In Vitro Maturation of Porcine Oocytes in a Dry Incubator without CO_2 Gas Supplement

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ABSTRACT

The present study was conducted to develop a simple method for porcine oocyte maturation without CO_2 regulation. In experiment 1, we evaluated that the effect of CO_2 non-supplement on porcine oocyte maturation. Cumulus-oocyte complexes (COCs) were collected from 2～6 mm follicles and divided into three groups (Control, tube-CO_2, and tube-non-CO_2). For control, COCs were cultured in 4-well multidish in a CO_2 incubator. For tube-CO_2, COCs were cultured in a round-bottom tube in a CO_2 incubator, and for tube-non-CO_2, COCs were cultured in a round-bottom tube sealed tightly without CO_2 supplement in a dry incubator. The proportion of oocytes reached to metaphase II (M-II) was not significantly different among three groups (87.9% to 91.4%). In experiment 2, we evaluated the effect of CO_2 non-supplement during oocyte maturation on development of embryos. Oocytes with a polar body were divided into two groups (Control and tube-non-CO_2) and applied 1.1 kV/cm or 1.2 kV/cm voltages for parthenogenetic activation. After activation, embryos were cultured for 6 days and examined the development. The proportion of embryos cleaved was not significantly different among treatment (86.3% to 91.5%). The proportion of embryo reached to blastocyst stage was not significantly different among treatment (13.9% to 25.2%). The cell number of blastocysts was not significantly different among treatment (29.0 to 32.4). In conclusion, oocytes cultured in a dry incubator without CO_2 supplement have enough competence to development after parthenogenetic activation. These results would be useful for transporting oocytes or embryos a long distance.

(Key words : Pig, Oocytes, *In Vitro* Maturation, Dry Incubator)

INTRODUCTION

Since the first successful embryo culture from eight-cell to blastocyst stage (Whitten, 1956), *in vitro* embryo culture has been reported in many species, including mice (Whitten and Biggers, 1968), rabbits (Kane, 1972), pigs (Menino and Wright, 1982), sheep (Gandolfi and Moor, 1987), and cows (Xu *et al*., 1987). *In vitro* maturation, fertilization, and culture of porcine oocytes and embryos have many potential in research and commercial use (Nagai 2001; Niemann and Rath 2001).

Ovaries were usually collected from a local abattoir and transported to the laboratory. Oocytes were collected from the ovaries. In some case, oocytes were transported to a long distance by using a portable incubator. Portable incubators were developed and used in several species including mice (Chen *et al*., 2005), sheep (Byrd *et al*., 1997), cattle (Varisanga *et al*., 2000), horse (Love *et al*., 2003), and mink whale (Iwayama *et al*., 2005). In porcine, several types of portable CO_2 chambers were developed and shown to be effective for porcine oocyte maturation and subsequent embryo development after *in vitro* fertilization (Suzuki *et al*., 1999; Karja *et al*., 2004; Fujii *et al*., 2010).

Some laboratories purchased oocytes from companies by using a portable box without CO_2 regulation, maintained an optimal temperature (Li *et al*., 2009; Krisher *et al*., 1998). Li *et al*. (2009) cultured porcine oocytes in Hepes-based TCM-199 medium in a portable incubator overnight and cultured in a CO_2 incubator in fresh medium on the following day.

The present study was conducted to develop a simple method for porcine oocyte maturation without CO_2 regulation by using common maturation medium. Developmental competence to the blastocyst stage was evaluated after electrical activation. This method would be useful for transporting oocytes or embryos a long distance.

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MATERIALS AND METHODS

In Vitro Maturation of Porcine Oocytes

Ovaries were collected from prepubertal gilts at a local abattoir and were transported to the laboratory in a 0.9% NaCl solution at 35℃. Cumulus-oocyte complexes (COCs) were aspirated from 2~6 mm diameter antral follicles with an 18-gauge needle fixed to a 10-ml disposable syringe. The COCs were washed three times in TCM 199 (31100-035, Gibco Grand Island, NY) supplemented with 0.1% polyvinylalcohol, 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 μg/ml LH (L-5269, Sigma Chemical Co, St. Louis, MO), 0.5 μg/ml FSH (F-2293, Sigma), 10 ng/ml epidermal growth factor (E-4127, Sigma), 75 μg/ml penicillin G, and 50 μg/ml streptomycin.

Approximately 20~30 COCs were transferred to 500 μl of the same medium which had been covered with mineral oil in a 4-well multidish (Nunc, Roskilde, Denmark) (Abeydeera et al., 2000). For tube culture, oocytes were cultured 500 μl of the same medium covered with 500 μl mineral oil in 5 ml polystyrene round-bottom tube (BD Falcon) in a CO_2 incubator or a dry incubator. When a tube was cultured in a dry incubator, the tube was tightly capped and sealed with paraffin film. All medium was equilibrated at 39℃ in an atmosphere of 5% CO_2 in air. After 42 to 44 h of culture, the oocytes were freed from the cumulus cells by vigorously vortexing the cells for 4 min in TL-Hepes supplemented with 0.1% PVA and 0.1% hyaluronidase. Oocytes were stained with Hoechst 33342 to determine metaphase II stage.

Activation and Embryo Culture

After 42 to 44 h culture, oocytes with a visible polar body were selected and used for the experiments. Oocytes were placed between two 0.2 mm-diameter platinum electrodes 1 mm apart in the medium, which consisted of 0.3 M mannitol, 1.0 mM $CaCl_2 \cdot H_2O$, 0.1 mM $MgCl_2 \cdot 6H_2O$ and 0.5 mM HEPES. Activation was induced with two successive DC pulses of 1.1~1.2 kV/cm for 30 μsec. After activation, 20 to 30 reconstructed embryos were transferred to 500 μl of North Carolina State University (NCSU)-23 medium (Petters and Wells, 1993) supplemented with 0.4 % BSA and covered with mineral oil in a 4-well multidish. After 6 days of culture all embryos were stained with Hoechst 33342 to determine the number of nuclei by using an epifluorescent microscope, and embryos with two or more nuclei were determined to have cleaved.

Experimental Design

In experiment 1, we evaluated the effect of CO_2 non-supplement on porcine oocyte maturation. Cumulus-oocyte complexes (COCs) were divided into three groups (Control, tube-CO_2, and tube-non-CO_2). For the control, COCs were cultured in 4-well multidish in a CO_2 incubator. For tube-CO_2, COCs were cultured in round-bottom tube in a CO_2 incubator, and for tube-non-CO_2, COCs were cultured in round-bottom tube without CO_2 supplement in a dry incubator.

In experiment 2, we evaluated the effect of CO_2 non-supplement during oocyte maturation on development of embryos. Oocytes with a polar body were divided into two groups (Control and tube-non-CO_2) and applied 1.1 kV/cm or 1.2 kV/cm voltages. After activation, embryos were cultured for 6 days and examined the development.

Statistical Analysis

We conducted four replicate trials for each treatment. Data were analyzed by analysis of variance and Duncan multiple-range test by using the general linear models in the Statistical Analysis System software to determine treatment differences. All percentage data were subjected to arcsine transformation before statistical analysis. Data are expressed as the mean ± SEM. A probability of $p<0.05$ was considered to be statistically significant.

RESULTS

Effect of CO_2 Non-Supplement on Oocyte Maturation *In Vitro*

The meiotic competence of COCs was examined after 42 to 44 h culture. The proportion (87.9% to 91.4%) of oocytes reached to metaphase II (M-II) was not significantly different among control, tube-CO_2, and tube-non-CO_2 (Table 1). These results suggest that porcine oocytes cultured in a dry incubator without CO_2 supplement can reach to nucleic maturation similar to common maturation culture. Nuclei maturation rate of oocyte between control and tube-CO_2 was not different and tube-CO_2 was deleted in the next experiments

Effect of CO_2 Non-Supplement during Oocyte Maturation *In Vitro* on the Development of Embryos

After activation, oocytes were cultured for 6 days. The proportion (88.7% to 91.5%) of embryos cleaved was not significantly different among treatment (Table 2). The proportion (13.9% to 25.2%) of embryo reached to blastocyst stage was not significantly different among treatment (Table 2). The cell number of blastocyst (29.0~32.4) was not significantly different among treatment (Table 3). Embryo development (cleavage rate, blastocyst formation rate, and cell number of blastocysts) was no difference between two voltages (1.1 and

1.2 kv/cm) supplied (Table 2 and 3). These results suggest that oocytes cultured in a dry incubator without CO_2 supplement have enough developmentablility compared to common maturation system.

DISCUSSION

Since the first successful embryo culture from eighth-cell to blastocyst stage (Whitten, 1956), *in vitro* embryo culture has been reported in many species, including mice (Whitten and Biggers, 1968), rabbits (Kane, 1972), pigs (Menino and Wright, 1982), sheep (Gandolfi and Moor, 1987), and cows (Xu *et al*., 1987). *In vitro* production of embryos involved with *in vitro* maturation, *in vitro* fertilization, and *in vitro* culture technique would be crucial for research and commercial use.

Most of researchers have been used a incubator with an atmosphere of 5% CO_2 in air, because culture media buffered with sodium bicarbonate are designed to maintain their pH at 7.4 under the condition (Geshi *et al*., 1999). Some researchers used portable CO_2 incubator to transport oocytes or embryos to a long distance (Chen *et al*., 2005; Byrd *et al*., 1997; Varisanga *et al*., 2000; Love *et al*., 2003). In porcine, Suzuki *et al*. (1999) developed a portable CO_2 camber to culture embryos and showed to be effective for porcine oocyte maturation and subsequent embryo development after *in vitro* fertilization (Karja *et al*., 2004).

Some laboratories purchased oocytes from companies by using a portable box without CO_2 regulation, maintained an optimal temperature (Li *et al*., 2009; Krisher *et al*., 1998). Li *et al*. (2009) cultured porcine oocytes in Hepes-based TCM-199 medium (TCM199 with 2.9 mM Hepes, 5 μg/ml insulin, 10 ng/ml EGF, 0.5 μg/ml p-FSH, 0.91 mM pyruvate, 0.5 mM cysteine, 10% porcine follicular fluid, 25 ng/ml gentamicin) in a portable incubator overnight and cultured in a CO_2 incubator in fresh medium on the following day. In the present study, we used common maturation medium which was not Hepes-based. Surprisingly, the proportion of oocytes reached to metaphase II (M-II) was 91.4% without CO_2 supplement in a dry incubator (Table 1). Li *et al*. (2009) cultured oocytes just overnight (for less than a day) in a portable box, but we cultured oocytes for 42 ~44 h without CO_2 regulation. We cultured oocytes for almost 1 day more than Li *et al*. (2009) without CO_2 supplement and oocyte maturation rate was comparable. These results indicate that Hepe-based medium might not be needed to culture oocytes without CO_2 regulation and that oocytes can be cultured in a dry incubator without CO_2 regulation.

A 5% CO_2 concentration in the incubator is usually used for embryo production, maintaining the pH of the medium at 7.4. The pH of the medium becomes significantly higher (7.6~7.7) when the CO_2 concentration is reduced from 5 to 2% (Geshi *et al*., 1999). It was reported that the pH rose to 8 without Hepes when porcine embryos were culture in a dry incubator (Ozawa *et al*., 2006). In the present study, we did not measure correctly pH of the medium but confirmed ridden pH examining the medium color changed. Therefore tight sealing might not be enough to maintain medium pH. Even though medium pH was increased, oocytes could be matured and developed to the blastocyst stage after parthenogenetic activation (Table 1, 2 and 3). Slight pH change in medium might not affect oocyte maturation. The intracellular pH was not always directly correlated with the pH in the culture medium (Bavister, 1995). Therefore, there is some possibility that intracellular pH of oocytes is different from pH of the medium. Further studies are needed to determine the relationship between intracellular pH of oocytes and the pH of medium used in the present.

Ozawa *et al*. (2006) also reported successful pig embryo culture without a CO_2 incubator. They cultured embryos in medium covered with paraffin oil in tightly closed glass tubes. They used paraffin oil to prevent

Table 1. Effect of CO_2 non-supplement on porcine oocyte maturation *in vitro*

Treatments	No. of oocytes	
	Examined	% Metaphase II
Control	85	87.9±3.5
Tube-CO_2	83	88.3±2.0
Tube-non-CO_2	84	91.4±2.5

Table 2. Effect of CO_2 non-supplement during porcine oocyte maturation *in vitro* on the development of embryos

Voltages	Treatments	No. of oocytes	% Cleaved	% Blastocysts
1.1 kv/cm	Control	87	88.7±3.9	20.1±4.0
	Tube-non-CO_2	94	91.5±1.0	13.9±2.8
1.2 kv/cm	Control	81	86.3±2.1	25.2±3.6
	Tube-non-CO_2	97	90.8±0.8	22.7±4.1

Table 3. Cell number of blastocysts

Voltages	Treatments	No. of blastocyts	Cell No. of blastocysts	Range
1.1 kv/cm	Control	17	29.0±4.8	18~6
	Tube-non-CO_2	13	29.2±5.9	20~61
1.2 kv/cm	Control	21	31.9±6.8	19~60
	Tube-non-CO_2	20	32.4±5.3	21~66

liquid evaporation to maintain osmolality. Li *et al*. (2009) cultured porcine oocytes in Hepes-based TCM-199 medium but did not use oil to cover medium during shipment. In the present study, we covered medium with mineral oil and the tube was tightly capped and sealed with paraffin film. This system might be better to maintain osmolality of medium.

In conclusion, we demonstrated that oocytes can be cultured in common maturation medium in a dry incubator without CO_2 regulation and can develop to the blastocyst stage after parthenogenetic activation. However, we did not evaluate whether oocytes cultured in a dry incubator can develop to embryos after *in vitro* fertilization. In future, this should be needed to elucidate. These results would be useful for transporting oocytes or embryos a long distance.

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