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Effects of follicular fluids on the growth of porcine preantral follicle and oocyte

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Summary

We examined the effect of supplementing the culture medium with follicular fluid (FF) on the growth of porcine preantral follicles and oocytes. Firstly, preantral follicles were retrieved from ovaries and then FF was collected from all antral follicles that were 2–7 mm in diameter (AFF), which included large follicles of 4–7 mm in diameter (LFF) and small follicles of 2–3 mm in diameter (SFF). When preantral follicles with a diameter of 250 μm were cultured in medium containing AFF, the growth of follicles and oocytes was greater than when follicles were cultured in medium containing fetal calf serum (FCS). When this growth-promoting effect in AFF was compared for LFF and SFF, the LFF were shown to be significantly more effective than SFF. This LFF effect was lost, however, when the concentration of LFF in the medium was decreased from 5% to 0.5% or when LFF were heat treated (60 °C for 30 min) or trypsin was added. In contrast, a decrease in SFF concentration from 5% to 0.5% and heat treatment of the SFF enhanced preantral follicle growth. Furthermore, proteins obtained from LFF that had molecular weights greater than 10 kDa (LFF > 10 kDa) had similar, but relatively reduced, growth-promoting properties. The remaining three LFF protein fractions (<10 kDa or <100 kDa or >100 kDa), however, did not have these growth-promoting properties. In conclusion, the supplementation of medium with LFF, rather than serum, enhanced preantral follicle and oocyte growth. Factors that enhanced follicle development in LFF and factors that suppressed follicle development in SFF were proteins and these LFF factors ranged in size from 10 kDa to over 100 kDa.

Keywords: Diameter, Follicular fluid, Oocyte, Pig, Preantral follicle

Introduction

Harvesting oocytes is a prerequisite for successful *in vitro* embryo production. Although the recovery of oocytes from large antral follicles is poor, because of their restricted number, many preantral follicles are present in ovaries; hence, development of an *in vitro* culture system for preantral follicles could efficiently provide large quantities of oocytes for embryo production. *In vitro* development of *in vitro* fertilized oocytes derived from preantral follicles does not proceed further than the blastocyst stage, however, and for pigs no live birth has yet been obtained (Wu *et al.*, 2001; Miyano & Manabe 2007). This finding suggests that culture conditions for preantral follicles are still inadequate. The medium for the first successful *in vitro* growth of porcine preantral follicles was supplemented with fetal calf serum (FCS) (Hirao *et al.*, 1994; Telfer *et al.*, 2000); and FCS has been proven to be beneficial for antrum formation, growth of the follicles and proliferation of granulosa cells (Shuttleworth *et al.*, 2002; Mao *et al.*, 2004). In the case of porcine oocyte maturation, supplementation of *in vitro* maturation (IVM) medium with follicular fluid (FF), instead of serum, was reported to improve the oocyte maturation ratio and subsequent male pronuclear formation and monospermic fertilization rates after *in vitro* fertilization (Naito *et al.*, 1988; Vatzias *et al.*, 1999; Algriany *et al.*, 2004). In addition, the supplementation of the IVM medium with FF collected from large follicles (3.1–8.0 mm in diameter) has been shown to improve the developmental competence of

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oocytes, as compared with supplementing FF collected from small follicles (<3.0 mm in diameter) (Yoon *et al.*, 2000). Factors that improved the oocyte maturation in LFF were found to be proteins with molecular weights ranging from 10 to 100 kDa (Ito *et al.*, 2007). The effect on preantral follicle growth of supplementing the medium with FF that have originated from follicles of varying diameters has not been examined previously. Furthermore, the factors in the FF responsible for this improved developmental competence have not been characterised.

In this study, we collected FF from follicles that had different diameter sizes, i.e. large follicles of 4–7 mm in diameter (LFF) and small follicles of 2–3 mm in diameter (SFF), and examined the effect of supplementing the medium. The development of preantral follicles, was assessed by their growth, as measured by follicle diameter and antrum formation, and the oocyte development was assessed by growth, as measured by oocyte diameter and the survival rate when compared with that of FCS supplementation. Furthermore, in order to characterise the factors in FF responsible for improved follicle and oocyte development and to define the molecular weight ranges of these factors, we subjected them to heat and trypsin treatments and ultrafiltration with either a 10 or a 100 kDa filter and also examined the effect of supplementing the medium with these treated FFs.

Materials and methods

Collection of preantral follicles

Porcine ovaries from prepubertal gilts were collected from a local slaughterhouse and transported to the laboratory in phosphate-buffered saline solution (PBS) that contained antibiotics, within 1 h of collection and kept at 35 °C. Ovarian cortical tissue (<1 mm thickness) was sliced from the ovarian surface, digested with collagenase (Wako) for 15 min at 38.5 °C and washed three times in minimum essential medium (MEM Gibco). Preantral follicles of approximately 250 μm in diameter were then isolated under a stereomicroscope.

Follicle culture

The follicles were embedded in collagen gel, according to the manufacture's protocol (Nitta gelatin). Briefly, follicles were washed with a culture medium and transferred into 500 μl of liquid collagen gel. After gelatinization, the gels containing 10 follicles were floated in 4 ml of the culture medium (Falcon 1008, Becton Dickinson). The culture medium was αMEM (Gibco) supplemented with 5% FF or 5% FCS and 2 mAU of follicle-stimulating hormone (FSH; Kawasaki). This concentration was determined during the course of the study (see below). Incubation was carried out at 38.5 °C in 5% CO_2 in air. Follicles were cultured for 14 days, half of the medium was replaced with freshly prepared medium every 48 h. Follicular diameter was measured at x and y dimensions (90°) to assess follicle development. At the end of the culture period, follicles were opened carefully with a fine needle and oocytes with normal morphology were picked, the diameter measured and the survival ratio was determined by trypan blue (Nacalai) staining. When oocytes were so severely degenerated that their diameter could not be assessed, they were considered to be dead. The culture was replicated three times using 10 oocytes each and a total of 30 oocytes were used per treatment group.

Preparation of follicular fluid (FF)

The FF was aspirated from all antrum follicles of 2–7 mm in diameter (AFF), including small antral follicles of 2–3 mm in diameter (SFF) and large antral follicles of 4–7 mm in diameter (LFF), respectively and centrifuged for 20 min (10 000 g) at 4°C. The supernatant was collected, filtered (0.2 μm) and stored at −20 °C until use.

Experimental design

Experiments 1 and 2: Comparisons of FCS and AFF (Experiment 1) or LFF and SFF (Experiment 2) on their effects on growth of follicles and oocytes were carried out.

Preantral follicles were cultured in the medium containing 5% FCS (INC) or 5% AFF (Experiment 1) and 5% LFF or 5% SFF (Experiment 2), respectively. Growth of preantral follicle was assessed by diameter measurement and antrum formation rate and oocyte growth was assessed by diameter measurement and survival ratio.

Experiment 3: To optimize FSH concentration in culture medium supplemented with 5% LFF or 5% SFF, the medium was supplemented with 0, 2 or 20 mAU FSH and the growth of follicles and oocytes was examined as described above.

Experiment 4: Effects of different concentrations (0.5 or 5%) of LFF and SFF in culture medium on growth of follicles and oocytes were assessed as described above.

Experiment 5: Characterization of molecules in FFs that affected the growth of follicles and oocytes was carried out. FFs were heated at 60 °C for 30 min or treated with 200 μg/ml trypsin for 60 min at 37 °C to denature the proteins. The effect of adding these treated FFs to the culture medium on the growth of preantral follicle and oocyte was assessed as described above. The molecular weight (MW) range of factors that affected follicle development in the FFs (LFF or SFF) was determined by centrifuging LFF and SFF in a

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VIVASPIN (Vivascience Ltd) and by separation of the fractions according to their MW; either more or less than 10 kDa or more or less than 100 kDa. The effect of supplementing the medium with the fractionated FFs on the growth of preantral follicles and oocytes was assessed as described above.

Statistical analysis

Changes in the follicle and oocyte diameters were compared using one-way ANOVA followed by Fisher's protected LSD. The rate of antrum formation and surviving oocytes and the proportion of oocytes categorized by their size were compared by chi-squared test; p values <0.05 were considered significantly different.

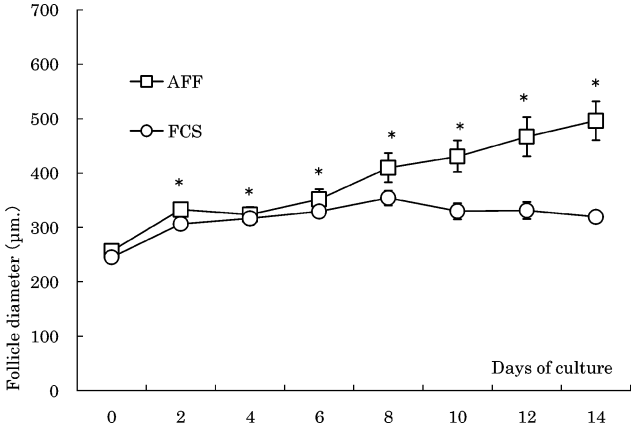

Figure 1 Changes in the diameter of porcine preantral follicles cultured in medium containing 5% FCS or 5% AFF (follicular fluid from antral follicle of 2–7 mm in diameter) for 14 days of culture.The diameter of follicles cultured with AFF was significantly (∗, $p < 0.05$) larger than that of follicles cultured with FCS. Bar represents S.E.

Results

Experiment 1

Changes in the diameters of follicles cultured with either FCS or AFF are shown in Fig. 1. Follicles cultured in medium containing AFF showed an increase in follicular diameter from 256.7 to 496.3 μm during the 14 days of culture. The diameters of follicles cultured in AFF-supplemented medium were significantly larger than those of follicles cultured in FCS-supplemented medium; the largest follicular diameter was 0.9 mm in AFF-supplemented medium. After 14 days of culture, six (20%) follicles formed antrum in AFF-supplemented medium, but none of the follicles formed antrum in FCS-supplemented medium. A significantly higher survival rate was obtained for oocytes cultured in AFF-supplemented medium (63.3%) than that rate obtained for oocytes cultured in FCS-supplemented medium (6.7%) (Table 1, $p < 0.05$).

Experiment 2

Follicles cultured in medium supplemented with LFF showed an increase in diameter from 245.0 to 713.3 μm and the largest follicular diameter was 1.1 mm. The diameters of follicles cultured in LFF-supplemented medium were significantly larger than those cultured in SFF-supplemented medium (Fig. 2). When cultured in LFF-supplemented medium, significantly higher antrum formation and survival rates of oocytes (86.7% and 86.7%, respectively) were obtained compared with those cells cultured in SFF-supplemented medium (0% and 10%, respectively) ($p < 0.05$) (Table 2).

Experiment 3

Only the addition of 2 mAU FSH to the culture medium supported follicle growth regardless of the origin of the FF added. When no FSH was added to the medium containing FF, follicles degenerated rapidly and a clear diameter could not be measured after 4 days of culture. In medium containing FF that was supplemented with 20 mAU FSH, follicles degenerated after several days of culture.

Experiment 4

As shown in Figs. 3 and 4, when the concentration of LFF in culture medium decreased from 5% to 0.5%,

Table 1 Effect of follicular fluid or serum on antrum formation of porcine preantral follicles and on growth of oocytes.

	Antrum[b]			Distribution of oocytes diameter (μm)					Survival[d]	
Protein[a] (5%)	No.	(%)	No. of oocytes[c]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
AFF	6/30	20	30	3	14	5	8	78.3	19/30	63.3[a]
FCS	0/30	0	9	0	1	3	5	72.6	2/30	6.7[b]

[a]Serum and antral follicle fluid (AFF) were added to the culture medium.
[b]Antrum formation was examined at the end of the culture period (14 days).
[c]Number of oocytes with normal morphology (14 days) that were examined for diameter.
[d]Survival rate of oocytes were determined by trypan blue staining.

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Table 2 Effect of follicular fluid origins on antrum formation of porcine preantral follicles and on growth of oocytes.

	Antrum[b]			Distribution of oocytes diameter (μm)					Survival[d]	
FF[a] (5%)	No.	(%)	No. of oocytes[c]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
LFF	26/30	86.7	30	5	10	7	8	77.9[a]	26/30	86.7[a]
SFF	0/30	0	28	2	4	7	15	65.3[b]	3/30	10.0[b]

[a]Follicle fluid (LFF and SFF) were collected from large follicles (4–7 mm in diameter) or small follicles (2–3 mm in diameter) and added to the culture medium.
[b]Antrum formation was examined at the end of the culture period (14 days).
[c]Number of oocytes with normal morphology (14 days) that were examined for diameter.
[d]Survival rate of oocytes were determined by trypan blue staining.

Table 3 Effect of different concentrations of follicular fluids on the antrum formation of porcine preantral follicles and growth of oocytes.

		Antrum[b]			Distribution of oocytes diameter (μm)					Survival[d]	
FF[a]	Con.[a] (%)	No.	(%)	No. of oocytes[c]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
LFF	5	22/30	73.3[a]	29	3	13	7	6	80.6[a]	22/30	73.3[a]
	0.5	10/30	33.3[b]	27	13	6	5	3	91.2[b]	14/30	46.7[b]
SFF	5	0/30	0	28	2	4	7	15	67.4	3/30	10.0[a]
	0.5	0/30	0	23	4	6	5	8	76.6	10/30	33.3[b]

[a]Follicle fluid (LFF and SFF) were collected from large follicles (4–7 mm in diameter) or small follicles (2–3 mm in diameter) and added to the culture medium.
[b]Antrum formation was examined at the end of the culture period (14 days).
[c]Number of oocytes with normal morphology (14 days) that were examined for diameter.
[d]Survival rate of oocytes were determined by trypan blue staining.

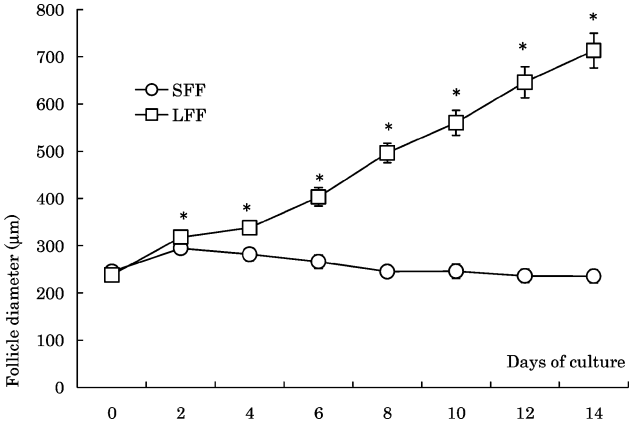

Figure 2 Changes in the diameter of preantral follicles cultured in medium containing 5% follicular fluid that had been collected from large follicles (LFF) of 4–7 mm in diameter and from small follicles (SFF) of 2.3 mm in diameter during 14 days of culture.The diameter of follicles cultured with LFF was significantly larger than that of follicles cultured with SFF (∗, $p < 0.05$). Bar represents S.E.

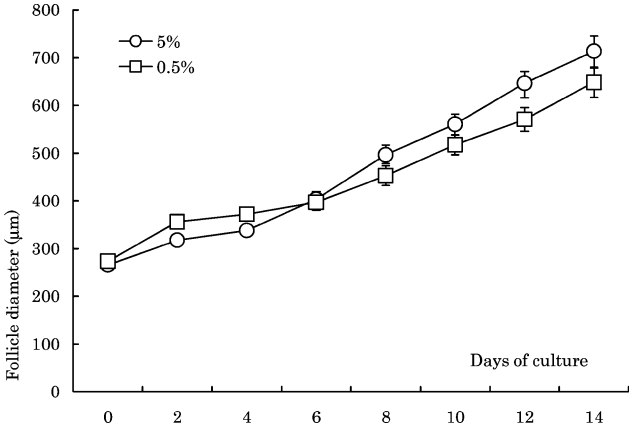

Figure 3 Changes in the diameter of porcine preantral follicles cultured in medium containing different concentrations of LFF. When the concentration of LFF in culture medium decreased from 5% to 0.5% the growth of preantral follicle did not change. Bar represents S.E.

growth of preantral follicle did not change (Fig. 3). oocyte diameter was significantly increased from 80.6 μm (5%) to 91.2 μm (0.5%), however, and both antrum formation and survival rates of oocytes were significantly decreased from 73.3% and 73.3% (5%) to 33.3% and 46.7% (0.5%), respectively (Table 3, $p < 0.05$). In the medium containing SFF, the growth rate of follicles was similar at both concentrations (5% and 0.5%), however there was a significant difference in follicle size between the two concentrations (Fig. 4). In addition, survival ratios of oocytes increased significantly at a lower concentration of SFF compared

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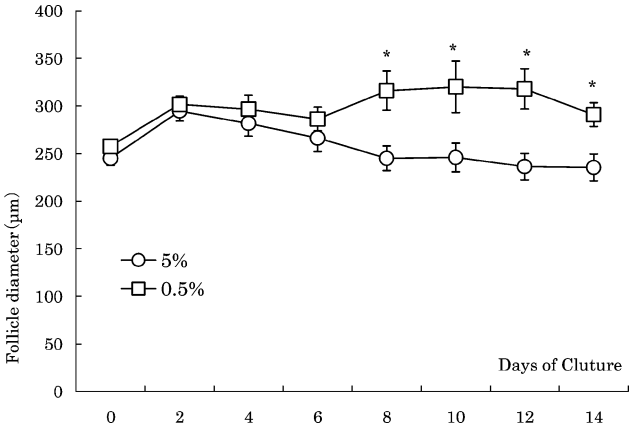

Figure 4 Changes in the diameter of porcine preantral follicles cultured in medium containing different concentrations of SFF during the 14 days of culture. Diameters of follicles cultured with 0.5% SFF were significantly (∗, $p < 0.05$) larger than of those cells cultured with FCS. Bar represents S.E.

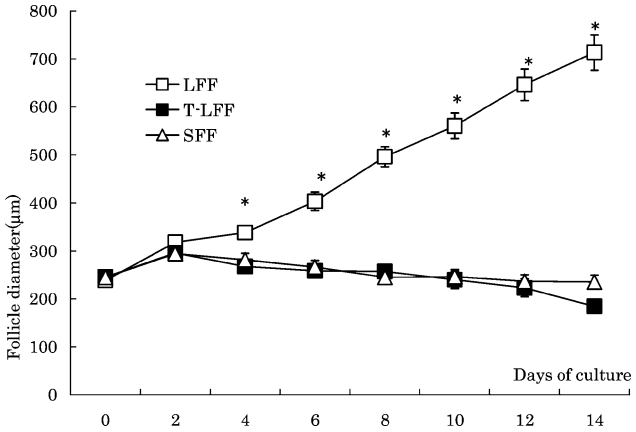

Figure 6 Changes in the diameter of preantral follicles cultured in medium containing LFF, trypsin-treated LFF (T-LFF) or SFF during 14 days of culture.When LFF was treated with trypsin, the growth rate of follicles was significantly reduced to a level similar to those seen for SFF (∗, $p < 0.05$). Bar represents S.E.

with a high concentration (10% for 5% SFF and 33% for 0.5% SFF, Table 3).

Experiment 5

Effect of protein denaturation

When preantral follicles were cultured in a medium containing LFF that had been heated to 60 °C, the follicle growth was reduced significantly (Fig. 5) and the rates of antrum formation and survived oocytes decreased significantly (LFF without heat treatment: 93.3% and 100.0%, LFF with heat treatment: 60.0% and 56.7%, respectively) (Table 4), when compared with those cells cultured with LFF without heat treatment. In contrast, heat treatment of SFF resulted in a significantly increased follicular diameter (Fig. 5). Moreover, heat treatment of SFF increased the antrum formation rate significantly (23.3%), when compared with non-heat-treated SFF (6.7%) (Table 4). Additionally, when LFF was treated with trypsin, the growth rate of follicles was reduced significantly to the levels similar to those seen with SFF (Fig. 6). Antrum formation and the survival rate of oocytes in medium supplemented with trypsin-treated SFF and LFF were 0% and 10% for SFF and 0% and 3.3% for LFF, respectively (Table 5).

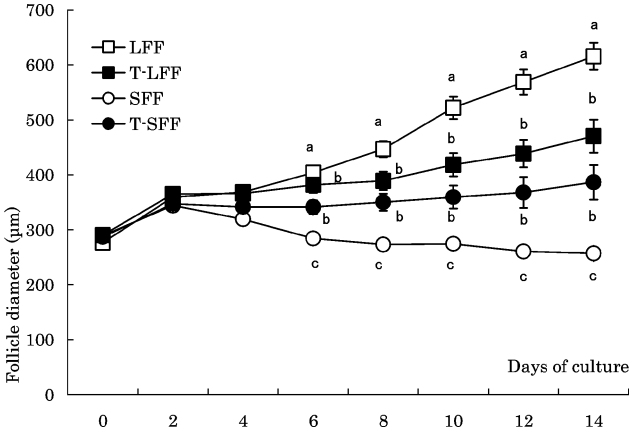

Figure 5 Changes in the diameter of porcine preantral follicles cultured in medium containing LFF after heat treatment (60 °C for 30 min, T-LFF) SFF and SFF after heat treatment (T-SFF) during 14 days of culture.When follicles were cultured with T-LFF, the growth rate of follicles was significantly reduced (a, b; $p < 0.05$). In contrast, heat treatment of SFF resulted in a significantly increased follicular diameter (b, c; $p < 0.05$).The diameter of follicles cultured with AFF was significantly different (∗, $p < 0.05$). Bar represents S.E.

Molecular weight range of proteins

The molecular weight range of the fraction responsible for enhanced growth rate of follicles is shown in Figs. 7 and 8. LFF proteins of molecular weight (MW) < 10 kDa (LFF < 10 kDa) did not support follicle growth and both the survival and antrum formation rates were 0% (Table 6). Follicles cultured in the medium supplemented with LFF proteins with a MW higher than 10 kDa (LFF > 10 kDa) showed similar follicular growth, antrum formation (80%) and oocyte survival rates (100%) (Table 6) when compared with those cells cultured with non-fractionated LFF (Fig. 7). Conversely, neither SFF > 10 kDa nor SFF < 10 kDa improved the follicle development and the survival rate of oocytes cultured with SFF > 10 kDa was only 20% (Fig. 8 and Table 6). Both fractionated LFFs

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Table 4 Effect of heat treatment of follicular fluids on antrum formation of porcine preantral follicles and growth of oocytes.

		Antrum[c]			Distribution of oocytes diameter (μm)					Survival[e]	
FF[a] (5%)	Heat[b] (60 °C)	No.	(%)	No. of oocytes[d]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
LFF	–	28/30	93.3	30	3	15	11	1	83.3[a]	30/30	100.0
	+	18/30	60	24	2	6	12	4	75.0[b]	17/30	56.7
SFF	–	2/30	6.7	17	1	1	5	10	62.5	11/30	36.7
	+	7/30	23.3	18	3	4	5	6	64.6[b]	12/30	40.0

[a]Follicle fluid (LFF and SFF) were collected from large (4–7 mm in diameter) or small follicles (2–3 mm in diameter) and added to the culture medium.
[b]LFF and SFF were heated at 60 °C for 30 min.
[c]Antrum formation was examined at the end of the culture period (14 days).
[d]Number of oocytes with normal morphology (14 days) that were examined for diameter.
[e]Survival rate of oocytes were determined by trypan blue staining.

Table 5 Effect of trypsin treatment of follicular fluids on antrum formation of porcine preantral follicles and growth of oocytes.

	Antrum[b]			Distribution of oocytes diameter (μm)					Survival[d]	
FF[a] (5%)	No.	(%)	No. of oocytes[c]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
LFF	26/30	86.7	30	5	10	7	8	77.9[a]	26/30	86.7[a]
SFF	0/30	0	28	2	4	7	15	67.4[b]	3/30	10.0[b]
LFF + Try	0/30	0	1	0	1	0	0	75[a,b]	1/30	33.3[b]

[a]LFF + Try: Large follicle fluid (LFF) was treated with trypsin (200 μg/ml) for 60 min at 37 °C. LFF, SFF or LFF + Try were added to the culture medium.
[b]Antrum formation was examined at the end of the culture period (14 days).
[c]Number of oocytes with normal morphology (14 days) that were examined for diameter.
[d]Survival rate of oocytes were determined by trypan blue staining.

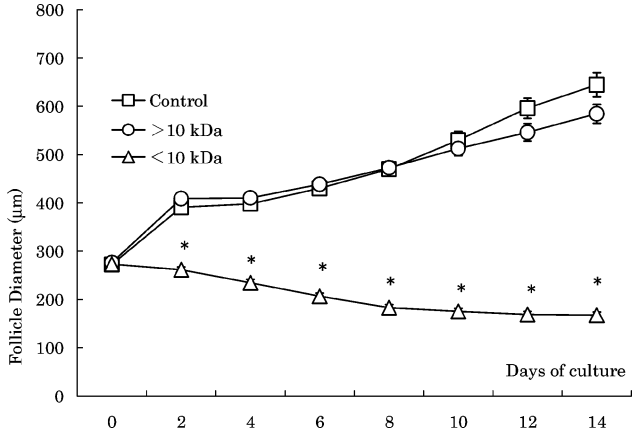

Figure 7 Changes in the diameter of porcine preantral follicles cultured in medium containing fractionated LFF (LFF, LFF > 10 kDa or LFF < 10 kDa) during 14 days of culture. Follicles cultured with LFF > 10 kDa showed similar follicle growth compared with those cells cultured with non-fractionated LFF; however LFF < 10 kDa did not support follicle growth (∗, $p < 0.05$). Bar represents S.E.

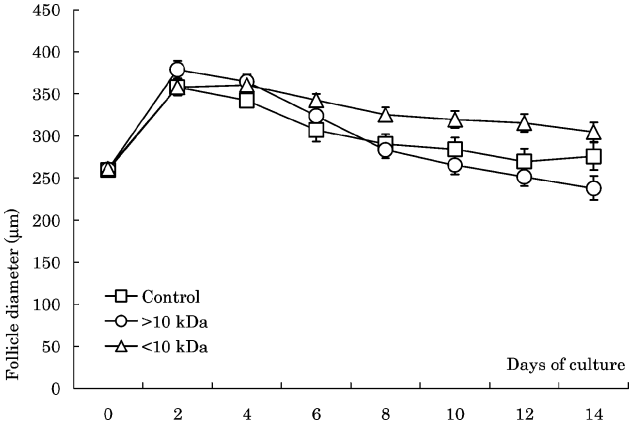

Figure 8 Changes in the diameter of porcine preantral follicles cultured in medium containing fractionated SFF (SFF, SFF > 10 kDa or SFF < 10 kDa) during 14 days of culture. Neither SFF nor SFF < 10 kDa improved follicular development. Bar represents S.E.

(>100 kDa or <100 kDa) lost the ability to improve follicle growth and showed a significantly lower survival rate and antrum formation when compared with that of non-fractionated LFF (Fig. 9, Table 7). Fractionation of SFF did not induce any notable changes in follicle and oocyte development (Fig. 10 and Table 7).

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Table 6 Effect of fractionation of follicular fluids with 10 kDa filter on the antrum formation of porcine preantral follicles and growth of oocytes.

		Antrum[b]			Distribution of oocytes diameter (μm)					Survival[d]	
FF[a] (5%)	10 kDa[a]	No.	(%)	No. of oocytes[c]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
LFF	–	30/30	100	30	1	13	13	3	80.0	30/30	100.0
	>	24/30	80	30	2	14	13	1	82.1	30/30	100.0
	<	0/30	0	30	12	7	5	6	85.4	1/30	3.3
SFF	–	1/30	3.3	13	0	3	3	7	61.5	0/30	0.0
	>	0/30	0	20	3	3	6	8	73.8	6/30	20
	<	0/30	0	24	4	5	3	12	66.7	0/30	0.0

[a]Follicle fluid (LFF and SFF) were separated with a 10 kDa filter, and these treated FFs were added to the culture medium.
[b]Antrum formation was examined at the end of the culture period (14 days).
[c]Number of oocytes with normal morphology that were examined for diameter.
[d]Survival rate of oocytes were determined by trypan blue staining.

Table 7 Effect of fractionation of follicular fluids with a 100 kDa filter on the antrum formation of porcine preantral follicles and growth of oocytes.

		Antrum[b]			Distribution of oocytes diameter (μm)					Survival[d]	
FF[a] (5%)	10 kDa[a]	No.	(%)	No. of oocytes[c]	>100	>87.5	>75.0	<75.0	Ave.	No.	%
LFF	–	29/30	96.7	30	1	13	13	3	80.0[a]	30/30	100.0[a]
	>	5/30	16.7	24	3	9	3	9	76.0[a,b]	8/30	26.7[b]
	<	0/30	0	27	0	5	8	14	67.6[b]	13/30	43.3[b]
SFF	–	0/30	0	28	2	4	7	15	67.4	3/30	10.0
	>	1/30	3.3	25	2	4	2	17	65.0	4/30	13.3
	<	0/30	0	30	0	4	3	23	61.3	5/30	16.7

[a]Follicle fluid (LFF and SFF) were separated with a 100 kDa filter, and these treated FFs were added to the culture medium.
[b]Antrum formation was examined at the end of the culture period (14 days).
[c]Number of oocytes with normal morphology that were examined for diameter.
[d]Survival rate of oocytes was determined by trypan blue staining.

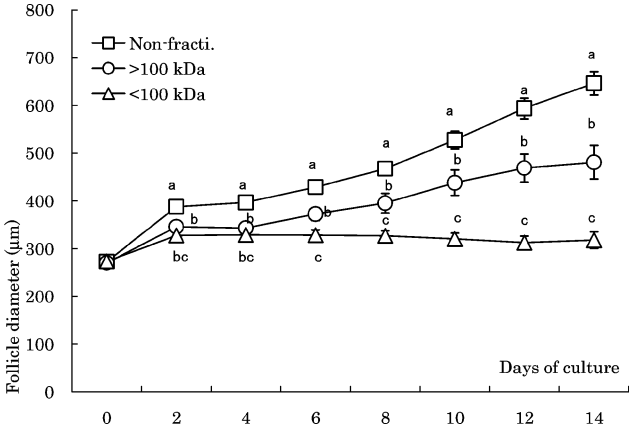

Figure 9 Both fractionated LFF (LFF > 100 kDa and LFF < 100 kDa) significantly lost the ability to support follicle development compared with those cells cultured with non-fractionated LFF (a–c; $p < 0.05$). Bar represents S.E.

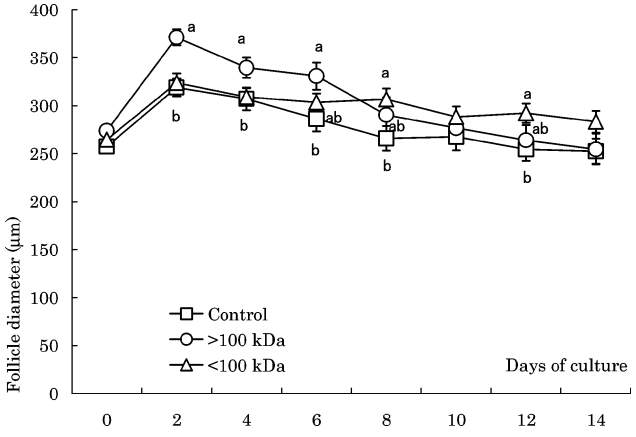

Figure 10 Changes in the diameter of porcine preantral follicles cultured in medium containing fractionated SFF (SFF, SFF > 100 kDa or SFF < 100 kDa) during 14 days of culture. Fractionation did not induce any notable changes in follicle size. Bar represents S.E. Letters (a–c) differ significantly ($p < 0.05$).

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Discussion

Serum has been added to the culture medium for preantral follicles to enhance the proliferation of granulosa cells and antrum formation (Hirao *et al.*, 1994; Telfer *et al.*, 2000; Mao *et al.*, 2004). Furthermore, the origin of serum (fetal calf serum or prepubertal gilt serum) has been reported to affect follicle growth (Mao *et al.*, 2002). In this study, however, we found that supplementation of the culture medium with FF, especially LFF, improved growth of preantral follicles and oocytes significantly when compared with FCS supplementation. This report is the first to show that supplementation of medium with FF is effective to improve growth of preantral follicles. Many factors are secreted from oocytes, cumulus cell and granulosa cells into FFs and these factors were reported to affect follicle and oocyte development (Albertini *et al.*, 2001). It was suggested, therefore, that there are more beneficial factors for follicle growth in FF than those in serum.

In previous studies on *in vitro* oocyte maturation, the supplementation of *in vitro* maturation medium with porcine FF instead of serum improved the maturation rate, male pronucleus formation and the monospermic fertilization of pig oocytes (Naito *et al.*, 1988; Vatzias & Hagen, 1999; Algriany *et al.*, 2004). Furthermore, Itoh *et al.*, (2007) reported that FF collected from follicles with large diameters (>5 mm) also improved oocyte maturation in comparison with that of FF collected from small follicles. In the present study, LFF also improved the growth of preantral follicles and oocytes, while SFF did not improve follicle growth. In this study when 30 preantral follicles were cultured with LFF, 26 (86.7%, Table 2) formed antrums and this rate compared favourably with previous reports using various media (αMEM, M199 or NCSU 27) supplemented with hormones, ascorbic acid or transferring (Wu *et al.*, 2001; Mao *et al.*, 2002, 2004; Wu & Tian 2007). Wu *et al.* (2001) reported that the optimal concentration of serum for preantral follicle culture is 7.5%. When the concentration of LFF in the medium was decreased from 5% to 0.5% in the study, growth rate of follicles did not change and the medium produced larger oocytes. Survival ratio of oocytes, however, was decreased. In addition, when SFF concentration was decreased from 5% to 0.5%, follicle size did not change and showed significantly smaller sizes when compared with those cultures with LFF. From these results, we suggest that LFF has beneficial factors on follicle growth even at a low concentration. Conversely, it was suggested that there may be some follicular growth suppressive factors in SFF. Although the reasons for the enhanced oocyte growth, even at a lower concentration of LFF in this study, are not clear, we consider that there may be an individual optimal concentration of FF for growth of preantral follicles and oocytes.

It has been reported that epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) and FSH improve preantral follicle development (Bolamba *et al.*, 2002; Diaz *et al.*, 2006; Wu & Tian 2007). Although we only examined the effects of FSH in the present study, when 0, 2 or 20 mAU of FSH was added to either LFF- or SFF-containing medium, the highest growth rate was observed when 2 mAU of FSH was added, regardless of the origin of FF. We, therefore, added FSH at this concentration to all experimental media. The optimal concentration of other hormones for each culture medium containing either SFF or LFF still needs to be determined.

In order to examine whether the factors in LFF are proteins, we treated FFs with 60 °C or trypsin. Both heat and trypsin treatments of LFF decreased growth of follicles and oocytes. In contrast, heat treatment of SFF actually increased the follicular growth. The results suggested that factors in LFF accelerated follicle growth and the factors involved in SFF that suppress follicle growth are proteins. Finally, we attempted to evaluate the range of molecular weight of factors in LFF by filtration through 10 and 100 kDa filters. When cultured in the medium supplemented with LFF > 10 kDa, follicles showed similar growth compared with that of non-treated LFF; however, LFF < 10 kDa decreased the ability to support follicular growth drastically. It can be suggested, therefore, that the MW of the beneficial factors for preantral follicle growth are more than 10 kDa. In addition, in the present study both LFF > 100 kDa and LFF < 100 kDa lost the ability to support follicle growth and oocyte development. It can be inferred, therefore, that there are several proteins in LFF whose MW were either >100 kDa or <100 kDa and these factors would contribute concomitantly to follicular growth and oocyte development.

In conclusion, supplementation of the culture medium with FF improves the growth of preantral follicles and oocytes and this effect was greater in LFF than in SFF. In addition, the factors in LFF are proteins with MW ranging from 10 to over 100 kDa.

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