Effects of the Presence and the Numbers of Corpora Lutea in Non-Delivered and Delivered Pigs on In Vitro Oocyte Maturation and Embryonic Development

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Abstract. The aim of this study was to investigate the effects of the presence and the numbers of corpora lutea (CL) in porcine ovaries on in vitro oocyte maturation and embryonic development following intracytoplasmic sperm injection (ICSI). At oocyte collection, the ovaries of non-delivered and delivered pigs were classified into four groups by CL presence. The effect of the number of CL was also investigated following re-division of the non-delivered groups into four groups. In addition, the progesterone (P4) concentrations in follicular fluid (FF) of all the groups were measured to confirm the relationship between the presence and numbers of CL. Throughout the present study, the oocytes recovered from the CL-holding ovaries showed high (P<0.05) oocyte maturation rates, blastocyst rates and P4 concentrations in FF. Furthermore, in the non-delivered groups, the blastocyst rates and P4 concentrations in FF seemed to coincide with the CL numbers in each ovary. From these findings, we concluded that the presence and number of CL in the ovary can be used as an indicator for estimation of the developmental competence of porcine oocytes. Additionally, the present study suggests that P4 in FF influences in vitro oocyte maturation and embryonic development in porcine in vitro production.

Key words: Corpus luteum, Embryonic development after ICSI, Oocyte maturation, Pig, Progesterone

T o date, in vitro production (IVP) of embryos has been attempted in many species including pigs [1–11], cattle [12–15] and sheep [16, 17]. In these trials, the oocytes used for IVP were obtained from animals in various stages of growth, e.g., sexual maturity. The usage of younger oocyte donors enables reduction of the generation interval and rapid expansion of a valuable genotype. In many cases, however, the oocytes obtained from prepubertal animals have had lower developmental competence than those of postpubertal or aged animals [1, 6, 8, 14–18]. The low quality of oocytes recovered from prepubertal animals is the principal problem for IVP.

In IVP of porcine embryos, prepubertal pigs without corpora lutea (CL) in their ovaries are commonly utilized as oocyte donors. The prepubertal individuals are characterized by the fact that they have not completed maturation or ovulated yet. Ovulation produces morphological and physiological changes in the ovaries. CL are formed after ovulation and secrete progesterone (P4), which seems to be transferred to follicular fluid (FF), leading to a change of the hormonal balance in the FF. Therefore, the prevulatory oocytes of prepubertal pigs without CL are exposed in FF with a lower P4 concentration than those of adult pigs [1].

Supplementation of the culture media with P4 accelerates the meiosis resumption in porcine oocytes [19]. Thus, P4 might be a key molecule in oocyte maturation and subsequent development. Pigs are multi-ovulatory animals. Therefore, it is likely that under the presence of a greater number of CL, oocytes might be exposed to a higher P4 concentration. Based on the facts described above, we hypothesized that the presence of CL in the ovary and their number influence oocyte maturation and embryonic development in in vitro culture systems. However, it is not fully understood whether or not the difference of ovarian conditions, especially the number of CL, positively affect in vitro oocyte maturation and embryonic development in porcine IVP.

Therefore, the aim of the present study was to investigate the effects of CL presence and number on in vitro porcine oocyte maturation and subsequent developmental competence up to the blastocyst stage.

Materials and Methods

Oocytes and porcine follicular fluid (FF) collection

Ovaries were obtained from non-delivered and delivered pigs at a local slaughterhouse and transported to the laboratory in a sterilized saline (0.9% NaCl) solution at 37 °C within 3 h and washed twice in sterilized saline solution. Follicular fluid including cumulus-oocyte complexes (COCs) was aspirated from 3–8-mm antral follicles of the ovaries using an 18-G needle attached to a 5-ml disposable syringe. Those COCs were washed three times in a HEPES-buffered Tyrode’s medium containing 0.05% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich, St. Louis, MO, USA) (TLH-PVA), and those with two or three layers of cumulus cells and uniform cytoplasm were selected for IVM culture. Follicular fluid (FF) was centrifuged at 1,200 × g for 15 min and stored at –20 °C.
until used for hormonal assay.

**In vitro maturation (IVM)**

The medium for IVM was medium 199 (with Earle’s salts, L-glutamine and 2,200 mg/l sodium bicarbonate; Sigma) supplemented with 0.05% PVA, 3.05 mM glucose (Wako Pure Chemical Industries, Osaka, Japan), 0.91 mM Na-pyruvate (Wako), 100 μM cysteamine (Sigma), 10 ng/ml epidermal growth factor (Sigma) and 75 mg/l kanamycin (Sigma). Selected COCs were washed three times in IVM medium, and 8–15 COCs were cultured in a 100 μl droplet of the IVM medium covered with mineral oil (Sigma) for 44 h at 39 C in a humidified atmosphere of 5% CO₂ in air. In the first 22 h of culture, COCs were cultured with 10 IU/ml PMSG (Teikoku-Zouki, Tokyo, Japan) and 10 IU/ml hCG (Teikoku-Zouki), and in the last half of the culture period, they were cultured without these hormones.

**Intracytoplasmic sperm injection (ICSI)**

After IVM culture, the oocytes were stripped of their cumulus cells by gentle pipetting in IVM medium. Denuded oocytes were washed three times and kept in fresh IVM medium until ICSI.

According to our previous reports [4, 5, 9], pelleted frozen semen was thawed in pre-warmed (39 C) Dulbecco’s phosphate buffered saline (PBS; Gibco-BRL, Grand Island, NY, USA) containing 0.1% PVA (PBS-PVA) and was washed by centrifugation at 300 × g for 3 min in the same medium. The procedure for ICSI was performed according to the method of Yong et al. [11] with some modification. Briefly, manipulation was conducted with the aid of a pair of micromanipulators (Leitz, Wetzlar, Germany) under an inverted microscope. One drop each (4 μl) of PBS-PVA containing spermatozoa and TLH-PVA containing denuded oocytes was placed on the lid of a 50 × 9-mm petri dish (Falcon 1006, Franklin Lakes, NJ, USA) and covered with mineral oil. A 1-μl supernatant of washed semen was added into PBS-PVA on a manipulation dish. From the edge of the sperm-containing droplet, a motile spermatozoon was aspirated into the injection pipette tail-first without an immobilizing treatment such as tail-scoring/cutting and transferred to the drop containing oocytes. An oocyte was held with its polar body at either the 6 or 12 o’clock position using a holding pipette. A spermatozoon was injected into the oocyte cytoplasm and mixed with cytoplasmic components gently using a mouth-regulated open tube.

**In vitro culture (IVC)**

After ICSI, oocytes were washed three times in Porcine Zygote Medium-4 (PZM-4) [20] supplemented with 2.77 mM myo-inositol (Sigma), 0.34 mM tri-sodium citrate (Merck, Darmstadt, Germany) and 50 μM β-mercaptoethanol (Sigma). Immediately after washing, 8–15 oocytes were cultured under 5% CO₂, 5% O₂ and 90% N₂ at 39 C in a 30-μl drop of PZM-4 covered with mineral oil.

**Observation of cleavage and blastocyst cell number**

The rates of cleavage and blastocyst formation were determined at 48 and 144 h from the onset of IVC, respectively. The produced blastocysts were treated with 0.5% protease (Actinase E; Kaken Pharmaceuticals, Tokyo, Japan) in PBS to remove the zona pellucida. Then, they were kept in a hypotonic solution consisting of equal volumes of 1% (w/v) sodium citrate (Merck) and 30% fetal calf serum (Gibco-BRL). The samples were prepared by the gradual-fixation/air drying method [21] using ethanol in place of methanol. The slides were stained with 2% (v/v) Gimsa (Merck) in buffered saline (pH 6.8) for 10 min. After washing, the cell numbers of blastocysts were determined by phase-contrast microscopy.

**Hormonal assay**

Progesterone (P₄) concentrations in FF were measured using ECLusys 2010 (Roche Diagnostics GmbH, Basel, Switzerland). The sample was diluted in the ECLusys Diluent Multi Assay (Roche Diagnostics GmbH) to 10 or 50 fold. The interassay CV was 6.8%.

**Experimental design**

In this study, the meiotic and developmental competences were examined using oocytes recovered from the ovaries of non-delivered and delivered pigs. At oocyte collection, the ovaries were classified into six subgroups by CL presence and number in each ovary (Fig. 1). The oocytes were subjected to IVM, ICSI and IVC with respect to each subgroup. Since oocytes recovered from the ovaries of non-delivered/CL- (CL0) pigs are commonly used for porcine IVP, this group was set as a control group. Therefore, the analyses were carried out using one main factor (ovary condition). A comparison of the data was performed between the control and all other groups.

In Experiment 1, the effect of CL presence on each ovary, e.g., non-delivered/CL-, non-delivered/CL+, delivered/CL- and delivered/CL+, on meiotic and subsequent developmental competence were investigated. In this experiment, the CL1-5, CL6-10 and CL11≤ subgroups were pooled as non-delivered/CL+ group and used for analysis.

![Fig. 1.](image_url) A diagram showing an image of the experimental design. In Experiment 1, the ovaries obtained from non-delivered or delivered pigs were classified according to the presence of CL as follows: non-delivered/CL- or CL+ and delivered/CL- or CL+. In Experiment 2, segmentation of ovaries obtained from the non-delivered pigs was performed based on the numbers of CL in the ovaries. The oocytes recovered from ovaries without CL in the non-delivered pigs, referred to as non-delivered/CL- (Exp. 1) and CL0 (Exp. 2), were considered the control group. All data were compared with the control (CL0).
Experiment 2 was conducted to investigate the effect of CL number in each ovary on meiotic and subsequent developmental competence. In this case, only the oocytes recovered from non-delivered pigs were used for an analysis because a limited number of delivered pigs were slaughtered compared with the non-delivered pigs. The group of non-delivered/CL- pigs was referred to as CL0.

The data of the non-delivered/CL+ group was used after re-division into the three groups, CL1-5, CL6-10 and CL11. The mean CL numbers of the non-delivered/CL+ and delivered/CL+ groups were 7.3 and 11.0, respectively. The effect of ovary condition on meiotic and developmental competence is summarized in Table 2. The two groups with CL showed higher (OR 1.56 and 4.82, P<0.05) maturation rates (84.3%, 94.2%) compared with that of the control (76.8%). In addition, the blastocyst rates in the non-delivered/CL+ (28.9%) and delivered/CL- (34.6%) and delivered/CL+ (44.0%) groups were higher (OR 1.94, 2.46 and 4.31, P<0.05) than that of the control (16.7%), whereas the cleavage rates were similar among all the groups. Only the non-delivered/CL+ group showed a higher (P<0.05) cell number in their blastocysts.

**Results**

**Meiotic and developmental competence and follicular fluid progesterone concentration**

All raw data in Experiments 1 and 2 are summarized in Table 1. The maturation rate was approximately 80%, while the blastocyst rates ranged from 16.7 to 44.0%. The results of statistical analyses of meiotic and developmental competence are described below. The distribution of P4 concentrations in FF was represented by a box and whisker plot and was conducted by Steel's test. Differences were considered significant when the P value was less than 0.05.

**Effect of CL presence on porcine IVP (Experiment 1)**

The mean CL numbers of the non-delivered/CL+ and delivered/CL+ groups were 7.3 and 11.0, respectively. The effect of ovary condition on meiotic and developmental competence is summarized in Table 2. The two groups with CL showed higher (OR 1.56 and 4.82, P<0.05) maturation rates (84.3%, 94.2%) compared with that of the control (76.8%). In addition, the blastocyst rates in the non-delivered/CL+ (28.9%) and delivered/CL- (34.6%) and delivered/CL+ (44.0%) groups were higher (OR 1.94, 2.46 and 4.31, P<0.05) than that of the control (16.7%), whereas the cleavage rates were similar among all the groups. Only the non-delivered/CL+ group showed a higher (P<0.05) cell number in their blastocysts.

**Effect of CL numbers on porcine IVP (Experiment 2)**

Table 3 shows the OR and 95% CI for meiotic and developmental competence. The mean CL numbers of the CL1-5, 6-10 and 11 ≤ groups were 3.4, 7.9 and 12.6, respectively. As shown in Table 3,
there was no significant difference (P=0.063) in the maturation rates, and all groups showed similar cleavage rates (57.6–73.2%). However, the blastocyst rates in the CL6-10 and 11 ≤ groups (31.9% and 37.7%, respectively) were higher (OR 2.33 and 2.77, P<0.05) than that of the control (16.7%). Also, a higher (P<0.05) cell number per blastocyst was found in the CL1-5 group.

Discussion

The ovaries of prepubertal and adult animals are distinguished by the presence of CL in the ovary. The developmental competences of oocytes recovered from these ovaries are different [1, 6, 8, 14–18]. In addition, it is noteworthy that pigs are multi-ovulatory animals. In the present study, we hypothesized that the presence of CL and the number in the ovary influence porcine oocyte maturation and embryonic development in in vitro culture systems. To our knowledge, this is the first report showing that the effects of the presence and number of CL positively enhance the efficiency for porcine IVP.

In porcine IVP, polyspermy of oocytes fertilized in vitro is a significant problem that induces embryonic polyploidization. While it has been reported that the incidence of polyspermy is increased in prepubertal animals compared with adults [6], Sherrer et al. [8] demonstrated that polyspermy is not affected by the sexual maturity of oocyte donors. This contradiction suggests that sexual maturity of the oocyte donors may not be a dominant factor for production of polyspermic zygotes. In our laboratory, IVP of porcine embryos following ICSI has been applied with success [4, 5, 9, 22, 23]. Embryo production by ICSI is the only method capable of completely eliminating the risk of polyspermy. Therefore, in the present study, the mono-spermic fertilized porcine embryos were undoubtedly produced by ICSI.

The hormonal balance, especially in P4, in FF changes during the estrous cycle. Bagg et al. [1] found higher P4 concentrations in FF of adult pigs than in prepubertal pigs due to the presence of CL. In the present study, as shown in Fig. 2, the groups with CL showed higher P4 concentrations in FF than the control group (without CL). Additionally, in the group of non-delivered pigs, the P4 concentrations in FF tended to increase depending on the number of CL. On the other hand, the ovaries of delivered pigs showed an interesting distribution for the P4 level in FF. As shown in Table 3, the delivered/CL+ group displayed a remarkably wide distribution with a high estradiol-17β (E2) concentration (more 430 ng/ml; data not shown). The P4 concentration in FF just after luteolysis is usually low and then increases with follicular growth; during this phase, the E2 concentration in FF is high (60–400 ng/ml) [24, 25]. Thus, the ovaries of the delivered/CL- group used in the present study might have been obtained during the period of luteolysis to ovulation.

Meiotic resumption for oocyte maturation begins from germinal vesicle breakdown (GVBD). In the previous study, it has been reported that P4 accelerates GVBD [19, 26]. The P4 concentrations in FF of the groups with CL were higher than those of groups without CL in the present and previous studies [1]. This distinction of P4 concentrations in FF supports the observation that the oocyte maturation rates in the groups with CL were higher than in the con-

| Table 2. Effect of CL presence in the ovary on the maturation, cleavage and blastocyst rates of porcine oocytes following ICSI (Experiment 1) |
|---|---|---|---|---|---|---|
| Group | MII | Cleavage | Blastocyst |
| | P value | OR | 95% CI | P value | OR | 95% CI |
| Non-delivered/CL- | 0.010 | Ref | Ref | Ref |
| Non-delivered/CL+ | 1.56 | 1.08–2.26 * | 1.14 | 0.71–1.82 | 1.94 | 1.11–3.40 * |
| Delivered/CL- | 0.97 | 0.50–1.85 | 1.12 | 0.53–2.35 | 2.46 | 1.09–5.54 * |
| Delivered/CL+ | 4.82 | 1.46–15.90 * | 1.96 | 0.94–4.07 | 4.31 | 2.09–8.86 * |
| OR: odds ratio. 95% CI: 95% confidence interval of odds ratio. * Statistical significance compared with the non-delivered/CL- group (P<0.05). |

| Table 3. Effect of CL number in the ovary on the maturation, cleavage and blastocyst rates of porcine oocytes following ICSI (Experiment 2) |
|---|---|---|---|---|---|---|
| Group | MII | Cleavage | Blastocyst |
| | P value | OR | 95% CI | P value | OR | 95% CI |
| CL0 | 0.063 | Ref | Ref | Ref |
| CL1-5 | 1.96 | 1.16–3.31 | 0.85 | 0.46–1.56 | 1.31 | 0.61–2.82 |
| CL6-10 | 1.28 | 0.82–2.00 | 1.19 | 0.63–2.22 | 2.33 | 1.16–4.70 * |
| CL11≤ | 1.82 | 0.68–4.86 | 2.25 | 0.85–5.95 | 2.77 | 1.11–6.94 * |
| OR: odds ratio. 95% CI: 95% confidence interval of odds ratio. * Statistical significance compared with the CL0 group (P<0.05). |
control group (without CL) in the present study, although there was no significant difference (P=0.063) in the maturation rates (Experiment 2, Table 3).

In the present study, the groups with CL showed higher (P<0.05) blastocyst rates and P₄ concentrations in FF than those of the control group (without CL). From this finding, we infer that there is a correlation between the blastocyst rates and P₄ concentrations in FF. However, there was no significant correlation between the two factors (r=0.033). This fact might indicate that the improvement of the blastocyst rates was related to a certain level of P₄ and not dose dependent. This may explain why the delivered/CL+ group showed the highest blastocyst rate with a relatively low P₄ concentration. Undoubtedly, we can propose that the presence and number of CL in the porcine ovary may be an indicator of whether or not the oocytes have a high developmental competence.

Interestingly, the delivered/CL- group showed the second highest blastocyst rate next to the delivered/CL+ group. As described above, the ovaries of the delivered/CL- group would be obtained during period of luteolysis to ovulation. Thus, the oocytes recovered from these ovaries should have received the effect of CL presence during the prior luteal phase as in the case of the oocytes of the delivered/CL+ group. In other words, the influence of CL on oocytes continued up to the onset of oocyte maturation. This would be a reasonable consideration for the high developmental competence of the oocytes recovered from the delivered/CL- group.

Throughout the present experiments, there were significant differences in the cell numbers of blastocysts (34.1–49.8). However, the cell numbers obtained from all groups in this study were comparable with those of blastocysts produced in vitro in previous studies (25.0–74.0) [2, 5, 6, 8] and lower than those of blastocysts produced in vivo (164.5 ± 51.9) [10]. Although, we do not know why these were a difference in the cell numbers of blastocysts examined in the present study, it is clear that we could produce blastocysts with a high proportion keeping comparable quality to those produced in previous studies [2, 5, 6, 8].

The intra-oocyte glutathione (GSH) concentration, an indicator of cytoplasmatic maturation, has been used for evaluation of formability in male pronuclei (MPN). The MPN formation rates of the three groups, CL0 (50%), CL1-5 (65%) and CL6-10 (74%), showed a stepwise increase (data not shown) along with the CL numbers in the ovary and the P₄ levels in FF (Fig. 2). Unfortunately, in the present study, the GSH concentration in the porcine oocytes was not determined. However, these observations indirectly suggest the possibility that the CL number in the ovary may influence the increase of the intra-oocyte GSH concentration and support embryonic development.

The present results demonstrate clearly that the porcine oocytes recovered from the ovaries with CL were highly competent for in vitro oocyte maturation and embryonic development. Also, they show that the ovaries with CL had a high P₄ concentration in the FF, suggesting that P₄ is a key molecule for porcine oocyte maturation and embryonic development. Furthermore, it is possible that P₄ exposure ended before the oocytes were subjected to IVM. Therefore, in our laboratory, a further study is being conducted to ensure the effect of pre-exposure to P₄ before IVM.

In conclusion, the presence of CL in ovary and their number seemed to be an indicator for estimation of the developmental competence of porcine oocytes. Additionally, the present study suggests that P₄ in FF affects in vitro oocyte maturation and embryonic development in porcine IVP.

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