Improve the Meiotic Competence of Growing Porcine Oocytes from Preantral Follicle

L.T. Hoai, N.X. Yen, L.K. Thoai, N. Van Thuan, and H-T. Bui

Abstract

In this study, we evaluated the effect of in vitro growth culture on meiotic competence and fertilization ability of porcine oocytes derived from preantral follicles (2–3 mm in diameter). It is reported that these growing oocytes were not able to obtain full meiotic competence after normal maturation culture. Therefore, oocyte—granulose cell complexes (OGCs) were cultured in vitro growth for 24 h in medium supplemented with 1 mM dbcAMP, 0.01 IU/mL FSH, 0.001 mg/ml 17β Estradiol and 10 ng/ml Androstenedione; then subsequent to in vitro maturation culture for 48 h before undergoing in vitro fertilization. After in vitro maturation, the percentage of growing oocytes reaching to metaphase II was similar to fully-grown oocytes collected from large antral follicles (4–6 mm in diameter). However, the fertilization ability of growing oocytes after in vitro growth was still lower compared to that of fully-grown oocytes (16 and 37%, respectively). Thus, the growing oocytes from 2 to 3 mm follicles should be pre-culture in vitro growth before undergoing in vitro maturation.

Keywords

Growing oocytes • Preantral follicles • In vitro growth (IVG) • In vitro maturation (IVM) • In vitro fertilization (IVF)

1 Introduction

The mammalian ovary contains a large number of primordial follicles with non-growing oocytes. However, only a few of these follicles can enter the growth phase and reach to ovulation, most of them undergo degeneration. Thus, many studies have been conducted to establish a model system for in vitro culture of growing oocytes from preantral follicles. However, the ability of growing oocytes that can acquire meiotic competence and undergo fertilization is still limited in porcine [1].

In some laboratories, oocytes collected from antral follicles (2–6 mm in diameter) by aspiration methods have been cultured for in vitro maturation. It has been reported that only fully grown oocytes (approximately 120 μm) from follicles of 4–6 mm can acquire full meiotic competence [2]. Growing oocytes (110–115 μm) from smaller follicles (2–3 mm) cannot complete maturation phase and obtain developmental competence under the same conditions as oocytes from larger follicles [3, 4]. Some researchers have claimed that there are some difficulties for growing oocytes isolated from preantral follicles to complete fully grown state and progress to maturation to obtain full meiotic competence after IVM. Therefore, the aim of this research is to pre-culture in vitro growth (IVG) of porcine oocytes from preantral follicles (2–3 mm) for 24-h. Then, these IVG oocytes were cultured for in vitro maturation (IVM) to examine meiotic competence. Finally, fertilization ability of oocytes after IVG and IVM were examined.

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2 Materials and Methods

2.1 Collection of Porcine Oocytes

Porcine ovaries were obtained at a local slaughterhouse. The ovaries were washed thrice with 0.9% sodium chloride and once with Dulbecco's PBS (PBS) containing 0.1% polyvinyl alcohol (PBS-PVA; Sigma Chemical Co.). Small follicles 2–3 mm in diameter were collected by dissection method, followed by Moor and Trounson [5]. Follicles 4–6 mm in diameter were aspirated from ovaries. OGCs were isolated from the follicles in 25 mM HEPES-buffered TCM-199 containing 0.1% PVA (HEPES-199).

2.2 In Vitro Growth of Oocytes

The OGCs from preantral follicles (2–3 mm) were cultured in vitro for 24 h in 96-well plate at 5% CO_2 and 38.5 °C and the time of OGCs collection was designated 0 h. The medium for IVG culture of oocytes was based on previous reports, with some modifications [6, 7]. The basic medium consisted of Minimum Essential Medium Alpha Medium (α-MEM; Sigma Chemical Co.), 10% fetal bovine serum (FBS), 0.1 mg/mL sodium pyruvate, 0.1 mg/ml Penicillin, 0.05 mg Streptomycin, 0.01 IU/ml follicle stimulating hormone (FSH), 1 mM dibutyl cyclic AMP (dbcAMP), 5% follicular fluid, 0.001 mg/ml 17β estradiol and 10 ng/ml androstenedione. After culture, some oocytes were denuded with mouth pipetting and fixed with acetic acid-ethanol (1:3, v/v), and stained with 1% (w/v) aceto-orcein to assess chromatin morphology which were classified as stringy chromatin (SC) and germinal vesicle I-IV (GVI-IV) stages [8]; while others were subsequent to maturation culture.

2.3 In Vitro Maturation of Oocytes

OCGCs collected from 2 to 3 mm preantral follicles and IVG oocytes were cultured for maturation. The fully-grown oocytes collected from antral follicles 4–6 mm were cultured as control. The maturation medium was bicarbonate buffered TCM-199 supplemented with 10% FBS, 0.1 mg/ml sodium pyruvate, 0.08 mg/ml kanamycin sulfate, 2.2 mg/ml sodium bicarbonate, and 0.1 IU/ml human menopausal gonadotropin. The OCGCs were cultured for 42 h under 5% CO_2 at 38.5 °C. Selected oocytes were denuded, fixed and stained to examine the stage of meiotic division.

2.4 In Vitro Fertilization of Oocytes

The IVF medium, modified Tris-buffered medium (mTBM), consisted of 113.1 mM NaCl, 3 mM KCl, 20 mM Tris, 7.5 mM $CaCl_2$, 11 mM glucose, 5 mM sodium pyruvate, 2 mM caffeine and 1 mg/ml BSA. After IVM, the matured oocytes were removed cumulus by 0.1% hyaluronidase. The denuded oocytes were washed and placed in 45 µl microdrops (10–15 oocytes/each)of IVF medium covered by paraffin oil at 38.5 °C and 5% CO_2 for 30 min. Oocytes were co-incubated sperm suspension for 30 min as described in Abeydeera and Day [9]. Oocytes were then washed and transferred to 50 µl microdrops of caffeine-free IVF medium covered by paraffin oil and incubated for 4 h. Presume zygotes were washed with in vitro development (IVD) medium and transferred to 50 µl microdrops of IVD medium covered by paraffin oil and incubated in 5% CO_2, 38.5 °C.

2.5 Statistical Analysis

At least three replications (≥ 30 oocytes) of experiments were performed. The statistical differences between groups were analyzed using one-way ANOVA followed by Tukey's multiple range test. Values of $P < 0.05$ were considered significant.

3 Results and Discussion

3.1 In Vitro Growth of Growing Oocytes

Growing oocytes from preantral follicles of 2–3 mm in diameter were collected and fixed at 0 h as a control group. There was a significant difference between oocytes fixed at 0 h and oocytes after undergoing IVG. After 24-h in vitro growth, the cumulus cell layers of growing oocytes increased rapidly compared to oocytes before cultured (Fig. 1a, b). Most of oocytes collected from 2 to 3 mm preantral follicles were found at SC stage (88.41%), which indicated that oocytes from these follicles are still in the growing phase. After culture, 66.43% (Table 1) of growing oocytes became fully grown (GVI). Only 18.88% of them remained stringy chromatin (Fig. 2a). The chromosome morphology of growing oocytes acquired GVI which is distinguished by heterochromatin ring morphology (Fig. 2b). Therefore, it is necessary for growing oocytes

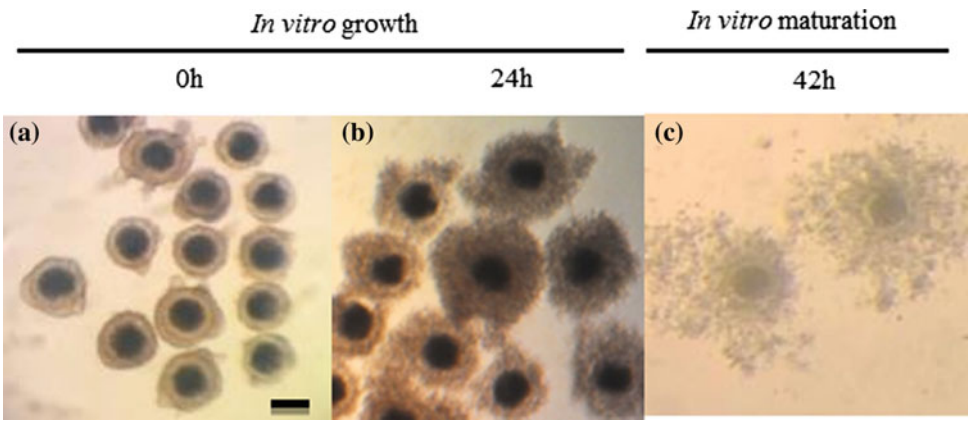

Fig. 1 Morphology of porcine oocytes-granulosa cell complexes (OGCs) before and after in vitro growth and maturation culture. After 24 h cultured in vitro growth medium, OGCs were subjected to in vitro maturation for 42 h. *Scale bar* is 110 μm

Table 1 The chromatin morphology of oocytes from preantral follicles with and without in vitro growth

Time of culture (hour)	No. of oocytes examined	Growing phase		DG (%)
		SC (%)	GVI(%)	
0 h	60	53[a] (88.41)	3[a] (5.03)	4[a] (6.55)
24 h	59	11[b] (18.88)	40[b] (66.43)	8[a] (15.52)

OGCs from preantral follicles (2–3 mm) were collected and cultured in vitro growth for 24 h. Stringy Chromatin stage (SC); Germinal Vesical I stage (GVI). [a,b]Within a column, value with different superscripts differ significantly ($P < 0.05$)

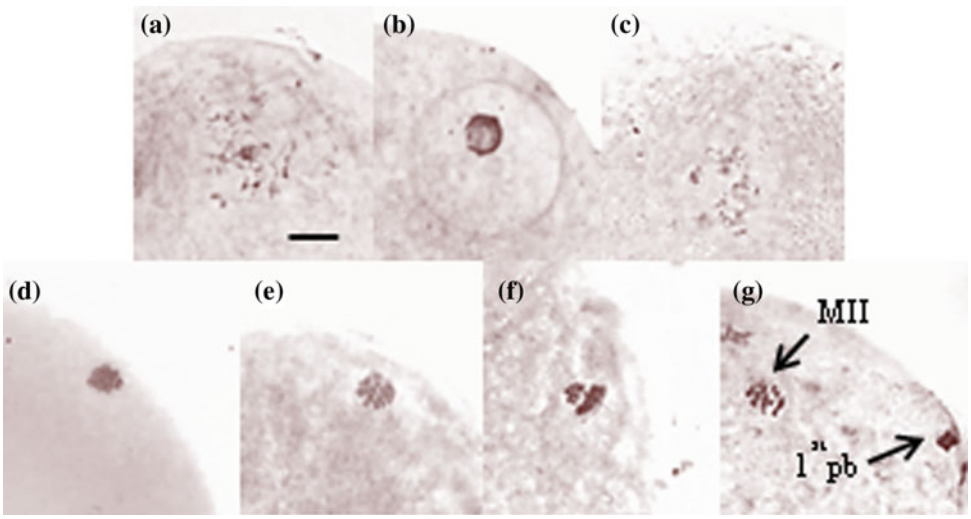

Fig. 2 Chromosomal morphology of oocytes during in vitro growth and in vitro maturation. The morphologies include stringy chromatin (SC, **a**), germinal vesicle I (GVI, **b**), germinal vesicle II-IV (GV II-IV, **c**), diakinesis (D, **d**), metaphase I (MI, **e**), anaphase I-telophase I (AI-TI, **f**) and metaphase II (MII, **g**). *Scale bar* is 10c

from preantral follicles of 2–3 mm in diameter to culture for 24 h of IVG in order to gain fundamental materials for development. In IVG culture condition, the growth of oocytes is promoted by dbcAMP, FSH, 17β estradiol and follicular fluid. The role of dbcAMP is to inhibit the germinal vesicle breakdown and inactivate maturation promoting factors (MPF) therefore prevent maturation. In addition, FSH stimulates the proliferation and differentiation of granulosa cells. It has been reported that the supplement of dbcAMP and FSH in culture medium promote the attachment of cumulus cells to oocytes [10]. Besides, 17β estradiol enhances the expression of growth differentiation factor 9 (GDF-9) which is important for the formation of transzonal projections (TZPs), one of the major factors for oocyte development [11]. Moreover, follicular fluid supplied macromolecules which may promote the development of oocytes.

3.2 In Vitro Maturation of Growing Oocytes

Growing oocytes from preantral follicles of 2–3 mm in diameter were collected and cultured for IVM as a control group. It indicated that with direct IVM, growing oocytes

Table 2 Nuclear chromosome stages of in vitro grown porcine oocytes after in vitro maturation

	No. of oocytes examined	GV stage		GVBD stage				DG (%)
		GVI (%)	GVII-GVIV (%)	D (%)	MI (%)	AI-TI (%)	MII (%)	
IVM (4–6 mm)	59	3^{a} (5.46)	4^{a} (7.22)	6^{a} (10.00)	10^{a} (16.88)	9^{a} (15.37)	24^{a} (40.64)	3^{a} (5.12)
IVM (2–3 mm)	57	11^{a} (19.23)	30^{b} (52.39)	8^{a} (14.70)	4^{b} (7.18)	1^{b} (1.52)	1^{b} (1.52)	2^{a} (3.48)
IVG + IVM (2–3 mm)	51	4^{a} (7.52)	4^{a} (7.86)	6^{a} (12.25)	11^{a} (21.50)	$3^{a,b}$ (6.00)	18^{a} (34.80)	5^{a} (9.94)

Oocytes after in vitro growth were subjected to in vitro maturation culture for 42 h. Germinal vesical breakdown stage (GVBD), germinal vesicle I (*GVI*), germinal vesicle II (*GVII*), germinal vesicle IV (*GVIV*), diakinesis (*D*), metaphase I (*MI*), anaphase I-telophase I (*AI-TI*), metaphase II (*MII*). $^{a-b}$Values with different superscript alphabets in same column differ significantly ($P < 0.05$)

Table 3 Fertilization ability of in vitro growth porcine oocytes after in vitro maturation

	No. of oocytes examined	Fertilized oocytes (%)				Unfertilized oocytes (%)				DG (%)
		Normal	Abnormal fertilization			MII	MI	Others	Total	
		2PN2PB	Polyspermy	Others	Total					
4–6 mm	33	12^{a} (36.92)	7^{a} (21.79)	8^{a} (23.59)	15^{a} (45.38)	5^{a} (14.36)	0 (0)	0 (0)	5^{a} (14.36)	1^{a} (3.33)
2–3 mm	31	5^{a} (16.06)	5^{a} (16.36)	9^{a} (29.09)	15^{a} (45.48)	4^{a} (13.03)	1 (3.03)	3 (9.7)	8^{a} (25.76)	4^{a} (12.73)

Growing oocytes from 2 to 3 mm follicles were cultured in vitro growth for 24 h and 42-h in vitro maturation before subjected to in vitro fertilization culture. Examined oocytes from 4 to 6 mm follicles were used as control. Normal fertilization: the oocytes that have two pronuclei and two polar bodies. Abnormal fertilization: other fertilizations such as two pronuclei and one polar body, more than two pronuclei.$^{a-b}$Values with different superscript alphabets in same column differ significantly ($P < 0.05$)

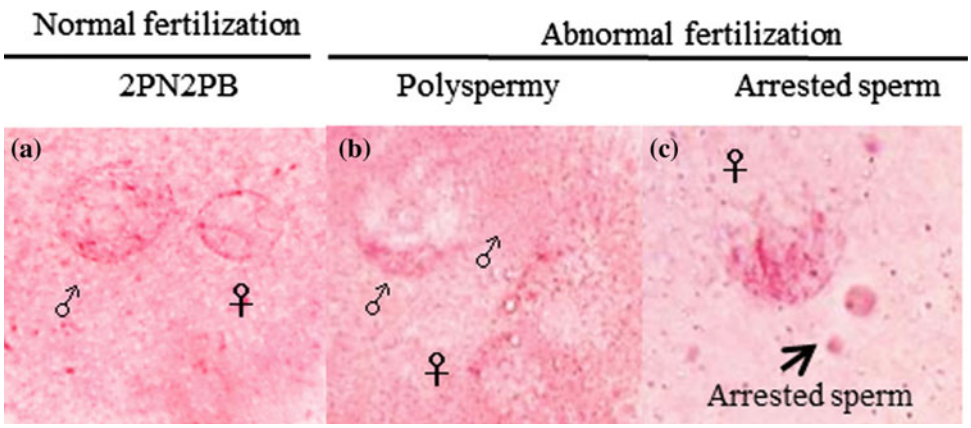

Fig. 3 Morphology of oocytes after in vitro fertilization. After 12 h of insemination, both groups had normal fertilization which had 2 p-ronulei and 2 polar bodies (2PN2PB, **a**) and abnormal fertilization including polyspermy (**b**) and arrested sperm (**c**). *Scale bar* is 10 μm

could not reach to metaphase II (MII). After 24 h of IVG culture, oocytes which had increasing levels of cumulus cells surrounding were subsequently cultured for IVM to determine the ability of oocytes to acquire meiotic competence. After 42-h culture, cumulus cells were expanded from oocytes (Fig. 1c). Growing oocytes collected from preantral follicles have shown the capability of meiotic competence since they had 34.80% reaching to MII, which was similar to that of 40.6% of fully grown oocytes from antral follicles of 4–6 mm (Table 2). Moreover, the growing oocytes after culturing IVG and IVM had shown many stages of chromosome morphology (Fig. 2c–g). Therefore, growing oocytes after undergoing both IVG and IVM were able to acquire full meiotic competence.

3.3 In Vitro Fertilization of Growing Oocytes

The oocytes from large antral follicles (4–6 mm) were collected as control. After IVM, both growing oocytes from 2 to 3 mm follicles and fully-grown oocytes from 4 to 6 mm groups were fertilized to assess the fertilization ability. After 12 h of insemination, the fully-grown oocytes were fertilized normally (36.9%—Table 3). Normal fertilization showed 2 pronulei and 2 polar bodies (Fig. 3a). The percentage of normal fertilization of IVG oocytes was 16.06%, which was still lower compared to that of fully-grown oocytes. Moreover, the abnormal fertilization (Fig. 3b, c) in both groups was similar (45.38 and 45.48% respectively). However, the unfertilized rate of IVG oocytes (25.76%) was higher than

that of fully-grown oocytes (14.36%). This pre-cultured IVG method maybe good model for application in single pregnant species such as bovine as well as human; since they produce one or two matured oocytes during menstrual cycles.

4 Conclusion

In conclusion, in vitro grown of oocytes isolated from preantral follicles with 2–3 mm in diameter have similar meiotic competence to fully grown oocytes from 4 to 6 mm follicles after in vitro maturation. Therefore, the pre-cultured IVG of oocyte from preantral follicle (2–3 mm) is important to improve the percentage of mature oocytes.

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