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Full Length Research Paper

A new tool for *in vitro* culture of porcine eggs

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Mineral oil is usually used to cover the microdrops of medium in oocytes or embryos culture system here designated as oil method. A large number of oocytes are needed for the production of porcine embryos for in vitro fertilization or somatic cell nuclear transfer (SCNT). The oil method not only wastes a lot of mineral oil, but needs tedious steps in the transferring of embryos. Here we designed a new method called nest dish, which need not mineral oil, to replace the oil method and improve the development rates of porcine eggs in vitro. The oocyte maturation rate with the mTCM199 (83.2%) was significantly higher than with the NCSU23 (75.5%, $P< 0.05$), although the parthenogenetic cleavage rates with two media were not significantly different (77.7 and 72.4%, $P< 0.05$). Chosing mTCM199 as base medium, the rate of maturation with concave dish (90.1%) was significantly higher than with the flat dish (82.6%, $P> 0.05$) in nest method, although no significant differences in the oocyte maturation were found between flat dish (82.6%) in nest method and oil method (80.0%). Parthenogenetic cleavage from nest method (80.1% for concave dish, 78.0% for flat dish) did not show any decrease compared to oil method (76.2%), but the developmental rate to blastocysts in the nest groups(17.9 and 19.5%) were significantly higher than the oil method (12.3%, $P< 0.05$). These results showed that mTCM199 presented higher maturation rate than that NCSU-23 did, and the nest method with concave dish significantly improved the maturation rate of porcine oocytes in vitro and can replace the conventional oil method.

Key words: Porcine oocytes, *in vitro* maturation (IVM), microdrop method, nest dish method.

INTRODUCTION

Porcine oocytes are cheap and abundant by *in vitro* maturation (IVM) of follicular oocytes from ovaries collected from slaughterhouse, and were are used in fertilization and cloning researches. However, the system for porcine oocyte IVM is not so perfect as demonstrated by some papers. In these studies, *in vivo* oocytes supported higher developmental ability of cloned embryos compared with *in vitro* maturated oocytes (Onishi et al., 2000; Polejaeva et al., 2000; Grupen et al., 1999). Optimizing IVM system of porcine oocytes to achieve both nuclear and cytoplasmic maturation is necessary (Niemann et al., 2003) although porcine oocytes IVM and *in vitro* fertilisation (IVF) techniques have been improved in recent years. For example, the high incidence of polyspermy penetration remains the obstacle to successful production of a large number of porcine embryos *in vitro* (Niwa, 1993; Hunter, 2000; Abeydeera, 2001; Matás et al., 2003; Wang et al., 1994).

Many studies believed that the cytoplasmic maturation on IVM of porcine oocytes needs to be improved (Yoshida et al., 1993; Yamauchi and Nagai, 1999). Some studies added β-mercapthonal (BME) (Yoshida et al., 1993), L-cysteine (Yamauchi and Nagai, 1999), cysteame (Abeydeera and Day, 1997), and follicular fluid (Segers et al., 2008) into the culture medium and made certain achievements. Other conditions in culture were also considered such as culture temperature, N2 concentration and mineral oil. Nearly all the researches used microdrops of medium covered by liquid paraffin for culture of oocytes and embryos (Brinster, 1963) (here we designated as oil method), but whether mineral oil had bad influence on IVM of oocytes remained controversial

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(Shimada et al., 2002; Deleuze et al., 2010). At the same time, a large number of oocytes are needed for the production of porcine embryos by IVF or somatic cell nuclear transfer (SCNT), which needs the preparation of dishes with mineral oil. The oil method not only wastes a lot of mineral oil, but requires tedious steps in transferring the embryos. Here we developed a new method called nest dish, which needs no mineral oil. This method not only saved workload and money, but also avoided the potential impact of mineral oil. The present study was conducted to explore the feasibility of nest dish method on porcine eggs development *in vitro*.

MATERIALS AND METHODS

Materials

Glass petri dishes (180 × 50 mm and 90 × 40 mm) were purchased from Xi'an Yanhe company China. Pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were produced bv hormone products factory in Ningbo, China. Dulbecco's phosphate buffered saline (DPBS) was purchased from Gibco company and other reagents were purchased from Sigma-Aldrich chemical co. unless otherwise noted.

Preparation of nest dish

Briefly, the glass petri dishes (35 × 30 mm) were put into a dish (90 × 40 mm) and they were kept in a bigger dish (180 × 50 mm). This set of dishes nest was named and placed in an incubator at 38.5°C, 5% CO_2 in air. (Figure 1A and B)

Preparation of porcine follicular fluid (pFF)

The pFF was collected from follicles (3 - 8 mm diameter) of porcine ovary using a 10- mL disposable syringe with a 16- gauge needle. After centrifugation at 1600 r for 20 min at 4°C, the suspension was filtered through 0.22 µm syringe filters and stored at -20°C until use.

Cumulus-oocyte-complexes (COCs) collection and oocytes matured *in vitro*

Ovaries were collected at local slaughter, transported to the laboratory in 0.9% (w/v) NaCl with antibiotics at 37°C and maintained at this temperature while being transported to the laboratory. Oocytes were aspirated from follicles larger than 3 mm in diameter using an 18- gauge needle fixed to a 10 ml disposable syringe. After washing four times with DPBS containing 0.1% (w/v) polyvinyl alcohol, oocytes were washed five times with culture medium.

In experiment 1, each group of 50 oocytes surrounded by compact cumulus were transferred to a 500 µl drop of the same medium (mTCM199 or NCSU-23) which had been previously covered with warm paraffin oil (Fisher Scientific, Pittsburgh, PA) in a polystyrene culture dish (Beckton Dickinson Labware, Lincoln Park, NJ) and equilibrated at 38.5°C in 5% CO_2 in humidified air. In experiment 2, each group of 200 oocytes were cultured by nest method with flat dish and concave dish containing mTCM199 (2 – 3 ml) which had been previously incubated around 2 h.

The oocytes were cultured in maturation medium with PMSG (10 IU/ml), HCG (10 IU/ml), 0. 57 mmol/L cysteine and 10% porcine follicular fluid (pFF) for 22 h at 38.5°C, 5% CO_2 in air, then the oocytes were cultured without hormonal supplements for an additional 22 h. The oocytes matured *in vitro* for 44 h and the cumulus cells were completely removed from the oocytes by treatment with 0.1% hyaluronidase and pipetting. Oocytes with extruded first polar body were judged matured and used for electric activation (Figure 2A and B).

Activation and embryos culture

Oocytes with extruded polar body were washed three times with activating fluid with 0.3 M mannitol, 1 mM $CaCl_2$ and 0.5 mM $MgSO_4$ and 0.05 mg/ml BSA, and transferred to a chamber containing the same fluid. Activation were induced by application of an alternating current (AC) pulse of 10 V for 5 s followed by a single direct current (DC) pulses of 1.6 kV/cm for 60 µs using an electro cell manipulator 2001 (BTX Inc., San Diego). After activation treatment, the embryos were washed five times with NCSU-23 containing 4 mg/ml bovine serum albumin (BSA), were then cultured in the same medium which had been previously covered with paraffin oil in a polystyrene culture dish and equilibrated at 38.5°C in an atmosphere of 5% CO_2 in air. The rate of cleavage and blastocyst formation were assessed on days 2 and 7 (Figure 3).

Statistical analysis

All data were obtained from five replicates. Percentage data were analyzed by chi-square tests ($P<0.05$).

RESULTS

Effect of medium on COCs *in vitro* developement

Using the conventional mineral oil microdrop method, COCs were separately cultured into mTCM199 and NCSU-23 media every time. Although the rate of maturation with mTCM199 (83.2%) were significantly higher than with NCSU-23 medium (75.5%, $P<0.05$), the developmental rate to cleavage with mTCM199 and NCSU-23 were not significantly different (77.7 and 72.4%, $P>0.05$) (Table 1).

Nest dish methods of IVM

The rates of maturation and cleavage applying nest method with flat dish (82.6 and 78.0%) were not significantly different with oil method (80.0 and 76.2%, $P>0.05$). There were no significant differences in the cleavage rates applying nest method with concave dish (80.1%) and flat dish (78.0%), but the rate of maturation with concave dish (90.1%) were significantly higher than the flat dish groups (82.6%, $P<0.05$), and the developmental rate to blastocysts in the nest groups (17.9 and 19.5%) were significantly higher than the oil method (12.3%, $P<0.05$) (Table 2).

DISCUSSION

mTCM199 and NCSU-23 were the most widely used and

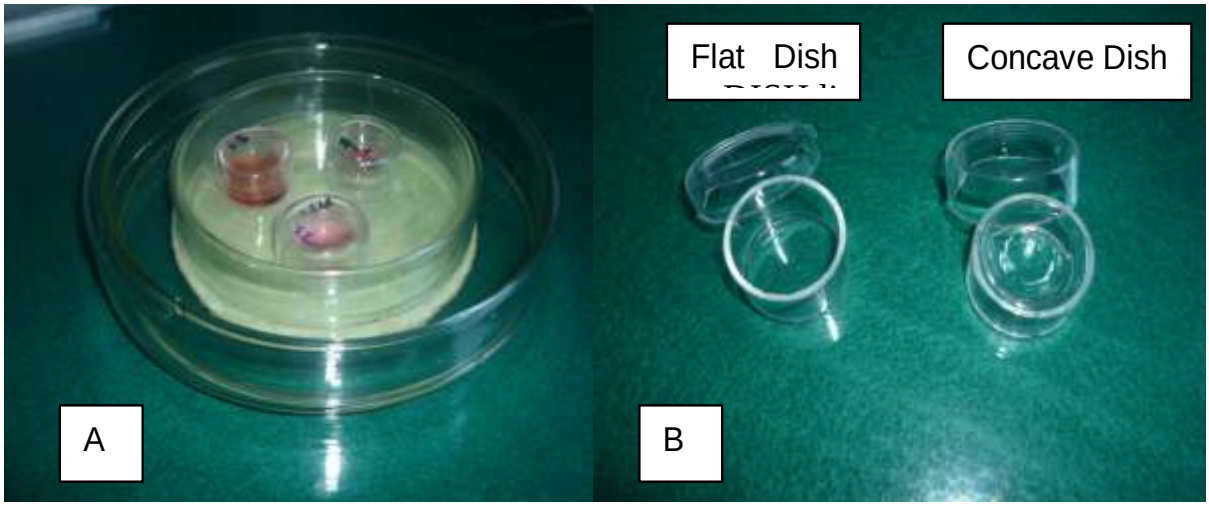

Figure 1. Nest dish methods. A, Glass dishes of diameter 180 and 90 mm; B, Glass petri dishes of diameter 35 mm.

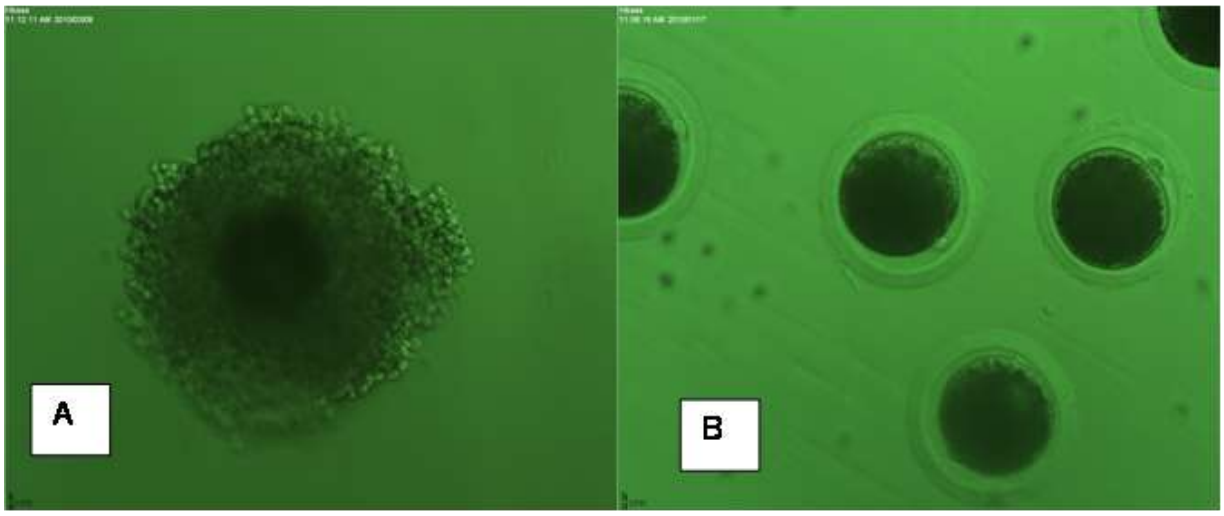

Figure 2. Pig oocytes. A, COCs; B,oocytes matured *in vitro*.

also most successful culture media in IVM of porcine oocytes (Funahashi and Day, 1997). The effects of mTCM199 and NCSU-23 medium on the maturation of porcine oocytes were compared in the laboratory. The results showed better maturation rate in mTCM199, which was inconsistent with the results by others (Hyunet al., 2003). Some researchers had shown that NCSU-23 was superior to mTCM199 (Zhang, 2006; Marques et al., 2007; Wang et al., 2002). The different results would be related to the use of hormones, growth factors in follicular fluid and oocyte genetic background in our laboratory. Anyway, we chose the mTCM199 as base medium for the activation experiment.

Oil method could affect oocytes maturation (Shimada et al., 2002), and the influence of mineral oil on oocytes development remained controversial. Some had tried mineral oil-free culture system but failed to get satisfactory results (Shimada et al., 2002; Segers et al., 2008). This study was conducted to explore the feasibility of oil-free nest method on *in vitro* development of porcine oocytes. There were no significant differences in the maturation rates between nest method with flat dish and oil methods, but the maturation rate appling nest method with concave dish were significantly higher than the other two groups and the developmental rate to blastocysts in the nest groups were significantly higher than the oil method. We speculated this would be related to the temperature, pH value and humidity which can be maintained by nest method with concave dish, although we did not detect detailly. The nest dish system was formed by a set of dishes from diameter 90 to 180 mm, which were flooded by distilled water, with diameter 35 mm of Petri dishes with culture medium inside. After the culture conditions of incubator stabilized, the nest plays a buffer role by

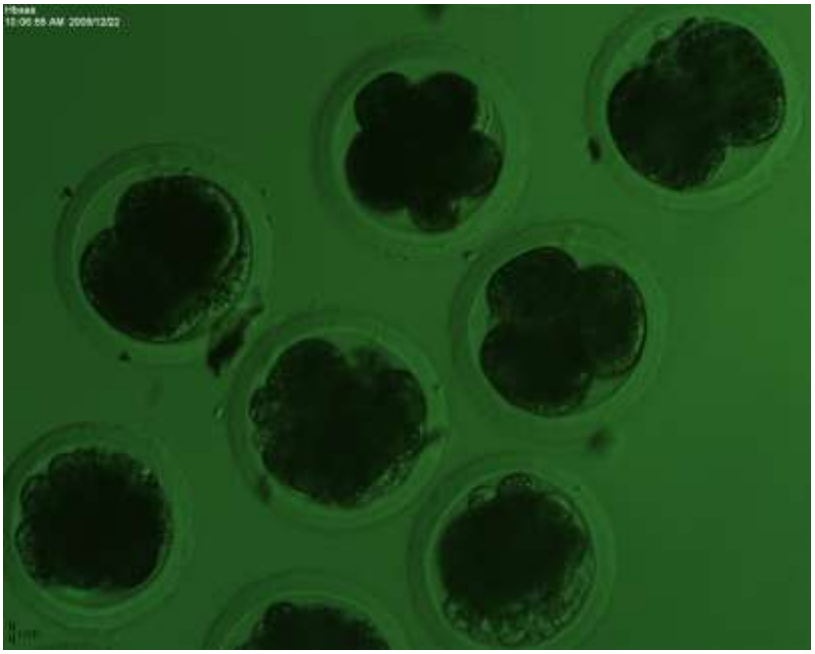

Figure 3. Embryos of parthenogenetic activation. A, 2 cella; B, blastocyst

Table 1. Effect of medium on oocytes IVM.

	No. of COCs	No. of oocytes matured (%)	No. embryos cleaved (%)
mTCM199	750	624 (83.2%)[a]	485 (77.7%)[a]
NCSU-23	600	453 (75.5%)[b]	328 (72.4%)[a]

Within the same column, values with same superscript are not significantly different (P>0.05), values with different superscript are significantly different (P>0.05).

Table 2. Effect of culture method on pig oocytes IVM.

Culture method	No. of embryos treated	NO. of oocytes matured (%)	No. embryos cleaved (%)	NO. of blastocyst (%)
Oil	1550	1240(80.0%)[a]	945(76.2%)[a]	191(12.3%)[a]
Flat dish	2000	1652(82.6%) [a]	1288(78.0%) [a]	358(17.9%)[b]
Concave dish	2000	1802(90.1%) [b]	1443(80.1%) [a]	390(19.5%)[b]

forming a gradient humidity and pH value within dishes of diameter 35 mm.

So the nest dish can effectively prevent water evaporation, and maintain the relatively stable pH value and osmotic pressure of the medium. Furthermore, the concave dish was curved at the bottom, which made connection closer between oocytes and cumulus cells, and regulated signal communication between many chemical substances inside and outside oocyte membrane, such as amino acids, nucleotide acid, phosphatidic acid, hormones, proteins and so on. The interaction of oocytes and cumulus cells made better development in the course of maturation *in vitro*. After parthenogenesis, the cells interaction led to the disappearance of the connection in concave dish due to the removing of cumulus cells; this may explain the cleavage rates which do not differ no matter what methods are used.

Conclusions

In conclusion, the present study indicated that the nest method was a feasible method for porcine oocytes maturation and embryos development *in vitro*, the nest method with concave dish significantly improved *in vitro*

maturation rates of porcine oocytes and can replace the conventional oil method.

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Abbreviations

IVM, *In vitro* maturation; **IVF,** *in vitro* fertilisation; **BME,** β-mercapthonal; **SCNT,** somatic cell nuclear transfer; **PMSG,** pregnant mare's serum gonadotropin; **hCG,** human chorionic gonadotropin; **DPBS,** Dulbecco's phosphate buffered saline; **pFF,** porcine follicular fluid; **COCs,** cumulus-oocyte-complexes; **AC,** alternating current; **BSA,** bovine serum albumin.

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