The Size of Perivitelline Space in Mouse Oocytes Cultured in the Medium with Decreased Concentration of NaCl

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Summary

The size of each part of mouse oocytes cultured in TYH medium and modified TYH medium with decreased concentration of NaCl 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 oocytes matured in modified TYH medium with decreased concentration of NaCl was also examined, in order to ascertain a relationship between the size of perivitelline space and the incidence of polyspermy.

When immature oocytes were cultured in modified TYH medium containing NaCl at 95.82, 82.15 or 68.49 mM, 92.6 to 100.0 % of the oocytes matured to the metaphase Ⅱ stage, showing no difference from the maturation rate (93.8 %) of control oocytes cultured in TYH medium. The maturation rate of oocytes cultured in modified TYH medium with NaCl at 44.50 mM was 0 %, which was significantly lower than that of the control oocytes. The size of perivitelline space in oocytes matured in modified TYH medium containing NaCl at 95.82 or 82.15 mM was 5.34 and 5.40 μm, respectively, showing no difference from the 5.40 μm of control oocytes. On the other hand, the size of perivitelline space of oocytes matured in modified TYH medium containing NaCl at 68.49 mM was 6.12 μm, which was significantly larger than those of the oocytes matured in TYH medium and modified TYH media containing NaCl at 95.82 and 82.15 mM.

The size of perivitelline space in oocytes immediately after collection was 0.28 μm. In oocytes cultured in TYH medium and modified TYH medium containing NaCl at 68.49 mM (low NaCl-TYH medium), 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 low NaCl-TYH medium for 14 hrs was 6.12 μm, which was significantly larger than 5.40 μm of control oocytes cultured in TYH medium. 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 the increase in inner diameter of their zonae pellucidae.

The rate of fertilization after insemination was similar between oocytes matured in low NaCl-TYH medium (92.7 %) and control oocytes matured in TYH medium (92.2 %), while the incidence of polyspermy was significantly lower in oocytes matured in low NaCl-TYH medium (40.4 %) than in control oocytes (57.6 %). From these findings, it was confirmed that there is a close relationship between the size of perivitelline space and the incidence of polyspermy in mouse oocytes.

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Key words : culture medium with decreased concentration of NaCl, incidence of polyspermy, mouse oocyte, size of perivitelline space

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 (GV) stage, the perivitelline space is known to start forming when meiotic maturation resumes and to enlarge in matured oocytes at the metaphase Ⅱ (MⅡ) stage (Kaufman *et al*., 1989; Okada *et al*., 1993).

Recently, it has been reported that the perivitelline space is significantly large and the incidence of polyspermy after insemination is significantly low in porcine and mouse oocytes matured *in vivo* than in those matured *in vitro* (Wang *et al*., 1998; Ueno *et al*., 2006). It has also been reported that the perivitelline space is significantly small and the incidence of polyspermy after insemination is significantly high in mouse oocytes matured in TYH medium containing tunicamycin or 4-methylumbelliferone, compared to control oocytes matured in TYH medium (Kitagawa and Niimura, 2006a; Ueno *et al*., 2006). Therefore, it is suggested that a relationship exists between the size of perivitelline space and the incidence of polyspermy in porcine and mouse oocytes (Wang *et al*., 1998; Kitagawa and Niimura, 2006a; Ueno *et al*., 2006).

On the other hand, it is known that the perivitelline space is significantly large in porcine oocytes matured in the medium with decreased concentration of NaCl, compared to control oocytes matured in the medium with usual concentration of NaCl (Funahashi *et al*., 1994; Kitagawa and Niimura, 2006b). From the results of porcine oocytes cultured in NCSU 37 medium with decreased concentration of NaCl, we suggested that the enlargement in the perivitelline space following treatment with low NaCl concentration is attributed

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to the reduction in the oocyte cytoplasm (Kitagawa and Niimura, 2006b). The size of perivitelline space in mammalian oocytes cultured in the medium with decreased concentration of NaCl has been investigated only in pigs but not in any other animals.

In the present study, the size of each part of mouse oocytes cultured in modified TYH medium with decreased concentration of NaCl was measured and compared to that of control oocytes cultured in TYH 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 oocytes matured in modified TYH medium with decreased concentration of NaCl was also examined, in order to ascertain a relationship between the size of perivitelline space and the incidence of polyspermy.

MATERIALS AND METHODS

Animals

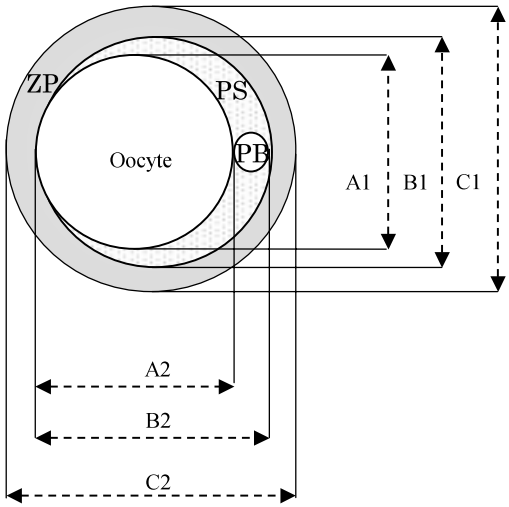
One hundred and thirty-eight female mature mice of ICR strain were used in the present study. They were housed in autoclaved metal cages and were given a standard chow (MF, Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum* in an air-conditioned room (24℃), under controlled-lighting conditions (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 i.u. of PMSG (Serotropin®, Teikoku Hormone Manufacturing Co. Ltd., Tokyo, Japan). Mice were sacrificed under anesthesia by diethyl ether in the present investigation.

Determination of NaCl concentration necessary to enlarge perivitelline space

In the present study, a concentration of NaCl at which NaCl had no effect on the maturation, but significantly enlarged perivitelline space in the resultant matured oocytes, was first determined. Immature oocytes covered with cumulus cells (COCs) were collected from antral follicles 48 hrs after the PMSG injection and cultured in modified TYH medium (Toyoda *et al*., 1971) containing 5 % (v/v) fetal bovine serum (FCS, Gibco BRL, NY, USA) and 10 i.u./ml PMSG at 37℃ in a CO_2 incubator (5 % CO_2 in air). The concentration of NaCl in modified TYH medium was 95.82, 82.15, 68.49 or 44.50 mM. COCs cultured in TYH medium with 119.37 mM NaCl, 5 % FCS and 10 i.u./ml PMSG were used as controls. Composition of culture medium for maturation of mouse oocytes was shown in **Table 1**.

Table 1. Composition of culture medium for maturation of mouse oocytes

Components	mM
NaCl	119.37, 95.82, 82.15, 68.49 or 44.50
KCl	4.78
KH_2PO_4	1.19
$CaCl_2 \cdot 2H_2O$	1.70
$MgSO_4 \cdot 7H_2O$	1.19
$NaHCO_3$	25.07
Glucose	5.55
Sodium pyruvate	1.00
Bovine serum albumin（mg/ml）	4.00
Penicillin G potassium salt（μg/ml）	50.00
Streptomycin（μg/ml）	75.00



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

Diameter of oocyte（A）=（A 1+A 2）/ 2
Inner diameter of zona pellucida（B）=（B 1+B 2）/ 2
Outer diameter of zona pellucida（C）=（C 1+C 2）/ 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

After cultured for 14 hrs, COCs were immersed in modified TYH medium with decreased concentration of NaCl or TYH medium containing 0.1% (w/v) hyaluronidase to disperse their cumulus cells. Then, the number and % of oocytes with 1st polar body in the perivitelline space (matured oocytes) were examined. The size of each part in matured oocytes was measured using a micrometer under a microscope, and the size of perivitelline space was calculated according to the method described in **Fig.1**.

Measurement of perivitelline space in oocytes cultured in modified TYH medium with decreased concentration of NaCl

In order to observe the effect of low NaCl on the size of perivitelline space, COCs were collected from antral follicles 48 hrs after the PMSG injection, and cultured in modified TYH medium containing 5 % (v/v) fetal bovine serum (FCS, Gibco BRL, NY, USA) and 10 i.u./ml PMSG at 37℃ in a CO_2

incubator (5 % CO_2 in air). Concentration of NaCl in modified TYH medium was lowered from 119.37 to 68.49 mM (low NaCl-TYH medium). COCs cultured in TYH medium containing 5 % FCS and 10 i.u./ml PMSG were used as controls.

After cultured for 0, 2, 4, 6, 8, 10, 12 and 14 hrs, cumulus cells were dispersed from oocytes by pipetting in low NaCl-TYH medium or TYH medium containing 0.1% (w/v) hyaluronidase. Then, the size of each part in oocytes was measured using a micrometer under a microscope, and the size of perivitelline space was calculated according to the method described in **Fig.1**.

Observation of nuclear maturation

In order to investigate nuclei, oocytes cultured in TYH medium or low NaCl-TYH medium for various periods were stained with Hoechst 33342 (Molecular Probes, Oregon, USA) and examined under a reflected-light fluorescing microscope (Nikon Corporation, Tokyo, Japan).

Observation of polyspermy

In order to observe the incidence of polyspermy, COCs were collected from antral follicles 48 hrs after the PMSG injection, and cultured in low NaCl-TYH medium containing 5 % (v/v) fetal bovine serum (FCS, Gibco BRL, NY, USA) and 10 i.u./ml PMSG at 37℃ in a CO_2 incubator (5 % CO_2 in air). COCs cultured in TYH medium containing 5 % FCS and 10 i.u./ml PMSG were used as controls.

After cultured for 14 hrs, cumulus cells were dispersed from oocytes by pipetting in low NaCl-TYH medium or TYH medium containing 0.1% (w/v) hyaluronidase. Then, only oocytes with 1st polar body in perivitelline space were inseminated *in vitro*. Sperm suspension was prepared by minutely cutting caudal epididymis of mature males in TYH medium for 1 hr. A small volume of the sperm suspension was introduced into 100 μl droplets of TYH medium, so that 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 hrs at 37℃ in a CO_2 incubator. After cultured 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 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 rates of nuclear maturation in cultured oocytes and the rates of polyspermy in inseminated oocytes were statistically analyzed by Chi-square test.

RESULTS

Maturation rate and size of perivitelline space in mouse oocytes cultured in modified TYH medium containing various concentrations of NaCl

When immature mouse oocytes were cultured in modified TYH medium containing NaCl at 95.82, 82.15 or 68.49 mM, 92.6 to 100.0 % of the oocytes matured to the MⅡ stage, showing no difference from the maturation rate (93.8 %) of control oocytes cultured in TYH medium (**Table 2**). The rate of maturation to the MⅡ stage of oocytes cultured in modified TYH medium with NaCl at 44.50 mM was 0 %, which was significantly lower than that of the control oocytes. The size of perivitelline space in oocytes matured in modified TYH medium containing NaCl at 95.82 or 82.15 mM was 5.34 and 5.40 μm, respectively, showing no difference from the 5.40 μm of control oocytes matured in TYH medium. On the other hand, the size of perivitelline space of oocytes matured in modified TYH medium containing NaCl at 68.49 mM was 6.12 μm, which was significantly larger than those of the oocytes matured in TYH medium and modified TYH media containing NaCl at 95.82 and 82.15 mM. In view of these results, the medium containing NaCl at 68.49 mM was used in subsequent experiments because NaCl did not affect the maturation of immature oocytes to the MⅡ stage and had the effect on enlargement of perivitelline space at this concentration.

Nuclear maturation in mouse oocytes cultured in low NaCl-TYH medium

Immediately after collection, nuclei of all oocytes were in the GV stage. When oocytes were cultured in TYH medium or low NaCl-TYH medium, the percentage of oocytes at the GV stage decreased over time during culture, and it reached 0 % after cultured for 4 hrs in TYH medium and 6 hrs in low NaCl-TYH medium, respectively. Oocytes in the MⅡ stage appeared at 8 hrs of culture, and the percentage of oocytes in this stage increased as the time of culture was prolonged, and reached 100 % after cultured for 10 hrs (TYH medium) and 12 hrs (low NaCl-TYH medium), respectively. In oocytes cultured for 12 and 14 hrs in TYH medium and low NaCl-TYH medium, there were no statistically significant

Table 2. The rate of maturation and the size of perivitelline space in mouse oocytes cultured in medium containing various concentrations of NaCl

Concentrations of NaCl (mM)	No.of oocytes cultured	No. and (%) of oocytes matured	Size of perivitelline space (μm)
119.37	32	30 (93.8)[a]	5.40 ± 0.1[b] *
95.82	32	30 (93.8)[a]	5.34 ± 0.1[b]
82.15	27	25 (92.6)[a]	5.40 ± 0.2[b]
68.49	29	29 (100.0)[a]	6.12 ± 0.1[a]
44.50	35	0 (0.0)[b]	n.d.

Maturation and perivitelline space in oocytes were observed after 14 hrs of culture.

* Mean ± S.E.

n.d.: Size of perivitelline space was not measured in oocytes cultured in the medium with 44.50 mM NaCl.

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

Table 3. The size of each part in cultured mouse oocytes

Kinds of culture media	Hours of culture	No. of oocytes examined	Inner diameter of zona pellucida (μm)	Diameter of oocyte (μm)	Thickness of zona pellucida (μm)	Size of perivitelline space (μm)
TYH medium	0	30	76.46 ± 0.6 [a**]	75.89 ± 0.6 [a]	8.53 ± 0.2 [a]	0.28 ± 0.1 [d]
	2	30	73.47 ± 0.5 [b,c]	68.93 ± 0.7 [b]	9.25 ± 0.2 [a]	2.28 ± 0.2 [c]
	4	33	73.65 ± 0.6 [a,b,c]	67.31 ± 0.4 [b,c,d]	9.28 ± 0.2 [a]	2.62 ± 0.2 [c]
	6	30	72.80 ± 0.6 [c]	67.52 ± 0.3 [b,c]	9.24 ± 0.2 [a]	2.67 ± 0.3 [c]
	8	33	74.22 ± 0.6 [a,b,c]	66.09 ± 0.3 [c,d,e]	8.83 ± 0.2 [a]	3.91 ± 0.2 [b]
	10	30	73.81 ± 0.3 [a,b,c]	64.04 ± 0.2 [e]	8.59 ± 0.2 [a]	4.99 ± 0.1 [a]
	12	30	75.38 ± 0.4 [a,b,c]	64.81 ± 0.3 [e]	8.32 ± 0.2 [a]	5.32 ± 0.2 [a]
	14	30	76.11 ± 0.6 [a,b]	65.10 ± 0.4 [d,e]	8.58 ± 0.2 [a]	5.40 ± 0.1 [a]
Modified TYH medium with decreased concentration of NaCl*	2	30	78.40 ± 0.6 [a]	68.43 ± 0.4 [a,b]	8.09 ± 0.2 [a,b]	4.65 ± 0.2 [b,c]
	4	37	77.67 ± 0.5 [a]	68.91 ± 0.5 [a,b]	8.37 ± 0.1 [a,b]	4.37 ± 0.2 [b,c]
	6	35	78.34 ± 0.5 [a]	70.32 ± 0.3 [a]	8.70 ± 0.1 [a]	4.02 ± 0.2 [c]
	8	35	78.38 ± 0.6 [a]	68.24 ± 0.5 [a,b]	8.30 ± 0.1 [a,b]	5.07 ± 0.3 [a,b,c]
	10	32	77.88 ± 0.5 [a]	68.78 ± 0.4 [a,b]	7.78 ± 0.1 [b]	4.53 ± 0.3 [b,c]
	12	31	79.29 ± 0.5 [a]	68.70 ± 0.3 [a,b]	7.58 ± 0.2 [b]	5.27 ± 0.2 [a,b]
	14	29	79.98 ± 0.5 [a]	67.73 ± 0.2 [b]	7.83 ± 0.2 [b]	6.12 ± 0.2 [a]

* Concentration of NaCl in modified TYH medium was lowered from 119.37 to 68.49 mM.
** Mean ± S.E.
Values with different superscripts in the same column in each experimental lot are significantly different ($P < 0.05$).

differences in the percentages of oocytes reaching the MII stage between the culture media examined.

Size of each part in mouse oocytes cultured in low NaCl-TYH medium

The size of each part in mouse oocytes cultured in low NaCl-TYH medium or TYH medium is shown in **Table 3**.

The perivitelline space in oocytes immediately after collection **(Fig.2)** was 0.28 μm, but in oocytes cultured in low NaCl-TYH medium and TYH medium it gradually enlarged as the time of culture was prolonged, even in the use of either medium examined. At 2, 4, 6, 8 and 14 hrs of culture, the perivitelline space of oocytes cultured in low NaCl-TYH medium **(Fig.3)** was 4.65, 4.37, 4.02, 5.07 or 6.12 μm, respectively, which was significantly larger than 2.28, 2.62, 2.67, 3.91 and 5.40 μm of control oocytes cultured in TYH medium **(Fig.4)**.

The inner diameter of the zona pellucida was 76.46 μm in oocytes immediately after collection. In oocytes cultured in low NaCl-TYH medium, the inner diameter of the zona pellucida was 77.67 to 79.98 μm, which was significantly larger than those in oocytes cultured for any period in TYH medium.

Immediately after collection, the diameter of oocytes was 75.89 μm, which was significantly larger than those of oocytes

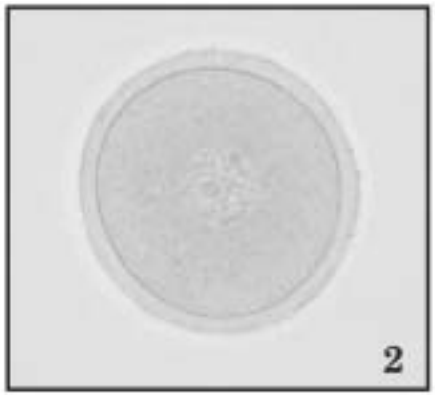


Fig.2. No perivitelline space of mouse oocyte immediately after collection.

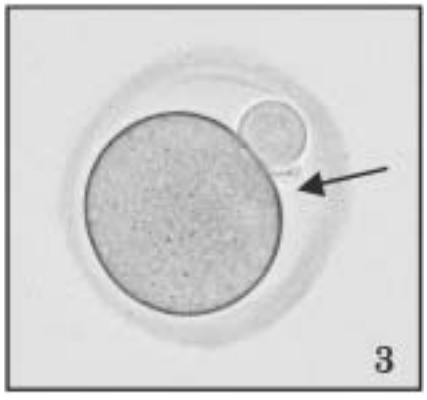


Fig.3. A large perivitelline space (an arrow) of mouse oocyte cultured for 14 hrs in modified TYH medium with decreased concentration of NaCl (68.49 mM).

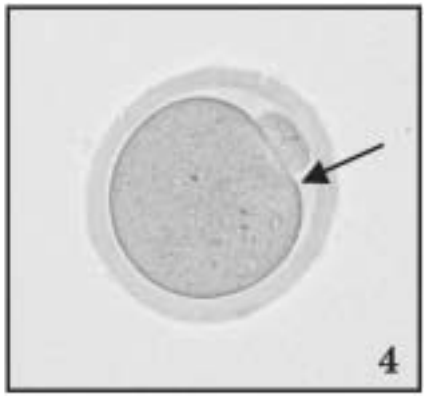


Fig.4. A small perivitelline space (an arrow) of control mouse oocyte cultured for 14 hrs in TYH medium.

cultured for any period in TYH medium. On the other hand, the diameter of oocytes cultured in low NaCl-TYH medium was 67.73 to 70.32 μm. The diameter was significantly larger in oocytes cultured in low NaCl-TYH medium than in those cultured in TYH medium at 4, 6, 8, 10, 12 and 14 hrs of culture.

The thickness of zona pellucida was 8.53 μm in oocytes immediately after collection, which was no different to those of oocytes cultured for any period in TYH medium. The thickness of zona pellucida was 7.58 to 8.70 μm in oocytes cultured in low NaCl-TYH medium. Although thickness of zona pellucida did not differ between oocytes cultured in low NaCl-TYH medium and TYH medium at 14 hrs of culture, it was significantly thinner in oocytes cultured in low NaCl-TYH medium than in those cultured in TYH medium at 2, 4, 6, 8, 10 and 12 hrs of culture.

Incidence of polyspermy in mouse oocytes matured in low NaCl-TYH medium

The rate of fertilization and the incidence of polyspermy in mouse oocytes matured in low NaCl-TYH medium and TYH medium were shown in **Table 4**. Although no significant difference was observed in the fertilization rate between oocytes matured in low NaCl-TYH medium (92.7 %) and control oocytes matured in TYH medium (92.2 %), the incidence of polyspermy was significantly lower in oocytes matured in low NaCl-TYH medium (40.4 %) than in control oocytes matured in TYH medium (57.6 %).

DISCUSSION

It has been known that the perivitelline space is significantly large and the incidence of polyspermy after insemination is significantly low in porcine oocytes matured in the medium with decreased concentration of NaCl, compared to control oocytes matured in the medium with usual concentration of NaCl (Funahashi *et al*., 1994; Kitagawa and Niimura, 2006b). From the results of porcine oocytes cultured in NCSU 37 medium with decreased concentration of NaCl, we suggested that the enlargement in the perivitelline space following treatment with low NaCl concentration is attributed to the reduction in the oocyte cytoplasm (Kitagawa and Niimura, 2006b). The size of perivitelline space in mammalian oocytes cultured in the medium with decreased concentration of NaCl has been investigated only in pigs but not in any other animals.

From the present observation concerning the measurements of perivitelline space in mouse 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 in low NaCl-TYH medium compared to that of the oocytes cultured in TYH medium. Since the inner diameter of the zona pellucida significantly increased in oocytes cultured in low NaCl-TYH medium, although the diameter of the same oocytes also significantly increased compared to oocytes cultured in TYH medium, it was suggested that the enlargement in the perivitelline space of oocytes following treatment with low NaCl was attributed to the increase in inner diameter of their zonae pellucidae.

Because nuclear maturation was found to progress in the oocytes cultured in low NaCl-TYH medium in the present study, as in the oocytes cultured in THY medium, nuclear maturation was considered to progress normally. Also, there was no difference in the fertilization rate of oocytes matured in low NaCl-TYH medium and TYH medium, confirming that the abilities to mature and fertilize in oocytes cultured in the medium with decreased concentration of NaCl were maintained normally. On the other hand, the incidence of polyspermy after insemination significantly decreased in oocytes cultured in low NaCl-TYH medium whose perivitelline space significantly enlarged. 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 mouse oocytes.

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Table 4. The incidence of polyspermy in inseminated mouse oocytes matured *in vitro*

Kinds of culture media	No. of oocytes inseminated	No. and (%) of oocytes fertilized	No. and (%) of monospermic oocytes	No. and (%) of polyspermic oocytes
TYH medium	128	118 (92.2)[a]	50 (42.4)[b]	68 (57.6)[a]
Modified TYH medium with decreased concentration of NaCl *	123	114 (92.7)[a]	68 (59.6)[a]	46 (40.4)[b]

* Concentration of NaCl in modified TYH medium was lowered from 119.37 to 68.49 mM.
Values with different superscripts in the same column are significantly different ($P < 0.05$).

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低 NaCl 濃度の培養液で培養したマウス卵母細胞の囲卵腔の大きさ

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要　約

NaCl の濃度を低くした低 NaCl-TYH 培養液で培養したマウス卵母細胞について、各部位の大きさを計測して通常の TYH 培養液で培養した卵母細胞のものと比較し、低 NaCl 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。併せて、低 NaCl-TYH 培養液で成熟させたマウス卵母細胞の媒精後の多精子侵入の頻度を調べた。

胞状卵胞から採取した卵母細胞を、NaCl の濃度を95.82、82.15および68.49mM に低下した修正 TYH 培養液で14時間培養すると、92.6ないし100％が成熟し、成熟率は対照の TYH 培養液（NaCl 濃度は119.37mM）で培養した卵母細胞の93.8％と相違なかったが、NaCl の濃度を44.50mM に低下した培養液で培養したものでは成熟したものはみられなかった。一方、囲卵腔の大きさは、NaCl の濃度を95.82および82.15mM に低下した培養液で培養した卵母細胞では5.34および5.40μm であり、対照の卵母細胞の5.40μm と相違なかったが、NaCl の濃度を68.49mM に低下した培養液で培養したものでは有意に大きく、6.12μm になった。

囲卵腔は、採取直後の卵母細胞では0.28μm あったが、TYH 培養液および NaCl の濃度を68.49mM に低下した修正 TYH 培養液（低 NaCl-TYH 培養液）で培養したものでは、いずれも培養時間の経過に伴って徐々に拡大した。なお、培養後２、４、６、８および14時間では、囲卵腔は、TYH 培養液で培養した卵母細胞に比べ、低 NaCl-TYH 培養液で培養した卵母細胞で有意に大きかった。透明帯の内径は、採取直後の卵母細胞と TYH 培養液でいずれの時間培養した卵母細胞との間で大きく変化しなかったが、低 NaCl-TYH 培養液で培養した卵母細胞では77.67ないし79.98μm となり、すべての培養時間で、TYH 培養液で培養したものに比べて有意に大きかった。卵母細胞の直径は、TYH 培養液および低 NaCl-TYH 培養液で培養したものの両者で、採取直後の卵母細胞のものに比べて有意に小さくなるとともに、培養後４、６、８、10、12および14時間では、TYH 培養液で培養したものに比べて低 NaCl-TYH 培養液で培養したもので有意に大きかった。透明帯の厚さは、採取直後の卵母細胞と TYH 培養液で培養した卵母細胞との間で相違なかった。一方、低 NaCl-TYH 培養液で培養した卵母細胞の透明帯は、7.58ないし8.70μm あり、培養後14時間では TYH 培養液で培養したものと相違なかったが、培養後２、４、６、８、10および12時間では TYH 培養液で培養したものに比べて有意に薄かった。これらのことから、低 NaCl-TYH 培養液で培養した卵母細胞で囲卵腔が大きくなるのは、透明帯の内径が大きくなるためであると考えられた。

一方、低 NaCl-TYH 培養液で成熟させた卵母細胞に媒精したところ、受精率は92.7％であり、対照の TYH 培養液で成熟させた卵母細胞の92.2％と相違なかったが、多精子侵入率は40.4％であり、対照の卵母細胞の57.6％に比べて有意に低かった。従って、低 NaCl 濃度の培養液で成熟させて囲卵腔を有意に拡大すると、媒精後の多精子侵入の頻度が有意に低下することが確かめられ、卵母細胞の囲卵腔の大きさと媒精後の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かめられた。

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