Comparison of the Size of Perivitelline Space in Mouse Oocytes Cultured with and without Cumulus Cells and Possible Hyaluronan Production in Oocytes

Sayaka UENO[1] and Sueo NIIMURA[2]*

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Summary

The size of perivitelline space was measured in mouse oocytes cultured without cumulus cells (DOs) and compared with that in oocytes cultured with cumulus cells. In addition, the size of perivitelline space of DOs cultured in the medium containing 4-methylumbelliferone (MU), an inhibitor of hyaluronan synthase, was observed to examine whether oocytes are involved in the synthesis and secretion of hyaluronan which plays a role in the enlargement of perivitelline space.

Of oocytes cultured with and without cumulus cells for 14 hrs, 96.7 % (29/30) and 93.5 % (29/31) held 1st polar bodies in their perivitelline space, respectively. 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 perivitelline space in oocytes cultured with cumulus cells (5.40 μm) did not differ from that (5.08 μm) of DOs. When DOs were cultured in the medium containing 0.25 mM MU, 88.2 % of the oocytes held 1st polar bodies in the perivitelline space, showing no difference from the percentage (85.7 %) of control DOs cultured in the medium without MU. The mean size of perivitelline space in DOs treated with 0.25 mM MU was 3.58 μm, which was significantly smaller than 4.65 μm in the control DOs. When DOs were cultured in the media containing 0.25 mM MU and hyaluronan at 0.05, 0.25 or 0.50 mg/ml, 86.9 to 92.8 % of the DOs held 1st polar bodies in the perivitelline space, showing no difference from the percentage (88.2 %) of the DOs cultured in the medium with only 0.25 mM MU. The mean sizes of perivitelline space in DOs cultured in the media containing 0.25 mM MU and hyaluronan at 0.05 or 0.50 mg/ml were 4.24 and 4.29 μm, respectively, showing no difference from the size (3.85 μm) of the DOs cultured in the medium containing only MU. However, the mean size of perivitelline space in DOs cultured in the medium containing 0.25 mM MU and 0.25 mg/ml hyaluronan was 4.67 μm, which was significantly larger than that of the DOs cultured in the medium containing only MU.

These findings strongly suggested that hyaluronan involved in the enlargement of perivitelline space of oocytes is synthesized and secreted by the oocytes themselves.

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Key words : denuded oocyte, hyaluronan production, mouse, oocyte with cumulus cell, size of perivitelline space

Perivitelline space is the gap between the plasma membrane and zona pellucida of oocytes. Recently, it has been reported that the incidence of polyspermy in porcine and mouse oocytes with larger perivitelline space is significantly lower than those with small perivitelline space (Funahashi *et al*., 1994; Wang *et al*., 1998; Kitagawa and Niimura, 2006; Ueno and Niimura, 2008). Therefore, it has been suggested that there could be a relationship between the size of perivitelline space and the incidence of polyspermy in porcine and mouse oocytes.

It is known that glycosaminoglycans including hyaluronan synthesized in cumulus cells by the stimulation of FSH and LH are secreted (Eppig, 1982; Ball *et al*., 1985) and the matrix of cumulus cells is occupied by the secreted hyaluronan (Eppig, 1979; 1980; Ball *et al*., 1982; Salustri *et al*., 1992). Hyaluronan has been found to also exist in the perivitelline space (Talbot and Dicarlantonio, 1984a; Dandekar and Talbot, 1992; Dandekar *et al*., 1992; Talbot and Dandekar, 2003), and one of its properties is retention of large volumes of water (Talbot and Dandekar, 2003). Therefore, when hyaluronan accumulates in the perivitelline space, its role is to absorb water causing the perivitelline space to enlarge. On the other hand, with regard to the secretory source of hyaluronan in the perivitelline space, it has been confirmed by electron microscopy that hyaluronan exists in the pore of the zona pellucida (Talbot and Dicarlantonio, 1984b). From these results, it was suggested that hyaluronan secreted from cumulus cells enters the perivitelline space through the zona pellucida (Talbot,1984). However, when the stimulation of FSH and LH occurs, glycosaminoglycans including hyaluronan are secreted not only from cumulus cells but also from oocytes (Pivko *et al*., 1982; Tesarik and Kopecny, 1986; Fléchon *et al*., 2003). In addition the mRNA of *HAS3*, which is a hyaluronan synthase, is present in porcine oocytes (Kimura *et al*., 2002). Moreover, when ultra thin sections of hamster cumulus-oocyte complex were stained with immunogold-labeled hyaluronidase, deposition of gold particles was observed in cumulus cells, perivitelline space and the matrix

[1] Graduate School of Science and Technology, Niigata University

[2] Faculty of Agriculture, Niigata University

* Corresponding author: niimura@agr.niigata-u.ac.jp

of cumulus cells, but not in the zona pellucida (Kan, 1990). From these findings, Kan (1990) suggested that hyaluronan in the matrix of cumulus cells derives from cumulus cells and that in perivitelline space derives from oocytes. However, there have been no reports confirming that oocytes synthesize and secrete hyaluronan. Moreover, the source of hyaluronan that plays a role in the enlargement of the perivitelline space of oocyte is unknown.

In the present study, the size of the perivitelline space was measured in mouse oocytes cultured without cumulus cells (DOs) and compared with that in oocytes cultured with cumulus cells. In addition, the size of perivitelline space of DOs cultured in the medium containing 4-methylumbelliferone (MU), an inhibitor of hyaluronan synthase (Nakamura *et al*., 1995; Itano, 2004), was observed to examine whether oocytes are involved in the synthesis and secretion of hyaluronan which plays a role in the enlargement of perivitelline space.

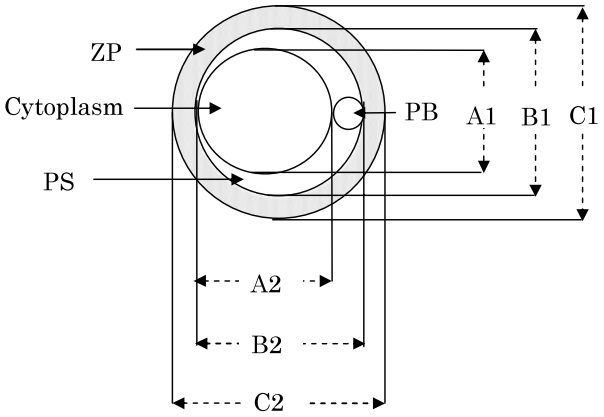
MATERIALS AND METHODS

Animals

Ninety female mature mice of 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 ℃), 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).

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

In order to observe the maturation and the size of perivitelline space in oocytes cultured with and without cumulus cells, immature oocytes covered with cumulus cells (COCs) were collected from antral follicles 48 hrs after the PMSG injection. Cumulus cells were dispersed from about half COCs by pipetting in TYH medium (Toyoda *et al*., 1971) containing 0.1 % hyaluronidase (Sigma-Aldrich, MO, USA), and COCs and denuded oocytes (DOs) were respectively cultured for 14 hrs in TYH medium containing 5% fetal bovine serum (FCS; Gibco BRL, NY, USA) and 10 IU/ml PMSG (PEAMEX®) at 37 ℃ in a CO_2 incubator (5 % CO_2 in air). After culture, cumulus cells were dispersed from COCs by pipetting in culture medium containing 0.1 % hyaluronidase (Sigma-Aldrich). The number of oocytes with 1st polar body in perivitelline space (maturation rate) was examined. On the other hand, only oocytes with 1st polar body in perivitelline space were selected, and 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**.



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 in oocyte.

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

In order to observe the size of perivitelline space in DOs in which hyaluronan synthesis was inhibited, DOs were cultured for 14 hrs in culture medium containing MU (Wako Pure Chemical Industries, Ltd., Osaka, Japan) at 0.25 mM (Niimura, 2008) at 37 ℃ in a CO_2 incubator (5 % CO_2 in air). The MU had previously 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 hrs in the medium containing DMSO at 0.1 % were used as vehicle controls. After culture, the maturation and the size of perivitelline space were observed by the above mentioned method.

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

In order to observe the effect of hyaluronan on the size of perivitelline space in DOs in which hyaluronan synthesis was inhibited, DOs were cultured for 14 hrs in the culture medium containing 0.25 mM MU (Wako Pure Chemical Industries, Ltd.) and hyaluronan (MP Biomedicals, Inc., OH, USA) at 0.00, 0.05, 0.25 or 0.50 mg/ml at 37 ℃ in a CO_2 incubator. The hyaluronan was directly dissolved in the culture medium. After culture, the maturation and the size of perivitelline space were observed by the above mentioned method.

Statistical analysis

The size of perivitelline space was statistically analyzed by One-way analysis of variance (ANOVA). The rate of maturation in cultured oocytes was statistically analyzed by

Chi-square test.

RESULTS

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

Of oocytes cultured with and without cumulus cells for 14 hrs, 96.7 % (29/30) and 93.5 % (29/31) held 1st polar bodies in their perivitelline space, respectively (**Table 1**). 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 perivitelline space in oocytes cultured with cumulus cells was 5.40 μm, showing no significant difference from 5.08 μm in DOs.

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

When DOs were cultured in the medium containing 0.25 mM MU, 88.2 % of the DOs held 1st polar bodies in perivitelline space, showing no difference from the percentage (85.7 %) of control DOs cultured in a medium without MU (**Table 2**). The mean size of perivitelline space in DOs cultured in the medium containing 0.25 mM MU was 3.58 μm, which was significantly smaller than 4.65 μm in the control DOs.

The maturation and the size of perivitelline space in denuded oocytes treated with MU and hyaluronan

When DOs were cultured in the medium containing 0.25 mM MU and hyaluronan at 0.05, 0.25 or 0.50 mg/ml, 86.9 to 92.8 % of the DOs held 1st polar bodies in perivitelline space, showing no difference from the percentage (88.2 %) of DOs cultured in the medium with only 0.25 mM MU (**Table 3**).

The mean sizes of perivitelline space in DOs cultured in the medium containing 0.25 mM MU and hyaluronan at 0.05 or 0.50 mg/ml were 4.24 and 4.29 μm, respectively, showing no difference from the size (3.85 μm) of DOs cultured in the medium containing only MU. However, the mean size of perivitelline space in DOs cultured in the medium containing 0.25 mM MU and 0.25 mg/ml hyaluronan was 4.67 μm, which was significantly larger than that of DOs cultured in the medium containing only MU.

DISCUSSION

It is known that glycosaminoglycans are present in the perivitelline space of oocytes before ovulation (Pivko *et al*., 1982; Stastna and Dvork, 1983; Tesarik and Kopecny, 1986; Hassan-Ali *et al*., 1998; Fléchon *et al*., 2003), and secretions from the oviducts (Kapur and Johnson, 1985; 1986; Buhi *et al*., 1993; 1997; 2000) are also present in the perivitelline space of oocytes after ovulation. Of these substances in perivitelline space of oocytes, hyaluronan, which has the property of retaining large volumes of water, is considered to play a role in the enlargement of perivitelline space (Talbot and

Table 1. The maturation and the size of perivitelline space in mouse oocytes cultured with and without cumulus cells

Oocytes	No. of oocytes cultured	No. and (%) of oocytes matured	Size of perivitelline space (μm)
With cumulus cells	30	29(96.7)[a]	5.40 ± 0.12* [a]
Without cumulus cells	31	29(93.5)[a]	5.08 ± 0.17[a]

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

Table 2. The maturation and the size of perivitelline space in denuded mouse oocytes cultured with 4-methylumbelliferone

Concentrations of MU (mM)	No. of oocytes cultured	No. and (%) of oocytes matured	Size of perivitelline space (μm)
0	35	30(85.7)[a]	4.65 ± 0.2* [a]
0.25	34	30(88.2)[a]	3.58 ± 0.2[b]

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

Table 3. The maturation and the size of perivitelline space in denuded mouse oocytes cultured with 0.25 mM 4-methylumbelliferone and various concentrations of hyaluronan

Concentrations of HA (mg/ml)	No. of oocytes cultured	No. and (%) of oocytes matured	Size of perivitelline space (μm)
0	34	30(88.2)[a]	3.85 ± 0.2[b]
0.05	45	39(86.9)[a]	4.24 ± 0.2[a,b]
0.25	44	39(88.6)[a]	4.67 ± 0.2[a]
0.50	42	39(92.8)[a]	4.29 ± 0.2[a,b]

The oocytes were observed after 14 hrs of culture.
* Mean ± S.E.
HA: hyaluronan.
Values with different superscripts in the same column are significantly different ($p<0.05$).

Dandekar, 2003). This hyaluronan was generally considered to be synthesized and secreted from cumulus cells after the stimulation of FSH or LH (Eppig, 1979; 1980; Ball *et al*., 1982; Salustri *et al*., 1992), and the hyaluronan accumulated between the cumulus cells and subsequently entered the perivitelline space through the zona pellucida (Talbot, 1984).

In the present study, the size of perivitelline space of matured DOs was measured and compared with that of oocytes cultured with cumulus cells. As a result, there was no difference in the size of the perivitelline space of both the oocytes matured with and without cumulus cells. This suggested that hyaluronan, which plays a role in enlarging perivitelline space, is secreted from the oocytes themselves. We also attempted to examine the size of the perivitelline space of DOs treated with MU, an inhibitor of hyaluronan synthase, in order to determine the ability of hyaluronan synthesis in oocytes. As a result, the size of perivitelline space of DOs following treatment with 0.25mM MU was significantly smaller than that of the control DOs. In addition, it was confirmed that there is no difference in the maturation rate between MU-treated DOs and control DOs. Furthermore, the perivitelline space of DOs treated with 0.25 mM MU and 0.25mg/ml hyaluronan was significantly large compared to that of DOs treated with only MU. Small sized perivitelline space of MU-treated DOs did not enlarge when DOs were cultured with MU and hyaluronan at a concentration of 0.5mg/ml. Although the reason for that is unclear, it has been reported that viscosity of hyaluronan rises with increase in concentration, and a decrease in the self diffusion coefficient (Gribbon *et al*., 2000; Cowman and Matsuoka, 2005) and self-association occurs (Turner *et al*., 1988). Therefore, since the concentration of 0.5mg/ml was too high, hyaluronan could not enter perivitelline space through the zona pellucida.

The mRNA of *HAS2*, a hyaluronan synthase, has been demonstrated in cumulus cells of the mouse, pig and cattle (Fulop *et al*., 1997; Kimura *et al*., 2002; Schoenfelder and Einspanier, 2003; Russell and Salustri, 2006). Compared to *HAS3* which may be present in porcine oocytes (Kimura *et al*., 2002), *HAS2* synthesizes hyaluronan that has a longer molecular mass (Itano *et al*., 1999). Therefore, hyaluronan synthesized and secreted by *HAS2* is less likely to be able to pass through zona pellucida. The results of the present study and these findings strongly suggested that hyaluronan involved in the enlargement of perivitelline space of oocytes is synthesized and secreted by the oocytes themselves. However, the results of the present study do not provide the information needed to determine the amount of hyaluronan secreted from oocytes themselves. Therefore, hyaluronan secretion by oocytes must be clarified in the future.

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培養したマウスの裸化卵母細胞における囲卵腔の大きさとヒアルロナン合成の可能性

上野紗也香[1]・新村末雄[2]*

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要　約

培養したマウスの裸化卵母細胞（DO）について、囲卵腔の大きさを計測して卵丘卵母細胞複合体（COC）の卵母細胞のものと比較するとともに、ヒアルロナン合成を阻害する作用のある4- メチルウンベリフェロン（MU）で処置したDOの囲卵腔の大きさを観察し、囲卵腔の拡大に役割を果たすヒアルロナンの合成と分泌に卵母細胞が関与しているのかどうかを検討した。

採取したDOとCOCを14時間培養したところ、卵母細胞の成熟率は93.5および96.7％であり、両者で有意な差はなかった。DOの囲卵腔は5.08μmあり、COCにおける卵母細胞の5.40μmとの間に有意な差はなかった。また、0.25mMのMUを含むTYH液で14時間培養したDOの成熟率は88.2％であり、対照のDOの85.7％と有意な差はなかったが、0.25mMのMUを含む培養液で成熟させたDOの囲卵腔は3.58μmであり、対照のDOの4.65μmに比べて有意に小さかった。さらに、0.25mMのMUとともに１ml中に0.05、0.25あるいは0.5mgのヒアルロナンを含む培養液で14時間培養したDOの成熟率は86.9ないし92.8％であり、0.25mMのMUのみで処置したDOの成熟率（88.2％）と相違なかった。一方囲卵腔は、MUのみで処置したDOでは3.85μmあり、MUとともに0.05あるいは0.5mg/mlのヒアルロナンを添加した培養液で培養したDOの4.24および4.29μmとの間に有意差はなかったが、MUとともに0.25mg/mlのヒアルロナンを添加した培養液で培養したDOでは有意に大きく、4.67μmになった。

以上の結果から、囲卵腔の拡大に役割を果たすヒアルロナンは卵母細胞自身が合成・分泌していることが推察された。

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キーワード：囲卵腔の大きさ、ヒアルロナン合成、マウス、裸化卵母細胞、卵丘を持つ卵母細胞

[1] 新潟大学大学院自然科学研究科

[2] 新潟大学農学部

*代表著者：niimura@agr.niigata-u.ac.jp