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—Original Article—

Prolonged exposure to hyaluronidase decreases the fertilization and development rates of fresh and cryopreserved mouse oocytes

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Abstract. Hyaluronidase is generally used to remove cumulus cells from mouse oocytes before oocyte cryopreservation, intracytoplasmic sperm injection or DNA injection. In general, use of cumulus-free mouse oocytes decreases *in vitro* fertilizing ability compared with cumulus-surrounded oocytes. The effect of hyaluronidase exposure on the quality of mouse oocytes is not fully understood. Here, we investigated the effect of hyaluronidase exposure time on the fertilization rate of fresh and vitrified mouse oocytes and their subsequent developmental ability *in vitro*. We found that the fertilization rate decreased with hyaluronidase treatments. This reduction in the fertilization rate following treatment with hyaluronidase was fully reversed by removal of the zona pellucida. In addition, oocytes treated with hyaluronidase for 5 min or longer had a reduced capacity to develop to the morula and blastocyst stage. The survival, fertilization, and developmental rates of vitrified-warmed oocytes were also reduced by longer exposure to hyaluronidase. In conclusion, these results suggest that prolonged exposure to hyaluronidase decreases the quality of mouse oocytes and shorter hyaluronidase treatment times may help achieve a stable and high fertilization rate in fresh and cryopreserved oocytes.

Key words: Hyaluronidase, *In vitro* fertilization, Mouse oocytes, Oocyte cryopreservation

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In bioscience research, genetically engineered mice (GEM) are frequently used to investigate gene functions and as models of human disease. Mouse banks have been established to produce, collect, archive and distribute GEM worldwide [1, 2]. Researchers can efficiently manage their mice and easily access deposited GEM via the mouse banks.

In mouse banks, many reproductive techniques, such as *in vitro* fertilization [3], sperm cryopreservation [4], oocyte and embryo cryopreservation [5–7] and embryo transfer, are applied to efficiently manage the numerous GEM strains [8, 9]. In some of these techniques (e.g., oocyte cryopreservation [6, 7], *in vitro* maturation [10], intracytoplasmic sperm injection (ICSI) [11], DNA injection [12]), cumulus cells surrounding the oocytes must be removed to improve oocyte manipulation.

Hyaluronidase is generally used to detach cumulus cells from oocytes during reproductive techniques [13, 14]. Hyaluronidase is an enzyme that hydrolyzes hyaluronan (also termed hyaluronic acid or hyaluronate), a polysaccharide consisting of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid [15]. Hyaluronan is a highly hydrated and viscoelastic matrix in the extracellular space of cumulus–oocyte complexes (COCs) [16]. Treatment with hyaluronidase causes decomposition of the hyaluronan-based matrix surrounding COCs and disperses the cumulus cells from oocytes. However, removing the cumulus cells can result in reduced fertilization of the oocytes [17, 18]. In some reports, treatment of human oocytes with hyaluronidases decreased oocyte survival, fertilization rates and developmental rates following ICSI [19–21]; however, the effect of hyaluronidase on mouse oocytes is not as well understood.

In this study, we examined the effect of hyaluronidase on the viability, fertilizing ability, and developmental ability of mouse oocytes. In addition, we investigated the influence of hyaluronidase on survivability, fertilizing ability and developmental ability of vitrified oocytes.

Materials and Methods

Animals

Mature female (8–12 weeks old) and male (12–16 weeks old) C57BL/6J mice were used as oocytes and sperm donors, respectively. The mice were maintained with a 12:12 h light-dark cycle (lights switched on at 0700 h and off at 1900 h). The room temperature was maintained at 22 C ± 2 C. The animals were given free access to food and water. All animal experiments were approved by the Animal Care and Use Committee at Kumamoto University.

Media

Sperm were preincubated in a modified Krebs-Ringer bicarbon-

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ate solution (TYH) containing 0.75 mM methyl-β-cyclodextrin (MBCD, Sigma-Aldrich, St, Louis, MO, USA) that strongly promotes sperm capacitation by facilitating cholesterol efflux from the plasma membrane of the sperm [3, 22]. Modified human tubal fluid (mHTF) with a high concentration of calcium (5.14 mM), which is bicarbonate-buffered solution, was used as the cumulus removal and fertilization mediums [23, 24]. Potassium simplex optimization medium (KSOM) was used to culture embryos to the blastocyst stage [25]. Hyaluronidase was purchased from Sigma-Aldrich (400–1000 unit/mg of hyaluronidase from bovine testes, Type I-S; Sigma-Aldrich). The titer of hyaluronidase (Lot No. 029K7001V), measured by Sigma-Aldrich, was 801 unit/ml. A stock solution of 1% hyaluronidase was prepared in mHTF and stored at – 20 C until use.

Oocyte collection

For superovulation treatment, female mice were injected intraperitoneally with 7.5 IU of equine chorionic gonadotropin (eCG) (serotropin; Aska Pharmaceutical, Tokyo, Japan), followed by 7.5 IU of human chorionic gonadotropin (hCG) (gonadotropin; Aska Pharmaceutical), which was administered 48 h after eCG injection. After 15−17 h, female mice were euthanized, and the oviducts were quickly collected. Under paraffin oil, oviducts were opened with a surgical needle, and the cumulus oocyte complexes (COCs) were transferred into a drop of mHTF.

Removal of cumulus cells by hyaluronidase treatment

Hyaluronidase (1% w/v) from bovine testes was diluted in mHTF to a final concentration of 0.1% (801 unit/ml). The COCs were incubated in 5% CO_2 at 37 C for 1, 5, 10 or 30 min. Many cumulus cells remained attached to oocytes at 1 min after treatment with hyaluronidase. Thus, the cumulus cells were completely removed by pipetting using a glass capillary during washing. The cumulus-free oocytes were then used for IVF or oocyte cryopreservation.

Oocyte cryopreservation

Oocytes were cryopreserved with a vitrification procedure, as described previously by Nakagata *et al.* [7]. Briefly, cumulus-free oocytes were transferred into 20% FBS in mHTF. Thereafter, 40–100 cumulus-free oocytes were suspended in one drop (100 µl) of 1 M dimethyl sulfoxide (DMSO) in phosphate buffered medium (PB1) at room temperature. Thereafter, 5 µl of this solution containing the oocytes was transferred into a cryotube at 0 C. After 5 min, 45 µl of vitrification solution (2 M DMSO, 1 M acetamide and 3 M propylene glycol in PB1:DAP213) was added to each cryotube to cover the oocytes. The cryotubes were directly plunged into liquid nitrogen and stored for at least 5 days in a liquid nitrogen tank. Subsequently, the cryotubes containing the oocytes were removed from the liquid nitrogen tank and incubated at room temperature for 30 sec. A prewarmed (37 C) 900-µl aliquot of sucrose solution (0.25 M) in PB1 was pipetted into each cryotube. The sucrose solution containing the oocytes was then transferred to a plastic dish. The recovered oocytes were serially washed in mHTF, and the survival of the vitrified-warmed oocytes was assessed.

IVF and embryo culture

Fresh sperm were collected from the cauda epididymides of male mice. The collected sperm were transferred into a 100-µl drop of sperm preincubation medium ($8–12 \times 10^4$ sperm/µl) and incubated in 5% CO_2 at 37 C for 60 min. After incubation, the sperm suspension was introduced into mHTF containing either COCs, cumulus-free oocytes, vitrified-warmed oocytes or zona-free oocytes. The final motile sperm concentration in the fertilization medium was 800–1200 sperm/µl. Six hours after insemination, the oocytes were washed in freshly prepared drops of mHTF to separate normally fertilized two-pronuclear and unfertilized oocytes. Twenty-four hours after insemination, two-cell embryos were observed, and the fertilization rate was calculated as the total number of two-cell embryos divided by the total number of inseminated oocytes and multiplied by 100. Some of the two-cell embryos were cultured to blastocysts in KSOM medium.

Removal of the zona pellucida

To examine the inhibitory effect of the zona pellucida after hyaluronidase treatment, we performed IVF using oocytes with or without the zona pellucida after hyaluronidase treatment. After removal of cumulus cells by hyaluronidase treatment, the zona pellucida was removed using acid Tyrode's solution. Oocytes without the zona pellucida were washed three times in drops of mHTF. The fertilizing ability of oocytes with or without the zona pellucida was examined by IVF.

Statistical analysis

Statistical analysis [analysis of variance (ANOVA) with Fisher's protected least significant differences (PLSD)] was performed using the StatView-5.0J for Windows statistical software (SAS Institute, Cary, NC, USA). The level of statistical significance was defined as $P < 0.05$.

Results

Effect of the duration of hyaluronidase treatment on the fertilizing ability of oocytes

Figure 1 shows the appearance of cumulus–oocytes complexes with time after adding hyaluronidase. After a 5-min treatment, most of the oocytes were engaged in complexes. Moreover, after 10- and 30-min treatments, most of the oocytes were denuded, but a smidgen of cumulus cells remained surrounding the oocytes. Prolonged hyaluronidase treatment at 0.1% decreased the fertilizing ability of oocytes *in vitro* (Table 1). Oocytes treated with hyaluronidase for 1 min maintained the same high fertilization rate as oocytes with cumulus cells. However, the fertilizing ability of hyaluronidase-treated oocytes was significantly reduced after incubation times longer than 5 min.

Development to the two-cell stage

The developmental ability of two-cell embryos produced by IVF using oocytes treated with hyaluronidase for 1 min was equal to that of oocytes with cumulus cells (Fig. 2). Treatment of oocytes with hyaluronidase for 5 min or longer adversely affected the developmental ability of embryos ($P < 0.05$).

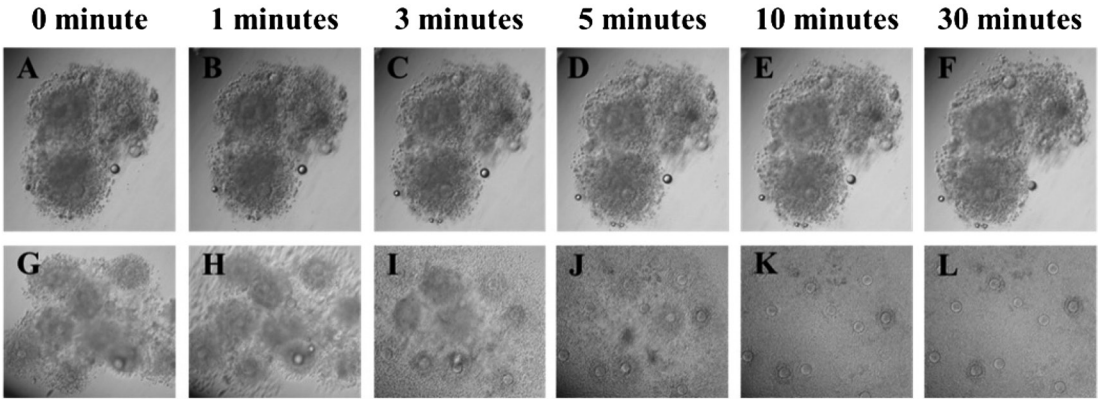

Fig. 1. Time course of the removal of cumulus cells from oocytes in 0.1% hyaluronidase solution. The figures shows the time-dependent change in cumulus-oocytes complexes in mHTF without (A–F) or with 0.1% hyaluronidase solution (G–L). A–F) Cumulus cells were attached to oocytes in mHTF without hyaluronidase for 30 min. G–L) Cumulus cells were gradually removed from oocytes in mHTF containing 0.1% hyaluronidase for 30 min. A number of oocytes were surrounded by cumulus cells without pipetting.

Table 1. Effect of 0.1% hyaluronidase on the fertilization rate of cumulus-free oocytes

Cumulus cells	Concentration of hyaluronidase (%)	Exposure time (min)	No. of inseminated oocytes	No. of two-cell embryos	Fertilization rate (%)
+	0	0	355	307	86.5 ± 15.8
–	0.1	1	306	255	83.3 ± 23.4
–		5	452	307	67.9 ± 9.8*
–		10	450	186	41.3 ± 16.3*
–		30	84	40	47.6 ± 7.7*

There were no parthenogenetic oocytes in this experiment. Values are expressed as means ± SD (n = 3–7). * Values differ significantly compared with the control, cumulus cells (+) without hyaluronidase ($P<0.05$).

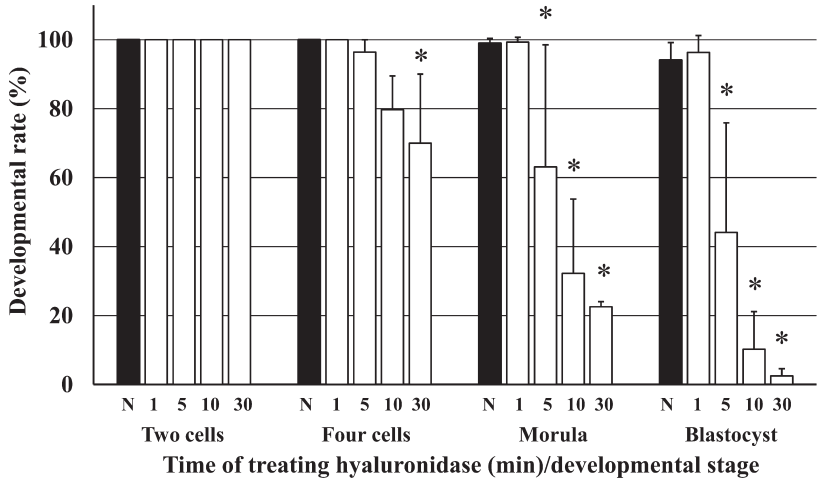

Fig. 2. Effect of treatment time in 0.1% hyaluronidase on the ability of two-cell embryos to develop to blastocysts. After IVF, two-cell embryos were cultured in KSOM. Developmental rates were calculated as the number of four-cell embryos, morulae or blastocysts divided by the number of two-cell embryos ×100 (%). Control (no treatment, N) indicates COCs without hyaluronidase treatment. Results are expressed as the mean ± SD (n = 4–7). * Values differ significantly compared with the control (N) ($P<0.05$).

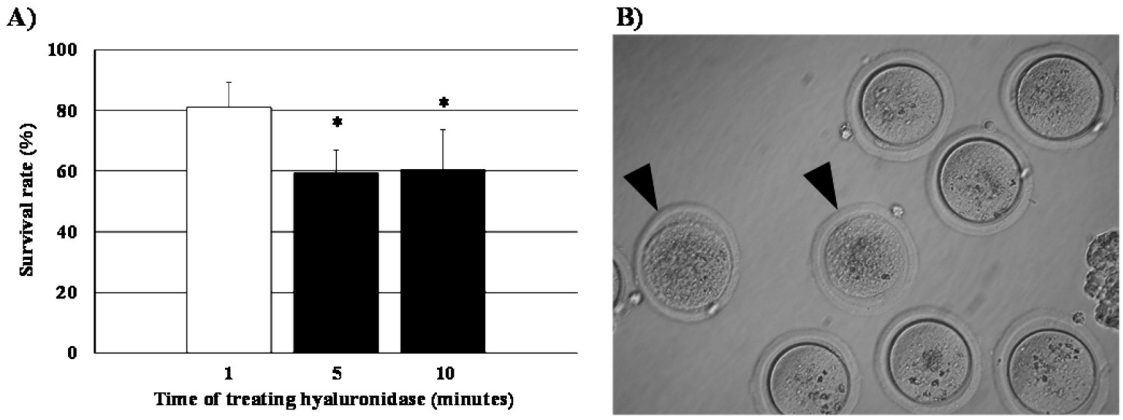

Fig. 3. Effect of treatment time in 0.1% hyaluronidase on the survival rate of vitrified-warmed oocytes. Oocytes were treated with hyaluronidase for 1, 5 or 10 min before cryopreservation. After warming the oocytes, the survival rate of the oocytes was examined (A). The survival rate was calculated as the number of morphologically normal oocytes divided by the number of vitrified oocytes ×100 (%). The image shows the morphology of normal oocytes and dead oocytes after cryopreservation (B). Results are expressed as the mean ± SD (n = 4). * Values differ significantly compared with the 1-min treatment ($P<0.05$).

Survival, fertilization, and development of vitrified-warmed oocytes

The survival rates of vitrified-warmed oocytes were significantly decreased when the oocytes were treated with hyaluronidase for 5 or 10 min (Fig. 3). In addition, longer hyaluronidase treatment times decreased the fertilizing ability of vitrified-warmed oocytes, and the treatment for 10 min yielded statistically significant difference (Table 2).

The developmental ability of two-cell embryos produced by vitrified-warmed oocytes treated with hyaluronidase for 1 min was significantly higher than that of the embryos produced by oocytes treated with hyaluronidase for 5 or 10 min (Fig. 4).

Table 2. Fertilization rate of vitrified-warmed oocytes treated with 0.1% hyaluronidase

Time (min)	No. of inseminated oocytes	No. of two-cell embryos	Fertilization rate (%)
1	230	182	79.1 ± 6.8
5	272	166	61.0 ± 6.5
10	199	75	37.7 ± 19.7*

There were no parthenogenetic oocytes in this experiment. Values are expressed as means ± SD (n = 4). * Values differ significantly compared with 1-min hyaluronidase treatment ($P<0.05$).

Effect of the zona pellucida.

To examine the functional modifications of the zona pellucida caused by hyaluronidase, the fertilizing ability of oocytes with or without the zona pellucida was investigated. Removal of the zona pellucida with acid Tyrode's solution restored the fertilization rate of oocytes treated with hyaluronidase for 10 min (Table 3).

Discussion

In this study, we demonstrated that prolonged exposure to hyaluronidase degraded fresh and cryopreserved mouse oocytes. Similarly, the length of exposure to hyaluronidase affected the survivability and fertilizing and developmental ability of vitrified-warmed oocytes. These results showed that conservation of high fertilizing ability in cryopreserved oocytes requires maintenance of high fertilizing ability before cryopreservation. Namely, shortening the hyaluronidase treatment is important for maintaining high fertilizing ability after cryopreservation. In addition, the oocyte fertilization rate was fully restored by removal of the zona pellucida.

There are some previous reports on the effect of cumulus cell removal using hyaluronidase on the quality of oocytes in mammals. Cross and Brinster used 300 units/mg of hyaluronidase to remove cumulus cells with mechanical disturbance by suction and release of the oocytes in small bore pipettes [17]. On the other hand, Itagaki *et al.* incubated cumulus surrounded oocytes in TYH containing 150 units/mg hyaluronidase [26]. Also, 0.03% of hyaluronidase has been used for removing cumulus cells [16]. In this study, we used 0.1% (801 unit/ml) hyaluronidase because this concentration has been generally used to remove cumulus cells before oocyte cryopreservation. In the preliminary study, treatment with a low concentration of hyaluronidase (0.03%) also decreased the fertilizing ability at 5 and 10 min as well as 0.1% (data not shown). Based on the above findings, the exposure period for hyaluronidase during the removal of cumulus cells may strongly influence the reduction in fertilizing ability.

We showed that removing the zona pellucida restored the fertilizing ability of oocytes treated with hyaluronidase for 10 min to rates similar those of untreated oocytes. This result indicates that hyaluronidase may modify the characteristics of the zona pellucida, resulting in decreased fertilization rates. During fertilization, glucosaminidase is released from cortical granules, and the N-acetylglucosamine residue on the zona pellucida protein 3 is removed. This is the mechanism by which polyspermic fertilization is avoided through inhibition of sperm-zona binding [27]. Treatment with a commercial

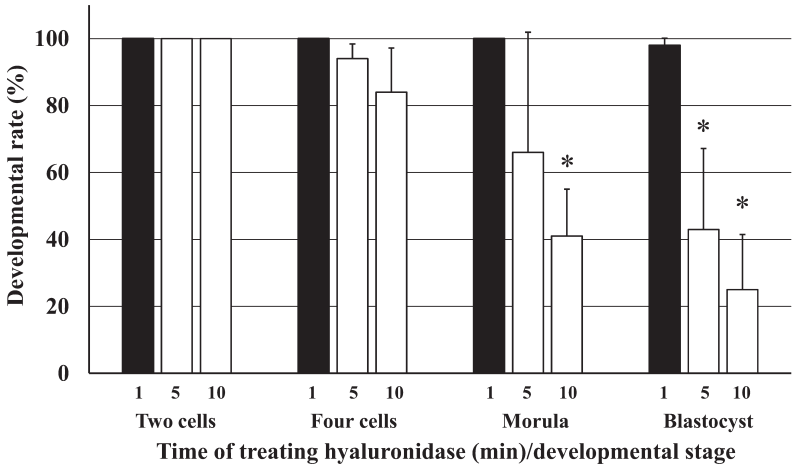

Fig. 4. Effect of treatment time in 0.1% hyaluronidase on the development of two-cell embryos derived from vitrified-warmed oocytes to blastocysts. Developmental rates were calculated as the number of four-cell embryos, morulae or blastocysts divided by the number of two-cell embryos × 100 (%). Results are expressed as the mean ± SD (n = 4). * Values differ significantly compared with 1 min (P<0.05).

Table 3. Effect of zona pellucida removal on the fertilization rate oocytes with 0.1% hyaluronidase

Zona pellucida	No. of inseminated oocytes	No. of fertilized oocytes	Fertilization rate (%)
–	70	64	91.4 ± 3.4
+	81	30	37.0 ± 3.9*

There was no parthenogenetic oocyte in this experiment. Values are expressed as means ± SD (n = 3). * The values differed significantly between the two groups (P<0.05).

hyaluronidase may similarly modify the oligosaccharide residues on the zona pellucida of oocytes and lower the fertilization rate.

In this study, we demonstrated that prolonged exposure to hyaluronidase decreased the developmental ability *in vitro*. Previously, Tachibe *et al.* reported that incubation in culture medium containing a high concentration of hyaluronidase (3 mg/ml, 0.3%) for >1 h dramatically decreased the developmental ability of fertilized mouse eggs or embryos [28]. These results suggest that hyaluronidase has an adverse effect on the early stages of oocyte or embryo development. In particular, before fertilization, oocytes may be easily influenced by short hyaluronidase treatments compared with embryos.

The low fertility of hyaluronidase-treated oocytes may imply the existence of contaminants with adversary effects in the reagent. The hyaluronidase used in this study was derived from bovine testes. Biological reagents may contain undefined components. To investigate the effects of the contaminants in hyaluronidase, we additionally examined the effects of different batches of hyaluronidase on the fertilizing and developmental ability of mouse oocytes. This experiment showed that treatment with 0.1% hyaluronidases of different batches for 10 min had no effects on the fertilization ability of oocytes (data not shown). However, a reduction was observed for treatment with the different batches of hyaluronidase at higher concentrations (0.8% and 1.0%) for 10 min. Previously, Zuccotti *et al.* reported that collagenase contaminated with various proteases contributed to dissolution of the zona pellucida [29]. The effects of the commercially available collagenase they used depended on the types and concentrations of the contaminating proteases in the batch. Hyaluronidase prepared from bovine testes may contain contaminants resulting in different responses between the types and batches of hyaluronidase. To elucidate the reason for the different responses, further investigations to determine which contaminants reduce the fertilizing and developmental ability of oocytes are required. This result strongly suggests that quality checks should be performed on hyaluronidase to obtain high fertility of oocytes before removing cumulus cells.

Detaching cumulus cells from oocytes by treatment with hyaluronidase treatment has been used in the field of reproductive technology. Mouse oocyte cryopreservation is currently a very important technique for effectively producing GEM [7, 30], and there are many benefits of using cryopreserved oocytes and IVF for GEM for the purposes of archiving cryopreserved embryos of mice or expanding mouse colonies. Previously, we developed a system for the cryopreservation of cumulus-free mouse oocyte and IVF that achieves a high fertilization rate [7]. However, some factors cause variability of survivability and fertilizing ability of cryopreserved mouse oocytes. In this study, we demonstrated that prolonged exposure to hyaluronidase apparently reduced the survivability, fertilization and developmental ability of vitrified-warmed oocytes. Therefore, a short hyaluronidase treatment is critical for maintaining high survivability and fertilizing ability of frozen–thawed oocytes. To prepare high-quality cryopreserved oocyte, a small number of cumulus–oocyte complexes should be handled to shorten the duration of exposure to the medium containing hyaluronidase.

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