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Xanthan gum and Locust bean gum gel supports *in vitro* development of porcine oocytes derived from early antral follicles

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Abstract. Early antral follicle (EAF)-derived porcine oocytes develop more readily on polyacrylamide-gel (PAG) than on plastic plates. Xanthan gum (XG) and locust bean gum (LBG) are edible polysaccharides. We investigated XG-LBG gel supports in the development of EAF-derived porcine oocytes. XG and LBG were mixed in a 1:1 ratio to form a substrate. We cultured oocyte granulosa cell complexes (OGCs) from the EAFs on XG-LBG gels of various concentrations. The oocyte diameters were comparable among the 0.3, 0.5, and 1.0% gels; granulosa cell proliferation was greater on the 1.0% gel. The proliferation and survival rates of the granulosa cells, and the histone H4 at lysine 12 acetylation levels were higher in OGCs cultured on 1.0% XG-LBG than those grown on 0.3% PAG. Development to the blastocyst stage was 13.8% for the XG-LBG gels and 9.4% for PAG. In conclusion, XG-LBG are safe and efficient substrates for *in vitro* culture of oocytes.
Key words: Early antral follicle, Locust bean gum, Oocyte, Pig, Xanthan gum

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Oocyte development is well orchestrated with that of the surrounding granulosa cells. In the last stage of oocyte development, oocytes and granulosa cells form an antrum. When oocyte-granulosa cell complexes (OGCs) collected from the preantral follicles of cows and pigs are cultured *in vitro*, the granulosa cells proliferate and form antrum-like structures that are similar to antral follicles [1]. Therefore, maintaining the 3D structure of the OGCs and proliferation of the granulosa cells are important for oocyte development. To date, several culture methods have been developed to support the growth of oocytes of large animals [2], but the oocytes grown *in vitro* are inferior to those developed *in vivo.* This might be due to inappropriate culture environments.

Proper *in vitro* tissue formation is likely to be achieved by manufacturing an artificial biomaterial structure that mimics the biological cellular environment. The use of a soft culture substrate consisting of 0.3% poly acrylamide gel (PAG) instead of a stiff plastic culture plate enhances granulosa cell proliferation and the antral formation of OGCs, and oocytes cultured on PAG gel have greater developmental ability than those grown in a plastic culture well [3]. Furthermore, oocytes cultured on PAG have developmental markers such as large diameters, high histone acetylation levels, and high lipid contents. PAG is a useful substrate because its stiffness can be modified and it is easily processed; however, it is well known that the acrylamide monomer is neurotoxic and is a potential carcinogen [4]. Xanthan gum (XG) is a product of the fermentation of glucose by a plant-associated bacterium. It contains repeats of four monosaccharides (two D-glucose, two mannose, and one D-glucuronic acid) and has useful properties, i.e., it is cost effective, easily processed, has high pseudoplastic flow behavior, and is stable over a wide ranges of pH values, temperatures, and salt concentrations [5]. Furthermore, XG is nontoxic, does not cause any eye or skin inflammation, and has been approved by the United States Food and Drug Administration [6]. XG becomes a hydrogel when soaked in water and crosslinked with ions such as Na^+ and Ca^{2+}. However, in our previous experiments, the XG gel was too fragile to create culture substrates for the *in vitro* culture of OGCs. Therefore, we enhanced the stiffness of the XG gel by mixing it with Locust Bean Gum (LBG). LBG is an edible polysaccharide derived from the carob bean (*Ceratonia siliqua*, a Mediterranean tree), and is a linear galactomannan with different contents and distributions of galactose [7]. By mixing XG with LBG in a 1:1 ratio, then heating and cooling, it is possible to form a highly elastic gel [8, 9]. In the present study, we examined the effect of a polysaccharide hydrogel consisting of XG and LBG on the *in vitro* growth of oocytes derived from early antral follicles (EAFs), and compared oocyte development on XG-LBG gels and PAG culture systems.

First, we examined the effects of three concentrations of XG–LBG gel (0.3, 0.5 and 1.0%) on OGC development. As the concentration of XG-LBG increased, the stiffness of the gel increased; when the gel was over 1.0%, the gel was too sticky to be handled for *in vitro* OGCs incubation. Therefore, we used three concentrations of XG-LBG gel from 0.3% to 1.0%. Oocyte diameter is a fundamental marker of oocyte growth, but the diameters of the oocytes grown *in vitro* did not differ among the three gel concentrations (Table 1). The number of granulosa cells surrounding the oocytes is also a useful marker of OGC development, both *in vitro* and *in vivo* [1, 10]. We found that the proliferation and survival rates of the granulosa cells constituting the OGCs was greatest on the 1.0% XG-LBG gel compared to the other

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groups (Table 1). On the basis of these data, we used 1.0% XG-LBG gel for all subsequent experiments, and further addressed the question of whether 1.0% XG-LBG gel is more suited to supporting *in vitro* oocyte growth than PAG. In previous in vitro trials, we found that a PAG gel culture system yielded the highest quality oocytes [3]. A comparison between PAG and XG-LBG gel culture conditions revealed that the survival rate of the granulosa cells was significantly higher in the XG-LBG group (Table 2). Although XG has a beneficial effect on the survival rate of chondrocytes [11], the mechanism underlying this effect remains unclear. To investigate the quality of oocytes cultured on the XG-LBG gel, oocytes were subjected to parthenogenetic activation followed by *in vitro* culture; the oocytes cultured on the XG-LBG gel were more capable of developing to the blastocyst stage than those cultured on PAG, although the difference was not statistically significant ($P = 0.2$, Table 3). We have reported that the level of acetylation of histone H4 at lysine 12 (H4K12) is a good marker of *in vitro* oocyte growth and the acetylation level is reported to reflect energy sufficiency (ATP and lipid content) of the oocytes grown *in vitro* [3, 12]; and this notion is supported by Lin *et al*. [13]. They reported that histone modifications including H4K12 acetylation reflected oocyte competence. Also, the suboptimal culture conditions of germinal vesicle stage oocytes reduced the acetylation levels and rate of SN chromatin configuration; the extent of the reduction was greater for acetylation levels of H4K12. The level of H4K12 acetylation in the oocytes developed on XG-LBG gel was significantly higher than in those developed on PAG ($P < 0.05$, Fig. 1B), indicating the XG-LBG gel more induced more H4K12 acetylation or protected oocytes from certain adverse factors in culture environment.

In conclusion, the XG-LBG gel system provides a new means of culturing oocytes derived from EAFs, *in vitro*, and can support oocyte development more efficiently than the previous culture systems.

Materials and Methods

Chemicals and media

We purchased all regents from Nacalai Tesque (Kyoto, Japan), unless otherwise stated. To culture the OGCs, we used minimum essential medium Eagle-alpha modification (α-MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 26 mM $NaHCO_3$, 10 mM taurine, 0.1 mAU/ml follicle-stimulating hormone (Kyoritsu Seiyaku, Tokyo, Japan), 2% polyvinylpyrrolidone (K-90), 2 mM hypoxanthine (Sigma-Aldrich), 1% insulin transferrin selenium (Gibco BRL, Paisley, UK), 1 μg/ml 17β-estradiol, 3 mg/ml BSA (Fraction-V), and antibiotics. The *in vitro* maturation (IVM) medium was Medium 199 (Gibco), supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.5 mM L-cysteine, 0.9 mM sodium pyruvate, 1 mM L-glutamine, 10 ng/ml epidermal growth factor, 5% fetal calf serum, 10 IU/ml equine chorionic gonadotropin (ASKA Pharma, Tokyo, Japan), and 10 IU/ml human chorionic gonadotropin (Fuji Pharma, Tokyo, Japan). We carried out *in vitro* embryo culture and oocyte activation in porcine zygote medium 3 (PZM3) [14]. The *in vitro* OGC culture and oocyte maturation were performed at 38.5°C in 5% CO_2 and 95% air, whereas the *in vitro* embryo culture was performed at 38.5°C in 5% O_2, 5% CO_2, and 90% N_2. We aspirated the pFF for the IVM medium from the antral follicles (AFs; diameter 3–5 mm) of 100 gilts, and centrifuged it at 10,000 × *g* for 20 min. The resulting supernatants were collected, sterilized, and stored at

Table 1. Effect of differential concentration of xanthan gun (XG) and locust bean gun (LBG) mix gel on oocyte granulosa cell complexes *in vitro*

Groups	No. of trials	No. of oocytes	Rate of AF (%, Day 14)	Diameter of oocytes (μm)	Granulosa cell number	Survival rate of granulosa cells (%)
XG-LBG 0.3%	3	32	88.9 ± 5.6	120.2 ± 0.7	179,067 ± 8,187 [a]	83.3 ± 1.8
XG-LBG 0.5%	3	29	80.6 ± 2.8	118.8 ± 0.5	203,103 ± 10,129 [ab]	82.6 ± 2.4
XG-LBG 1.0%	3	29	80.6 ± 2.8	118.4 ± 0.7	213,862 ± 9,988 [b]	88.6 ± 1.2

AF, antral formation. [a–b] $P < 0.05$.

Table 2. Effect of differential substrate gel on oocyte granulosa cell complexes *in vitro*

Substrates	No. of trials	No. of oocytes	Rate of AF (%, Day 14)	Diameter of oocytes (μm)	Granulosa cell number	Survival rate of granulosa cells (%)
PAG 0.3%	4	34	83.3 ± 9.9	122.0 ± 0.9	195,030 ± 8,701	88.2 ± 1.0 [a]
XG-LBG 1.0%	4	35	81.0 ± 8.9	120.9 ± 1.2	198,182 ± 9,305	94.1 ± 0.7 [b]

XG, xantan gum. LBG, locast bean gum. AF, antral formation. [a–b] $P < 0.01$.

Table 3. Effect of differential substrate gel on developmental ability of oocyte grown *in vitro*

Substrates	No. of trials	No. of oocytes	Rate of blasturaton (%)	No. of blastomere
PAG 0.3%	7	85	9.4 ± 2.2	41.3 ± 3.2
XG-LBG 1.0%	7	73	13.8 ± 2.8	45.2 ± 4.6

XG, xantan gum. LBG, locast bean gum.

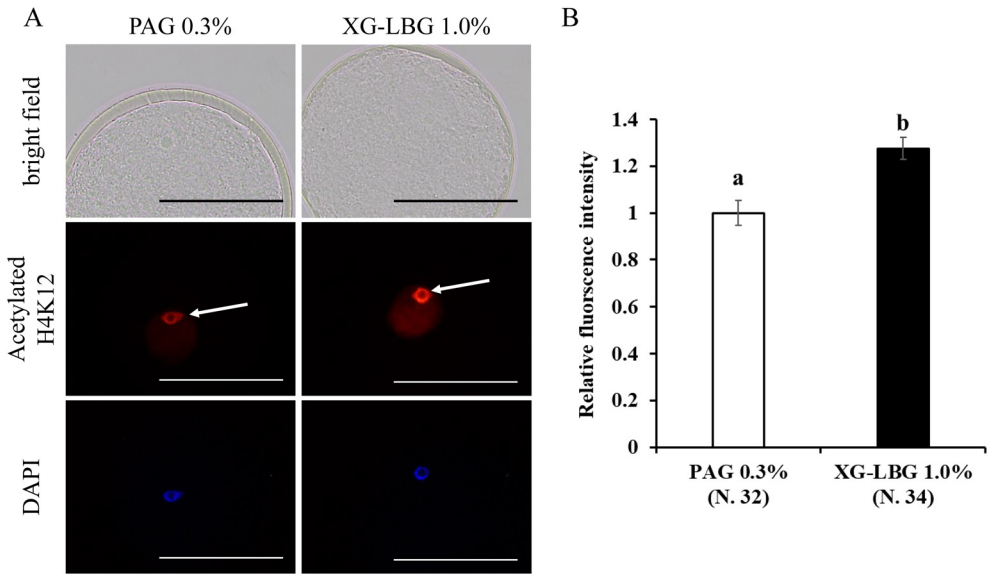

Fig. 1. Acetylation levels of oocytes cultured on 0.3% polyacrylamide gel (PAG) or 1.0% xanthan gum and locust bean gum (XG-LBG) gel culture systems. A: Representative pictures. Oocytes grown *in vitro* were immunostained for acetylated histone H4 at lysine 12 (H4K12). Arrows indicate H4K12 positive rim surrounding the nucleolus (SN) of oocytes. Bar represents 100 µm. B: Relative acetylation levels of oocytes; the mean value for oocytes developed on 0.3% PAG was defined as 1.0. Data are presented as the mean ± SEM. [a–b] $P < 0.05$.

–20°C until required.

Collection of ovaries, and collection of oocytes from early antral follicles (EAFs)

We collected ovaries from prepubescent gilts at a local slaughterhouse and transported them within 1 h to the laboratory at approximately 35°C in phosphate buffer saline (PBS) containing antibiotics. The ovarian cortical tissues were excised from the ovarian surfaces under a stereomicroscope, and the OGCs were collected from the EAFs (diameter 0.5–0.7 mm).

Preparation of 0.3% polyacrylamide gel (PAG) sheets

We prepared PAGs comprising 0.3% N,N'-methylenebisacrylamide, 10% acrylamide, ammonium peroxodisulfate, N,N,N',N'-tetramethylethylenediamine, and water according to the general method for western blot analysis. Detailed information on PAG preparation for the culture of OGCs derived from EAFs can be found in the literature [3].

Preparation of XG and LBG gel

XG and LBG (Sansho, Osaka, Japan) were heated in an autoclave at 120°C and 2 atm for 20 min, then cooled to room temperature. We injected 50 µl of the gel into each well of a 96-well plate and equilibrated the gel with the culture medium for one night. Before incubation, we replaced the OGCs culture medium with fresh culture medium (Fig. 2).

In vitro maturation and activation, and in vitro culture (IVC)

After IVG for 14 days, we subjected OGCs that had formed an antrum to IVM for 48 h. After IVM, we denuded the oocytes from the surrounding granulosa cells (GCs), and parthenogenetically activated them for 5 min in IVC medium containing 10 µg/ml ionomycine, then incubated them for 4.5 h in PZM3 containing 10 µg/ml cytochalasin B and 10 µg/ml cycloheximide. After activation, we cultured the embryos for 8 days in culture medium, and determined the rate of blastulation and the total number of blastocysts. We fixed the blastocysts in 4% paraformaldehyde, mounted them on microscope slides using an antifade reagent (ProLong Gold antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI); Invitrogen, OR, USA), and counted them using a BZ-8000 fluorescence digital microscope (Keyence, Tokyo, Japan).

Measurement of the number and survival rate of granulosa cells surrounding the oocytes grown in vitro

After IVG, GCs were enzymatically dispersed in Accumax (Innovative Cell Technologies, San Diego, CA, USA) and stained with trypan blue. The total number of GCs was calculated based on the volume and concentration of the cellular suspension using a hematocytometer. Live and dead cells were calorimetrically determined; white, live and blue, dead cells.

Measurement of oocyte diameters

We measured the diameters of the ooplasm (horizonal and vertical diameter) of each oocyte under a digital microscope (Keyence). The averages of the two diameters were used as the diameter of the oocytes.

Measurement of the levels of H4K12 acetylation in the oocytes

To measure the H4K12 acetylation levels, we collected oocytes from the OGCs at the end of the culture period (14 days). We fixed the oocytes in 4% paraformaldehyde, incubated them with a primary

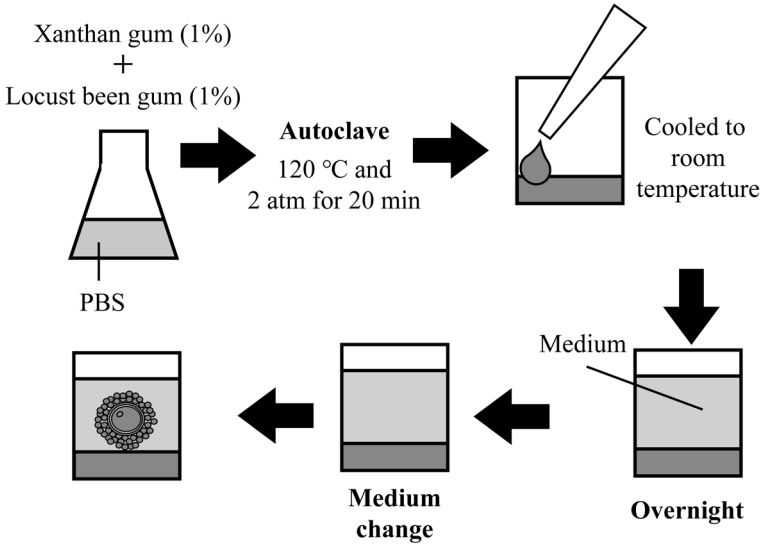

Fig. 2. Xanthan gun (XG) and locust bean gun (LBG) (Sansho, Osaka, Japan) were dissolved in phosphate buffer saline (PBS) by heating in an autoclave at 120°C and 2 atm for 20 min, then cooled to 50°C. Completely dissolved XG-LBG solution (50 µl) was transferred to each well of a 96-well plate and cooled to room temperature to form gels. The gel was equilibrated in culture medium (200 µl) for one night. Before experiment, the medium was replaced with fresh culture medium. Oocyte granulosa cell complexes (OGCs) collected from early antral follicles were placed on the gel and cultured for 14 days.

antibody (rabbit polyclonal anti- histone H4 acetyl K12, 1:200; Abcam, Cambridge, UK), then incubated them with a secondary antibody (fluorescein-conjugated goat anti-rabbit IgG, 1:500; Cell Signaling Technology, Danvers, MA). The oocytes were then mounted on slides with an anti-fade reagent containing DAPI (Invitrogen). Images of oocytes were captured under a fluorescence microscope (Keyence). We quantified the fluorescence intensities of the whole oocytes using ImageJ software (NIH, Bethesda, MD, USA).

Data analysis

We compared the data from the three culture conditions using analysis of variance (ANOVA) followed by a post hoc Tukey's test. Comparison of the data obtained for 0.3% PAG and XG-LBG was performed using Student's *t*-test. Percentages were arcsine-transformed before analysis. Values less than 0.05 were considered significantly different.

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