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FROZEN STORAGE OF PORCINE ZYGOTE MEDIUM 3 FOR *IN VITRO* CULTURE OF CLONED PORCINE EMBRYOS

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ABSTRACT

The present study was designed to examine whether the frozen storage of porcine zygote medium (PZM) with 3 mg/ml of BSA was feasible for culturing porcine embryos. In experiment 1, the effect of PZM3 that was frozen and stored within one week on the *in vitro* developmental competence of porcine parthenotes was evaluated. PZM3 that was stored at 4°C served as control. The results show that the cleavage (83.9±1.2% vs. 84.5±1.8%, $P>0.05$) and blastocyst (65.2±2.1% vs. 63.1±3.8%, $P>0.05$) rates are similar between frozen-warmed PZM3 and the control. In addition, the total cell numbers per blastocyst (50±7 cells vs. 47±5 cells, $P>0.05$) were similar between the groups. In experiment 2, we tested whether PZM3 in frozen storage for 5 months was able to support the *in vitro* development of parthenotes similar to that supported by freshly made PZM3. The results show that no statistical differences were observed although the cleavage (97.8±2.7% vs. 90.7 ±3.1%, $P>0.05$) and blastocyst (75.4 ±1.6% vs. 65.1±2.3,$P>0.05$) rates in the control group were slightly higher than that in the test group. Also, we found no significant differences in the total cell numbers per blastocyst (48±7 cells vs. 46±6 cells, $P>0.05$) between the groups. In experiment 3, the effect of PZM3 frozen storage on the pre-implantation development of porcine-cloned embryos was investigated. Our results indicate no significant differences in the rates of cleavage (71.5±5.1% vs. 78.1±1.9%, $P>0.05$) and blastocyst formation (34.6±7.6% vs. 38.2±3.5%, $P>0.05$) as well as the total cell numbers per blastocyst (40±11 vs. 48±9, $P>0.05$) between the test and control groups. Taken together, our results imply that the frozen storage of PZM3 is feasible and of practical value for culturing parthenote and cloned porcine embryos.

Key words: Frozen storage, *in vitro* culture, porcine embryo, pre-implantation development, PZM3.

INTRODUCTION

Somatic cell nuclear transfer includes complicated procedures in which the cloned embryo is cultured *in vitro*, which is essential for successful production of transgenic cloned animals. Liquid culture media preparation is an important and indispensable step for human assisted reproductive technologies or domestic animal embryo engineering laboratories. Generally, culture media include stable components such as salts, amino acid and vitamins and labile compounds such as antibiotics, glutamine, calcium lactate and sodium pyruvate(Weathersbee *et al.*, 1995). The shelf life of liquid culture media, which are often prepared from individual components or reconstituted from a commercially available powder (Davidson *et al.*, 1988), can only be used within a limited time frame of 1-2 months even when stored at 2-8°C, which may be caused by the natural breakdown process of many labile components in the media (Weathersbee *et al.*, 1995). Many laboratories have resorted to freezing media and storing them at -20°C or -80°C, which can overcome the instability problems of the media.

Freeze-thawed media are apparently effective for human IVF (Bernart *et al.*, 1990), bovine IVF (Franco *et al.*, 2005) and the *in vitro* development of the one-cell murine embryo (De Silva, 1993). However, the freeze-thawed media require specialized storage facilities such as ultra-low temperature freezer before the media are ready to use (Weathersbee *et al.*, 1995). Moreover, an easy and simple preparation method of frozen media that is stored for several months without the necessity for re-adjusting and testing pH, osmolarity, sterility and endotoxin levels after thawing saves time and is cost-effective (Bernart *et al.*, 1990).

Although the shelf life of frozen liquid medium can be extended for some days, the maximal shelf life of different frozen liquid medium has not been determined. Therefore, medium that may be supportive of *in vitro* fertilization and early embryo development may be discarded based on the arbitrary guidelines regarding the storage limitations, which is wasteful and an inefficient use of personnel and materials (Bell *et al.*, 1995). To avoid waste, we must establish concrete guidelines for

frozen media that consider the shelf life of different culture media. However, no data have been reported in the literature to suggest a specific time point at which porcine culture media, such as PZM3, NCSU23, are no longer supportive for porcine embryos.

A cost-effective, efficient, reliable, consistent and easily controlled protocol for the preparation and storage of the culture media of porcine embryos is needed. However, few studies have been conducted in this area. Therefore, the present study aimed to examine whether *in vitro* culture of porcine embryo is feasible using freeze-thawed PZM3 medium.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation and storage of porcine zygote medium 3 (PZM3): Three liters of complete PZM3 were made using the protocols by Yoshioka *et al*.(2002) and by Lai *et al*.(2003). After the medium was prepared, the liquid PZM3 was evenly distributed into 50ml aliquots per Nunc centrifuge tube (Cat. No. 339497,NalgeNunc International, Roskilde, Denmark).Half of the tubes with PZM3 were placed into the -80°C freezers, and the rest were placed into the 4°C refrigerator. Generally, aliquots of frozen medium were thawed at least one day before use. Culture dishes were prepared using a Nunc 4-well dish (Cat. No. 144444, NalgeNunc International, Roskilde, Denmark) with 400 µl of thawed or non-frozen 'ready-to-use' media. The dishes were covered with 400 µl of mineral oil per well. The media in 4-well dishes were kept overnight in the CO_2 incubator for equilibration (38.5°C, 5% CO_2, 5% O_2 and 90% N_2) until use.

Preparation for somatic cells: The animal experiments in the current study have been approved by the Animal Care and Use Committee of Anhui Agricultural University. Porcine fetal fibroblast cells were isolated from an approximately 40-day-old Duroc fetus. The procedures of primary cell culture and subculture of the cell line were performed as previously described (Zhang *et al.*, 2007). Cells at passage 3-8 were grown for 1 week to 100% confluence in wells of 4-well dishes (Nunc, Denmark) in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum at 39°C with 5% CO_2 and 100% humidity. Just before nuclear transfer (NT), attached cells were washed twice with Ca^{2+}- and Mg^{2+}-free phosphate buffered saline (PBS) and incubated at 39°C for 5 min with 100 µl of 0.25% Trypsin-EDTA. The dissociated cells were then moved to a centrifuge tube containing 500 µl of Hepes-buffered tissue culture medium 199 (TCM-199) that was supplemented with 2% adult cattle serum (CS). The fibroblast suspension was stored for approximately 30 min at room temperature (24-29°C) until use.

***In vitro* maturation of porcine oocytes:** Sow and peripubertal gilt ovaries were collected from a local slaughterhouse and transported in physiological saline solution with antibiotics at 25-35°C to the laboratory within 4 h after slaughter. Cumulus-oocyte complexes (COCs) were aspirated from follicles that were 3-6 mm in size with an 18-gauge needle using vacuum suction or a disposable syringe. COCs with at least 3 layers of enclosed compact cumulus and cytoplasm were selected and washed twice in Hepes-buffered TCM-199 with 0.3%(w/v) heparin (5000 IE/ml, LEO Pharma A/S, Denmark), 5% amphotericin (w/v) and 10%(v/v) CS. Afterwards, the selected COCs were matured in groups of 50-70 in 400 µl of IVM media in 4-well dishes at 38.5°C with 5% CO_2 and humidified air for 40-46 h. The IVM medium was bicarbonate-buffered TCM-199 that was supplemented with 15% (v/v) CS, 10% (v/v) porcine follicular fluid, 10 IU/ml of eCG, 5 IU/ml of hCG (both from Suigonan Vet; Denmark), 0.8 mM L-glutamine and 0.05 mg/ml of gentamicin.

COCs were then transferred to 1 mg/ml of hyaluronidase and pipetted repeatedly for 2 min to denude cumulus cells. Oocytes with intact cell membrane and clear perivitelline space were selected for future use.

Production of parthenogenetically activated embryos: Denuded oocytes were washed three times followed by balancing in activation medium for 2-3 min. 20-30 MII oocytes were then placed in a fusion chamber, which had been filled with activation medium. Two direct current pulses of 1.6 kv/cm were then applied at 100 µsec with a 1-sec interval using a CFS-150/B BLS fusion machine (BLS Ltd.,Budapest, Hungary) to stimulate oocyte activation. The activation medium was 0.3 M mannitol, 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% (w/v) polyvinyl alcohol. Subsequently, oocytes were washed three times and cultured in PZM3 with 10 µg/ml of cytochalasin B (CB) and 10 µg/ml of cycloheximide. Four hours later, activated oocytes were washed three times and cultured in PZM3.

Production of cloned embryos: The solution used for micromanipulation was Hepes-buffered TCM-199 that was supplemented with 5 µg/ml of CB and 2% CS. Micromanipulation was performed using an inverted microscope that was equipped with manipulation systems and a warmed stage. The procedure of micromanipulation was performed as previously reported (Zhang *et al.*, 2007). A single 50 µl micromanipulation medium drop was made in the central area on a lid of a 60 mm culture dish (Nunc, Denmark) and covered with mineral oil. Each group of 20-30 oocytes and nuclear donor cells were placed in this drop and incubated for 5-10 min. Subsequently, one oocyte was secured with a holding

pipette (inner diameter: 25-35 µm and outer diameter: 80-100 µm). The first polar body and 20% of the adjacent cytoplasm presumptive containing metaphase plate were aspirated out with a beveled pipette (inner diameter: 20µm). A somatic cell with a diameter at approximately 20 µm was injected into the perivitelline space through the same slit. Reconstructed couplets were transferred into drops of Hepes-buffered TCM-199 with 2% CS and covered with mineral oil for recovery for 0.5-1.5 h until fusion and activation.

A simultaneous fusion and activation (FAS) protocol was employed using 0.3 M mannitol that was supplemented with 0.05 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.01% (w/v) polyvinyl alcohol. Reconstructed couplets were equilibrated in the activation solution for 4 min, and then groups of 5 couplets were placed in the fusion chamber (BTX microslide 1-mm fusion chamber, model 450; BTX, USA) that had been filled with fusion solution. Couplets were aligned manually using a fine needle to make the contact plane parallel to electrodes, and then two direct current pulses of 1.65 kv/cm for 100 µsec with a 1-sec interval were applied using a CFS-150/B fusion machine. Subsequently, couplets were washed three times and cultured in PZM3 with 10 µg/ml of CB and 10 µg/ml of cycloheximide. Four hours later, the fusion results were examined under a stereomicroscope. Fused couplets, i.e., reconstructed embryos, were transferred to PZM3 according to the description of individual experiments.

***In vitro* culture and quality assessment of embryos:** Embryo culture was conducted at 39°C with 5% CO_2, 5% O_2, 90% N_2 and 100% humidity. Cleavage and blastocyst rates were determined under a stereomicroscope after 48 h and 144-168 h of *in vitro* culture, respectively. Some blastocysts were stained by 20 µg/ml of Hoechst33342 for 10 min and then photographed under UV light using an inverted microscope. Total cell numbers per blastocyst were counted using Image J.

Experimental designs: In Experiment 1, prepared liquid PZM3 was aliquoted into 50 ml Nunc centrifuge tubes. Half of the tubes with PZM3 were stored in -80°C freezers, and the rest were placed into the 4°C refrigerator. Within one week after storage, a tube of frozen PZM3 was warmed to 38.5°C in the CO_2 incubator. In addition, a tube of PZM3 that was stored at 4°C served as a control. A total of 400 µl of PZM3 per well was added to at least three 4-well culture dishes, which were balanced for at least 4 h in the incubator before being used to culture embryos.

In Experiment 2, we tested whether PZM3 that was kept in frozen storage for 5 months supported the *in vitro* development of parthenotes comparable to that supported by freshlypreparedPZM3. After frozen storage for 5 months, PZM3 was thawed using the same method as Experiment 1. PZM3 that was freshly prepared and stored less than 1 week at 4°C acted as control.

In Experiment 3, we used somatically cloned embryos to investigate the effect of freeze-thawed PZM3 on the pre-implantation development of porcine embryos.

Statistical analysis: Data were expressed as the mean±SEM and analyzed using the chi-square module in SPSS 11.0 with $P<0.05$ denoting a significant difference.

RESULTS

Effect of frozen storage of PZM3 on the *in vitro* development of porcine parthenote embryos: Within the first week of storage at 4°C and -80°C, PZM3 was used to culture porcine embryos that were derived from parthenogenetic activation to investigate whether freeze-thawed PZM3 had a negative effect on the *in vitro* developmental competence of porcine embryos. As shown in Table 1, no significant difference existed between the two test groups based on the cleavage rate (83.9±1.2% vs. 84.5±1.8%) and the blastocyst formation rate (65.2±2.1% vs. 84.5±1.8%). Moreover, similar total cell numbers per blastocyst were observed in these two groups (50±7 vs. 47±5). We prolonged the duration of the frozen storage of PZM3 at -80°C to 5 months and compared its ability to support preimplantation development of porcine parthenotes to freshly prepared PZM3, which was stored at 4°C for no longer than 1 month. As shown in Table 2, although the cleavage (90.7±3.1% vs. 97.8±2.7%) and blastocyst rates (65.1±2.3% vs. 75.4±1.6%) in the control group were both slightly higher than that in the test group, no statistical differences were observed. We also found no significant difference in total cell numbers (48±23 vs. 46 ±19) per blastocyst between the groups.

Effect of frozen storage of PZM3 on the *in vitro* development of porcine somatic cell-cloned embryos: As shown in Table 3, the rates of cleavage (71.5±5.1% vs. 78.1±1.9%) and blastocyst formation (34.6±7.6% vs.38.2±3.5%) were similar between the freeze-thawed PZM3 and the fresh PZM3 using cultured porcine embryos that were produced from somatic cell nuclear transfer instead of parthenotes. No significant difference in the total cell number (40 ± 11 vs. 48 ± 9) was observed.

Table 1. Effect of frozen PZM3 on the *in vitro* development of porcine PAEs (parthenogenetically activated embryos).

Group	No. of Embryos cultured	[1)]No. of Embryos cleaved (%, Mean±SEM)	[2)]No. of Blastocysts (%, Mean±SEM)	Total cell number per blastocyst (No. of embryos examined)
One week frozen storage of PZM3	93	78（83.9±1.2）	60 (65.2±2.1)	50±7（16）
Fresh PZM3	103	87 (84.5±1.8)	65 (63.1±3.8)	47±5（10）

[1)]Cleavage rate= No. embryos cleaved / No. embryos cultured; [2)]Blastocyst rate=No. blastocyst / No. Embryos cultured. Significant difference at $P<0.05$ was not observed between treatment groups.

Table 2. Effect of frozen PZM3 on the *in vitro* development of porcine PAEs (parthenogenetically activated embryos).

Group	No. of Embryos cultured	[1)]No. of Embryos cleaved (%, Mean±SEM)	[2)]No. of Blastocysts (%, Mean±SEM)	Total cell number per blastocyst (No. of embryos examined)
Five months frozen storage of PZM3	129	117 (90.7±3.1)	84 (65.1±2.3)	48±7（15）
Fresh PZM3	138	135 (97.8±2.7)	104 (75.4±1.6)	46±6（19）

[1)]Cleavage rate= No. embryos cleaved / No. embryos cultured; [2)]Blastocyst rate=No. blastocyst / No. Embryos cultured. Significant difference at $P<0.05$ was not observed between treatment groups.

Table 3.Effect of frozen PZM3 on the *in vitro* development of porcine somatic cell nuclear transfer embryos.

Group	No. of Embryos cultured	[1)]No. of Embryos cleaved (%, Mean±SEM)	[2)]No. of Blastocysts (%, Mean±SEM)	Total cell number per blastocyst (No. of embryos examined)
One week Frozen storage of PZM3	95	68 (71.5±5.1)	33 (34.6±7.6)	40±11（12）
Fresh PZM3	100	78 (78.1±1.9)	38 (38.2±3.5)	48±9（15）

[1)]Cleavage rate= No. embryos cleaved / No. embryos cultured; [2)]Blastocyst rate=No. blastocyst / No. Embryos cultured. Significant difference at $P<0.05$ was not observed between treatment groups.

DISCUSSION

In the present study, we found that the use of PZM3, which was placed in frozen storage and warmed and buffered in the CO_2 incubator, supported rates of *in vitro* development of both porcine parthenotes and cloned embryos that were comparable to that of fresh, ready-for-use PZM3.

PZM3 is suitable for the *in vitro* culture of embryos in a low-oxygen environment (Yoshioka *et al.*, 2002). Moreover, PZM3 is convenient and easily made and does not require medium change during *in vitro* culture. Therefore, PZM3 is superior to traditional media such as NCSU23 and NCSU37, not only because the births of viable piglets from IVF, cloned embryos that were cultured in PZM3 have been documented (Yoshioka *et al.*, 2003; Du Y*et al.*, 2007a), but also for saving more energy and labor input related to media preparation.

A major task of regular management in embryo laboratories or clinical reproductive centers is to prepare liquid culture media for embryos. Based on the guidelines provided by manufacturers, commercial medium is used within a recommended time, which usually ranges from 1-2 months between the first opening and the last use. Meanwhile, self-prepared liquid culture media is frequently discarded by scientists or technicians after 2 weeks of use. The liquid culture medium must be prepared once a month, which increases labor and cost. In addition, batch variation and contamination are also inevitable. However, if the use of liquid culture medium that was frozen were feasible to support the embryonic development, frozen storage would be helpful to reduce batch variation, contamination (from frequent handling), cost and labor.

Our results show that freeze-thawed PZM3, either frozen stored for 1 week or 5 months, performed similarly to freshly prepared PZM3 in the regards of supporting pre-implantational development of porcine parthenogenetically activated embryos. Then we extended our findings to nuclear transferred embryos using PZM3 that was frozen for 1 week and freshly prepared PZM3. Moreover, when PZM3 that frozen stored at -80°C for 5 months were used to culture cloned embryos, the *in vitro* development rate was comparable to that when PZM3 frozen stored or kept at 4°C for 1 week (data not shown). The births of cloned piglets also

confirmed that nuclear transferred blastocysts that developed after being cultured in freeze-thawed PZM3 are of good quality (Du *et al.*, 2007b). Thus, our results imply that it is feasible to culture porcine embryos using PZM3 that had been previously frozen. However, protocols that involve freezing media must carefully consider the types of containers such as centrifuge tubes that are used. The original material of the centrifuge tubes, which contain aliquots of media, must withstand lower temperatures such as -80°C. The present results suggest that there was no loss of nutrient quality in the frozen PZM3 after 5 months. However, further investigation is required to determine the maximum duration for the storage of frozen PZM3 that is useful for culturing parthenote and cloned porcine embryos at least for up to 5 months.

Taken together, the frozen storage of PZM3 is feasible and of practical value for culturing parthenote and cloned porcine embryos.

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