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ORIGINAL ARTICLE

Sperm Biology

Sperm hyaluronidase is critical to mammals' fertilization for its ability to disperse cumulus–oocyte complex layer

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Glycosylphosphatidylinositol-anchored sperm hyaluronidases have long been believed to assist in sperm penetration through the cumulus–oocyte complex (COC); however, their role in mammalian fertilization remains unclear. Previously, we have shown that hyaluronidase 5 (*Hyal5*)/*Hyal7* double-knockout (dKO) mice produce significantly fewer offspring than their wild-type (WT) counterparts because of defective COC dispersal. Male infertility is mainly caused by a low sperm count. It can be further exacerbated by the deficiency of sperm hyaluronidase, which disperses the cumulus cells of the outer layer of the COC. In the current study, we evaluated the effects of a low count of *Hyal*-deficient sperm and conditions of ovulated oocytes on the fertilization rate using a mouse model. Our results demonstrated that a low sperm count further decreases the *in vitro* fertilization (IVF) rate of *Hyal*-deficient dKO spermatozoa. In addition, the dKO spermatozoa resulted in a fertilization rate of 12.5% upon fertilizing COCs with a thick cumulus layer, whereas the IVF rate was comparable to that of WT spermatozoa when oocytes with a thin or no cumulus layer were fertilized. Finally, we proved that the IVF rate of dKO spermatozoa could be recovered by adding rat spermatozoa as a source of sperm hyal. Our results suggest that a deficiency of proteins involved in fertilization, such as sperm hyal, has a vital role in fertilization.

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INTRODUCTION

An ovulated cumulus–oocyte complex (COC) in the abdominal cavity is picked up by fimbriae of the fallopian tube and transported into the ampulla where it may be fertilized.[1–3] In mammals, the ovulated COC contains metaphase II-arrested oocytes surrounded by the zona pellucida (ZP), corona radiata cells, and cumulus cells that are embedded in the extracellular matrix which is rich in hyaluronic acid (hyaluronan) and is composed of repeating disaccharide units of N-acetyl-D-glucosamine and D-glucuronic acid.[4] Because fertilization requires spermatozoa to enter the COC and pass through the cumulus matrix, sperm hyaluronidase (hyals) is thought to enable acrosome-intact spermatozoa to reach the ZP through hyaluronan hydrolysis.[2] The role of sperm hyals in mammalian fertilization is a topic of ongoing debate. The presence of hyals protein on the surface of mammalian spermatozoa was first reported in the 1980s.[5] It was named PH-20 because of its original location on the posterior head of the spermatozoon, which migrates to the inner acrosomal membrane after acrosome reaction.[6–9] Although PH-20 was initially known to possess a hyals domain and ZP-binding domain, *PH-20*-knockout analysis results showed that it was hardly involved in ZP binding.[10] The PH-20 molecule was first named HYAL7 in this paper because it is highly homologous to the previously reported sperm hyals and possesses hyals activity.

Hyals must be present on the surface of spermatozoa for natural fertilization because the ovulated oocytes are surrounded by cumulus cells with high molecular weight hyaluronic acid. In the 1990s, different researchers confirmed the presence of hyals in the spermatozoa of mammals, including humans, and reported that sperm hyals is important for the interaction between spermatozoa and oocytes *in vitro*.[11,12] In mice, the seven hyals-like genes, *Hyal1, Hyal2, Hyal3, Hyal4, Hyal5, Hyal6,* and sperm adhesion molecule 1 (*Spam1*; *Hyal7*), are clustered as two tightly linked multiplets on chromosomes 9F1-9F2 and 6A2.[13,14] The two sperm-specific hyal isoforms, HYAL5 and HYAL7, are glycosylphosphatidylinositol (GPI)-anchored enzymes that are attached to the plasma membrane or the inner acrosomal membrane.[15] Furthermore, in mice, both *Hyal5* and *Hyal7* are single-copy genes located side by side on chromosome 6.

Baba *et al.*[10] demonstrated the function of sperm hyals, HYAL7. They demonstrated that HYAL7-null male mice had normal fertility. Furthermore, spermatozoa from *Hyal7*-knockout (KO) mice still

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exhibited hyal activity, and these mice produced litters 1 day later than the normal birth date. In 2005, Kim *et al.*[15] reported that mouse sperm expresses a novel hyals, HYAL5. Interestingly, HYAL5 is found on both plasma and acrosomal membranes, whereas HYAL7 is found only on the plasma membrane. Furthermore, HYAL7-deficient mice exhibit normal fertility, probably because the loss of HYAL7 was complemented by another protein.[16] Finally, in 2019, we produced double-knockout (dKO) mice deficient in both *Hyal7* and *Hyal5*. The dKO mice were not only 90% less fertile than their normal counterparts but also had a fertilization rate of less than 5% with *in vitro* fertilization (IVF).[17] However, the dKO male mice were not completely infertile indicating that other factors, in addition to the sperm hyal activity, might be involved in fertilization. The goal of the current study was to examine the effects of a decreased count of hyal-deficient spermatozoa and the condition of ovulated oocytes on fertilization rates in a mouse model.

MATERIALS AND METHODS

Animals

Male *Hyal5*/*Hyal7* dKO mice were produced from Microgen (Seoul, Korea)[17] and bred with C57BL/6N mice purchased from KOATECH Inc. (Pyeongtaek, Korea). All animals were housed under a 12 light:12 dark cycle (lights on: 7 a.m.–7 p.m.) at a constant temperature of 23°C ± 1°C and humidity of 50% ± 20% with free access to food and water. All animal experiments were approved by the Institutional Animal Care and Use Committee of Daegu-Gyeongbuk Medical Innovation Foundation (Daegu, Korea; Approval No. DGMIF-21021602-00).

Sperm collection

The sperm dishes were prepared with a 100 μl drop of FERTIUP® mouse sperm pre-incubation medium (Cosmo Bio, Tokyo, Japan) that was covered with mineral oil (Fisher Scientific, Pittsburgh, PA, USA), placed in a 35-mm sterile plastic dish, and incubated at 37°C under 5% (*v*/*v*) CO_2 for at least 30 min before sperm collection. The spermatozoa were collected from the cauda epididymidis of 3–5-month-old male mice and rats. Briefly, male mice were killed with CO_2, both epididymides were removed and punctured with a 26-gauge needle, and the released spermatozoa were then transferred to the sperm dish. The spermatozoa were capacitated by incubating them for 1 h before insemination.

IVF

IVF was performed by following the procedure described by Matsumura *et al.*[18] In brief, the fertilization dishes were prepared using a 200-μl drop of Center for Animal Resources and Development (CARD) medium (Cosmo Bio), and the washing dishes were prepared using four 80-μl drops of modified human tubal fluid (mHTF) medium that were covered with mineral oil, placed in 60-mm sterile plastic dishes (Corning, Glendale, AZ, USA), and incubated at 37°C under 5% CO_2 for at least 30 min before oocyte collection. Female mice (6–8-week-old) were superovulated via the intraperitoneal injection of pregnant mare's serum gonadotropin (7.5 IU; Prospec-Tany TechnoGene Ltd., Rehovot, Israel) followed by human chorionic gonadotropin (hCG; 7.5 IU; Sigma-Aldrich, St. Louis, MO, USA) 48 h later. Subsequently, 12–14 h after hCG injection, the mice were killed with CO_2, and their oviducts were removed and transferred to a fertilization dish containing mineral oil. The COCs were collected from the ampulla by using a needle and forceps in a stereo microscope and placed in the fertilization dish containing CARD medium. The capacitated spermatozoa were counted in a Makler sperm counting chamber (Graticules Optics, Cambridge, UK) and then mixed with a drop of CARD medium containing oocytes in the fertilization dish. After 3 h, the oocytes were washed three times in the washing dish, and the fertility was estimated 6 h after insemination.

Competitive IVF

To confirm the fertilizing ability of the dKO spermatozoa, we performed IVF with different concentrations of spermatozoa. Fertilized oocytes were incubated at 37.5°C under 5% CO_2. After 72 h, single-celled, two-celled, and morula stage embryos were collected, transferred to 5 μl lysis buffer (50 mmol l^{-1} Tris-HCl, 0.5% Triton X-100, 200 μl ml^{-1} proteinase K, pH 8.0), and incubated for 2 h at 55°C and then for 10 min at 95°C to inactivate proteinase K. The nature of the fertilizing spermatozoon was evaluated using nested PCR of DNA obtained from single-celled embryos. The first nested PCR reaction was carried out in 20 μl reaction mixture containing *Hyal7* forward primer-1 (F1: 5′-cacatagatgcaaaccaagcag-3′) and reverse primer-1 (R1: 5′-ggatactaggtcgagatgc-3′) and *Hyal5* reverse primer (HR: 5′-ctcccatatccagaagagag-3′). Two microliters of the first PCR product were used as a DNA template for the second PCR reaction that was conducted with other primer sets, including *Hyal7* forward primer-2 (F2: 5′-caagctcatttgcgcaaagc-3′) and reverse primer-2 (R2: 5′-cattcccaaccttggacact-3′) and *Hyal5* reverse primer. The final PCR product size for wild-type (WT) and dKO mice was estimated to be 523 bp and 212 bp, respectively.

Fertilization rate estimation

Fertilization of the oocytes was assessed by staining the pronucleus with a fluorescent dye, Hoechst 33342, followed by visualization of the stained inseminated oocytes using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany). The presence of two pronuclei and two polar bodies was indicative of successful fertilization.

Statistical analyses

All data are representative of at least three independent experiments unless otherwise stated. The results are expressed as the mean ± standard error of mean (s.e.m.). Student's *t*-test and one-way analysis of variance (ANOVA) followed by Duncan test were used for statistical analyses, and $P < 0.05$ was considered statistically significant.

RESULTS

Relationship between low sperm count and IVF rate in Hyal5/ Hyal7 dKO mice

Previously, we reported that mouse spermatozoa lacking hyals had a low fertilization rate, confirming the importance of sperm hyals in fertilization. To examine how hyals-deficient mouse spermatozoa would affect fertilization at a reduced concentration, we carried out IVF assays with different *Hyal5*/*Hyal7* dKO sperm counts.

When IVF was performed with 1×10^6 WT spermatozoa, a fertilization rate of 74.4% was achieved, whereas with 1×10^5 and 1×10^4 WT spermatozoa, the rate was found to be 45.6% and 26.5%, respectively. Furthermore, IVF using different dKO sperm numbers resulted in fertilization rates of 13.7%, 6.5%, and 3.0% at 1×10^6, 1×10^5, and 1×10^4, respectively (**Figure 1** and **Supplementary Table 1**). Thus, a decrease in the fertilization rate was observed with decreased sperm number in both experimental groups; however, the fertilization rate of *Hyal5*/*Hyal7* dKO spermatozoa was significantly lower than that of WT spermatozoa.

Fertilization of COCs by Hyal5/Hyal7 dKO spermatozoa

Naturally ovulated oocytes are completely surrounded by a thick layer of cumulus. The thick layer of the oocytes collected in the current study was either symmetrical (type I, **Figure 2a**) or asymmetrical

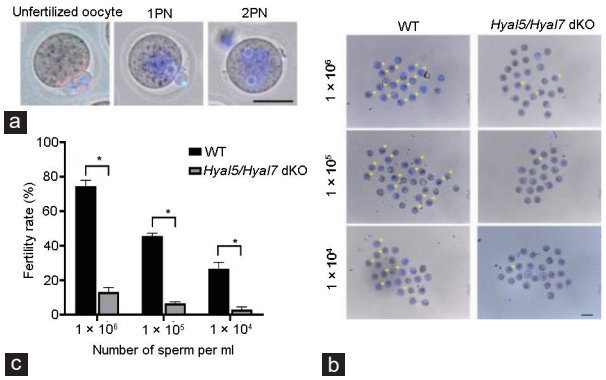

Figure 1: Fertilized oocytes following IVF by different sperm numbers from wild-type (WT) and *Hyal5/Hyal7* dKO mice. Cauda epididymidal spermatozoa at different numbers (1.5×10^6, 1.5×10^5, and 1.5×10^4 cells) from WT and *Hyal5/Hyal7* dKO mice were incubated with COCs in a 200 µl TYH drop for 6 h. (**a**) Three types of oocytes were stained with Hoechst 33342 dye. The presence of two pronuclei indicated fertilization, whereas a single pronucleus (haploid) in the oocyte indicated that a spermatozoon did not enter the oocyte. Scale bar = 50 µm. (**b**) The presence of oocytes with two pronuclei were defined as "fertilized oocytes". The oocytes with two pronuclei are indicated by asterisks. Pronuclei were stained with Hoechst 33342 dye (blue). Scale bar = 100 µm. (**c**) Rate of fertilization. Significant ($P < 0.05$) differences are indicated by an asterisk. The results of IVF were expressed as the mean ± s.e.m. of three separate experiments. *Hyal5*: hyaluronidase 5; IVF: *in vitro* fertilization; WT: wild-type; dKO: double-knockout; COCs: cumulus–oocyte complexes; s.e.m.: standard error of mean.

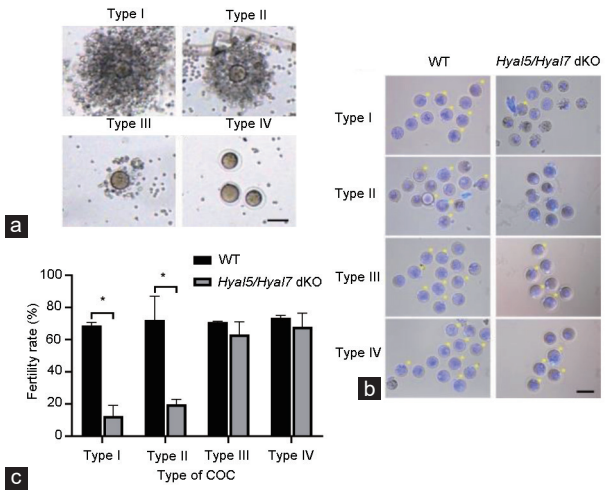

Figure 2: IVF by hyaluronidase-deficient mouse spermatozoa and various types of COCs. (**a**) Criterion for determining the status of mouse oocytes based on the surrounding cumulus cell mass. The status of the COCs was categorized as follows: type I, the oocytes were tightly packed with cumulus cells; type II, the oocytes had a loosely associated cumulus mass but still had many cumulus cells; type III, the oocytes had a few cumulus cells; and type IV, the oocytes completely lost cumulus cells (naked eggs). Scale bar = 100 µm. (**b**) Two pronuclei are indicated by asterisks. Pronuclei were stained with Hoechst 33342 (blue). Scale bar = 100 µm. (**c**) IVF assay of *Hyal5/Hyal7* dKO spermatozoa. The fertilization rate of four types of COCs inseminated with capacitated cauda epididymidal spermatozoa from WT (black column) and *Hyal5/Hyal7* dKO (shaded column) mice is shown. Significant ($P < 0.05$) differences are indicated by an asterisk. The results of IVF are expressed as the mean ± s.e.m. of three separate experiments. *Hyal5*: hyaluronidase 5; IVF: *in vitro* fertilization; WT: wild-type; dKO: double-knockout; COCs: cumulus–oocyte complexes; s.e.m.: standard error of mean.

(type II, **Figure 2a**). Furthermore, the oocytes were surrounded by a few cumulus cells in a few cases (type III, **Figure 2a**), and very few oocytes were completely naked (type IV, **Figure 2a**). Hence, we examined the effects of the thickness of COC on the fertilizing efficiency of spermatozoa (**Figure 2b** and **Supplementary Table 2**). The IVF rate between the WT and *Hyal5/Hyal7* dKO spermatozoa for these different types of oocytes was compared. As shown in **Figure 2c**, the *Hyal5/Hyal7* dKO spermatozoa showed a significantly lower fertilization rate than their WT counterparts when the COC layer was thick (type I and II), indicating that *Hyal5/Hyal7* dKO spermatozoa were inefficient in penetrating the thick cumulus layer. However, the fertilization rate of WT and *Hyal5/Hyal7* dKO spermatozoa was comparable (67.9% ± 8.5% and 63.1% ± 8.1%, respectively) when the oocytes were surrounded by a thin cumulus layer (type III) or had no cumulus layer at all (naked, type IV). Furthermore, mating a *Hyal5/Hyal7* dKO male mouse with a WT female mouse yielded 1.7 pups on an average. These results indicate that *Hyal5/Hyal7* dKO spermatozoa lacking hyals activity can fertilize oocytes with a thin or no cumulus layer.

Effect of the addition of rat spermatozoa on IVF with Hyal5/Hyal7 dKO spermatozoa

To evaluate the importance of hyals in mammalian fertilization, we performed IVF using *Hyal5/Hyal7* dKO spermatozoa mixed with different numbers of rat spermatozoa. Interestingly, *Hyal5/Hyal7* dKO mouse spermatozoa, which are deficient in hyals activity, could fertilize oocytes in the presence of rat spermatozoa (**Figure 3** and **Supplementary Table 3**). Moreover, by adding rat spermatozoa, the IVF rate of 1×10^4 *Hyal5/Hyal7* dKO spermatozoa, which is a low number, was increased from 4.2% to 21.5%, similar to that of WT mouse spermatozoa.

Competitive IVF with WT and Hyal5/Hyal7 dKO spermatozoa

We performed a competitive IVF assay using mixed populations of WT and *Hyal5/Hyal7* dKO spermatozoa at different numbers to ascertain

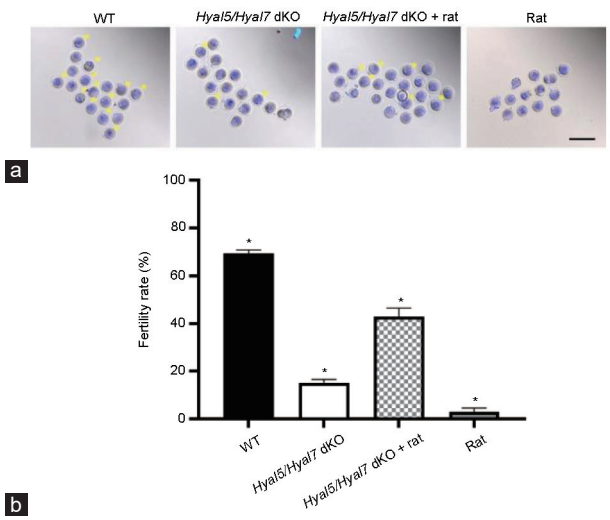

Figure 3: IVF using *Hyal5/Hyal7* dKO spermatozoa mixed with rat spermatozoa. Caudal epididymidal sperm of WT mice (black column), *Hyal5/Hyal7* dKO mice (open column), *Hyal5/Hyal7* dKO mice and rat spermatozoa (mosaic column), and only rat spermatozoa (gray) were incubated with mouse oocytes for 6 h. (**a**) The oocytes containing female and male pronuclei were defined as "fertilized oocytes". Scale bar = 50 µm. Pronuclei (indicated by an asterisk) were stained with Hoechst 33342 (blue). (**b**) The fertilization rate. Significant ($P < 0.05$) differences are indicated with an asterisk. *Hyal5*: hyaluronidase 5; IVF: *in vitro* fertilization; WT: wild-type; dKO: double-knockout.

whether *Hyal5/Hyal7* dKO spermatozoa are functionally inferior to WT spermatozoa in penetrating the cumulus matrix, even though

they were similar in structure and activity, except for the hyals activity. Thus, if the hyals do not affect fertilization, *Hyal5/Hyal7* dKO-derived fertilized oocytes will be produced when IVF is performed by mixing WT and *Hyal5/Hyal7* dKO spermatozoa in equal proportions. The IVF rate for the 1:1 ratio was 57.9%, and all morulae were from WT spermatozoa (**Figure 4** and **Table 1**). As the proportion of *Hyal5/Hyal7* dKO spermatozoa was increased, the formation of heterozygotes was observed among the developed morulae (8.3%, 9.1%, and 8.3% for 1:2, 1:3, and 1:10 of WT:*Hyal5/Hyal7* dKO, respectively). However, the majority of the morulae were still from WT spermatozoa, indicating that *Hyal5/Hyal7* dKO spermatozoa were comparatively less efficient in fertilization. The IVF rates for 1:2, 1:3, and 1:10 ratios were 66.7%, 52.3%, and 57.1%, respectively.

DISCUSSION

This study demonstrates why sperm hyals are important for mammalian male fertilization on the basis of *Hyal5*/*Hyal7* dKO mice. It is known that men remain fertile throughout most of their life, producing millions of spermatozoa everyday, whereas female fertility is limited to the period between puberty and menopause. Environmental and genetic factors not only affect the human reproductive system but also increase the risk of infertility by reducing sperm production.

In the present study, we investigated the effects of a decrease in the number of spermatozoa without hyals activity on fertilization. There are many proteins on the surface of the spermatozoon, and abnormalities in these proteins can lead to infertility. Ejaculated spermatozoa face some obstacles in meeting with the oocyte and subsequent successful fertilization. The first hurdle is whether it has the ability to pass through the uterotubal junction.[19] Cho *et al.*[20] and Nishimura *et al.*[21] reported that testis-specific knockout of a disintegrin and metalloprotease 1a (*ADAM1a*) resulted in infertile male mice. Although ADAM1a is a testicular cell-specific protein that does not exist on the sperm surface, it was found that infertility was caused by the lack of movement to the sperm surface during spermatogenesis of ADAM3. In addition, *ADAM3* knockout male mice also lacked reproductive ability because their sperm failed to pass through the uterotubal junction.[21] Therefore, they suggested that ADAM3 is important for sperm migration through the uterotubal junction. In the second hurdle, the fertilization is successful only when the spermatozoon passes through the COC. We confirmed in our last study that sperm hyals play an important role in dispersing the COC layer.[15]

The first purpose of this study was to determine how a small number of hyals-deficient spermatozoa affect fertilization. The results reveal that a decrease in the sperm number led to a decline in the IVF rate. Moreover, the degree of decline was higher in *Hyal5/Hyal7* dKO mice than that in WT mice, indicating that the decrease in sperm number exacerbated the low IVF rate caused by the poor sperm quality. The results demonstrate that the probability of infertility increases further if the sperm number decreases and the molecules involved in fertilization, such as hyals, are deficient. On the other hand, it has been reported that hyaluronic acid fragments (HAFs) mediate the activation of ovulated COCs by interacting with CD44 and inducing the nuclear factor kappa B (NF-κB) pathway.[22,23] Although direct evidence for the functional role of HAFs has not been established in this study, HAFs do not appear to be essential for other fertilization events, given that there is no difference between the fertilization rate of IVF with WT and *Hyal5/Hyal7* dKO spermatozoa when the COC layer was removed from the oocyte.

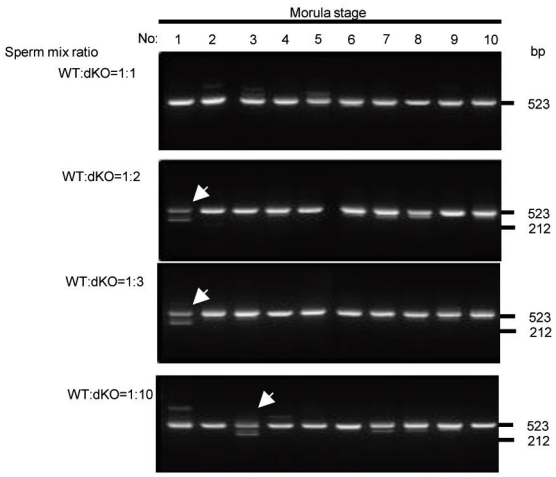

Figure 4: Competitive fertilization of COCs with a mixed population of WT and *Hyal5/Hyal7* dKO spermatozoa. PCR analysis of the genomic DNA obtained from the embryos resulting from *in vitro* fertilization. Metaphase II-arrested mouse oocytes with intact cumulus cells were incubated with an equally mixed suspension of WT and *Hyal5/Hyal7* dKO mouse spermatozoa. After initial incubation for 6 h, the fertilized oocytes were further incubated for 96 h. Genomic DNA was prepared from each of the developing embryos and then used as a template for PCR amplification. Two DNA fragments of 523 bp and 212 bp, originating from the WT and *Hyal5/Hyal7* KO alleles, respectively, were detected by 1.2% agarose gel electrophoresis. *Hyal*: hyaluronidase; WT: wild-type; dKO: double-knockout; COCs: cumulus–oocyte complexes.

Table 1: Heterozygous rate of double-knockout sperm mixed with wild-type sperm

Sperm mix ratio (WT: dKO)	*Total (n)*	*Morula*		
		Embryos analyzed (n)	*Heterozygous, n (%)*	*WT embryos, n (%)*
1:1 (1×10^5:1×10^5)	19	11	0 (0)	11 (100.0)
1:2 (1×10^5:2×10^5)	18	12	1 (8.3)	11 (91.7)
1:3 (1×10^5:3×10^5)	21	11	1 (9.1)	10 (90.9)
1:10 (1×10^5:1×10^6)	21	12	1 (8.3)	11 (91.7)

dKO: double-knockout; WT: wild-type

Next, we evaluated the effects of COC thickness on the IVF rate because, although sperm conditions are important in natural fertilization, ovulation conditions are also very important. When we obtained COCs from a female mouse, we could isolate four different types of oocytes with cumulus layers exhibiting varying degrees of thickness. The thickness and shape of the COC seem to be dependent on the ovulation condition and location of the oocytes in the female reproductive system. Oocytes with different COC thicknesses clearly showed a difference in the fertilization rate, proving that the *Hyal5/Hyal7* dKO male mice are not completely sterile.

Further, to study the role of sperm hyals in fertilization, we performed competitive IVF assays by mixing *Hyal5/Hyal7* dKO and WT spermatozoa. We examined whether the *Hyal5/Hyal7* dKO spermatozoa could result in fertilization when the two are mixed. When the WT:*Hyal5/Hyal7* dKO sperm ratios were 1:2 or 1:3, approximately 8% of the morulae were from dKO spermatozoa, whereas a 1:1 ratio did not yield any morula from *Hyal5/Hyal7* dKO genotype. Despite the higher proportion of dKO spermatozoa in the mixture, the *Hyal5/Hyal7* dKO spermatozoa participating in fertilization remained unchanged. The results indicate that hyals-deficient sperm cannot fertilize the

oocytes, even in the presence of neighboring WT spermatozoa. In industrialized societies, a low sperm count along with hyals-deficient sperm could be a more serious problem. However, a low IVF rate with *Hyal5/Hyal7* dKO spermatozoa could be raised by adding a source of functional hyals. For example, the addition of rat sperm improved the IVF rate of *Hyal5/Hyal7* dKO spermatozoa considerably.

The final stage of mammalian fertilization is the process by which two gametes fuse after the sperm penetrates the ZP layer. Earlier studies based on *in vitro* experiment suggested that sperm hyals has a functional ZP binding domain in the C-terminal region, in addition to the hyals domain in the N-terminal region. In type IV oocytes with nearly eliminated COC layers, IVF with spermatozoa from dKO mice lacking sperm hyals resulted in IVF rates similar to those when WT spermatozoa were used. Therefore, sperm hyals is not involved in binding with or penetrating the ZP. The final stage of fertilization is known to involve IZUMO1.[24] Mouse spermatozoa that are deficient in IZUMO1 lose their ability to fertilize owing to a lack of binding capacity with the oocyte plasma membrane.

CONCLUSIONS

In this study, we demonstrated that the function of sperm hyals and ovulation status are important factors in mammalian fertilization. In other words, as the number of *Hyal5/Hyal7* dKO spermatozoa decreased, the IVF fertilization rate decreased more than that with WT spermatozoa, and when rat spermatozoa were added, the fertilization rate was partially recovered. In addition, the IVF rates differed depending on the ovulation status of female mice. *Hyal5/Hyal7* dKO spermatozoa could not fertilize oocytes with a thick COC layer, whereas they could fertilize oocytes with a thin or no cumulus layer. However, further investigations are needed to establish whether monotocous animals without sperm hyals are infertile.

AUTHOR CONTRIBUTIONS

DWS, YHK, BSS, BWS, SUK, SP, and EK performed experiments. SHJ, GW, and EK contributed to the design, and writing draft and final manuscript. GW and EK contributed to the study concept, design, and administration. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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Supplementary Table 1: Fertility rate by sperm concentration

Sperm concentrations	*Control (%)*	*dKO (%)*
$1\times10^{6\dagger}$	74.4±3.43 (38/52)*	13.7±2.64 (8/64)
$1\times10^{5\dagger}$	45.6±1.60 (32/70)*	6.5±0.98 (4/65)
$1\times10^{4\dagger}$	26.5±3.85 (15/59)*	3.0±1.50 (2/61)

†Within the same low, *Significantly different at $P<0.05$. Data are shown as mean (%)±SEM of three replications. SEM: standard error of mean; dKO: double-knockout

Supplementary Table 2: Fertility rate by cumulus–oocyte complex type

COC type	*Control (%)*	*dKO (%)*
I†	68.7±2.04 (23/34)*	12.5±6.57 (4/28)
II†	72.2±14.70 (21/29)*	19.8±3.10 (6/30)
III†	71.0±0.48 (22/31)	63.1±8.06 (18/28)
IV†	73.5±1.47 (24/33)	67.9±8.54 (21/31)

†Within the same low, *Significantly different at $P<0.05$. Data are shown as mean (%)±SEM of three replications. SEM: standard error of mean; dKO: double-knockout; COC: cumulus–oocyte complex

Supplementary Table 3: Fertility rate by sperm type

Control (%)	*dKO*	*dKO + rat*	*Rat*
69.4±1.41 (29/42)[d]	15.2±1.33 (9/60)[b]	42.8±3.62 (20/46)[c]	3.1±1.55 (2/49)[a]

The different letters indicate $P<0.05$. Data are shown as mean (%)±SEM of three replications. SEM: standard error of mean; dKO: double-knockout