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—Full Paper—

Amount of Hyaluronan Produced by Mouse Oocytes and Role of Hyaluronan in Enlargement of the Perivitelline Space

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Abstract. Production of hyaluronan (hyaluronic acid: HA) was demonstrated in denuded mouse oocytes (DOs) by the enzyme-linked immunosorbent assay, and the role of HA in enlargement of the perivitelline space in the oocytes was examined. The incidence of polyspermy following insemination was also observed in DOs in which HA synthesis was inhibited. HA was not detected in culture medium containing DOs immediately after collection. After culture for 7 h, 4.75 pg of HA per DO was detected in the medium, and the mean amount of HA significantly increased to 20.78 pg 14 h after culture. When DOs were cultured in medium containing 0.25 mM 4-methylumbelliferone (MU), an inhibitor of HA synthase, the mean amount of HA in the culture medium with DOs was 8.61 pg, which was significantly smaller than the amount in the control medium with non-treated DOs (21.59 pg). The mean size of the perivitelline space in oocytes cultured with cumulus cells (5.40 μm) did not differ from that (5.08 μm) of DOs. The mean size of the perivitelline space was significantly smaller in the MU-treated DOs (3.58 μm) than in the control DOs (4.65 μm). The fertilization rate did not differ between the MU-treated DOs (84.9%) and control DOs (81.0%), whereas the incidence of polyspermy was significantly higher in the MU-treated DOs (13.3%) compared with the control DOs (2.1%). These findings clarified that the HA involved in enlargement of the perivitelline space in oocytes is synthesized and secreted by the oocytes themselves. They also suggest that there is a close relationship between the size of perivitelline space and the incidence of polyspermy in mouse oocytes.

Key words: Enzyme-linked immunosorbent assay, Hyaluronan production, Incidence of polyspermy, Mouse oocyte, Size of perivitelline space

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The perivitelline space is the gap between the plasma membrane and zona pellucida in oocytes. Recently, it has been reported that the incidence of polyspermy in porcine and mouse oocytes with larger perivitelline space is significantly lower than that in oocytes with a smaller perivitelline space [1–4]. Therefore, it has been suggested that there could be a relationship between the size of the perivitelline space and the incidence of polyspermy in porcine and mouse oocytes.

It is known that glycosaminoglycans including hyaluronan (hyaluronic acid: HA) synthesized in cumulus cells by the stimulation of FSH and LH are secreted [5, 6] and that the extracellular matrix in cumulus cells is occupied by the secreted HA [7–10]. HA has been found to also exist in the perivitelline space [11–14], and one of its properties is retention of large volumes of water [14]. Therefore, when HA accumulates in the perivitelline space, its role is to absorb water, which causes the perivitelline space to enlarge.

On the other hand, with regard to the secretory source of HA in the perivitelline space, Salustri *et al*. [15] used [^{3}H] glucosamine as a metabolic precursor to examine the production of HA in the mouse cumulus-oocyte complex (COC), cumulus cells and oocytes after 18 h of culture and reported that HA was detected in the medium with COCs or cumulus cells, but not in the medium with oocytes. However, when stimulation of LH occurs, glycosaminoglycans including HA are secreted not only from cumulus cells but also from oocytes [16–19]. In addition, the mRNA of *HAS3*, which is an HA synthase, is present in porcine oocytes [20]. Moreover, when ultrathin sections of hamster cumulus-oocyte complex were treated with immunogold-labeled hyaluronidase, deposition of gold particles was observed in cumulus cells, the perivitelline space and between cumulus cells, but not in the zona pellucida [21]. From these findings, we considered that HA in the matrix of cumulus cells derives from the cumulus cells and that HA in the perivitelline space derives from oocytes. Since synthesis and secretion of HA by oocytes has only been examined by Salustri *et al*. [15], we considered it still too early to conclude that oocytes are not involved in the production and secretion of HA.

In the present study, the amount of HA in a medium containing mouse oocytes cultured without cumulus cells (DOs) was examined using an enzyme-linked immunosorbent assay (ELISA), a different method from that used by Salustri *et al*. [15]. The size of the perivitelline space was measured in the cultured DOs and compared with that in oocytes cultured with cumulus cells. In addition, the changes in size of the perivitelline spaces of DOs cultured in a medium containing 4-methylumbelliferone (MU), an inhibitor of HA synthase [22, 23], were observed to examine whether oocytes are involved in the synthesis and secretion of HA and to examine the role of HA secreted by oocytes in enlargement of the perivitelline space. The incidence of polyspermy following insemination in DOs treated with MU was also observed in order to examine the relationship between the incidence of polyspermy and the size of the perivitelline space.

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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 given a standard chow (MF; Oriental Yeast, 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, Tokyo, Japan).

Collection of oocytes

COCs were collected from antral follicles 48 h after the PMSG injection. Cumulus cells were dispersed from COCs by pipetting in TYH medium [24] containing 0.1% hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) except for some COCs.

Demonstration of HA in a medium with denuded oocytes

Seventy to 80 DOs were cultured in 60 μl TYH medium containing 5% fetal bovine serum (FCS; Gibco BRL, Grand Island, NY, USA) and 10 IU/ml PMSG (PEAMEX®) at 37 C in a CO_2 incubator (5% CO_2 in air).

At 0, 7 and 14 h after culture, culture medium containing DOs was stored at –20 C until demonstration of HA. In order to demonstrate HA, the ELISA used by Kongtawelert and Ghosh [25] was employed. Activated polyvinyl chloride immunoassay plates (Nunc, Roskilde, Denmark) were precoated with poly-L-lysine (Sigma-Aldrich) by addition of 100 μl/well containing a solution of 50 μg/ml distilled water. After incubation for 1 h at 37 C, the solution was flicked out, the plates were air-dried and then 100 μl/well of bovine vitreous humor HA (MP Biomedicals, Cleveland, OH, USA; 25 μg/ml), which had been dissolved in PBS [26], was added to the precoated plates. The plates were then incubated overnight at room temperature and washed three times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween 20), and 100 μl PBS containing 1% (w/w) BSA (Sigma-Aldrich) was added to each well. The plates were further incubated at 37 C for 1 h, washed five times with PBS-Tween 20 and then air-dried. The coated plates were wrapped in polyethylene film and stored at 4 C until demonstration of HA.

The frozen media containing DOs cultured for various periods were thawed and centrifuged at 1500 rpm (× 176 g) for 10 min. The supernatants were collected and diluted twofold with TYH medium. Sixty μl of these diluted samples or 60 μl of TYH medium containing HA at 0, 0.195, 0.39, 0.78, 1.56, 3.12, 6.24 or 12.48 ng/ml (standard solution) was pipetted into small tubes with 60 μl of biotinylated HA binding protein (Seikagaku, Tokyo, Japan; 0.175 μg/ml distilled water) and incubated at 37 C for 90 min. One hundred μl of this reaction mixture was then applied to HA-coated and BSA-blocked plates and incubated at 37 C for 1 h. The plates were washed five times with PBS-Tween 20, and 100 μl of the appropriate dilution (1:5,000) of alkaline phosphatase-conjugated avidin (Sigma-Aldrich) was added to each well. After incubation for 1 h at 37 C, the plates were washed five times with PBS-Tween 20 and air-dried. One hundred μl of alkaline phosphatase substrate (p-nitrophenyl phosphate; Sigma-Aldrich) was added, and the plates were incubated at 37 C for 15 min. The reaction was stopped by addition of 80 μl of 0.2 M NaOH. The absorbance at 405 nm was determined using a microplate reader (BIO-RAD, CA, USA).

A standard curve for HA was constructed from the values for standard solution, and the amount of HA in the medium was determined by applying the value of the medium to the standard curve. For this assay, triplicate results were averaged.

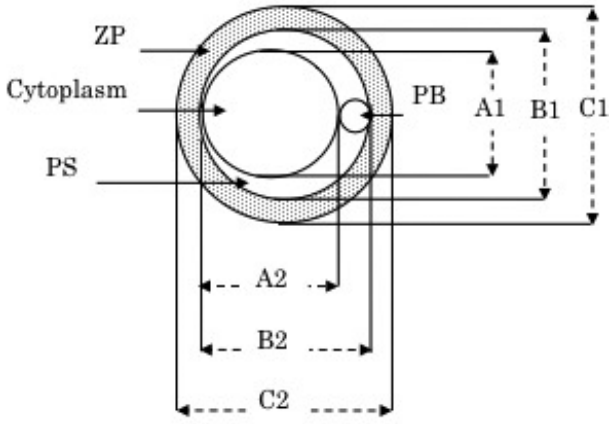

Fig. 1. Calculation method for the size of each part of the oocyte. ZP: Zona pellucida. PS: perivitelline space. PB: 1st polar body. Diameter of cytoplasm (A) = (A1+A2) / 2. Inner diameter of zona pellucida (B) = (B1+B2) / 2. Outer diameter of zona pellucida (C) = (C1+C2) / 2. Thickness of zona pellucida = (C–B) / 2. Size of perivitelline space = (B–A) / 2

Observation of maturation and size of the perivitelline space in cultured oocytes with and without cumulus cells

COCs and DOs were respectively cultured for 14 h in TYH medium containing 5% fetal bovine serum (FCS; Gibco BRL) and 10 IU/ml PMSG (PEAMEX®) at 37 C in a CO_2 incubator. In regard to COCs, cumulus cells were dispersed from COCs by pipetting in culture medium containing 0.1% hyaluronidase (Sigma-Aldrich) after culture. The number of oocytes with a 1st polar body in the perivitelline space (maturation rate) was examined. On the other hand, only oocytes with a 1st polar body in the perivitelline space were selected, and the size of each part of the oocytes was measured using a micrometer; the size of the perivitelline space was calculated according to the method described in Fig. 1.

Observation of maturation and size of the perivitelline space in cultured denuded oocytes treated with 4-methylumbelliferone

In order to observe the size of the perivitelline space in DOs in which hyaluronan synthesis was inhibited, the minimum concentration of MU at which MU had no effect on the maturation of DOs, but significantly decreased the size of the perivitelline space in the resultant matured oocytes, was determined. DOs were cultured for

14 h in culture medium containing 0.1, 0.2, 0.25, 0.3 or 0.4 mM MU (Wako Pure Chemical Industries, Osaka, Japan) at 37 C in a CO_2 incubator. The MU had previously been dissolved in dimethyl sulfoxide (DMSO) and then diluted with the culture medium. The concentration of DMSO in the culture medium was adjusted to 0.1% (v/v), and DOs cultured for 14 h in the medium containing 0.1% DMSO were used as vehicle controls. After culture, the maturation and size of the perivitelline space were observed.

Demonstration of HA in the medium with denuded oocytes treated with MU

The amount of HA was demonstrated in the medium containing DOs in which HA synthesis was inhibited to clarify the ability to synthesize and secret HA in oocytes. A group of 70 to 80 DOs was cultured for 14 h in 60 μl culture medium containing 0.25 mM MU (Wako Pure Chemical Industries) at 37 C in a CO_2 incubator. The concentration of DMSO in the culture medium was adjusted to 0.1% (v/v), and the medium containing DOs cultured for 14 h with DMSO at 0.1% was used as the control. After culture, the medium containing the DOs was stored at –20 C until analysis. The amount of HA in the medium with non-treated or MU-treated DOs was determined using the ELISA method mentioned above. For this assay, triplicate results were averaged.

Observation of the incidence of polyspermy in denuded oocytes treated with MU

In order to observe the incidence of polyspermy in DOs in which HA synthesis was inhibited, DOs were cultured in medium containing 0.25 mM MU for 14 h at 37 C in a CO_2 incubator. After culture, only DOs with a 1st polar body in the perivitelline space were selected and inseminated. A sperm suspension was prepared by minutely cutting the caudal epididymides of mature males in the culture medium for 1 h at 37 C in a CO_2 incubator. A small volume of the sperm suspension was introduced into 100 μl droplets of culture medium so that the final concentration of spermatozoa was adjusted to 2×10^6 /ml. The DOs were introduced into the droplets of sperm suspension and cultured for 12 h at 37 C in a CO_2 incubator. Inseminated DOs were observed under a phase contrast microscope. The DOs containing 2 or more pronuclei in their cytoplasm were judged to be fertilized, and those containing 3 or more pronuclei were judged to be polyspermic.

Statistical analysis

The size of each part including the perivitelline space was statistically analyzed by one-way analysis of variance (ANOVA). The rate of maturation in cultured oocytes, and the rates of fertilization and polyspermy in oocytes following insemination were statistically analyzed by Chi-square test. The amount of HA was statistically analyzed by Student's *t*-test.

Results

Amount of HA in the medium with denuded oocytes

As shown in Table 1, HA was not detected in the culture medium containing DOs immediately after collection. On the other hand, 4.75 pg of HA per DO was detected in the medium after 7 h of culture, and the mean amount of HA significantly increased to 20.78 pg per DO at 14 h after culture.

Table 1. Amount of hyaluronan in the medium with denuded mouse oocytes

Hours of culture	pg/oocyte
0	ND*
7	4.75 ± 0.63**[b]
14	20.78 ± 3.34[a]

*Not detectable. **Values are expressed as means ± S.E. of three independent assays. Values with different superscripts in the same column are significantly different ($P<0.05$).

Maturation and size of the perivitelline space in oocytes cultured with and without cumulus cells

Of the oocytes cultured with and without cumulus cells for 14 h, 86.1% (31/36) and 85.7% (30/35) held 1st polar bodies in their perivitelline spaces, respectively (Table 2). The percentages of oocytes with 1st polar bodies in the perivitelline space (maturation rates) did not differ between those cultured with and without cumulus cells. The mean size of the perivitelline space in oocytes cultured with cumulus cells was 5.40 μm, showing no significant difference from the mean size, 5.08 μm, in the DOs.

Maturation and size of the perivitelline space in denuded oocytes treated with MU

When DOs were cultured in the medium containing 0.1, 0.2, 0.25, 0.3 or 0.4 mM MU, 81.3 to 89.5% of the DOs held 1st polar bodies in the perivitelline space, showing no difference from the percentage (85.7%) of the control DOs cultured in a medium without MU (Table 3). The mean sizes of the perivitelline spaces in the DOs cultured in the medium containing 0.1 or 0.2 mM MU were 4.39 and 4.17 μm, respectively, showing no difference from the size (4.65 μm) of the control DOs. On the other hand, the sizes of the perivitelline spaces in the DOs cultured in the medium containing 0.25, 0.3 or 0.4 mM MU were 3.58 (Fig. 2a), 3.99 and 3.88 μm, respectively, which were all significantly smaller than that in the control DOs (Fig. 2b). In view of these results, the medium containing 0.25 mM MU was used in subsequent experiments because MU did not affect maturation and had a significant effect on the size of the perivitelline space at this concentration.

Amount of HA in the medium with denuded oocytes treated with MU

The mean amount of HA per DO in the medium containing MU-treated DOs (8.61 ± 1.80 pg) was significantly smaller than the 21.59 ± 3.19 pg in the control medium with non-treated DOs.

Incidence of polyspermy in denuded oocytes treated with MU

The fertilization rate was 84.9% (45/53) for the DOs (Fig. 3a) cultured in the medium containing 0.25 mM MU, showing no significant difference from the 81.0% (47/58) fertilization rate of the control DOs (Fig. 3b) cultured in the medium without MU. However, the incidence of polyspermy in the MU-treated DOs (Fig. 3a)

Table 2. Maturation and size of each part in mouse oocytes cultured with and without cumulus cells

Oocytes	No. of oocytes cultured	No. (%) of oocytes matured	Inner diameter of zona pellucida (μm)	Diameter of cytoplasm (μm)	Thickness of zona pellucida (μm)	Size of perivitelline space (μm)
With cumulus cells	36	31 (86.1)[a]	76.10 ± 0.60*[a]	65.10 ± 0.37[a]	8.58 ± 0.21[a]	5.40 ± 0.12[a]
Without cumulus cells	35	30 (85.7)[a]	73.88 ± 0.51[b]	63.72 ± 0.54[b]	9.05 ± 0.17[a]	5.08 ± 0.17[a]

The oocytes were observed after 14 h of culture. *Mean ± S.E. Values with different superscripts in the same column are significantly different (P<0.05).

Table 3. Effects of 4-methylumbelliferone on maturation and size of each part in denuded mouse oocytes

Concentrations of MU (mM)	No. of oocytes cultured	No. (%) of oocytes matured	Inner diameter of zona pellucida (μm)	Diameter of cytoplasm (μm)	Thickness of zona pellucida (μm)	Size of perivitelline space (μm)
0	35	30 (85.7)[a]	74.89 ± 0.49*[a,b]	65.64 ± 0.31[b]	8.63 ± 0.15[a]	4.65 ± 0.2[a]
0.1	43	35 (81.3)[a]	73.88 ± 0.41[a,b]	65.74 ± 0.32[b]	9.18 ± 0.20[a]	4.39 ± 0.2[a,b]
0.2	39	34 (87.1)[a]	73.52 ± 0.37[b]	65.12 ± 0.32[b]	8.88 ± 0.16[a]	4.17 ± 0.1[a,b]
0.25	34	30 (88.2)[a]	72.83 ± 0.45[b]	65.12 ± 0.38[b]	8.71 ± 0.16[a]	3.58 ± 0.2[b]
0.3	38	34 (89.5)[a]	75.22 ± 0.35[a]	67.17 ± 0.31[a]	8.80 ± 0.19[a]	3.99 ± 0.1[b]
0.4	28	23 (82.2)[a]	75.59 ± 0.48[a]	67.83 ± 0.36[a]	8.73 ± 0.21[a]	3.88 ± 0.2[b]

The oocytes were observed after 14 h of culture. *Mean ± S.E. MU: 4-methylumbelliferone. Values with different superscripts in the same column are significantly different (P<0.05).

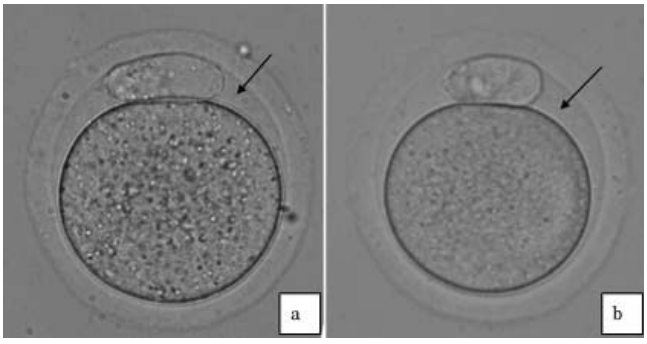

Fig. 2. Mouse oocytes cultured for 14 h in the medium with (a) and without (b) 4-methylumbelliferone. The perivitelline space (arrow) is smaller in the 4-methylumbelliferone-treated oocyte (a) than in the control oocyte (b).

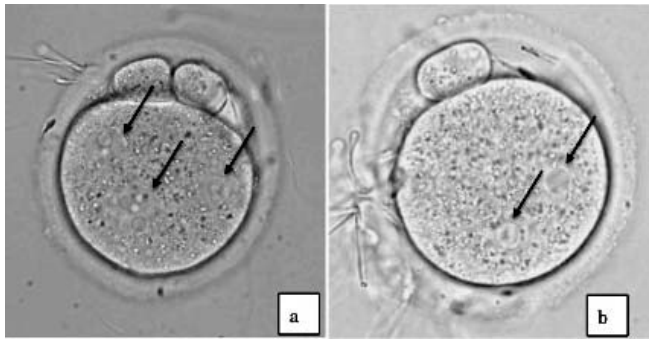

Fig. 3. Fertilized mouse oocytes matured in the medium with (a) and without (b) 4-methylumbelliferone. a: A polyspermic oocyte with three pronuclei (arrows) in the cytoplasm. b: A monospermic oocyte with two pronuclei (arrows) in the cytoplasm.

was 13.3% (6/45), which was significantly higher than the 2.1% (1/47) incidence in the control DOs.

Discussion

In the present investigation, HA was not detected in the medium with DOs immediately after collection from antral follicles but was detected from the medium with DOs after culture. It was also confirmed that the amount of HA increased as the time of culture was prolonged and that the amount of HA was significantly smaller in the medium with MU-treated DOs in which HA synthesis was inhibited than in the control medium with non-treated DOs. From these results, we surmised that oocytes themselves synthesize and secrete HA and that the amount of secreted HA increases as oocyte maturation progresses.

The results of the present study on HA detection are different from those of Salustri *et al.* [15]. Such a discrepancy in the results may be due to the method used for HA detection. We believe that the sensitivity of the ELISA method used in the present study is higher than that of the method used by Salustri *et al*. [15].

In the present investigation, there was no difference in the size of the perivitelline space in the oocytes matured with or without cumulus cells. We also attempted to examine the changes in the size of the perivitelline space in DOs in which HA synthesis was inhibited in order to examine whether oocytes are involved in the synthesis and secretion of HA, which plays a role in enlargement of

the perivitelline space. The results showed that the perivitelline space in the MU-treated DOs was significantly smaller than that of the control DOs.

The mRNA of *HAS2*, an HA synthase, has been demonstrated in the cumulus cells of the mouse, pig and cattle [20, 27–29]. Compared with *HAS3*, which may be present in porcine oocytes [20], *HAS2* synthesizes HA that has a longer molecular mass [30]. Furthermore, HA is known to bind with CD44, an HA receptor present on the surfaces of cumulus cells in preovulatory porcine, bovine and human antral follicles [28, 31–35], after which the HA binds with proteoglycans and forms the extracellular matrix of the cumulus cell [36]. This matrix has been confirmed electron microscopically to have a structure with a large amount of fibrous materials [37]. The HA secreted from cumulus cells therefore not only forms a network through self-binding, but also binds to the HA receptor to form glycocalyx on the cumulus cell surface [38]. These facts lead us to believe that the HA synthesized and secreted by *HAS2* in cumulus cells is less likely to be able to pass through the zona pellucida. These findings [20, 27–38], together with the results of the present study with regard to HA detection and the size of the perivitelline space in DOs after MU treatment, strongly suggest that the HA involved in enlargement of the perivitelline space in oocytes is not synthesized and secreted by cumulus cells but by the oocytes themselves.

Recently, it has been suggested that there could be a relationship between the size of the perivitelline space and the incidence of polyspermy in porcine and mouse oocytes [1–4]. In the present investigation, it was confirmed that the size of the perivitelline space in mouse DOs treated with MU was smaller and that the incidence of polyspermy in those after insemination was higher than those in the control DOs. These results suggest that there is a close relationship between the size of the perivitelline space and the incidence of polyspermy in mouse oocytes.

The reason for the high incidence of polyspermy after insemination in oocytes with small perivitelline spaces is unclear. On the other hand, it is known that proteins and HA are present in perivitelline spaces of oocytes before ovulation [11–14, 39] and that secretions from the oviducts [40–44] are also present after ovulation. When the perivitelline space decreases in zize, the quantities of substances in the perivitelline space also decrease. Therefore, it is suggested that these decreased substances may not obstruct physically the movement and attachment of sperm to the plasma membrane in oocytes [12, 14]. Moreover, HA has been reported to have an inhibitory effect on membrane fusion [45–47]. Therefore, it is considered that small amounts of HA in smaller perivitelline spaces may not prevent membrane fusion of sperm and oocytes, resulting in the higher incidence of polyspermy in oocytes with a smaller perivitelline space.

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