Size of Perivitelline Space and Incidence of Polyspermy in Mouse Oocytes Matured In Vivo and In Vitro

Sayaka Ueno[1] and Sueo Niimura[2]*

[1]Graduate School of Science and Technology,
[2]Faculty of Agriculture, Niigata University, Niigata-shi, Niigata 950-2181, Japan

Abstract: *In mouse oocytes soon after collection, perivitelline space could be seen in about half of them and its mean size was 0.28 μm. After 6 hrs of in vivo and in vitro maturation, perivitelline space appeared in all oocytes. In oocytes in the process of maturation in vivo, the mean size of perivitelline space significantly increased 6 hrs after hCG injection and reached 7.00 μm at 14 hrs after the hCG injection. On the other hand, perivitelline space in cultured oocytes enlarged significantly at 2 hrs culture. Thereafter, perivitelline space in oocytes continued to enlarge with culture time and reached 5.40 μm at 14 hrs of maturation culture. The size of perivitelline space in oocytes 14 hrs after the hCG injection was significantly larger than that in oocytes cultured for 14 hrs. The fertilization rates of oocytes matured in vivo (85%) did not differ from that of oocytes matured in vitro (80%). However, the incidence of polyspermy in oocytes matured in vivo was 4%, which was significantly lower than the 16% of oocytes matured in vitro. These findings suggest there could be a relationship between the size of the perivitelline space and the incidence of polyspermy in mouse oocytes.*

Key words: *Mouse oocyte, Maturation, Perivitelline space, Polyspermy*

Introduction

Perivitelline space is the gap between the plasma membrane and zona pellucida of oocytes. Recently, it has been reported that porcine oocytes matured in Whitten medium with decreased concentration of NaCl (low NaCl-Whitten medium) or low NaCl-NCSU37 medium have significantly larger perivitelline spaces, compared with oocytes matured in high NaCl-Whitten medium or NCSU 37 medium [1, 2]. It has also been reported that the incidence of polyspermy in porcine oocytes matured in low NaCl-Whitten medium or low NaCl-NCSU37 medium is significantly lower than that in those matured in high NaCl-Whitten medium and NCSU 37 medium [1, 2]. Furthermore, it is known that perivitelline space in porcine oocytes matured *in vivo* is larger and the incidence of polyspermy after insemination is lower than in oocytes matured *in vitro* [3]. Therefore, it has been suggested that a close relationship between the size of the perivitelline space and the incidence of polyspermy is present in porcine oocytes, although it is unclear whether this relationship exists in oocytes of other animals.

In mouse oocytes, perivitelline space starts to form at 8 to 9 hrs after an hCG injection and enlarges in the vicinity of the 1st polar body at 12 to 13 hrs after the hCG injection, reaching maximum size at 17 to 19 hrs after the hCG injection [4]. Kaufman *et al.* [5] also reported that the perivitelline space of mouse oocytes enlarges until the 1st polar body is released. However, there have been no detailed reports with regard to the change in size of the perivitelline space in mouse oocytes during maturation *in vivo* and *in vitro*.

In the present study, changes in the size of the perivitelline space were examined in mouse oocytes during maturation *in vivo* and *in vitro*. The incidence of polyspermy following insemination in mouse oocytes matured *in vivo* and *in vitro* was also observed, in order to examine whether a relationship between the incidence of polyspermy and the size of the perivitelline space exists in mouse oocytes.

Received: September 11, 2007
Accepted: January 15, 2008
*To whom correspondence should be addressed.
e-mail: niimura@agr.niigata-u.ac.jp

Materials and Methods

Animals

Ninety female mature mice of the ICR strain were used in the present study. They were housed in autoclaved metal cages and were given a standard chow (MF; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24°C), under controlled-lighting conditions (14L/10D). They received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (Niigata University Animal Care Committee). These mice were intraperitoneally injected with 5 IU of pregnant mare serum gonadotrophin (PMSG; PEAMEX®, Sankyo Yell Yakuhin Co. Ltd., Tokyo, Japan).

Collection and culture of oocytes

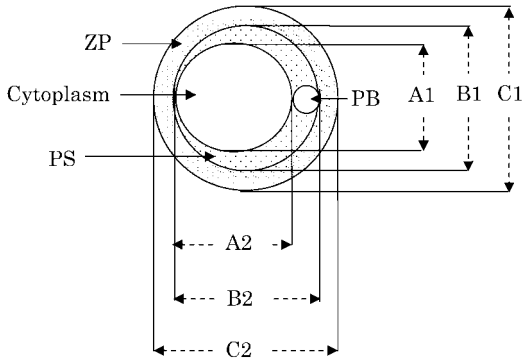
In order to observe oocytes in the process of maturation *in vitro*, immature oocytes covered with cumulus cells (COCs) were collected from antral follicles 48 hrs after a PMSG injection and cultured in TYH medium [6] containing 5% fetal bovine serum (FCS; Gibco BRL, NY, USA) and 10 IU/ml PMSG (PEAMEX®) at 37°C in a CO_2 incubator (5% CO_2 in air). PMSG-injected females were further intraperitoneally injected with 5 IU of hCG (Gonatropin®; Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan) 48 hrs after the PMSG injection to obtain oocytes in the process of maturation *in vivo*. COCs were collected from antral follicles 0, 2, 4, 6, 8, 10 and 12 hrs after the hCG injection and from oviducts 14 hrs after the hCG injection.

Measurement of perivitelline space in oocytes

At 0, 2, 4, 6, 8, 10, 12 and 14 hrs after the hCG injection or after maturation culture, cumulus cells were dispersed from oocytes by pipetting in TYH medium containing 0.1% hyaluronidase (Sigma-Aldrich, MO, USA). The size of each part of the oocyte was measured using a micrometer and the size of the perivitelline space was calculated according to the method described in Fig. 1.

Observation of nuclear maturation

In order to investigate nuclei of oocytes cultured for various periods and those collected from antral follicles and oviducts, oocytes in which the perivitelline space had been measured were stained with Hoechst 33342 (Molecular Probes, Oregon, USA) and examined for evidence of nuclear maturation under a reflected-light fluorescence microscope (Nikon Corporation, Tokyo, Japan).



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

Diameter of cytoplasm (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 of the oocyte.

Observation of polyspermy

At 14 hrs after the hCG injection and after maturation culture, cumulus cells were dispersed from oocytes by pipetting in TYH medium containing 0.1% hyaluronidase (Sigma-Aldrich). In order to observe the incidence of polyspermy, only oocytes with the 1st polar body in the perivitelline space were selected and inseminated. Sperm suspension was prepared by minutely cutting caudal epididymis of mature males in TYH medium for 1 hr at 37°C in a CO_2 incubator. A small volume of the sperm suspension was introduced into 100-μl droplets of TYH medium, and the final concentration of spermatozoa was adjusted to 2×10^6 /ml. The denuded oocytes were introduced into the droplets of sperm suspension and cultured for 6 to 12 hrs at 37°C in a CO_2 incubator. After culture with spermatozoa, inseminated oocytes were observed under a phase contrast microscope. The oocytes including 2 or more pronuclei in their cytoplasm were judged to be fertilized, and those including 3 or more pronuclei were judged to be polyspermic.

Statistical analysis

The size of the perivitelline space in oocytes was statistically analyzed by one-way analysis of variance (ANOVA). The rates of fertilization and polyspermy after insemination were statistically analyzed by the chi-square test.

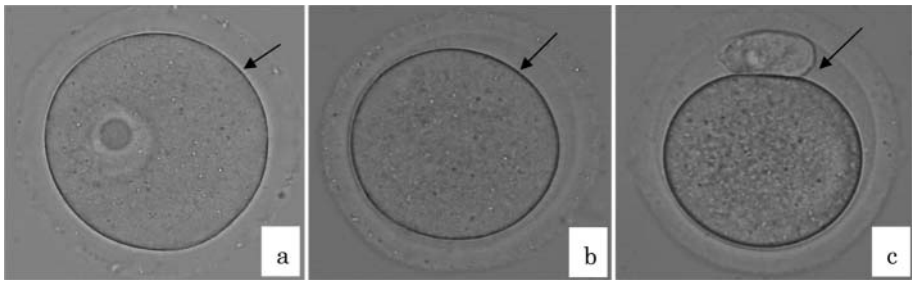


Fig. 2. Perivitelline space in mouse oocytes. a. A small perivitelline space (arrow) is seen between the plasma membrane and zona pellucida of an oocyte immediately after collection. b. A flat area (arrow) is seen in the plasma membrane of an oocyte cultured for 4 hrs. The perivitelline space enlarges in this area. c. A large perivitelline space is seen in the vicinity of the 1st polar body (arrow) of an oocyte 14 hrs after the hCG injection.

Table 1. Changes in the size of the perivitelline space in mouse oocytes during maturation *in vivo* and *in vitro*

Hours after hCG injection or culture	*In vivo* maturation		*In vitro* maturation	
	No. of oocytes examined	Size of perivitelline space (μm)	No. of oocytes examined	Size of perivitelline space (μm)
0	30	0.28 ± 0.07*[d]	30	0.28 ± 0.07*[d]
2	31	0.73 ± 0.18[d]	30	2.28 ± 0.22[c]
4	31	0.52 ± 0.12[d]	33	2.62 ± 0.23[c]
6	31	3.04 ± 0.31[c]	30	2.67 ± 0.26[c]
8	31	2.11 ± 0.22[b,c]	33	3.91 ± 0.21[b]
10	31	5.14 ± 0.26[b]	30	4.99 ± 0.12[a]
12	31	5.08 ± 0.31[b]	30	5.32 ± 0.17[a]
14	30	7.00 ± 0.23[a]	30	5.40 ± 0.11[a]

*Mean ± S.E. Values with different superscripts in the same column are significantly different ($p<0.05$).

Results and Discussion

Nuclear maturation

At 0 hrs after the hCG injection, nuclei of oocytes were in the germinal vesicle (GV) and diakinesis stages, mostly in the GV stage (93%). The percentage of oocytes at the GV stage decreased over time *in vivo* and *in vitro* and reached 0% after culture for 4 hrs and 6 hrs after the hCG injection, respectively. Nuclei of oocytes cultured for 8 hrs and 8 hrs after the hCG injection were almost at the metaphase I (MI) stage, 76 and 74%, respectively. At 8, 10, 12 and 14 hrs after maturation culture, the percentages of oocytes with MII stage nuclei were 9, 90, 97 and 97%, respectively. Of oocytes at 8, 10, 12 and 14 hrs after the hCG injection, 3, 74, 77 and 90%, were at the MII stage.

Changes in the size of the perivitelline space

The perivitelline space could barely be seen in 47% (14/30) of oocytes immediately after collection from antral follicles (Fig. 2a) and could not be seen in 53% of the oocytes. Perivitelline space could be seen in 52% (16/31) and 87% (26/30) of the oocytes at 2 hrs after the hCG injection or after culture, and in 55% (17/31) and 97% (32/33) at 4 hrs after the hCG injection and culture, respectively. From 6 hrs onwards after the hCG injection or after culture, perivitelline space was formed in all oocytes. A flat area of the plasma membrane appeared in the oocytes at 4 hrs after culture (Fig. 2b) or 6 hrs after the hCG injection, indicating that enlargement of perivitelline space took place in that area. From 8 hrs onwards after the hCG injection or after culture, release of the 1st polar body occurred and perivitelline space further enlarged in the vicinity of 1st polar body (Fig. 2c).

Changes in the size of the perivitelline space in mouse oocytes during maturation *in vivo* and *in vitro* are shown in Table 1 and Fig. 3.

The mean size of perivitelline space in oocytes immediately after collection from antral follicles was

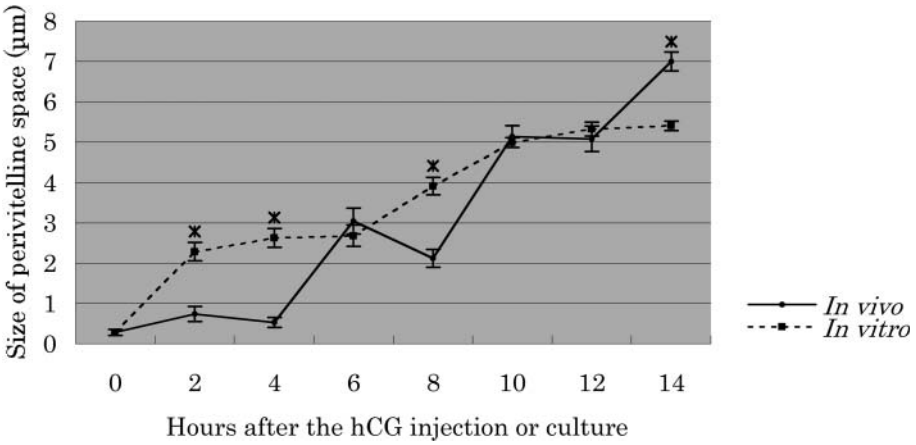


Fig. 3. Changes in the size of the perivitelline space in mouse oocytes during maturation *in vivo* and *in vitro*. *: $p<0.05$.

0.28 μm and increased significantly to 3.04 μm at 6 hrs after the hCG injection. Thereafter, perivitelline space continued to enlarge with time and reached 7.00 μm at 14 hrs after the hCG injection. On the other hand, perivitelline space in cultured oocytes was enlarged significantly at 2 hrs after culture (2.28 μm). Thereafter, it continued to enlarge with culture time, becoming 5.40 μm at 14 hrs after culture. At 6, 10 and 12 hrs after the hCG injection and after culture, there were no significant differences in the size of the perivitelline space between *in vivo* oocytes and cultured oocytes. However, the size of the perivitelline space in oocytes at 2, 4 and 8 hrs after the hCG injection was significantly smaller than that of oocytes at 2, 4 and 8 hrs after culture. In addition, the size of the perivitelline space was significantly larger in oocytes at 14 hrs after the hCG injection than in those at 14 hrs after culture.

The perivitelline space was reported to be significantly larger in porcine oocytes matured *in vivo* compared to those matured *in vitro* [3]. From the results of porcine [3] and mouse oocytes, we consider that the presence of a significantly large perivitelline space might be a factor common to mammalian oocytes matured *in vivo*. Although the reason why oocytes matured *in vivo* have larger perivitelline space is unclear, Okada [4] and Chung [7] reported that oviductal secretions are involved in the enlargement of perivitelline space in ovulated oocytes. Buhi [8, 9] reported that the substances in the perivitelline space of oocytes change after ovulation. In the present study, all the oocytes at 14 hrs after the hCG injection were collected from oviducts and were therefore affected by secretions from oviducts.

In the present investigation, the diameter of cytoplasm and the inner diameter of the zona pellucida in mouse oocytes immediately after collection were 75.9 and 76.5 μm, respectively. The inner diameter of the zona pellucida did not change throughout maturation *in vivo* and *in vitro*. On the other hand, the diameter of the cytoplasm in oocytes decreased significantly in the process of maturation *in vivo* and *in vitro*, reaching 67.7 and 65.1 μm at 14 hrs after the hCG injection and culture, respectively.

In mouse ovulated oocytes at 12.5 to 13 hrs after the hCG injection, perivitelline space occupied about 40% of the inner space of the zona pellucida [7]. It has been suggested that reduction in the diameter of the cytoplasm of oocytes with maturation and increase of the inner diameter of zona pellucida are factors responsible for formation and enlargement of perivitelline space [10]. Sorenson [11], however, suggested that enlargement of the perivitelline space is caused by an increase of the inner diameter of the zona pellucida rather than a reduction in the size of the oocyte cytoplasm. In the present investigation, the inner diameter of the zona pellucida did not change throughout maturation *in vivo* and *in vitro*, while the diameter of the cytoplasm decreased in oocytes during maturation both *in vivo* and *in vitro*. From these results concerning the changes in size of each part of the mouse oocyte during maturation, we consider that formation and enlargement of the perivitelline space are mainly caused by a reduction in the size of the oocyte cytoplasm.

Table 2. The incidence of fertilization and polyspermy in mouse oocytes matured *in vivo* and *in vitro*

Maturation	No. of oocytes inseminated	No. (%) of fertilized oocytes	No. (%) of monospermic oocytes	No. (%) of polyspermic oocytes
In vivo	53	45 (85)[a]	43 (96)[a]	2 (4)[b]
In vitro	55	44 (80)[a]	37 (84)[b]	7 (16)[a]

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

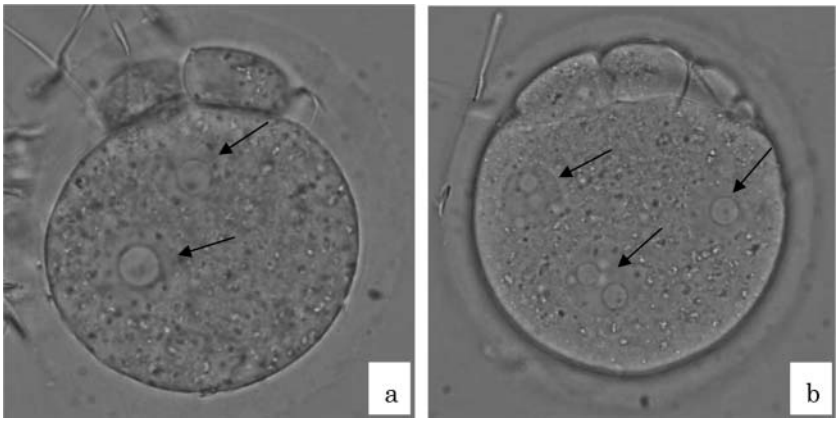


Fig. 4. Fertilized mouse oocytes matured *in vivo* (a) and *in vitro* (b). a. A monospermic oocyte with two pronuclei (arrows) in the cytoplasm. b. A polyspermic oocyte with three pronuclei (arrows) in the cytoplasm.

Incidence of polyspermy

The rates of fertilization and polyspermy after insemination in mouse oocytes matured *in vivo* and *in vitro* are shown in Table 2.

The fertilization rate was 85% in oocytes matured *in vivo* (Fig. 4a), and 80% in oocytes matured *in vitro*, showing no significant difference. However, the incidence of polyspermy in oocytes matured *in vivo* was 4%, which was significantly lower than the 16% of oocytes matured *in vitro* (Fig. 4b).

Recently, it has been suggested that there is a relationship between the size of perivitelline space in porcine oocytes and the incidence of polyspermy following insemination [1–3], but it is not clear whether the relationship is present in the oocytes of animals other than pigs. In the present investigation, we confirmed that the size of the perivitelline space in mouse oocytes matured *in vivo* was larger and that the incidence of polyspermy after insemination was lower than in oocytes matured *in vitro*. These findings suggest there could be a relationship between the size of the perivitelline space and the incidence of polyspermy in mouse oocytes.

The reason for the low incidence of polyspermy after insemination in oocytes with large perivitelline spaces is unclear. However, it is known that proteins and hyaluronan are present in the perivitelline space of oocytes before ovulation [12–14], and that secretions from oviducts [13–17] and the contents of cortical granules [1, 2, 13–15, 18] are also present after ovulation and fertilization, respectively. When the perivitelline space enlarges, the quantities of substances in the perivitelline space also increase. Therefore, it has been suggested that these increased substances may physically obstruct the movement and attachment of sperm to the plasma membrane of oocytes [13, 14]. Moreover, hyaluronan has been reported to have an inhibitory effect on membrane fusion [19, 20]. Therefore, it is considered that large amounts of hyaluronan in larger perivitelline spaces may also prevent membrane fusion of sperm and oocytes, resulting in lower incidences of polyspermy in oocytes with larger perivitelline spaces.

References

1) Funahashi, H., Cantley, T.C., Stumpf, T.T., Terlouw, S.L. and Day, B.N. (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.
2) Kitagawa, T. and Niimura, S. (2006): Relationship between the size of perivitelline space and the incidence of polyspermy in porcine oocytes. Bull. Facul. Agric. Niigata Univ., 59, 21–26.
3) Wang, W.H., Abeydeera, L.R., Prather, R.S. and Day, B.N. (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.
4) Okada, A., Inomata, K. and Nagae, T. (1993): Spontaneous cortical granule release and alteration of zona pellucida properties during and after meiotic maturation of mouse oocytes. Anat. Rec., 237, 518–526.
5) Kaufman, M.H., Fowler, R.E., Barratt, E. and McDougall, R.D. (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.
6) Toyoda, Y., Yokoyama, M. and Hoshi, T. (1971): Studies on the fertilization of mouse eggs *in vitro*. I. *In vitro* fertilization of eggs by fresh epididymal sperm. Jap. J. Anim. Reprod., 16, 147–151.
7) Chung, S.O. (1973): Volume changes during the preimplantation stages of mouse egg development. Yonsei Med. J., 14, 63–89.
8) Buhi, W.C., Alvarez, I.M. and Kouba, A.J. (2000): Secreted proteins of the oviduct. Cells Tissues Organs, 166, 165–179.
9) Buhi, W.C. (2002): Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein. Reproduction, 123, 355–362.
10) Miao, Y., Ma, S., Liu, X., Miao, D., Chang, Z., Luo, M. and Tan, J. (2004): Fate of the first polar bodies in mouse oocytes. Mol. Reprod. Dev., 69, 66–76.
11) Sorenson, R.A. (1973): Cinemicrography of mouse oocyte maturation utilizing Nomarski differential-interference microscopy. Am. J. Anat., 136, 265–276.
12) Talbot, P. and Dicarlantonio, G. (1984): Ultrastructure of opossum oocyte investing coats and their sensitivity to trypsin and hyaluronidase. Dev. Biol., 103, 159–167.
13) Talbot, P. and Dandekar, P. (2003): Perivitelline space: does it play a role in blocking polyspermy in mammals? Microsc. Res. Tech., 61, 349–357.
14) Dandekar, P. and Talbot, P. (1992): Perivitelline space of mammalian oocytes: extracellular matrix of unfertilized oocytes and formation of a cortical granule envelope following fertilization. Mol. Reprod. Dev., 31, 135–143.
15) Okada, A., Yanagimachi, R. and Yanagimachi, H. (1986): Development of a cortical granule-free area of cortex and the perivitelline space in the hamster oocyte during maturation and following ovulation. J. Submicrosc. Cytol., 18, 223–247.
16) Kim, H., Kim, H., Kim, S., Kim, M. and Schuetz, A.W. (1996): Oviductal protein produces fluorescence staining of the perivitelline space in mouse oocytes. J. Exp. Zool., 274, 351–357.
17) Kim, H. and Schuetz, A.W. (1993): Structural and functional differentiation of follicular and oviductal mouse oocytes visualized with FITC-protein conjugates. Zygote, 1, 297–307.
18) Hoodbhoy, T., Dandekar, P., Calarco, P. and Talbot, P. (2001): p62/p56 are cortical granule proteins that contribute to formation of the cortical granule envelope and play a role in mammalian preimplatation development. Mol. Reprod. Dev., 59, 78–89.
19) Orkin, R.W., Khudson, W. and Toole, B.P. (1985): Loss of hyaluronate dependent coat during myoblast fusion. Dev. Biol., 107, 527–530.
20) Kujawa, M.J. and Tepperman, K. (1983): Culturing chick muscle cells on glycosaminoglycan substrates: attachment and differentiation. Dev. Biol., 99, 277–286.