Relationship between the Size of Perivitelline Space and the Incidence of Polyspermy in Porcine Oocytes

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Summary

The size of each part of porcine oocytes cultured in NCSU37 medium, NCSU37 medium whose NaCl concentration was lowered from 108.76 to 61.6 mM and NCSU37 medium containing 0.1 % tunicamycin, an inhibitor of glycoprotein synthesis, was measured to investigate how the size of the perivitelline space changed as oocytes matured, and the reason(s) the size of the perivitelline space changed. The incidence of polyspermy in porcine oocytes after insemination was also observed, in order to ascertain a relationship between the size of the perivitelline space and the incidence of polyspermy.

The size of perivitelline space enlarged as the time of culture was prolonged, even in the use of either medium examined. The perivitelline space in oocytes cultured in NCSU37 medium for 44 hrs was 3.9 μm, but it was a significantly larger in oocytes cultured in NCSU37 medium with decreased concentration of NaCl (6.4 μm) and a significantly smaller in oocytes cultured in NCSU37 medium containing tunicamycin (2.4 μm). From the measurements of each part of oocytes during culture, it was suggested that the enlargement in the perivitelline space following treatment with low NaCl was attributed to reduction of the oocyte cytoplasm, while the decrease in the perivitelline space following treatment with tunicamycin was attributed to expansion of the oocyte cytoplasm.

Oocytes cultured for 44 hrs in NCSU37 medium, NCSU37 medium with decreased concentration of NaCl and NCSU37 medium containing tunicamycin showed the maturation rate of 88.8, 93.7 and 87.2 %, respectively, and the fertilization rate after insemination of 83.6, 87.0 and 88.2 %, respectively. There were no differences in either the maturation rate or the fertilization rate seen among these three culture media. On the other hand, the incidence of polyspermy of oocytes matured in NCSU37 medium was 52.1 %, which was significantly higher than 30.0 % of oocytes matured in NCSU37 medium with decreased concentration of NaCl, and was significantly lower than 71.7 % of oocytes matured in NCSU37 medium containing tunicamycin. From these findings, it was clarified that the higher incidence of polyspermy is closely related to the smaller perivitelline space in porcine oocytes.

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Key words : porcine oocyte, size of perivitelline space, incidence of polyspermy

Polyspermy is a phenomenon in which multiple sperm penetrate into a single oocyte during fertilization. It has been reported that the incidence of polyspermy is particularly high in porcine oocytes fertilized *in vitro* (Wang *et al*., 1991; 1994; 1997; Abeydeera and Day, 1997). Since it is known that polyspermic oocytes cannot develop to live offspring in mammals, polyspermy is a major problem that must be resolved in embryo production *in vitro*.

In porcine oocytes, it has been reported that the perivitelline space is significantly larger and the incidence of polyspermy after insemination is significantly lower in those matured *in vivo* than in those matured *in vitro* (Wang *et al*., 1998). Funahashi et al. (1994) have also reported that the perivitelline space is significantly large and the incidence of polyspermy after insemination is significantly low in porcine oocytes matured in Whitten medium whose NaCl concentration was lowered from 68.49 to 44.5 mM (low NaCl-Whitten medium), compared to control oocytes matured in usual Whitten medium. Therefore, it is suggested that a relationship exists between the size of perivitelline space and the incidence of polyspermy in porcine oocytes (Wang *et al*., 1998). However, since Funahashi et al. (1994) only measured the size of the perivitelline space in porcine oocytes, it remains unclear why the perivitelline space enlarged after cultured in the medium with decreased concentration of NaCl.

It is generally thought that glycoproteins synthesized and secreted by cumulus cells are involved in the enlargement of the perivitelline space (Talbot and Dandekar, 2003). Glycoproteins secreted from cumulus cells accumulate between cumulus cells and in the perivitelline space (Talbot, 1984). Hyaluronan, a major component of the glycoprotein synthesized and secreted by cumulus cells, in particular has a water absorbing property which results in enlargement of the matrix of cumulus and the perivitelline space (Talbot and Dandekar, 2003). In the cumulus-oocyte complex with cumulus cells whose glycoprotein synthesis was inhibited, therefore, it is supposed that the perivitelline space in oocytes

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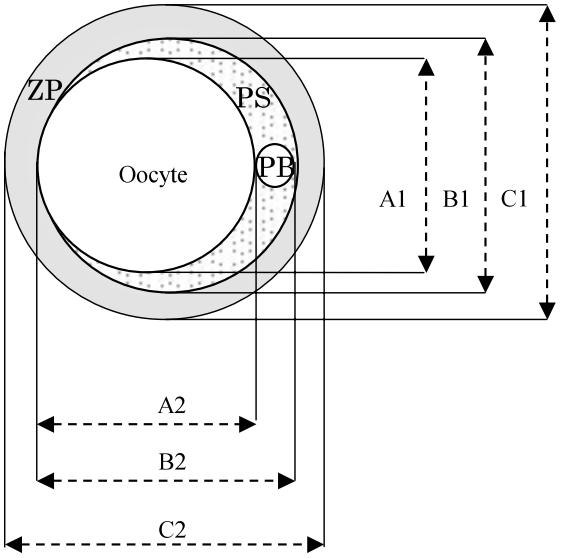

ZP: Zona pellucida, PS: perivitelline space, PB: 1st polar body.

Diameter of oocyte (A) = (A1+A2)/2
Inner diameter of zona pellucida (B) = (B1+B2)/2
Outer diameter of zona pellucida (C) = (C1+C2)/2
Thickness of zona pellucida = (C-B)/2
Size of perivitelline space = (B-A)/2

Fig.1. Calculation method of the size of each part in oocyte

fails to enlarge and the incidence of polyspermy after insemination increases.

In the present study, the size of each part of porcine oocytes cultured in NCSU37 medium with decreased concentration of NaCl or tunicamycin, an inhibitor of glycoprotein synthesis, was measured and compared to that of control oocytes cultured in NCSU37 medium, in order to examine how the size of the perivitelline space changed as oocytes matured, and the reason(s) the size of the perivitelline space changed. The incidence of polyspermy in porcine oocytes after insemination was also observed, in order to ascertain a relationship between the size of the perivitelline space and the incidence of polyspermy.

MATERIALS AND METHODS

Collection of oocytes and culture

Ovaries were obtained from prepubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9 % NaCl solution maintained at 39 ℃ . The ovaries were washed in 0.9 % NaCl solution containing 200 i.u./ml potassium penicillin G. Immature oocytes covered with cumulus cells (COCs) were aspirated from medium-sized follicles (3-6 mm in diameter) with a 21-gauge needle fixed to a 10-ml disposable syringe. Collected COCs were washed in phosphate buffered saline (PBS, pH 7.4) (Dulbecco and Vogt, 1954) and then in NCSU37 medium supplemented with 10 % (v/v) porcine follicular fluid, 10 % (v/v) fetal calf serum (FCS; Gibco BRL, Grand Island, NY, USA), 10 i.u./ml eCG (Serotropin; Teikoku Hormone Manufacturing Co. Ltd, Tokyo, Japan), 10 i.u./ml hCG (Gonatropin; Teikoku Hormone Manufacturing Co. Ltd) and 0.57 mM cysteine (Petters and Well, 1993).

In order to observe the effect of the treatment of tunicamycin and low NaCl on the size of perivitelline space, COCs were cultured at 39 ℃ in an atmosphere of 5 % CO_2 in air in NCSU37 medium containing 1.0 % (w/v) tunicamycin (Sigma-Aldrich, St. Louis, NJ, USA) (TM-NCSU37 medium) or NCSU37 medium whose NaCl concentration was lowered from 108.76 to 61.6 mM (low NaCl-NCSU37 medium). COCs cultured in NCSU37 medium were used as controls. Forty to 50 COCs were transferred into each well of a 4-well multidish (Nunc, Roskilde, Denmark) containing 400 μl/well of the culture medium as above mentioned, which had previously been covered with mineral oil (Sigma-Aldrich) and equilibrated in a CO_2 incubator (4020, Asahi Life Science, Tokyo, Japan).

Measurement of each part in oocytes

After cultured for 0, 22 and 44 hrs in each medium, cumulus cells were dispersed from the oocytes by pipetting in PBS containing 0.1 % hyaluronidase (Sigma-Aldrich). All the denuded oocytes cultured for 0 and 22 hrs, and about half the denuded oocytes cultured for 44 hrs were used for measurement of each part under a light microscope. Concerning the oocytes cultured for 44 hrs, measurement was done only for those with 1st polar body. The size of each part of oocytes was measured using a micrometer and the size of perivitelline space was calculated according to the method described in **Fig.1.**

Observation of polyspermy

The ejaculated boar semen was treated by the method of Funahashi et al. (1997), in order to induce capacitation of spermatozoa. The semen was washed three times in 0.9 % NaCl solution containing 63 mg/ml potassium penicillin G, 50 mg/ml streptomycin and 0.1 % bovine serum albumin (BSA; Sigma-Aldrich). Spermatozoa were resuspended in TCM-199 (Gibco BRL) containing 5 mM caffeine, 0.4 % (w/v) BSA, 0.01 % (w/v) sodium pyruvate, 0.0055 % (w/v) glucose and 0.009 % (w/v) calcium lactate to give a concentration of 1×10^6 live spermatozoa/ml, and a 400 μl of sperm suspension was covered with mineral oil in each well of a Nunc 4-well multidish.

In order to observe the incidence of polyspermy, only oocytes with 1st polar body were selected from the remaining denuded oocytes cultured for 44 hrs in each medium and were inseminated. These oocytes were washed twice in TCM-199 containing 5 mM caffeine, 0.4 % (w/v) BSA, 0.01 % (w/v) sodium pyruvate, 0.0055 % (w/v) glucose and 0.009 % (w/v) calcium lactate. Thirty to 50 denuded oocytes were introduced into the sperm suspension and cultured for 6 hrs

at 39 ℃ in a CO_2 incubator (5 % CO_2 in air).

After cultured with spermatozoa, inseminated oocytes were immersed in PBS containing 0.1 % pronase to dissolve their zonae pellucidae. These naked oocytes were stained with Hoechst 33342 and observed under a reflected-light fluorescing microscope. The oocytes including 2 or more pronuclei in their cytoplasm were judged to be fertilized ones, and those including 3 or more pronuclei were judged to be polyspermic ones.

Statistical analysis

The size of each part including perivitelline space was statistically analyzed by One-way analysis of variance (ANOVA). The rate of nuclear maturation in cultured oocytes, and the rates of fertilization and polyspermy in oocytes following insemination were statistically analyzed by Chi-square test.

RESULTS

Maturation rate in cultured porcine oocytes

As shown in **Table 1**, the maturation rates in porcine oocytes cultured for 44 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium were 88.8, 93.7 and 87.2 %, respectively, showing no significant difference by kinds of maturation media examined.

Size of each part in cultured porcine oocytes

The size of each part in porcine oocytes cultured in different media is shown in **Table 1**.

The perivitelline space in oocytes immediately after collection **(Fig.2)** was 1.2 μm, but in oocytes cultured for 22 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium it was 1.7, 3.2 and 1.3 μm, respectively. It was significantly larger in the oocytes cultured in low NaCl-NCSU37 medium than in those immediately after collection. However, there was no difference between oocytes cultured in NCSU37 medium and TM-NCSU37 medium. The size of the perivitelline space of oocytes cultured for 44 hrs in NCSU37 medium, low NaCl-NCSU37 medium **(Fig.3)** and TM-NCSU37 medium **(Fig.4)** was 3.9, 6.4 and 2.4 μm, respectively, which was each significantly larger than that of the oocytes immediately after collection. The perivitelline space was also significantly larger in oocytes cultured in low NaCl-NCSU37 medium than in oocytes cultured in NCSU37 medium or TM-NCSU37 medium.

The outer diameter of the zona pellucida was 145.5 μm in oocytes immediately after collection, which was no different to those of oocytes cultured for 22 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium. However, the outer diameter of the zona pellucida in oocytes cultured for 44 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium was 147.9, 151.8 and 149.1 μm, respectively, showing that it was no different between oocytes immediately after collection and those cultured in NCSU37 medium, but significantly larger in oocytes cultured in low NaCl-NCSU37 medium and TM-NCSU37 medium. On the other hand, the thickness of the zona pellucida in oocytes cultured in any of the culture media did not differ from that in oocytes immediately after collection.

The diameter of oocytes cultured for 22 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium was 118.1, 119.3 and 114.3 μm, respectively, which was no different to the 114.4 μm in oocytes immediately after collection. However, the diameter of oocytes cultured for 44 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium was 111.6, 109.1 and 116.2 μm, respectively, showing that it was no different between oocytes immediately after collection and those cultured in NCSU37 medium and TM-NCSU37 medium, but significantly smaller in oocytes cultured in low NaCl-NCSU37 medium.

Table 1. The rate of maturation and the size of each part in porcine oocytes cultured in different media

Kinds of culture media	Hours of culture	No. of oocytes examined	Maturation rates (%)	Outer diameter of zona pellucida (μm)	Diameter of oocyte (μm)	Thickness of zona pellucida (μm)	Size of perivitelline space (μm)
NCSU37	0	62		145.5 ± 0.5 ^{c} *	114.4 ± 0.4 ^{a,b}	14.4 ± 0.2 ^{a}	1.2 ± 0.1 ^{e}
	22	30		146.8 ± 0.6 ^{b,c}	118.1 ± 0.6 ^{a}	14.4 ± 0.3 ^{a}	1.7 ± 0.2 ^{c,d,e}
	44	71	88.8 ^{a}	147.9 ± 0.8 ^{b,c}	111.6 ± 1.2 ^{b}	14.3 ± 0.2 ^{a}	3.9 ± 0.3 ^{b}
Low NaCl-NCSU37	22	35		148.0 ± 0.6 ^{b,c}	119.3 ± 0.6 ^{a}	14.4 ± 0.2 ^{a}	3.2 ± 0.3 ^{b,c}
	44	59	93.7 ^{a}	151.8 ± 0.8 ^{a}	109.1 ± 0.7 ^{c}	14.9 ± 0.3 ^{a}	6.4 ± 0.4 ^{a}
TM-NCSU37	22	32		145.7 ± 0.8 ^{b,c}	114.3 ± 0.7 ^{a,b}	14.4 ± 0.2 ^{a}	1.3 ± 0.4 ^{d,e}
	44	68	87.2 ^{a}	149.1 ± 0.5 ^{a,b}	116.2 ± 0.6 ^{a}	14.0 ± 0.1 ^{a}	2.4 ± 0.2 ^{c,d}

* Mean ± S.E.

Values with different superscripts in the same column are significantly different ($P < 0.05$).

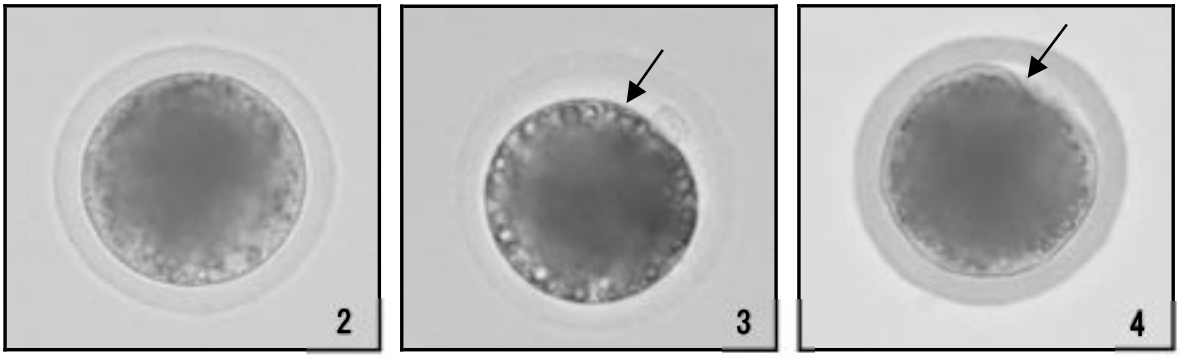

Fig.2. A very narrow perivitelline space of porcine oocyte immediately after collection.

Fig.3. A large perivitelline space (an arrow) of porcine oocyte cultured for 44 hrs in NCSU37 medium with decreased concentration of NaCl.

Fig.4. A small perivitelline space (an arrow) of porcine oocyte cultured for 44 hrs in NCSU37 medium containing tunicamycin.

Table 2. The incidence of polyspermy in porcine oocytes matured in different media

Kinds of culture media	No.of oocytes inseminated	No. and (%) of oocytes fertilized	No. and (%) of monospermic oocytes	No. and (%) of polyspermic oocytes
NCSU37	55	46(83.6) [a]	22(47.8) [b]	24(52.1) [b]
Low NaCl-NCSU37	46	40(87.0) [a]	28(70.0) [a]	12(30.0) [c]
TM-NCSU37	68	60(88.2) [a]	17(28.3) [c]	43(71.7) [a]

Values with different superscripts in the same column are significantly different ($P < 0.05$).

Incidence of polyspermy in porcine oocytes after insemination

As shown in **Table 2**, oocytes cultured for 44 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium showed a fertilization rate of 83.6, 87.0 and 88.2 %, respectively. There were no differences in the fertilization rate seen among these three culture media. On the other hand, the incidence of polyspermy of oocytes matured in NCSU37 medium was 52.1 %, which was significantly higher than 30.0 % of oocytes matured in low NaCl-NCSU37 medium, and was significantly lower than 71.7 % of oocytes matured in TM-NCSU37 medium.

DISCUSSION

The perivitelline space is the gap between the plasma membrane and the zona pellucida of the oocyte. Although not present in the oocytes in the germinal vesicle stage, the perivitelline space is known to start forming when meiotic maturation resumes and to enlarge in matured oocytes at the metaphase Ⅱ stage (Kaufman *et al*., 1989; Okada *et al*., 1993).

It has been reported that the perivitelline space enlarged when porcine oocytes were cultured in the medium with decreased concentration of NaCl (Funahashi *et al*., 1994). However, it remains unclear why the perivitelline space of porcine oocytes enlarged after cultured in the medium with decreased concentration of NaCl. From the present observation concerning the measurements of perivitelline space in porcine oocytes during a process of *in vitro* maturation, it was confirmed that the size of perivitelline space enlarges as the oocytes matured. It was also confirmed that the perivitelline space significantly enlarged when the oocytes were cultured for 44 hrs in low NaCl-NCSU37 medium compared to that of the oocytes cultured in NCSU37 medium, and that it significantly decreased in oocytes treated with tunicamycin which inhibited glycoprotein synthesis in cumulus cells.

The outer diameter of the zona pellucida significantly increased in oocytes cultured for 44 hrs in low NaCl-NCSU37 medium but the diameter of the same oocytes significantly decreased compared to oocytes cultured in NCSU37 medium. Furthermore, the outer diameter of the zona pellucida was no different in oocytes cultured for 44 hrs in TM-NCSU37 medium but the diameter of the same oocytes significantly increased compared to oocytes cultured in NCSU37 medium. Since the thickness of the zona pellucida in oocytes cultured in any of the culture media was no different to that in oocytes

immediately after collection, it was suggested that the enlargement in the perivitelline space of oocytes treated with low NaCl is attributed to reduction of the oocyte cytoplasm, and that the decrease in the perivitelline space of oocytes treated with tunicamycin is attributed to expansion of the oocyte cytoplasm.

In the present investigation, there were no differences in the maturation rate and fertilization rate of ooctyes cultured for 44 hrs in NCSU37 medium, low NaCl-NCSU37 medium and TM-NCSU37 medium, confirming that the abilities to mature and fertilize in oocytes cultured in the medium with decreased concentration of NaCl and tunicamycin were maintained normally. On the other hand, the incidence of polyspermy after insemination significantly decreased in oocytes cultured in low NaCl-NCSU37 medium whose perivitelline space significantly enlarged, and it significantly increased in oocytes cultured in TM-NCSU37 medium whose perivitelline space significantly decreased. From these findings, it was confirmed that there is a close relationship between the size of the perivitelline space and the incidence of polyspermy in porcine oocytes. Funahashi et al. (1994) and Wang et al. (1998) also previously reported that the size of the perivitelline space and the incidence of polyspermy are related, which strongly supports the present finding that these are closely related in porcine oocytes.

REFERENCES

Abeydeera, L.R. and B.N. Day. 1997. In *vitro* penetration of pig oocytes in a modified tris-buffered medium: effect of BSA, caffeine and calcium. *Theriogenology*, **48**:537-544.

Dulbecco, R. and M. Vogt. 1954. Plaque formation and isolation of pure lines with poliomyelitis viruses. *J.Exp. Med*., **99**:167-174.

Funahashi, H., T.C. Cantley, T.T. Stumpf, S.L. Terlouw and B.N. Day. 1994. Use of low-salt culture medium for *in vitro* maturation of porcine oocytes is associated with elevated oocyte glutathione levels and enhanced male pronuclear formation after in *vitro* fertilization. *Biol. Reprod*., **51**:633-639.

Kaufman, M.H., R.E. Fowler, E. Barratt and R.D. McDougall. 1989. Ultrastructural and histochemical changes in the murine zona pellucida during the final stages of oocyte maturation prior to ovulation. *Gamete Res*., **24**:35-48.

Okada, A., K. Inomata and T. Nagae. 1993. Spontaneous cortical granule release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes. *Anat.Rec*., **237**:518-526.

Petters, R.M. and K.D. Wells. 1993. Culture of pig embryos. *J.Reprod.Fertil.Suppl*., **48**:61-73.

Talbot, P. 1984. Hyaluronidase dissolves a component in the hamster zona pellucida. *J.Exp.Zool*., **229**:309-316.

Talbot, P. and P. Dandekar. 2003. Perivitelline space: does it play a role in blocking polyspermy in mammals? *Microsc. Res.Tech*., **61**:349-357.

Wang, W.H., L.R. Abeydeera, T.C. Cantley and B.N. Day. 1997. Effects of oocyte maturation media on development of pig embryos produced by *in vitro* fertilization. *J.Reprod. Fertil*., **111**:101-108.

Wang, W.H., L.R. Abeydeera, K. Okuda and K. Niwa. 1994. Penetration of porcine oocytes during maturation *in vitro* by cryopreserved, ejaculated spermatozoa. *Biol.Reprod*., **50**:510-515.

Wang, W.H., L.R. Abeydeera, R.S. Prather and B.N. Day. 1998. Morphologic comparison of ovulated and in *vitro*-matured porcine oocytes, with particular reference to polyspermy after *in vitro* fertilization. *Mol.Reprod.Dev*., **49**:308-316.

Wang, W.H., K. Niwa and K. Okuda. 1991. *In-vitro* penetration of pig oocytes matured in culture by frozen-thawed ejaculated spermatozoa. *J.Reprod.Fertil*., **93**:491-496.

ブタ卵母細胞における囲卵腔の大きさと多精子侵入の頻度との関係

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要　約

NaClの濃度を低くした低NaCl-NCSU37培養液およびツニカマイシン添加NCSU37培養液で培養したブタの卵丘卵母細胞複合体について、卵母細胞の各部位の大きさを計測して通常のNCSU37培養液で培養した卵母細胞のものと比較し、低NaCl処置およびツニカマイシン処置した卵母細胞の囲卵腔の大きさが変化する原因について検討した。また、媒精後の多精子侵入の頻度を観察し、ブタ卵母細胞の囲卵腔の大きさと多精子侵入の頻度との間に関係があるのか否かを検討した。

囲卵腔は、いずれの種類の培養液で培養した卵母細胞においても、成熟に伴って拡大した。なお培養後44時間において、囲卵腔は、通常のNCSU37培養液で成熟させた卵母細胞では3.9μmあったが、低NaCl-NCSU37培養液で成熟させた卵母細胞では有意に拡大して6.4μmになるとともに、ツニカマイシン添加NCSU37培養液で成熟させた卵母細胞では有意に縮小して2.4μmになった。一方、卵母細胞の各部位の計測から、低NaCl-NCSU37培養液で培養した卵母細胞で囲卵腔が大きくなったのは、卵母細胞の細胞質が収縮したことによるものと思われた。また、ツニカマイシン添加NCSU37培養液で培養した卵母細胞の囲卵腔が小さくなったのは、卵母細胞の細胞質が拡大したことによるものと思われた。

一方、NCSU37培養液、低NaCl-NCSU37培養液およびツニカマイシン添加NCSU37培養液で44時間培養した卵母細胞の成熟率および媒精後の受精率は、それぞれ88.8、93.7、87.2％および83.6、87.0、88.2％であり、これら3種の培養液の間で成熟率と受精率に相違はみられなかった。また多精子侵入の頻度は、NCSU37培養液で成熟させた卵母細胞では52.1％であったが、低NaCl-NCSU37培養液で成熟させて囲卵腔が大きくなった卵母細胞では有意に低く30.0％であるとともに、ツニカマイシン添加NCSU37培養液で成熟させて囲卵腔が小さくなった卵母細胞では有意に高く71.7％であった。以上の結果から、ブタ卵母細胞の囲卵腔の大きさと多精子侵入の頻度との間には密接な関係のあることが確認された。

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キーワード：ブタ卵母細胞、囲卵腔の大きさ、多精子侵入の頻度

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