM /IVF embryos repleacing BOEC, which is easily maintained and established for embryo culture.

Summary

The effect of various gas environment for development of bovine embryos produced by IVM /IVF was examined by the use of two different media and two co-culture system. The embryos were in the 2-to 8-cell stage collected at 40 to 44 hours after *in vitro* fertilization and were alloted randomly to experimental culture media under various gas condition. In Experiment 1, the percentage of embryos developing beyond morulae stages in 5%, 10% and 20% O_2 with 5% CO_2 in high humidified air was 22.1%, 15.0% and 12.1%($P<0.05$), respectively.

More embryos developed into morulae plus blastocysts(19.1%) in KSOM medium than in B2 medium(13.7%; $P<0.05$). In Experiment 2, The proportion of 2- to 8-cell produced from IVM /IVF developing into blastocysts in 5%

CO_2 and 10% CO_2 was 15% and 8%($P<0.05$), and the other hand in the 10% O_2(17%) was lower than those of 5% O_2(26%) and 20% O_2 (20%), respectively. In Experiment 3, 2- to 8-cell embryos derived from IVM/IVF oocytes were cultured simple defined medium and two co-culture system as bovine oviduct epithelial cell and buffalo rat liver cell under 5%, 10% and 20% O_2 with 5% CO_2. The proportion of embryos reaching morulae and blastocysts was not different depending on media and co-culture system ($P>0.05$), but O_2 concentration was affects the development of morulae plus blastocysts(33.2% vs 41.2%; $P<0.05$).

These experiments indicate that carbon dioxide and oxygen concentration can affect the development of bovine embryos produced by IVM/IVF when in a simple medium with and without co-culture system.

References

Batt PA, Gardner DK, Cameron AWN. 1991. Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. Reprod. Fertil. Dev. 3:601-607.

Bavister BD, Rose-Hellekant TA and Pinyopummintr T. 1992. Development of *in vitro* matured/*in vitro* fertilized bovine embryos into morulae and blastocysts in defined culture media. Theriogenology 37:127-146.

Biggers JD. 1987. Pioneering mammalian embryo culture. In:The mammalian preimplantation embryo. Ed. by Bavister, BD. Plenum Press New York pp 1-22.

Bize I, Santander G, Cabelo P, Driscoll D and Sharpe C. 1991. Hydrogen peroxide is involved in hamster sperm capacitation *in vitro*. Biol. Reprod. 44:398-403.

Borland RM, Biggers JD and Lechene CP. 1977. Fluid tansport by rabbit preimplantation blastocysts. J. Reprod. Fertil. 52:131-135.

Brackett BG and Oliphant G. 1975.Capacitation of rabbit spermatozoa *in vitro*. Biol. Reprod. 12:260-274.

Brinster RL. 1971. *In vitro* culture of the embryo. In:Pathways to conception. Ed. by Sherman A and Charles C. Tomas, pp. 245-277.

Carney EW and Bavister BD. 1987. Regulation of hamster embryo development *in vitro* by carbon dioxide. Biol. Reprod. 36:155-1163.

Cerutti PA. 1991. Oxidant stress and carcinogenesis. Eur. J Clin. Invest. 21:1-5.

Dulak NC and Temin HM. 1973. A partilly purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. J. Cell. Physiol. 81:153-160.

Ellington JE, Farrel PB, Simkin ME, Foote RH, Goldman EE and McGrath AB. 1990. Development and survival after transfer of cow embryos cultured from 1-2 cells to morulae or blastocysts in rabbit oviducts in a simple medium with bovine oviduct epithelial cells. J. Reprod. Fertil. 89:293-299.

Fry RC, Batt PA, Fairclough RJ and Parr RA. 1992. Human leukemia inhibitory factor improves the viability of cultured ovine embryos. Biol. Reprod. 46:470-474.

Fridhandler L. 1961. Pathways of glucode metabolism in fertilized rabbit ova at various pre-implantation stages. Exp. Cell Res. 22:303-316.

Fukui Y, McGowan LT, James RW, Pugh PA and Tervit HR. 1991. Factors affecting the in vitro development to blastocysts of bovine oocytes matured and fertilized *in vitro*. J. Reprod. Fertil. 92:125-131.

Gough NM, Williams RL, Hilton DJ, Pease S, Willson TA, Stahl J, Gearing DP, Nicola

NA and Metcalf, D. 1989. LIF:a molecule with divergent actions on myeloid leukaemic cells and embryonic stem cells. Reprod. Fertil. Dev. 1:281-288.

Graves CN and Biggers JD. 1970. Carbon dioxide fixation by mouse embryos prior to implantation. Science 167:1506-1508.

Hallden K, Li J, Carney EW and Foote RH. 1992. Increasing carbon dioxide from five percent to ten percent improves rabbit blastocyst development from cultured zygotes. Mol. Reprod. Dev. 33:276-280.

Legge M and Sellens MH. 1991 Free radical scavengers ameliorate the 2-cell block in mouse embryo culture. Hum. Reprod. 6:867-871.

Li J and Foote RH. 1993. Culture of rabbit zygotes into blastocysts in protein- free medium with one to twenty percent oxygen. Mol. Reprod. Dev. (in press).

Marquardt H, Todaro GJ, Henderson LE and Oroszian S. 1981. Purification and primary structure of a polypeptide with multiplication stimulating activity from rat liver cell cultures. J. Biol. Chem. 256:6859-6865.

Mass DHA, Storey BT, Mastroianni L, Jr. 1976. Oxygen tension in the oviduct of the Rhesus monkey. Fertil. Steril. 27:1312-1317.

Mastroanni L and Jones R. 1965. Oxygen tension within the rabbit fallopian tube. J. Reprod. Fertil., 9:99-102.

Mckierman SH and Bavister BD. 1990. Environmental variables influencing *in vitro* development of hamster 2-cell embryos to the blastocyst stage. Biol. Reprod. 43:404-413.

McLinans WF. 1972. The gaseous environment of the mammalian cell in culture. In: Growth, nutrition and metabolism of cells in culture. Ed. by Rothbalt, GH, Cristofalo VJ. New york Academic Press. pp. 138-170.

Miyazaki T, Sueoka K, Dharmarajan AM, Atlas SJ, Bulkley GB and Wallach EE. 1991. Effect of inhibition of oxygen free radical on ovulation and progesterone production by the in vitro perfused rabbit ovary. J. Reprod. Fert. 91:207-212.

Moses AC, Nissley SP, Short PA and Rechler MM. 1980. Immunological cross-reactivity of multiplication-stimulating activity polypeptides. Eur. J. Biochem. 103:401-408.

Nakao H and Nakasuji N. 1990. Effect of co-culture, medium components and gas phase on *in vitro* culture of *in vitro* matured and *in vitro* fertilized bovine embryos. Theriogenology 33:591-600.

Noda Y, Matsumoto H, Umaoka Y, Tatsumi K, Kishi J and Mori T. 1991. Involvement of superoxide radicals in the mouse two-cell block. Mol. Reprod. Dev. 28:356-360.

Overstrom EW. 1987. *In vitro* accessment of blastocyst differentiation. In: The mammalian preimplation embryo. Ed. by Bvister, BD. Plenum Press, New York. pp. 95-116.

Overstrom EW, Bones DJ, Biggers JD and Godkin JD. 1984. Transtrophectodrmal sodium transport during porcine blastogenesis. BIol. Reprod. 30(Suppl. 1):46.

Pabon JE. Jr., Findley WE and Gibbons WE. 1989. The toxic effect of short exposures to the atmospheric oxygen concentration on early mouse embryonic development. Fertil. Steril. 51:896-900.

Quinn P and Harlow GM. 1978. The effect of oxygen on the development of preim- plantation mouse embryos in vitro. J. Exp. Zool. 206:73-80.

Sawada M and Carlson JC. 1991. Rapid plasma membrane changes in superoxide radical formation, fluidity and phospholipase-A2 activity in the corpus luteum of the rat during induction of luteolysis. Endocrinology 128:

2992-2998.

Smith AG and Hooper ML. 1987. Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. Dev. Biol. 121:1-9.

Sohal RS and Allen RG. 1990. Oxidative stress as a causal factor in differentiation and aging: a unifying hypothesis. Exp. Gerontol. 25:499-522.

Temin HM, Pierson RW. Jr. and Dulak NC. 1972. The role of serum in the control of multiplication of avian and mammalian cells in culture. In:Growth, nutrition, metabolism of cells in culture Ed. by Cristafalo and Rothblat G. New York, Academic Press. pp. 50-81.

Tervit HR, WHittingham DG and Rowson LEA. 1972. Successful culture in vitro of sheep and cattle ova. J. Reprod. Fertil. 30:493-497.

Thompson JGE, Simpson AC, Pugh PA, Donnelly PE and Tervit HR. 1990. Effect of oxygen concentration on in vitro development of preimplantation sheep and cattle embryos. J. Reprod. Fertil. 89:573-578.

Ukaoka Y, Noda Y, Narimoto K and Mori T. 1992. Effect of oxygen toxicity on early development of mouse embryos. Mol. Reprod. Dev. 31:28-33.

Voelkel SA and Hu YX. 1992. Effect of gas atmosphere on the development of one-cell bovine embryos in two culture systems. Theriogenology 37:1117-1131.

Wang WL, Jiang HS, Lu KH, Gordon I and Polge C. 1992. The effect of gas phase on the *in vitro* development of bovine embryos derived from *in vitro* maturation and fertilization of ovarian oocytes. Theriogenology 37:320(Abstr.)

Wright RW Jr. 1977. Successful culture *in vitro* of swine embryos to the blastocyst stage. J. Anim. Sci. 44:854-858.

Wright RW Jr. and Bondioli KB. 1981. Aspects of in vitro fertilization and embryo culture in domestic animals. J. Anim. Sci. 53:702-728.

Yang BK, Yang X and Foote RH. 1993. Effect of growth factors on morula and blastocyst development of in vitro matured and *in vitro* fertilized bovine embryos. Theriogenology 40:521-530.

Yochim JM and Mitchell JA. 1968. Intrauterine oxygen tension in the rat during progestation: Its possible relation to carbohydrate metabolism and the regulation of oxydation. Endocrinology 83:706-713.