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—Original—

Predictive Value of the Area of Expanded Cumulus Mass on Development of Porcine Oocytes Matured and Fertilized *In Vitro*

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Abstract. The objective of the present study was to investigate the correlation between the degree of cumulus expansion and *in vitro* development of porcine cumulus-oocytes complexes (COCs) matured and fertilized *in vitro*. The COCs were matured in the maturation medium (IVMM) supplemented with 15% or 5% of porcine follicular fluid (PFF) from small, medium and large follicles (<2 mm, 2–5 mm and >5 mm, respectively). COCs cultured in IVMM with PFF for 48 h displayed less expansion than those cultured in IVMM alone ($P<0.05$), irrespective of follicle size. After culture for 24 h in IVMM with PFF and for another 24 h in IVMM alone, the degree of cumulus expansion was more prominent than culture in the presence of PFF for the entire 48 h period ($P<0.05$), but the percentages of oocytes with PB I showed no significant difference between the control and experimental groups ($P>0.05$). After *in vitro* fertilization, the oocytes failed to develop to the morula/blastocyst stages except for those matured in IVMM supplemented with 15% or 5% PFF obtained from >5 mm follicles for the first 24 h and followed by in IVMM alone for the second 24 h (12.5% and 11.1% of the embryos developed to morulae and blastocysts, respectively). The expanded cumulus areas of COCs were significantly positively correlated with their *in vitro* development ($p=0.0058$, 0.0001 and 0.0348 for the percentages of embryos developed to 2–4 cell, beyond 4 cell and morula and blastocyst stages, respectively). In conclusion, PFF had an inhibiting effect on cumulus expansion, and the inhibitory effect decreased progressively with the increase in size of follicles from which PFF was obtained, and the action of PFF on cumulus expansion was affected by the PFF culture time. The areas of the expanded cumulus mass may be used as a parameter to predict development of porcine oocytes matured and fertilized *in vitro*.

Key words: Porcine, Cumulus-oocytes complexes, *In vitro* maturation, Cumulus expansion, *In vitro* development

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In mammals, meiotic division of the oocyte starts during prenatal life and stops at the diplotene stage of prophase I just before or immediately after birth. The oocyte remains under meiotic arrest until the ovulatory luteinizing hormone (LH) surges, which stimulates the resumption of meiosis in the Graafian follicle. It is also known that mammalian follicular oocytes undergo spontaneous meiotic maturation when liberated from follicles and cultured in an appropriate medium [1]. Establishing a successful *in vitro*

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technique for oocyte maturation and fertilization in farm animals could provide a sufficient supply of oocytes and embryos for use in basic research and biotechnology.

Hyttel *et al.* [2] described how the oocyte from the dominant follicle underwent further ultrastructural modifications and attained full developmental competence through a process that might be termed "capacitation". Full oocyte maturation involves two components, that is, nuclear and cytoplasmic maturation. Nuclear maturation is indicated by the completion of the first meiotic division, marked by the extrusion of the first polar body (PB I). The cytoplasmic maturation is referred to as the process of oocytes obtaining factors required for full developmental potential [3, 4]. Normally, nuclear and cytoplasmic maturation are two series of events that occur simultaneously during oocyte maturation. Therefore, a nuclear matured oocyte normally means that it has obtained the full developmental potential if fertilized. Nevertheless, in some situations such as oocyte maturation *in vitro*, these two series of maturation events can be dissociated, resulting in the loss of developmental potential of oocytes. It has been postulated that the impaired embryo development of oocytes obtained after *in vitro* maturation might be due to impaired cytoplasmic maturation and/or asynchrony of nuclear and cytoplasmic maturation [5]. Although the nuclear maturation of porcine oocytes is easily assessed morphologically by the presence of PB I, there are no non-invasive methods to evaluate cytoplasmic maturation. Until now, no study has focused on the relationship between the morphology of *in-vitro* matured porcine oocytes and their quality after fertilization.

It has been reported that the cumulus expansion and physiological significance of this process are important in the study of developmental competence of mammalian oocytes matured and fertilized *in vitro* [6–8]. During the past few years, many researchers found that porcine follicular fluid (PFF) affected cumulus expansion and cytoplasmic development of porcine follicular oocytes [9–13]. Therefore the objectives of the present study were 1) to investigate the effects of different concentrations of PFF from different sized follicles on cumulus expansion, nuclear maturation and *in vitro* development of porcine cumulus-oocyte complexes (COCs), 2) to test the effects of PFF culture time during IVM on cumulus expansion, nuclear maturation and *in vitro* development of porcine COCs, and 3) to assess the coefficients of correlation between expanded cumulus areas and nuclear maturation, and development of porcine COCs matured and fertilized *in vitro*.

Materials and Methods

Collection of follicular oocytes

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory within 2 h in saline (9 g NaCl/l, 100 IU penicillin GK/ml and 100 IU streptomycin sulphate/ml) at 37–38 C. The ovaries were then immediately freed from their hilus and connective tissues, and washed 2–3 times in saline at 37 C. COCs were aspirated with a 10-ml syringe equipped with a 19-gauge needle. Only oocytes with compact cumulus cells were used in the experiments. They were washed 3 times in Dulbecco's-PBS (D-PBS: Sigma; D4031) with 5% newborn calf serum (NCS: Gibco; Cat. No. 16010–159) (pH: 7.48) before use.

In-vitro maturation (IVM)

COCs were washed 3 times with *in vitro* maturation medium (IVMM), which consisted of TCM199 (Gibco; Cat. No. 31100–035), 10% fetal calf serum (FCS: Gibco; Cat. No. 16000–036), 10 IU/ml eCG (Ninbo, China; Cat. No. 981018), 10 IU/ml hCG (Ninbo, China; Cat. No. 980630), 1 μg/ml estradiol-17β (Fluka, Switzerland; EEC No. 2000238), 100 mg/l sodium pyruvate, 900 mg/l calcium lactate, 550 mg/l D-glucose, 5958 mg/l Hepes, 100 IU/ml penicillin GK and 100 IU/ml streptomycin sulphate (pH: 7.38). They were then transferred into droplets of IVMM (10–15 oocytes/100 μl drop) under mineral oil (Sigma; Lot. 103H10455) in a polystyrene dish (35 mm: Nunclon, Denmark; Cat. No. 153066), and cultured for 48 h at 39 C under 5% CO_2 in air. After culture, oocytes with an expanded cumulus mass were selected and subjected to insemination.

Assay of cumulus expansion

Cumulus expansion was assessed according to the method of Daen *et al.* [14]. The area occupied by a COC was measured and calculated with the formula: area = length $\times$ width $\times$ 0.7854. The length

was the distance between the two most widely separated points and the width was the distance between the two closest points. At least five replicates were performed in each experiment.

Sperm preparation

The sperm preparation was carried out by a modified method described by Nagai *et al.* [15]. The epididymides of a boar (Landrace × Meishan × Yorkshire, 8 months old) were collected at the slaughterhouse and transported to the laboratory in saline at 37 C within 15 min. The epididymal spermatozoa were extruded from the distal portion of the cauda by pressure with a 20-ml syringe, then diluted with BF-3 solution (1:3) (BF-3 solution: 40 g/l lactose, 20 g/l casein, 20 g/l Tris, 10 g/l citric acid monohydrate, 10 g/l sucrose) and kept for 2 h at 4 C. At the same temperature, the solution was diluted with BF-3 solution with 4% glycerol (1:1) and balanced for 2 h at 4 C. After balancing, the sperm suspensions were frozen in 0.1 ml pellets and stored in liquid nitrogen until required for IVF.

Frozen spermatozoa (3 pellets) were thawed in 10 ml D-PBS containing 5% NCS and 2 mg/ml BSA-V (Böchringer, Germany) (pH: 7.48) at 37 C. Immediately after thawing, the suspensions were centrifuged for 4 min at 200 g, the supernatant was discarded and the spermatozoa were subsequently diluted to 2–4 × 10^8 cells/ml and preincubated in sperm preincubation medium which consisted of TCM199 with Earle's salts, 12% FCS , 0.9 mg/l calcium lactate, 0.1 g/l sodium pyruvate, 0.55 g/l D-glucose, 5.958 g/l Hepes, 100 IU/ml penicillin GK, and 100 IU/ml streptomycin sulfate (pH: 7.80) at 37 C under 5% CO_2 in air for 1 h. After preincubation, the proportion of spermatozoa with progressive forward motility was greater than 60%.

In-vitro fertilization(IVF), examination of oocytes and embryo culture

The oocytes with expanded cumulus mass were washed three times, then transferred to fertilization medium (BO solution containing 10 mg/ml BSA-V, and 2 mM caffeine, pH: 7.48). A portion of the preincubated sperm suspension was introduced into the medium so that the final concentration for IVF was 2–4 × 10^7 cells/ml.

After incubation with sperm for 12–16 h, the oocytes were removed from the surrounding cumulus mass and spermatozoa by pipeting with a narrow-bore glass pipette, and then washed twice with an embryo culture medium [TCM199 with Earle's salts, 10% FCS, 3.7 ml/l sodium lactate (60% syrup), 40 mg/l sodium pyruvate, 5958 mg/l Hepes, 100 IU/ml penicillin GK, and 100 IU/ml streptomycin sulfate, pH: 7.40]. Then 10–15 oocytes were transferred to a droplet of the embryo culture medium (100 μl) containing granulosa cells prepared by the method described below.

To examine the fertilizability of the IVM/IVF system in the present study, the oocytes after incubation for 12–16 h with spermatozoa were randomly selected from each group and fixed for 3–4 days with acetic alcohol (methanol and acetic acid, 3:1, v/v) at 4 C, stained with 1% aceto-orcein and examined under a phase-contrast microscope (Olympus, Japan). Oocytes with enlarged sperm heads and/or male pronuclei with sperm tails were regarded as fertilized. Among the fertilized oocytes, fertilization was considered normal if a sperm-tail was observed adjacent to a male pronucleus.

Preparation of porcine follicular fluid (PFF)

PFF aspirated from follicles of different size was centrifuged at 1,000g for 20 min at room temperature to remove blood cells and debris. The supernatant was transferred to a sterile centrifuge tube and stored at –20 C until use. The PFF was passed through 0.22-μm membrane filters before use.

Preparation of granulosa cells

The oocytes with an expanded cumulus mass were treated with 0.1% hyaluronidase (Sigma; H6254), and the suspended granulosa cells were washed 2–3 times with the embryo culture medium. The cells were then cultured at a concentration of 1 × 10^5 cells/ml in the same medium until confluency before being used for the co-culture with embryos.

Experimental design

In Experiment 1, COCs were cultured in IVMM without PFF supplementation (Control Group), or cultured in IVMM supplemented with 5% or 15% of PFF obtained from small (<2 mm), medium (2–5 mm) and large (>5 mm) follicles, respectively. After 48 h of culture, cumulus-expansion, nuclear maturation and *in vitro* development were investigated. The area of expanded cumulus of COCs was measured as an index of cumulus

expansion. Then a portion of the oocytes were denuded to examine the presence of PB I. The remainder of the cumulus enclosed oocytes were inseminated with preincubated, frozen-thawed epididymal spermatozoa to evaluate fertilization (12 to 16 h post insemination) and development. The oocytes used for IVF were co-cultured with granulosa cell monolayers.

In Experiment 2, the effects of culture time with PFF during IVM on cumulus-expansion, nuclear maturation and *in vitro* development of porcine COCs were tested. The COCs were cultured in IVMM with PFF for the first 24 h followed by IVMM alone for the second 24 h. Oocytes cultured in IVMM supplemented with 15% PFF obtained from >5 mm follicles for the entire 48 h served as the control. The areas of expanded cumulus of COCs were measured at 24 h and 48 h, respectively. Nuclear maturation, fertilizability and development of *in vitro* matured COCs were tested as described in Experiment 1.

Statistical analysis

Data were analyzed with ANOVA, χ^2-test and REG, CORR with an SAS system for windows v6.12. The differences were considered to be significant at $P \leq 0.05$ or very significant at $P \leq 0.01$.

Results

The percentage of oocytes penetrated after co-incubation with spermatozoa for 12–16 h was 75.6% (34/45), the rate of polyspermy was 23.5% (8/34) and the normal fertilization rate was 61.8% (21/34).

Experiment 1

The results of Experiment 1 are shown in Tables 1 and 2. There were no significant differences in COC areas between groups before culture. The addition of PFF to IVMM significantly decreased the degree of cumulus expansion as compared with the control medium ($P<0.05$), and the areas of expanded cumulus of COCs increased progressively with the increase in the size of the follicles from which the same PFF concentrate was obtained. The differences between treatment groups on expanded cumulus areas were not

Table 1. Effect of different concentrations of PFF from different sized follicles during IVM on cumulus expansion and nuclear maturation of porcine COCs

Follicle size	PFF conc.	No. of oocytes cultured	Cumulus areas (Means±SD mm^2)		Percentages of oocytes with PB I
			Before culture	After 48 h culture	
Control	0%	38	0.0351 ± 0.032	0.8436 ± 0.325[b]	81.6(31/38)
Small	15%	36	0.0391 ± 0.016	0.4212 ± 0.096[a]	91.7(33/36)
	5%	39	0.0387 ± 0.027	0.4464 ± 0.089[a]	82.1(32/39)
Medium	15%	41	0.0298 ± 0.035	0.4847 ± 0.065[a]	85.4(35/41)
	5%	47	0.0305 ± 0.011	0.4752 ± 0.092[a]	91.5(43/47)
Large	15%	32	0.0379 ± 0.024	0.6959 ± 0.104[a]	90.6(29/32)
	5%	44	0.0404 ± 0.018	0.5864 ± 0.128[a]	84.1(37/44)

[a,b] Means in the same column with different superscripts differ significantly($p<0.05$).

Table 2. Developmental competence of porcine oocytes matured for 48 h in IVMM with different concentrations of PFF from different sized follicles

Follicle size	PFF conc.	No. of oocytes used for IVF	Percentages of embryos cleaved	Percentages of embryos developed to		
				2–4 cells	> 4 cells	Morulae and Blastocysts
Control	0%	36	36.1 (13/36)	46.2 (6/13)	23.1 (3/13)	0
Small	15%	33	45.5 (15/33)	40.0 (6/15)	13.3 (2/15)	0
	5%	32	31.3 (10/32)	20.0 (2/10)	0	0
Medium	15%	35	45.7 (16/35)	37.5 (6/16)	6.3 (1/16)	0
	5%	43	44.2 (19/43)	42.1 (8/19)	10.5 (2/19)	0
Large	15%	27	48.1 (13/47)	46.2 (6/13)	15.4 (2/13)	0
	5%	37	45.9 (17/37)	41.2 (7/17)	17.6 (3/17)	0

Table 3. Cumulus expansion and nuclear maturation of porcine COCs matured for 24 h in IVMM with PFF followed by IVMM for 24 h

Follicle size	PFF conc.	No.of oocytes cultured	Cumulus areas (Means ± SD mm^2) culture for the first 24 h in IVMM with PFF	Cumulus areas (Means ± SD mm^2) culture for the second 24 h in IVMM	Percentages of oocytes with PB I
Control	15%	33	0.5837 ± 0.099*b	0.6877 ± 0.152**f	84.8(28/33)
Small	15%	45	0.2004 ± 0.026*a	0.9853 ± 0.059**c	88.9(40/45)
	5%	46	0.2508 ± 0.100*a	0.8099 ± 0.252**d	82.6(38/46)
Medium	15%	50	0.3556 ± 0.084*ab	1.1241 ± 0.145**ce	86.0(43/50)
	5%	35	0.3909 ± 0.115*ab	0.9967 ± 0.217**c	88.6(31/35)
Large	15%	36	0.5748 ± 0.129*b	1.1796 ± 0.189**e	91.7(33/36)
	5%	46	0.4867 ± 0.033*b	1.1350 ± 0.240**ce	84.8(39/46)

*, ** means are significantly different in the first and second 24 h at the same PFF concentration ($p<0.05$). [a,b] and [c,d,e,f] means in the same column with different superscripts differ significantly($p<0.05$).

Table 4. Developmental competence of porcine oocytes matured for 24 h in IVMM with PFF followed by IVMM for 24 h

Follicle size	PFF conc.	No.of oocytes used for IVF	Percentages of embryos cleaved	Percentages of embryos developed to 2–4 cells	Percentages of embryos developed to > 4 cells	Percentages of embryos developed to Morulae and Blastocysts
Control	15%	44	47.7 (21/44)	42.9 (9/21)	14.3 (3/21)	0
Small	15%	40	45.0 (18/40)	44.4 (8/18)	22.2 (4/18)	0
	5%	38	36.8 (14/38)	42.9 (6/14)	14.3 (2/14)	0
Medium	15%	43	46.5 (20/43)	45.0 (9/20)	25.0 (5/20)	0
	5%	31	48.4 (15/31)	46.7 (7/15)	33.3 (5/15)	0
Large	15%	33	48.5 (16/33)	50.0 (8/16)	31.3 (5/16)	12.5 (2/16)
	5%	39	46.2 (18/39)	50.0 (9/18)	33.3 (6/18)	11.1 (2/18)

significant (P>0.05). The percentages of oocytes with PB I showed no significant difference between groups (P>0.05) (Table 1). The cleavage rates and the percentages of 2–4 cell and >4 cell embryos showed no significant difference between groups (P>0.05). No embryos developed to morulae or blastocysts (Table 2).

Experiment 2

The results of Experiment 2 are shown in Tables 3 and 4. After 48 h culture, there was a significantly greater cumulus expansion in oocytes matured in IVMM with PFF for the first 24 h and then followed by IVMM alone for the second 24 h compared with those in the control group (P<0.05). The percentages of oocytes with PB I showed no significant difference between groups (P>0.05) (Table 3). The cleavage rates and the percentages of 2–4 cell and >4 cell embryos showed no significant difference between groups. Only embryos matured in IVMM with PFF abtained from >5mm follicles for the first 24 h of the 48h-culture period developed to morulae and blastocysts (11.1–12.5%, Table 4).

In Experiments 1 and 2, the coefficients of correlation between expanded cumulus areas and nuclear maturation (percentages of oocytes with PB I), *in vitro* development (percentages of embryos developed to 2–4 cells, >4 cells and morulae and blastocysts) of COCs were –0.0073, 0.6946, 0.8845 and 0.5661, respectively. The expanded cumulus areas of COCs were positively correlated to the percentages of embryos developed to 2–4 cells (P= 0.0058), >4 cells (P=0.0001) and morulae and blastocysts (P=0.0348).

Discussion

Meiotic maturation of the oocytes occurs within the follicles. The microenvironment around the oocyte, including follicular fluid, may play a major modifying role in determining the fate of the oocyte [4]. Follicular fluid is composed partly of secretions from the follicle, especially from the granulosa cells, and partly of exudates from plasma [9].

Tsafriri and Channing [16] reported on the presence of an oocyte maturation inhibitor (OMI) in follicular fluid that reduced the meiotic activity of sheep and cattle oocytes and prevented final oocyte maturation in pigs, sheep and cattle. A soluble cAMP-dependent protein kinase was also purified from bovine follicular fluid, suggesting that a highly phosphorylated state would sustain the meiotic arrest [6]. Protein fractions were also isolated from bovine follicular fluid and a very high concentration of the 60-KDa fraction (2.0 mg/ml) was necessary to maintain bovine oocytes at the GV stage [17]. In contrast, Leibfried and First [18] and Racowsky and McGaughey [19] did not confirm the presence of OMI in bovine and porcine follicular fluid, which was shown to support IVM of immature COCs. Some researchers found that PFF promoted cumulus expansion and cytoplasmic development of follicular oocytes, reduced the incidence of polyspermy and enhanced the ability of male pronuclear formation [9–13]. Our results indicated that, when porcine COCs were cultured in IVMM with PFF for 48 h, PFF had an inhibiting effect on cumulus expansion, while not affecting the resumption of meiosis and the extrusion of PB I. The inhibiting factor(s) in PFF may be bound to the surface of granulosa cells to affect cumulus expansion [20, 21].

In the present study, when porcine COCs were matured in the presence of PFF for the first 24 h followed by IVMM only for another 24 h, the cumulus expanded more significant than those matured for the entire 48 h in IVMM and IVMM with PFF. So the action of PFF on cumulus expansion of porcine COCs during IVM may be affected by the PFF culture time. As reported concerning bovine COCs, it showed "a direct time-dependent correlation" of the effects of follicular fluid with the surge of gonadotropins. The synergistic action of follicular fluid and gonadotropins may be affected by the culture time of follicular fluid during IVM [22]. Although Sirard *et al.* [23] found that in the cattle there is a linear inverse relationship between the concentration of follicular fluid and nuclear maturation, the same relationship between the concentration of PFF and cumulus expansion of porcine COCs was not significant in the present study. Ayoub and Hunter [24] concluded that the inhibition of bovine follicular fluid from small and medium follicles was greater than from large follicles. The results reported here also demonstrated that the inhibitory effects of PFF on cumulus expansion progressively decreased with the increase in the size of follicles from which PFF was obtained. Possible explanations of the results may be due to the component difference in PFF in different sized follicles and/or the change in the concentration of the inhibitory factor(s) with the growth of follicles.

The physiological significance of cumulus expansion is important in the study of the developmental competence of mammalian oocytes matured and fertilized *in vitro*. The "quality" of the cumulus is often cited as a major criterion in choosing oocytes for IVF protocols, with particular emphasis placed on the degree of cumulus expansion [8]. Our results indicate that the expanded cumulus areas of COCs were positively correlated with the percentages of embryos developed to 2–4 cells, beyond 4 cells and morulae and blastocysts ($p=0.0058$, 0.0001 and 0.0348, respectively). Evidence produced from several studies supported the idea that the synthesis of an extracellular matrix composed, in part, of hyaluronic acid and gap junction endocytosis is necessary for maximal expansion of the cumulus mass. Until now, the molecular and cellular bases of positive effects of the cumulus on development are not known. The cumulus cells surrounding the oocytes might be required for supplying nutrients and regulating metabolism for the oocyte during the final maturation period. It is known that the oocyte requires cumulus cells for the provision of pyruvate and oxaloacetic acid during development [25] and also draws on the cumulus for the provision of amino acids [26], ribonucleosides [27], or cyclic adenosine monophosphate, which has been demonstrated to have inhibitory effects on the nuclear maturation of intact and cultured oocytes [28]. Several studies also have provided indirect evidence that cumulus expansion may be functionally related to the nuclear or cytoplasmic maturation of the oocyte through the production of factors by the cumulus mass [25,29]. Especially the time course of maturational changes in the nuclei of cultured oocytes appears to be related to the presence [30] or to the degree of expansion of the cumulus [31]. Therefore, there are several possible explanations for the relationship between the degree of cumulus expansion and *in vitro* development of porcine oocytes matured and fertilized *in vitro*. 1) The cumulus mass may

produce some factors to temporarily inhibit the resumption of meiosis, and then synchronize factors to promote the nuclear and cytoplasmic maturation to improve the developmental competence of porcine oocytes. Fouladi Nashta *et al.* [32] and Blondi *et al.* [33] also reported that if oocytes can be cultured *in vitro* under conditions that maintain meiotic arrest at the GV stage for some time, they may have the opportunity to acquire greater developmental competence in cattle. The degree of cumulus expansion may affect the concentrations of these factors, and then affect the synchronization of the nuclear and cytoplasmic maturation. 2) Cumulus cells may also excrete some factors, which may benefit the developmental ability of porcine oocyte after fertilization *in vitro*, and the concentration of these factors may increase with the increase in the degree of cumulus expansion. 3) More message exchange pathways may be activated when more cell-to-cell junction losses occur. So more of the factors mentioned above would act upon the oocytes through activated message exchange pathways when the cumulus mass expands more significantly. To the best of our knowledge, the present study is the first to demonstrate a clear correlation between cumulus expansion and the development of porcine oocytes matured and fertilized *in vitro*. Our results suggest that the areas of the expanded cumulus mass may be used as a parameter to predict the development potential of porcine oocytes matured and fertilized *in vitro*.

It was concluded that: 1) To some extent, PFF had inhibitory effects on cumulus expansion, but did not affect the resumption of meiosis and the extrusion of PB I in porcine oocytes, and the inhibitory effects of PFF decreased progressively with the increase in the size of follicles from which PFF was obtained; 2) there was "a direct time-dependent correlation" between PFF action and cumulus expansion of porcine COCs during IVM, and the action of PFF on cumulus expansion may be affected by the culture time of PFF; and 3) the area of expanded cumulus of porcine COCs had a significantly positive correlation with the percentages of embryos developed to 2–4 cells, beyond 4 cells and morulae and blastocysts; and the areas of the expanded cumulus mass may be used as a parameter to predict development of porcine oocytes matured and fertilized *in vitro*.

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