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Effects of Luteinizing Hormone on Nuclear Maturation of Pig Oocytes In Vitro

Yasuhiro Tsuzuki[1]*, Tomoyasu Sakamoto[1], Koji Ashizawa[1] and Noboru Fujihara[2]

[1]Laboratory of Animal Reproduction, Faculty of Agriculture, Miyazaki University, Miyazaki 889-2192 and
[2]Laboratory of Animal Reproduction, Faculty of Agriculture, Kyushu University, Fukuoka 812-8581, Japan

Abstract: *This research was designed to investigate the effects of luteinizing hormone (LH, 100 ng/ml) on pig oocyte maturation in vitro (Experiment 1), and the relationship between the gap junction and LH (Experiment 2). In Experiment 1, cumulus-enclosed oocytes (CO) and denuded oocytes (DO) were cultured in the presence or absence of LH. After culture for 42–43 hours, nuclear maturation rates which reached the metaphase stage of the second meiotic division (M-II stage) were observed. In Experiment 2, we evaluated whether the gap junction, formed between cumulus cells and oocytes, was associated with the presence of LH in the maturation medium. The addition of LH to the maturation medium significantly enhanced nuclear maturation of both CO and DO ($P<0.05$) compared with those oocytes which were cultured without LH. The addition of 1-heptanol (5 mM), a blocker of coupling in the gap junction, to the maturation medium supplemented with LH significantly inhibited ($P<0.05$) the number of CO and DO reaching the M-II stage. These results suggest that the addition of LH to the maturation medium could improve the nuclear maturation of pig oocytes.*

Key words: *LH, IVM, pig oocytes*

Pig embryos can be derived by maturation and fertilization *in vitro* [1, 2]. It is well known that the addition of luteinizing hormone can enhance nuclear and cytoplasmic maturation of oocytes *in vitro* in cow [3–6] and pig oocytes [7]. It has been suggested that LH may lead to germinal vesicle breakdown (GVBD) of oocytes *in vitro* if granulosa or cumulus cells are present [8]. Some researches suggested that gap junctions formed between oocytes and cumulus cells or cumulus and granulosa cells are related to gonadotropin-stimulated meiotic resumption of mouse oocytes [8, 9], but no research report on the relationship between gonadotropin stumulation and gap junctions in the pig was not found. In view of this, further investigation seemed to be needed to determine how LH acts on oocyte maturation.

The present study was undertaken to assess the influence of LH on the maturation of porcine cumulus-enclosed oocytes and denuded oocytes *in vitro*.

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*To whom correspondence should be addressed.

Materials and Methods

Oocyte preparation

Ovaries were obtained from a slaughterhouse and brought to the laboratory in physiological saline within 2 hr. The oocytes were aspirated from superficial follicles (1–6 mm in diameter) with a 20-G needle (Terumo, Tokyo, Japan) attached to a 6 ml plastic syringe (Top, Tokyo, Japan). After aspiration, oocytes surrounded by more than one layer of cumulus cells were selected and washed with TCM-199 (Gibco BRL Products, MD., U.S.A.) more than 4 times. The oocytes were transferred to 100 μl drops of maturation medium and cultured for 42–43 hr in 5% CO_2, 95 % air, 100% humidity at 39°C. The maturation medium contained 25 mM Hepes buffered TCM-199 supplemented with 10% (V/V) fetal calf serum (Gibco), 0.35 mM D-glucose, 2.29 mM calcium lactate, 0.91 mM sodium pyruvate, 1 μg/ml estradiol-17β (Sigma, E-8875, St Louis, Mo., U.S.A.), 5 iu/ml pregnant mare's serum gonadotropin (Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Katayama Chemical Co., Osaka, Japan).

Experiment 1

Oocytes were assigned to four groups: oocytes enclosed in cumulus cells (CO) were loaded into the maturation medium mentioned above, with (+CO) or without (−CO) 100 ng/ml LH (from Human Pituitaries, L-5259, Sigma). Another group of CO were loaded into

Table 1. The effect of luteinizing hormone (LH, 100 ng/ml) on the nuclear maturation of pig oocytes *in vitro* for 42–43 hr

Treatment			Percentage of oocytes reaching each nuclear maturation stage after *in vitro* maturation						
	LH	N	GV	PM-I	M-I	A-I	T-I	M-II	Degeneration
CO	–	45	4.5	47.7[a]	0.0[a]	4.5	4.5	38.6[a]	0.0
DO	–	143	7.0	42.7[a]	13.3[b]	4.9	11.2	18.9[b]	2.1
CO	+	45	0.0	8.9[b]	2.2[a]	0.0	6.7	80.0[c]	2.2
DO	+	146	2.7	6.8[b]	2.1[a]	0.7	6.2	81.5[c]	0.0

GV, Germinal Vesicle; PM-I, Prometaphase of the first meiotic division; M-I, Metaphase of the first meiotic division; A-I, Anaphase of the first meiotic division; T-I, Telophase of the first meiotic division; M-II, Metaphase of the second meiotic division; CO, Cumulus-enclosed oocytes; DO, Denuded oocytes; +, Presence of LH; –, Absence of LH.

a,b,c; Percentage with different superscripts are significantly ($P<0.05$) different within a column.

Dulbecco's phosphate buffer saline (Gibco BRL Products) supplemented with 0.2% sodium citrate and their cumulus cells were removed mechanically with a vortex mixer and by pipetting (denuded oocytes, DO). The oocytes were transferred to the maturation medium, supplemented with (+DO) and without (−DO) LH. The effect of LH on CO and DO was evaluated by observing nuclear maturation. After the maturation culture, the oocytes in each group were fixed with ethanol and acetic acid for more than 6 hr and stained with 1% orcein (Merk, Darmstadt, Germany) in 40% acetic acid.

To observe the nuclear stage of the oocytes before maturation, some cumulus-enclosed oocytes aspirated from ovaries were immediately denuded, fixed and stained as mentioned before.

Experiment 2

We observed the intact structure of junctional communication of projections of cumulus cells and oolemma in both CO and DO before maturation culture by transmission electron microscopy (data not shown). To evaluate whether gap junctions could influence oocyte maturation, CO and DO were introduced into maturation medium contained 100 ng/ml LH, supplemented with or without 5 mM 1-heptanol (Nacalai Teques, Kyoto, Japan), which blocks the coupling of gap junctions [9]. In our preliminary experiment, more oocytes were prevented from reaching the metaphase stage of the second meiotic division (M-II stage) in the medium supplemented with 5 mM 1-heptanol than those at 0.1, 1 and 10 mM. After the maturation culture, the oocytes in each group were fixed and stained as described previously.

Statistical analysis

All data were analyzed by chi-square test [10].

Results

Experiment 1

We observed the nuclear morphology of pig oocytes before the maturation culture. A large proportion of oocytes (76/86, 88.4%) were the same as in the germinal vesicle stage (GV stage).

The nuclear maturation of the oocytes cultured with and without LH is shown in Table 1. Non −CO was arrested at M-I stage although 13.3 % of −DO remained at this stage. On the other hand, the number of −CO which reached the M-II stage was significantly ($P<0.05$) higher than that in −DO. Maturation to the M-II stage was higher ($P<0.05$) in CO and DO cultured with LH than in those cultured without LH. There were no significant differences in the percentage of CO and DO oocytes reaching M-II when they were cultured with LH.

Experiment 2

As shown in Table 2, the addition of 1-heptanol to the maturation medium supplemented with LH significantly inhibited ($P<0.05$) the nuclear maturation in both CO and DO. No significant differences were found ($P>0.05$) between CO and DO cultured with or without 1-heptanol.

Discussion

In the present study, most of the oocytes were at the GV stage before the maturation culture. The addition of LH to the maturation medium resulted in a considerable improvement in the nuclear maturation to the M-II stage regardless of the presence or absence of cumulus cells (CO and DO). Downs [8] postulated that LH can inhibit the coupling of gap junctions forming between granulosa and cumulus cells or cumulus cells and oocytes to

Table 2. The effect of 1-heptanol (5 mM) on the nuclear maturation of pig oocytes *in vitro* for 42–43 hr with LH (100 ng/ml)

Treatment	1-heptanol	N	Percentage of oocytes reaching each nuclear maturation stage after *in vitro* maturation						
			GV	PM-I	M-I	A-I	T-I	M-II	Degeneration
CO	–	90	0.0[a]	10.0[a]	4.4[ab]	2.2[a]	6.7	76.7[a]	3.3
DO	–	98	0.0[a]	9.2[a]	3.1[b]	4.1[a]	7.1	76.5[a]	5.1
CO	+	110	7.2[b]	28.2[b]	11.8[a]	12.7[b]	12.7	27.3[b]	5.5
DO	+	125	16.8[c]	31.2[b]	11.2[a]	9.6[ab]	6.4	24.8[b]	3.2

GV, Germinal Vesicle; PM-I, Prometaphase of the first meiotic division; M-I, Metaphase of the first meiotic division; A-I, Anaphase of the first meiotic division; T-I, Telophase of the first meiotic division M-II, Metaphase of the second meiotic division; CO, Cumulus-enclosed oocytes; DO, Denuded oocytes; +, Presence of 1-heptanol; –, Absence of 1-heptanol.

a,b,c: Percentage with different superscripts are significantly ($P<0.05$) different within a column.

induce GVBD in the mouse. Most CO and DO in each group underwent GVBD in the present study. A high percentage of CO and DO cultured without LH reached to the prometaphase stage. These results indicate that pig oocytes can progress from the GV stage without LH or cumulus cells, and that LH could stimulate the nuclear maturation of pig oocytes after GVBD without cumulus cells.

In Experiment 1, the absence of LH, even though some −DO were arrested at the M-I stage, more −CO reached the M-II stage. This suggested that cumulus cells have a stimulating affect in maturing pig oocytes without adding gonadotropins to the medium. In the light of this, Isobe *et al.* [11] indicated that cumulus cells suppress the meiotic progression of pig oocytes matured *in vitro*, but we could not find any inhibitory effect of cumulus cells during oocyte maturation *in vitro*. This contradiction might be due to the difference between medium components. Compared to their maturation medium, we added three more reagents, sodium pyruvate, calcium lactate and D-glucose, to the maturation medium. Leese and Barton [12] indicated that mouse oocytes required sodium pyruvate in the medium. One possibility suggested is that the addition of pyruvate might be needed to stimulate the cumulus-enclosed oocytes in pig.

1-heptanol inhibited nuclear maturation even in the presence of LH in the medium. This indicates that gap junctions can be related to the stimulating effect of LH on the nuclear maturation of oocytes. Gap junctions can pass through components up to 1,000 Da in size [13]. It is considered that LH cannot pass through gap. junctions formed between cumulus cells and oocytes because of the great molecular weight (approximately 30,000). Therefore, it is postulated that uncoupling of the gap junction caused by the addition of 1-heptanol might suppress the passing of important factor(s) less than 1,000 Da, not LH, regarded as necessary for pig oocyte maturation.

In the present study the percentage of nuclear maturation in DO treated with LH and 1-heptanol was shown to be similar to that in CO matured with both reagents. Loewastein [14] suggested that the pores of the gap junctions in cumulus-oocyte complexes are likely to close suddenly after rupture of cumulus cell projections reaching to the oocytes. It is suggested that although DO were mechanically removed from enclosed cumulus cells in the present study, the pores of the gap junctions may not be closed for a longer period like those of CO.

In conclusion, LH has a positive effect on the nuclear maturation of pig oocytes *in vitro*. Gap junctions can be associated with stimulation of nuclear maturation of oocytes, but we could not clarify which factor(s) can pass through the gap junctions in the culture medium supplemented with LH. Further investigation seems to be needed to elucidate the factor(s) including molecles.

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