

## TECHNICAL NOTE

# Screening high-quality fetal bovine serum for porcine oocyte maturation in vitro

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## Abstract

Fetal bovine serum (FBS) is widely used in cell cultures due to its high stability and easy access. It was also used as a substitute for porcine follicular fluid (PFF) in previous studies. However, FBS components are unclear, and the presence of FBS in culture media may introduce a variation from batch to batch. This study aimed to establish an effective method to screen FBS in place of PFF in the culture media for porcine oocytes in vitro. We screened FBS from different sources by using porcine fetal fibroblast cells. The effects of six FBS samples on porcine fetal fibroblast cell growth were tested via frozen cell survival assay, cell clone formation assay, cell growth curve, and cell passage activity assay. The best serum that we called GFBS (heat-inactivated FBS, cat. no. 10500-64; Gibco) showed a similar effect on the maturation and development of porcine oocytes to that of PFF and can be used as a good substitute for PFF. These results suggested that the porcine fetal fibroblast cell culture test can be used as a valuable method to screen FBS for porcine oocyte maturation and embryonic development in vitro.

## KEYWORDS

fetal bovine serum, maturation rate, porcine oocytes

## 1 | INTRODUCTION

Porcine follicular fluid (PFF) is employed during porcine oocyte maturation in vitro.<sup>1-4</sup> PFF plays a key role in porcine oocyte maturation in vitro, and improves development of porcine parthenogenetic embryo and somatic cell nuclear transfer embryo in vitro. However, it is not a commercially available reagent and is usually derived from the ovaries obtained from slaughter houses. PFF has considerable

variability for many influencing factors, such as temperature change or long-distance transport stress. It is possible that PFF be collected from the ovarian tissue of infected pig and carrying the diseases.<sup>5</sup>

Fetal bovine serum (FBS) is the most widely used in cell cultures and can also be used as a substitute of follicular fluid in porcine oocyte maturation in vitro.<sup>6,7</sup> The stability of the same batch of FBS is higher than that of porcine follicular fluid. However, FBS components are unclear and contain many unknown factors.

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Considerable variability among sources or even among batches FBS is observed. Therefore, the process of serum prescreening is essential before using the serum for porcine oocyte maturation in vitro.

In this study, we screened FBS from different sources through porcine fetal fibroblast cell culture. Then, we tested the high-quality serum in porcine oocyte maturation and parthenogenetic embryo development in vitro. The purpose of this study was to establish a method to screen FBS in porcine oocyte maturation and embryo development in vitro.

## 2 | MATERIALS AND METHODS

### 2.1 | Chemicals

All chemicals were purchased from Sigma Aldrich unless stated otherwise. Washing buffer was prepared by dissolving 6.663 g NaCl, 0.237 g KCl, 0.168 g NaHCO<sub>3</sub>, 0.041 g NaH<sub>2</sub>PO<sub>4</sub>, 1.868 mL of C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub>, 0.102 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 2.383 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.065 g penicillin G, 0.010 g phenol red, 0.294 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.100 g polyvinyl alcohol (PVA), 2.186 g sorbitolum, 0.025 g gentamicin, and 0.022 g C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub> in 1000 mL of dH<sub>2</sub>O. Then, the pH was adjusted to 7.4. The solution was filtered with a 0.22 μm pore filter and stored at 4°C for 3 weeks. Mature culture medium (MM) was prepared by dissolving 9.5 g TCM-199, 1 g PVA, 2.2 g NaHCO<sub>3</sub>, 0.5496 g D-glucose, 0.1 g C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub>, 0.075 g penicillin G, 0.05 g streptomycin, 0.07 g L-cysteine, 0.5 mg luteinizing hormone, 0.5 mg follicle-stimulating hormone, and 10 μg epidermal growth factor in 1000 mL of dH<sub>2</sub>O. The solution was filtered with a 0.22 μm pore filter and stored at 4°C. Dulbecco's modification of Eagle's medium (DMEM-I) was prepared by adding 1 mM C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub> solution, 1× Non-Essential Amino Acid (MEM NEAA), 1× GlutaMAX-1, 1× penicillin-streptomycin into DMEM (cat. no. 10829018; Gibco). The activation solution was prepared by dissolving 2.73 g mannitol, 0.015 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.002 g MgCl<sub>2</sub>·6H<sub>2</sub>O, and 0.013 g HEPES in 100 mL of dH<sub>2</sub>O. The solution was filtered with a 0.22 μm pore filter and stored at 4°C for 2 weeks. Porcine zygote medium 3 (PZM-3) was prepared by dissolving 0.6312 g NaCl, 0.2106 g NaHCO<sub>3</sub>, 0.0746 g KCl, 0.0048 g KH<sub>2</sub>PO<sub>4</sub>, 0.0098 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.0616 g Ca-lactate·5H<sub>2</sub>O, 0.0022 g C<sub>3</sub>H<sub>3</sub>NaO<sub>3</sub>, 0.0146 g L-glutamine, 0.0546 g hypotaurine, 2 mL of BME, 1 mL of MEM and 1 mL of penicillin G + streptomycin in 100 mL of embryo water. The solution was filtered with 0.22 μm pore filter and stored at 4°C for 2 weeks.

Fetal bovine serum samples used in this study are as follows: FBS (cat. no. 10099-141, Gibco) that was heat inactivated at 56°C for 30 minutes, GFBS (heat-inactivated FBS, cat. no. 10500-64; Gibco), FCS (heat-inactivated FBS, cat. no. SH30070.03; Hyclone), SJQ (cat. no. 11012-8611; Sijiqing) that was heat inactivated at 56°C for 30 minutes, BI (Certified FBS, cat. no. 04-002-1A; Biological Industries), and heat-inactivated BI (HiBI).

### 2.2 | Frozen cell survival assay

Frozen porcine fetal fibroblast cells were resuscitated and washed with 7 mL of DMEM in a 15 mL centrifuge tube. Then, the cells were delivered to 0.1% gelatin-coated 6-well culture plates (50 000 cells/well). After culturing in complete medium for 72 hours, the cells were digested using 0.25% trypsin, and the viability was analyzed via trypan blue staining.

### 2.3 | Cell clone formation assay

Porcine fetal fibroblast cells were digested by using 0.25% trypsin, and then the number of cells was calculated using a cell counting chamber. The cells were delivered into 6-well cell plates (100 cells/well). The complete medium was refreshed every 3 days until cell clones could be observed with the naked eye. The diameter of cell cluster size of >50 μm was counted as a cell clone.<sup>8</sup>

### 2.4 | Cell growth curve

Cells were seeded in 0.1% gelatin-coated 96-well culture plates (5000 cells/well). After culturing for 1, 2, 3, 4, 5, 6, or 7 days, Cell Counting Kit-8 was used to determine the cell growth rate.

### 2.5 | Cell passage activity

Frozen porcine fetal fibroblast cells were resuscitated in DMEM-I with 10% BI as described in frozen cell survival assay. After the cells became 85% confluent, they were digested with 0.25% trypsin. Cells (3 × 10<sup>4</sup>/well) were seeded in 0.1% gelatin-coated 6-well culture plates and cultured in with DMEM-I with 10% FBS (SJQ, BI, HiBI, GFBS, FCS, or FBS). After culturing at 37°C in 5% CO<sub>2</sub> in air for 7 days, cells were digested using 0.25% trypsin, and the cell numbers were counted. The cell passage step was repeated thrice to stabilize the cell in this kind of complete medium, and then the number of cells was counted.

### 2.6 | Porcine oocyte collection and in vitro maturation

During the breeding season (April-May), porcine ovaries were obtained from the Rongchang slaughterhouse. All ovaries were transported to the laboratory by using physiological saline containing penicillin (100 IU/mL) and streptomycin (50 mg/mL) at 38.5°C. After washing with physiological saline, porcine ovaries were transferred into physiological saline at 38.5°C. Follicles that were 2-8 mm in diameter were punctured using a 20 mL syringe, and all the fluid containing cumulus-oocyte complexes (COCs) was aspirated. The contents of the syringe were transferred into a 50 mL centrifuge

tube and incubated for 5 minutes at 38.5°C. After sedimentation, the supernatant liquid was carefully discarded, and approximately 5 mL of lower liquid and precipitation were obtained. After washing thrice with 20 mL of washing buffer, the contents were transferred into a 60 mm diameter culture dish. COCs enclosed by more than three layers of compact cumulus cells with evenly granulated cytoplasm were selected using a stereomicroscope. After washing thrice with MM, 70 COCs were transferred into 500  $\mu$ L of MM incubated at 38.5°C in a humidified atmosphere of 5% CO<sub>2</sub> in air for at least 4 hours. Then, these COCs were cultured with MM supplemented with 10% PFF or FBS at 38.5°C in 5% CO<sub>2</sub> in air for 42–46 hours. After 45 hours of maturation, the COCs were transferred to 1 mg/mL of hyaluronidase in D-PBS and denuded cumulus cells by pipetting for 3 minutes. Under a stereo microscope, the oocytes with full yolk membrane, and first polar body extrusion were counted as mature oocytes.

## 2.7 | Activation and culture of porcine oocytes

Denuded oocytes were equilibrated for 20 seconds in the activation solution, and they were washed with activation solution thrice. Every 20 denuded oocytes were transferred to the chamber overlaid with activation solution and between two electrodes 500  $\mu$ m apart. After activation with an electric stimulus (55 V for 30  $\mu$ s) twice, oocytes were transferred and cultured in PZM-3 containing 1% bovine serum albumin at 38.5°C in 5% CO<sub>2</sub> in air. The blastocyst formation rates were counted at 168 hours.

## 2.8 | Statistical analysis

Statistical analysis data were expressed as mean  $\pm$  SEM. Each experiment was replicated at least thrice. The data were analyzed using *t* test. Statistical significance was accepted at  $P < .05$ . Data were analyzed with SPSS 22.0 software (SPSS Inc).

# 3 | RESULTS

## 3.1 | Screening for good FBS batches

To obtain FBS which can increase the quality of porcine oocyte matured in vitro, we screened FBS from different sources through porcine fetal fibroblast cell culture. The frozen cell survival assay, cell clone formation assay, cell growth curve, and cell passage activity were analyzed during porcine fetal fibroblast cell culture in cell culture medium contained different FBS. In our laboratory, BI was used as an additional FBS in porcine fetal fibroblast cell culture before cryopreserving cells. SJQ was always used for bacterial culture. The frozen cell survival rate, cell clone formation ratio, cell growth curve, and cell passage activity were detected during porcine fetal fibroblast cell generating stage in cell culture medium

containing different FBS groups ( $P > .05$ ) (Figure 1A). In the cell clone formation assay, the colony number was significantly higher in the GFBS group than in the BI group; and was significantly lower in the SJQ group than in other groups (Figure 1B). As shown in Figure 1C, the porcine fetal fibroblast cells grew better in GFBS, FBS, and FCS groups than in the BI group and fastest in the GFBS group. The GFBS group showed outstanding ability to support porcine fetal fibroblast cell growth in the cell passage activity assay (Figure 1D).

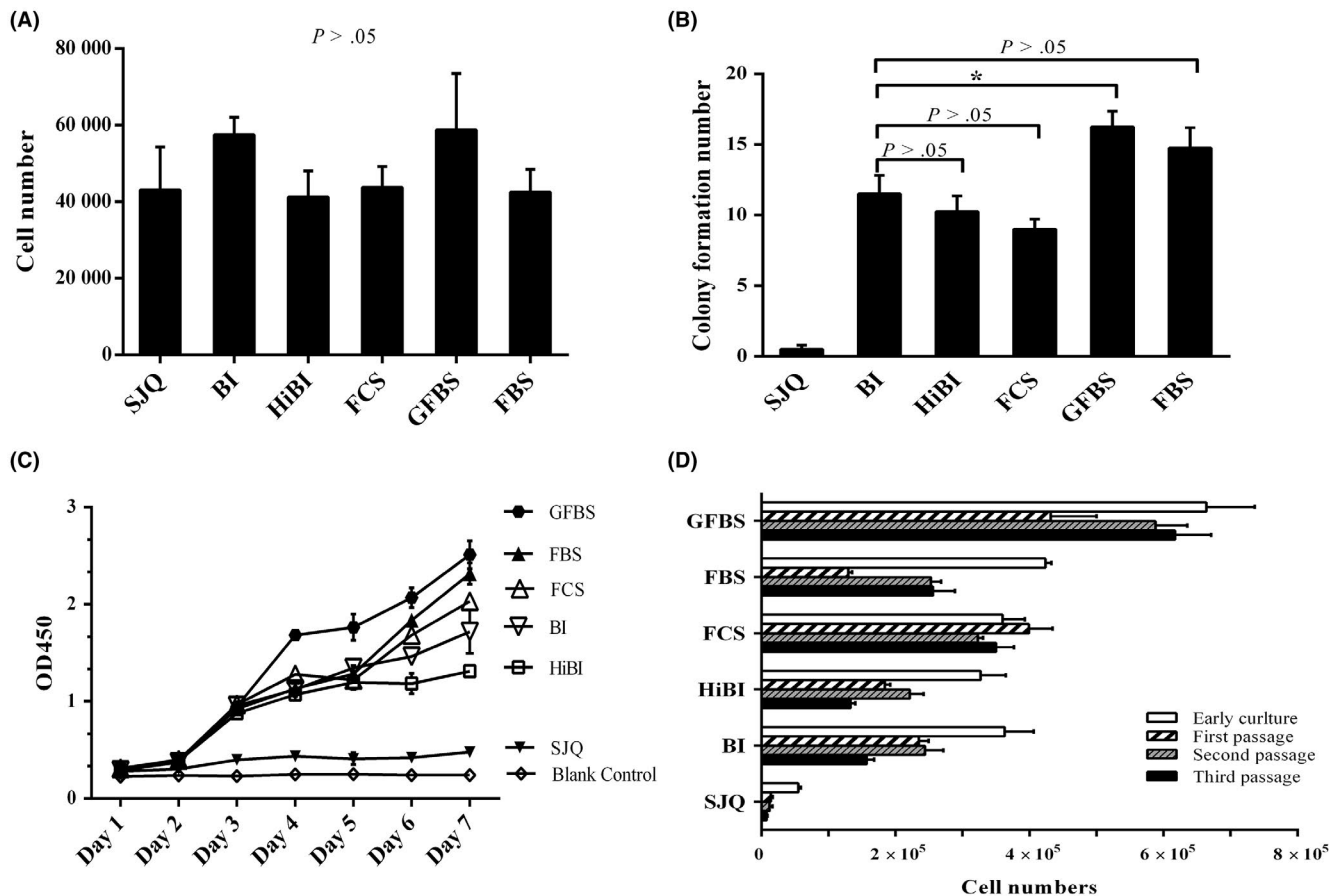
## 3.2 | Effects of FBS on porcine oocyte maturation and embryo development in vitro

The effects of best FBS GFBS and general FBS FCS found with porcine fetal fibroblast cells on porcine oocytes were tested. This experiment was performed with three groups, as follows: (a) MM was supplemented with 10% PFF as a control; (b) MM was supplemented with 10% GFBS; and (c) MM was supplemented with 10% FCS. As shown in Table 1, similar to the group with 10% PFF (maturation rate was approximately 79.50%), the maturation rate of porcine oocytes in MM supplemented with 10% GFBS was 79.67%, which was significantly higher than that in MM supplemented with 10% FCS (63.52%). The quality of porcine oocytes and PFF was affected by the temperature and season. The experiment shown in Table 1 was completed in the second half of the year. To investigate whether the screened out GFBS could substitute PFF, we compared the effects of 10% PFF, 10% GFBS, and their mixture (ie, 5% PFF + 5% GFBS) on porcine oocyte maturation and embryo development in the first half of the year. The oocyte maturation rates and blastocyst formation rates were similar (Table 2). The blastocysts were normal in all groups (Figure 2).

# 4 | DISCUSSION

Fetal bovine serum is not only widely used in cell cultures; but also used as a substitute of follicular fluid in oocytes maturation in vitro in previous studies.<sup>9–12</sup> However, serum is arguably the most common supplement in cell culture media, and also the least consistent.<sup>13</sup> Sikora<sup>14</sup> suggested that researchers need to report exactly how they screen serum to enable others to reproduce the work. So to establish a method for serum screening in vitro, porcine oocyte maturation and embryo development are important.

For the purpose of screening the best FBS, we detected the frozen cell survival rate, cell clone formation ratio, cell growth curve, and cell passage activity. In the frozen cell survival assay, after culturing for 72 hours, insignificant difference was found between the FBS groups ( $P > .05$ ). FBS did not affect the frozen cell survival rate, and the frozen cell survival assay may not be fit for serum screening. In the cell clone formation assay research, the BI group had a certain amount of cell clones, but only a few of them were found in the SIQ group. The colony formation number in HiBI, FCS or FBS was similar



**FIGURE 1** Fetal bovine serum screening in porcine fetal fibroblast cell. A, Frozen cell survival assay. B, Cell clone formation assay. C, Cell growth curve. D, Cell passage activity assay. All data are expressed as mean  $\pm$  SEM for at least three independent experiments. \**P* < .05

**TABLE 1** Effects of fetal bovine serum on porcine oocyte maturation in vitro

Medium	Supplement	Oocytes (n)	Mature oocytes (n)	Oocyte maturation rate (%)
MM	10% PFF	619	499	79.50 $\pm$ 3.11 <sup>a</sup>
MM	10% GFBS	348	275	79.67 $\pm$ 2.96 <sup>a</sup>
MM	10% FCS	465	290	63.52 $\pm$ 3.00 <sup>b</sup>

Note: Data are expressed as mean  $\pm$  SEM of the triplicate.

All experiments in this table were conducted in the second half of the year.

<sup>a,b</sup>Different letters indicate statistical difference within each column (*P* < .05).

Abbreviations: MM, mature culture medium; PFF, porcine follicular fluid.

to that in BI group. Only the GFBS group showed significantly high colony formation number in this test. In the cell growth curve test, the cell proliferation was did not show any significant difference in all groups on the days 1 and 2. However, on the third day, the cell growth rate of the SJQ group slowed down and was even similar to that of the blank control. From day 4, the cells in GFBS group grew more rapidly, and the cell growth rate was higher than in the other groups. In the cell passage activity assay, the GFBS group was the best one out of all the groups. Frozen porcine fetal fibroblast cells were resuscitated in DMEM-I with 10% BI, as described in the frozen cell survival assay. As shown in Figure 1D, a significant

difference in the cell number in the early culture was observed. Porcine fetal fibroblast cell is a primary cell that has a very limited passage number. In the BI, SJQ, FBS, and HiBI groups, as the number of passages increased, the cell number decreased. However, the cells maintained a cell number of approximately  $3 \times 10^5$  in the FCS group and  $5.5 \times 10^5$  in the GFBS group, although the cell number of the GFBS group significantly decreased after the first passage to adapt to this serum and soon increased up to  $5.5 \times 10^5$  after the second and third passages. These results indicated that the GFBS and FCS can make the cell remain their normal morphology in a long-term culture. It is also more suitable for this cell culture than BI.

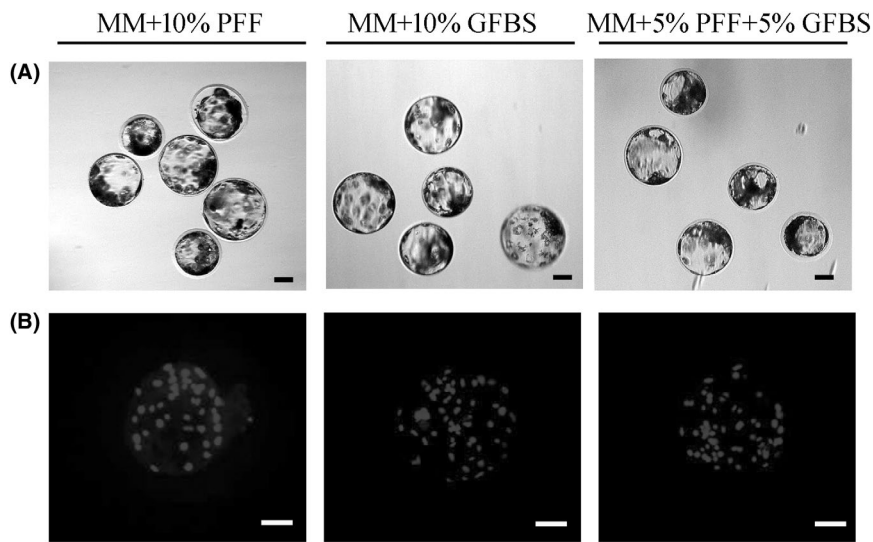
**TABLE 2** Effects of fetal bovine serum on porcine blastocyst formation rate in vitro

Medium	Supplement	Oocytes (n)	Oocyte maturation rate (%)	Blastocyst formation rate (%)	Mean cell number per blastocyst (n)
MM	10% PFF	249	84.06 ± 5.35	35.56 ± 5.09	51.73 ± 6.12
MM	10% GFBS	251	87.97 ± 4.60	36.65 ± 2.11	49.93 ± 5.44
MM	5% PFF and 5% GFBS	273	88.38 ± 5.99	38.58 ± 5.71	47.80 ± 2.04

Note: Data are expressed as mean ± SEM of three replicates.

All experiments in this table were conducted in the first half of the year.

Abbreviations: MM, mature culture medium; PFF, porcine follicular fluid.



**FIGURE 2** Development of porcine parthenogenetic embryo in vitro. A, Representative photographs of porcine parthenogenetic blastocysts under the microscope at day 7 of the culture. Bar = 100 μm. B, 4',6'-diamidino-2-phenylindole (DAPI) staining of porcine parthenogenetic blastocysts. Blastocysts were treated with DAPI on day 7 of the culture. Gray indicates the cell nucleus; bar = 100 μm

In this study, four methods were used to screen the sera. Comparatively, the frozen cell survival rate detection is more convenient and instant, but it is not capable of detecting the quality of serum. This may be due to the shortest time of cell contact with serum. Other methods are based on the detection of cell growth activity. Cell clone formation assessment requires a lot of work to calculate the number of clones under microscope, while the sizes of clones is mainly dependent on the operator's judgement, which lead to the lower efficiency and occurrence of error. Although the operation of cell passage activity analysis seems to be very complex, the results are the most effective and reliable. Cell growth curve assay is an easy method to operate and its results were similar to that of cell passage activity analysis. In summary, to reduce the work done, it is suggested that an enormous amount of serum samples could be preliminarily screened using the cell growth curve assay, and then confirmed with the cell passage activity analysis and cell clone formation assay.

To investigate whether the GFBS and FCS screened could substitute PFF in porcine oocyte maturation and development in vitro, we compared their effects on porcine oocyte maturation with PFF. The data showed that the GFBS group had similar oocyte maturation rates to PFF groups, but is significantly higher than FCS group. This result was similar to the prescreening results of serum in porcine fetal fibroblast cell testing. We also detected their effects on porcine parthenogenetic embryo development. Parthenogenetic blastocysts were collected on day 7 of culture,

and there was no difference between PFF, GFBS, and PFF + GFBS groups. It indicated that GFBS could be used as a good substitute for PFF for maturation of porcine oocytes and the embryonic development in vitro.

In conclusion, an effective method to screen FBS for porcine oocyte maturation and embryonic development in vitro has been developed. FBS from different sources could be screened by using the frozen cell survival assay, cell clone formation assay, cell growth curve, and cell passage activity assay on porcine fetal fibroblast cells. The screened high-quality serum was a good substitute of PFF for the maturation and development of porcine oocytes in vitro. The method to screen FBS is not only simple and rapid, but also cost-effective.

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#### CONFLICT OF INTEREST

None.

## AUTHOR CONTRIBUTION

XY and LG designed the study. QL and MW performed the experiments. XL and XY worked on the statistical analysis of the data and wrote the paper. The other authors provided help during the experiment. All authors read and approved the final manuscript.

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