EFFECT OF GAS ATMOSPHERE ON THE DEVELOPMENT OF BOVINE EMBRYOS USING A SIMPLE PORTABLE INCUBATOR

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ABSTRACT

The objective of this study was to examine the effect of gas atmosphere on the development of IVMFC of bovine embryos in a simple portable carbon dioxide incubator under negative and positve pressure of air. Incubation was carried out in small plastic boxes (15L ×10W ×4H cm). For CO_2 production, 0.25 g of effervescent granules in distilled water were used for successful IVMFC. In experiment I, the blastocyst rate observed in treatment 1 with negative pressure was higher ($p < 0.05$) than that in treatment 2 and 3 (43.5% and 42.9%, 23.9% and 4.8% for treatment 1 (i) and (ii), 2, 3, respectively). In experiment II (treatment 1), the survival rate of simple incubator-produced blastocysts, following cryopresevation with 1.8M EG +0.05M T in the absence of PVP had no difference from that in 5% PVP. It was observed that the rate in 5% PVP was higher ($p < 0.01$) than that in 20% PVP and higher ($p < 0.05$) than that in 10% PVP (81.8%, 67.5%, 56.5%, 6.3%; without PVP, 5% PVP, 10% PVP, and 20% PVP, respectively). In experiment II (treatment 2), the comparative survival rate of blastocysts and hatched blastocysts produced by a simple incubator did not show any significant difference between program freezer and deep freezer. In experiment II (treatment 3), the total cell number and live and dead ratio of blastocysts from a simple incubator showed no significant difference between program freezer and deep freezer. In experiment III, the pH of culture medium in all the gas treatments was compared with CO_2 incubator and there was no significant difference.

These results suggest that gas atmosphere with the negative pressure of air in a simple portable incubator increased the blastocyst formation rate. Hence, it can be successfully used for production of bovine IVMFC embryos and could be used at farm level.

Key words: IVMFC, Simple incubator, Deep freezer, Negative and positive pressure of air

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INTRODUCTION

After the advent of embryo transfer technology and in vitro fertilization, in recent years there has been rapid progress in the development of systems particularly for in vitro production of bovine embryos for both experimental and commercial

purposes.

In this experiment, as a first step to trasfer and popularise IVF embryos in field for the benefit of farmers; a simple, portable and economically viable CO_2 incubator developed (16) was used for the production of IVF bovine embryos. In this experiment, we created negative pressure in the small boxes where incubation was carried out by aspirating air, having in view that there may be negative pressure in the genital tract during maturation and fertilization of oocyte in the oviduct and during transfer of embryos from oviduct to uterus. Regarding this, it is the first report.

The composition of the gas atmosphere in which bovine embryos develop is one of the most important factors in their developmental processes (8, 21). It has been found that 2.5-5% CO_2 is optimum condition for in vitro matured, fertilized and cultured (IVMFC) bovine oocyte. Whitten (21, 22) reported on the minimum oxygen requirement for in vitro survival of mouse embryos and also on the toxicity which occurs if the concentration is high. More recently, researchers have evaluated the effect of altering the gas atmosphere on development of embryos in mice (9), hamster (6), swine (24), sheep (19, 20) and cattle (7, 23). While there is general agreement that oxygen concentration of 5% to 10% is optimal for culture of embryos, there are a few reports (1, 24) that do not support this view.

The purpose of this experiment is to determine the effect of two different gas atmospheres on the development of bovine embryos, with negative pressure of air in a portable incubator, so as to make in vitro maturation, fertilization and culture economically feasible in the field.

MATERIALS AND METHODS

In this experiment, a simple portable CO_2 incubator of metallic make (29L × 26W × 21H) is electrically heated to maintain the chamber temperatures at 38.5°C (Figure 1). Incubation was performed in small plastic boxes (15L × 10W × 4H) placed in the incubator. CO_2 requirement is fullfilled by adding 5ml of distilled water to 0.25g of effervescent granules (Tartaric acid, 420mg; carbonated hydrogen natrium 460mg; Silicone fiber 10mg per gram) for the induction of 2 to 5% CO_2 gas placed in a small plastic dish of 3cm diameter.

In vitro maturation of oocytes

Holstein ovaries were obtained from the local abbatoir and carried in Ringers solution supplemented with pencillin-g 100μg/ml and streptomycin sulfate 0.1μg/ml at 30 to 32°C. Oocytes with in follicles 2 to 6mm in diameter were aspirated with an 18-G needle and washed with modified PBS solution. The aspirated oocytes were washed 3 times in maturation medium TCM-199 (Earles salt, Gibco, NY, USA) supplemented with superovulated cow serum (5) 0-0.1mg/ml follicle stimulating hormone (FSH, Denka Pharmaceuticals Co, Kawasaki, Japan), and 50μg/ml Gentamycin (Sigma Chemicals, St Louis, USA). Oocytes sorrounded by cumulus cells were incubated in maturation medium for 22h at 38.5°C in container 1, 2 with only 0.25g of effervescent granules, with negative pressure, in container 3 with 0.25g of effervescent granules and air without negative pressure as gaseous atmosphere and in container 4 effervescent granules with air and 25ml of air injected from out side after closure of the box, so as to create positive pressure of air in the container.

In vitro fertilization

Frozen-thawed semen were used for fertilization in vitro After thawing in water (30-35°C) the spermatozoa were diluted to about 6ml of 2.5M caffein added to Brackett and Oliphant's medium (Caff-BO, (2)) and washed twice by centrifuga-

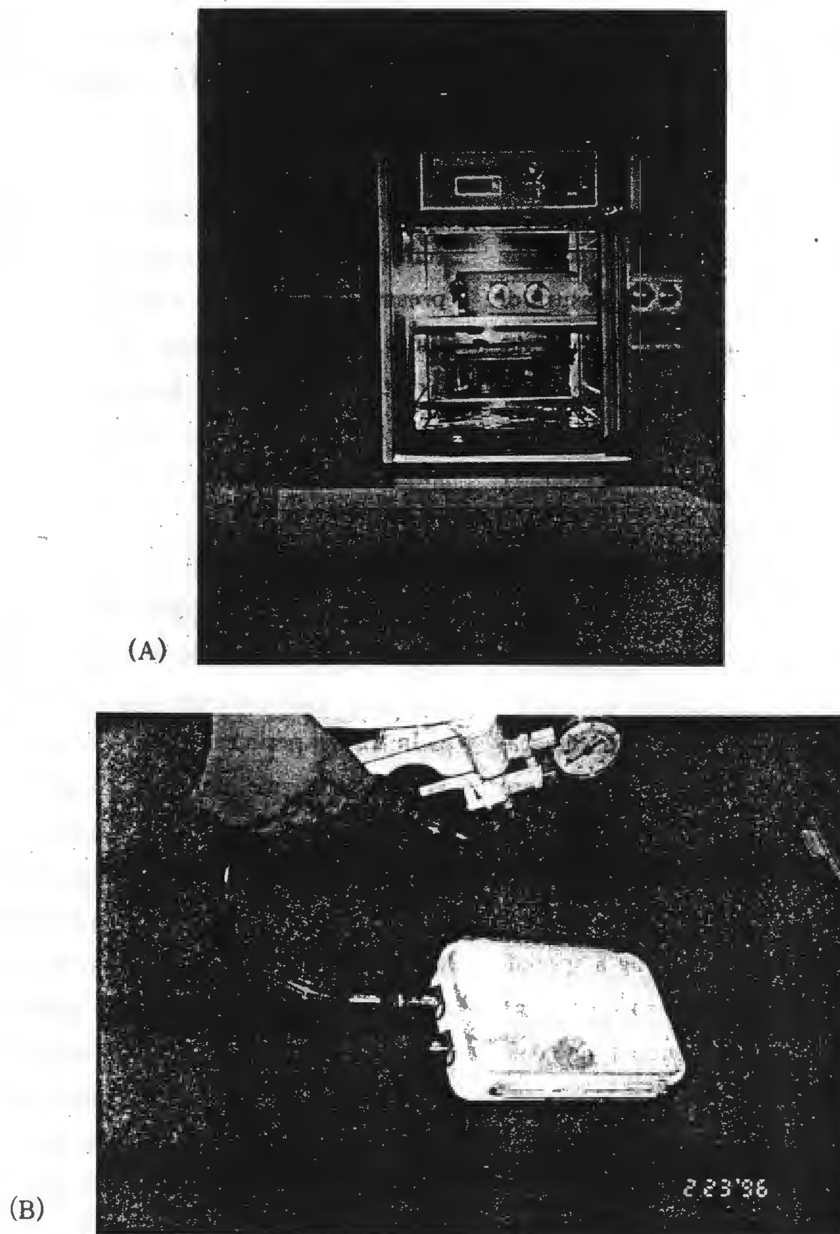

Figure 1. Simple portable incubator; (A) incubator (B) incubation box with handy aspirator.

tion at 500g for 5 min each. The resultant sperms were resuspended in Caff-BO supplemented with 0.3% bovine serum albumin (BSA, Sigma Chemicals Co, St Louis Mo, USA) and 20 μg/ml heparin (Shimizu pharmaceuticals, Shimizu, Japan) to a final concentration of 5×10^6 spermatozoa per ml. A 100 μl aliquot of the sperm suspension was covered with mineral oil. Oocytes matured in vitro were transfered into the sperm drops (20-30 oocytes per microdrop) for insemination.

After 5 h of insemination, oocytes with adherent cumulus cells were washed by repeated pipeting in culture medium TCM-199 supplemented with 5 μg/ml insulin (Wako Pure Chemical Industries, Osaka, Japan), 50 μg/ml gentamycin sulfate and 5% superovulated cow serum for further devolepment. The culture medium in 0.5ml polysterene dish 4-well multidish (NUNCLON, Roskilde,

Denmark) was covered with mineral oil (0.5ml). The culture medium was replaced with new medium 4 days later.

Experimental design

Three separate treatments were performed each using completely different design to compare the effect of only CO_2 gas with negative pressure of air, mixture of CO_2 and air without negative pressure and mixture of CO_2, air with positive pressure of air for the culture of in vitro produced bovine embryos.

EXPERIMENT I

Treatment-1: design (i)

In all the steps of incubation during IVMFC, after placing the culure dishes in the plastic boxes described above a small container kept along with the culture dishes and 0.25g of effervescent granules mixed with 5ml of distilled water were placed in the box. After addition of distilled water the box was closed immediately and simultaneosly to create negative pressure with only CO_2 atmosphere, 25ml of air is aspirated by using a specially designed handy aspirator (Fujihara Co, Japan) from, the inlet provided at one end of the box. Vaseline (Wako Pure Chemical Co, Japan) is applied around the rubber bedding of the plastic container to keep the box air tight. This experiment was replicated 6 times using a total of 375 oocytes. The embryos were observed at day 2 and again at day 7, 8 and 9 post fertilization to determine the cleavage rates and the percentage developing to the blastocyst stage.

Design (ii)

In this treatment, the same procedure as in treatment-1 was followed during IVMFC incubation except that aspiration of air was carried out by using 50ml syringe through a rubber stopper fixed on the upper surface of the box. About 150ml of air is aspirated to create negative pressure and maintain CO_2 gas atmosphere in the box. This experiment was replicated 6 times using a total of 295 oocytes.

Treatment-2

In this treatment a mixture of gas atmosphere of CO_2 and air was created by addition of effervescent granules into distilled water without negative pressure of air. This experiment was replicated 6 times using a total of 413 oocytes.

Treatment-3

In this treatment positive pressure is created by infusing 25ml of air into the box after addition of effervescent granules into distilled water. This experiment was replicated 6 times using 276 oocytes.

EXPERIMENT II

Treatment 1

Freezing of blastocysts

In this experiment blastocysts produced by the above treatments were frozen by using a simple, economically feasible freezing protocal developed in this laboratory. A Simple medical deep freezer (Y. Kikkawa and others, unpublished) is used for this purpose and in vitro produced bovine blastocysts were evaluated in different cryoprotectant solutions (Figure 2).

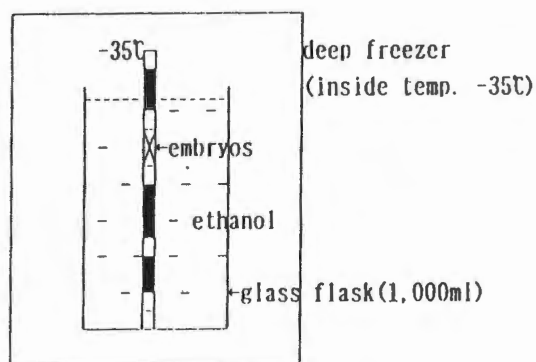

Figure 2. Diagram of a simple feezing system by using deep freezer.

In all the treatments of this experiment expanded blastocysts grade A (excellent) were washed in phosphate buffer saline (PBS) sup-

plemented with 1.8M ethylene glycol (EG; 15, 18) + 0.5M trehalose with 0%, 10% and 20% PVP were randomly used (10).

Treatment 2

Embryos were exposed to each cryoprotectant at room temperature. After 10 min of equilibration, the embryos were loaded into 0.25ml straws. The straws were suspended directly into a glass flask containing 99% ethanol at 4°C. This glass flask with straws was placed into a deep freezer container (Sanyo, MDF-135, Japan) and kept at −35°C. The distal end of the staws in cryoprotectant solutions were exposed to −35°C. After 90-100 min, when the temperature of ethanol reached to −31°C or −32°C, straws containing embryos were plunged into liquid nitrogen (−196°C). On the other hand, we have frozen some of the embryos by programme freezer also. Firstly, the embryos were equilibrated in room temprature for 10 min with the same cryoprotectants used as in medical freezer and were loaded in 0.25ml straws. Straws were then placed in an ethanol bath of programme freezer (Fujihira Co Ltd, Japan) and cooled to −7°C at a rate of 1°C per min. In this stage the straws were kept for 10 min and then cooled at the rate of 0.3°C until −33°C. Then the straws were plunged in liquid nitrogen for storage (15, 18).

After 2 to 5 days storage period in liquid nitrogen, the cryopreserved embryos were thawed in 30-35°C water bath and cryoprtectents were removed by the one step procedure (15). Embryos were washed with culture medium and cultured on feeder layers of bovine cumulus cells at 38.5°C under different gaseous atmosphere described in the above experiment. To examine the survival rate, frozen-thawed blastocysts were examined morphologically and the number of re-expanded blastocysts was recorded 24h to 48h after culture in vitro.

Treatment 3

Staining procedure

After thawing, embryos were exposed to 10mg/ml hoechst (Hoechst 333342, Sigma) and 10mg/ml propidium iodine (Wako) under mineral oil and incubated in a portable incubator for 30 min, then the embryos were washed in PBS supplemented with 0.3% BSA and mounted on a glass slide under a cover slip. The glass slide was examined under the flourescence microscope. This resulted in vital blue (bisbenzimide-positive) and non vital pink (propidium iodide) in microscope and live and dead cells were counted (13, 17).

EXPERIMENT III

Determination of pH in a portable incubator

Culture media used in different gas atmospheres during all the stages of IVMFC were randomly examined for pH and compared with the culture medium used in CO_2 incubator as a control. The pH examination was replicated 5 times in each of the treatments.

RESULTS

The effect of gas atmospher on early embryo development was shown in Tabel 1. The proportion of 2-cell stage embryos in treatment 1 design (i) was higher ($p < 0.01$) than that in design (ii), treatments II (8.7) and III (7.3). However, there were diferences between treatment 3 and the others regarding development of 4- and 8-cell stage embryos.

The blastocyst rate observed in the treatment 1 (43.5 and 42.9) was different from that in treatment II (23.9; $p < 0.05$) and treatment III (4.8; $p < 0.01$).

Table 2 shows the survival and blastocyst rate of IVF embryos produced in a simple portable incubator following cryopreservation in 1.8M EG + 0.05M T and different concentrations of PVP. The percentage of embryos that developed to hatched

Table 1. Development of IVMFC embryos using different gas atmosphere in portable incubator

Treatment	No of oocytes Inseminated	Develop to			Cleavage Rate (%)	Blastocyst Rate (%)
		2-cell	4-cell	8-cell		
Treatment-I (i) (with handy aspirator)	375	74(19.7)[a]	102(27.2)[a]	86(22.9)[a]	262(69.4)[a]	114(43.5)[a]
Treatment-I (ii) (with syringe)	295	23(7.80)[c]	63(21.4)[a]	84(28.5)[a]	170(57.6)[a]	73(42.9)[a]
Treatment-II	413	36(8.7)[c]	89(21.5)[a]	76(18.4)[a]	221(53.5)[a]	53(23.9)[b]
Treatment-III	276	20(7.3)[c]	37(13.4)[b]	6(2.2)[c]	63(22.8)[c]	3(4.8)[c]

Within columns, mean with different superscript are significantly different (anova T-student test, a-b; $P < 0.05$, a-c; $P < 0.01$)

Table 2. Comparative survival rate of blastocyst developed by using simple portable incubator follofwing cryopres ervation in the various concentration of cryoprotectants

Cryoprotectants	No. of freezing	No. of survival (%)	No. of Hatched blastocyst (%)
1.8M EG +0.05M T	22	19(86.4)	18(81.8)[a]
1.8M EG +0.05M T +5% PVP	40	35(87.5)	27(67.5)[ab]
1.8M EG +0.05M T +10% PVP	23	17(73.9)	13(56.5)[b]
1.8M EG +0.05M T +20% PVP	32	21(65.6)	2(6.3)[c]

Within colums, mean with different superscripts are significantly (anova, T-student test, a-b; $p < 0.05$, a-c; $p < 0.01$)

Table 3. Comparative survival rate of blastocyst developed by using simple portable incubator following cryopreservation in different freezing protocol*

Type freezing system	No. of freezing	No. of survival (%)	No. of Hatched blastocyst (%)
Program freezer (ET-1 FHK)	25	22(88.0)	19(76.0)
Deep freezer (Sanyo medical)	23	20(87.5)	19(82.6)
Control**	23	22(95.7)	21(91.3)

* Cryoprotectant medium: 1.8M EG +0.05M T; ** Control: Embryos were suspended in cryoprotectant solution without freezing and cultured in vitro

in 1.8M EG +0.05M T without PVP was similar to that with 5% PVP, whereas it was observed that the percentage was higher ($p < 0.01$) compared to that of 10% PVP and higher ($p < 0.05$) than that of 20% PVP (81.8%, 67.5%, 56.5% and 6.3%: without PVP, 5% PVP, 10% PVP and 20% PVP, respectively).

Table 3 shows comparative survival rate of embryos developed in a simple incubator using different freezing protocol and the results confirm that the survival rate and hatched blastocyst rate in the deep freezer and programe freezer had no difference from the control.

Table 4 shows comparative total cell number

Table 4. The total cell number and the live:dead ratio of blastocyst produced by simple portable incubator after frozen-thawed using different freezing protocol*

Type of freezing system	Total cell number	Live:dead cell ratio	Percent live
Program freezer (ET-1 FHK)	119±14	111:8	93.3%
Deep freezer (Sanyo medical)	108±15	102:6	94.4%
Control**	120±14	119:1	99.2%

* Cryoprotectant medium: 1.8M EG +0.05M T

** Control: Embryos were suspended in cryoprotectant solution (without freezing) and cultured in vitro

Table 5. pH of culture medium in different gas atmosphere in simple incubator

Treatment	pH range
CO_2 gas incubator	7.4~7.6
Treatment I	7.2~7.7
Treatment II	7.3~7.8
Treatment III	7.5~7.8
Treatment IV	7.6~7.9

and percentage of live cells in embryos produced in a simple incubator using different freezing protocol and the results confirm that the total cell number and percentage of live cells in the new deep freezer and programe freezer had no difference from the control.

We measured the pH of media in the portable. As shown in Table 5, there was no difference in pH among the treatment groups and the control.

DISCUSSION

In this experiment we tried to make optimum gas atmosphere of 2.5% to 5% CO_2 in air for IVMFC of bovine oocyte using effervescent granules in small incubation boxes placed in a simple portable incubator. The whole process of IVMFC, freezing, survival rate after thawing, live and dead cell ratio, pH of the medium were meticulously examined to transfer the technology of IVF to field and also to make IVF economically viable particularly in developing countries.

The results of our experiments demonstrate that incubation of cattle embryos in first two treatments under 2.5% to 5% CO_2 which is optimal (8, 14) with negative pressure of air (not reported so far), in which O_2 concentration may be reduced due to aspiration of air (20) had good developmental effect in IVMFC of early stage embryos, whereas in treatment 3 in the presence of increased concentration of O_2 in extra amount of air, in treatment 4 in the increased concentration of O_2 under positive pressure of air and relatively less concentation of CO_2, both the treatments resulted in low level of embryo development (Figure 3).

When the embryos were put in the cryoprotectant medium substantial amounts of ethylene glycol entered the embryonic cells, and at the same time trehalose helps to dehydrate the embryonic cells, a process which is very important for successful cryopreservation (11). During thawing trehalose helps to prevent the osmotic shock (13).

Mar.y reaserchers have established the beneficial effect of PVP during slow freezing (4), rapid freezing (3, 4) or vitrification (10, 12, 13). But in this experiment the addition of extra amount of PVP (20%) were not helpful when medical freezer was used for freezing. It might be due to delay in the formation of ice even after seeding, which is of

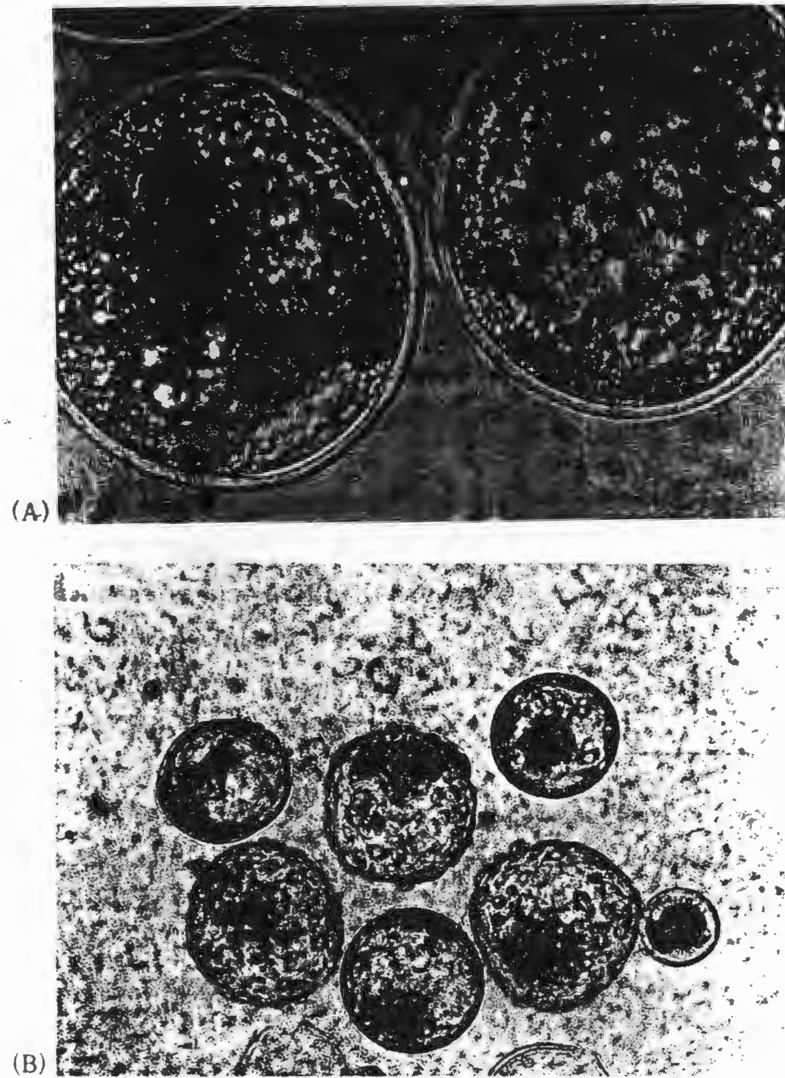

Figure 3. (A) Expanded and (B) hatched blastocyst produced by simple portable incubator.

natural kind in medical freezer. It might not been helpful for the freezing process.

The survival rate of blastocyst was compared in two freezing protocols and there was no significant difference in the number of survival of blastocysts and hatched blastocysts when cryoprotectant medium used was without PVP (Figure 4).

The live and dead cell ratio of blastocysts from a simple incubator was compared between deep freezer and program freezer. The live cells of the embryos get blue colour due to Hoechst stain but only the dead cells have access to the pink colour of the stain due to propodium iodide. The live and dead cell ratio showed no significant difference between the two freezing protocols.

There is no difference in the pH among all the treatments and control. it may be due to the prevalence of the same gas atmosphere as that of a simple incubator.

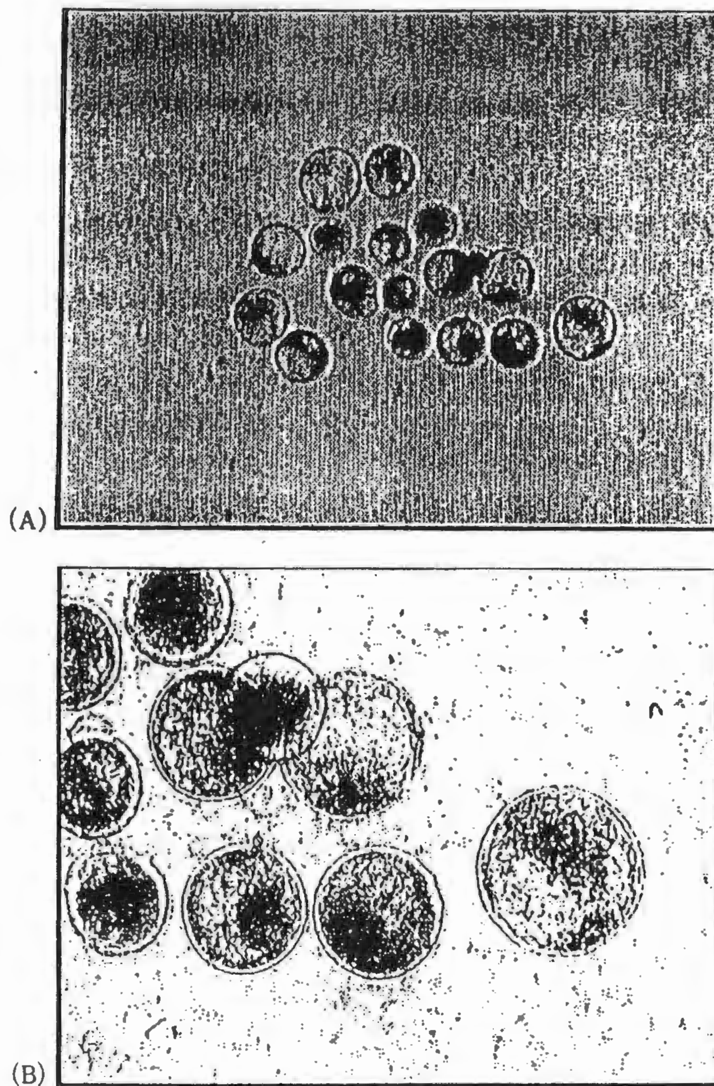

Figure 4. Development of (A) blastocysts and (B) expanded blastocytsts produced by simple incubator after freezing with medical freezer and thawing.

CONCLUSION

These results suggest that the whole process of IVMFC has yielded good results in a portable incubator, which makes it economically feasible in the field and also applies to deep freezer because the survival rate after freezing and thawing of embryos and ratio of live and dead cells of the blastocysts developed in a portable incubator were no different from those of program freezer.

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