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Effect of N-Acetyl-D-Glucosamine on Bovine Sperm-Oocyte Interactions

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Abstract. N-Acetyl-D-glucosamine (GlcNAc) is a major component of glycosaminoglycan, which is involved in sperm-oocyte interactions. We examined the effect of adding GlcNAc and other monosaccharides, D-mannose and D-fucose, to the *in vitro* fertilization (IVF) medium on bovine sperm-oocyte interactions. In medium in which sperm and a zona pellucida (ZP) were co-incubated with monosaccharides for 5 min, addition of GlcNAc (5 or 25 mM) significantly reduced the number of sperm that attached to the ZP. Pretreatment of gametes with GlcNAc (5 mM) prior to co-incubation also suppressed sperm-ZP attachment. Addition of GlcNAc (5 or 25 mM) to the medium in which sperm and a ZP were co-incubated for 5 h, however, significantly increased the number of sperm binding to and penetrating the ZP in a concentration-related manner. The other monosaccharides, D-fucose and D-mannose, did not have this effect. Supplementation of the sperm-oocyte co-incubation medium with 5 mM GlcNAc also enhanced the rate of polyspermic fertilization. When the ZPs were removed from the oocytes, GlcNAc did not affect the fertilization rate. Furthermore, incubation of sperm with 5 mM GlcNAc induced sperm membrane destabilization and an acrosome reaction, as evidenced by the hypo-osmotic swelling test and fluorescein isothiocyanate-labeled peanut agglutinin/propidium iodide (FITC-PNA/PI) staining. Finally, GlcNAc suppressed ZP hardening following fertilization, as determined by measuring the time required for pronase to dissolve the ZP. In conclusion, supplementation of IVF medium with GlcNAc has various effects on sperm-oocyte interactions including suppression of initial attachment, induction of sperm membrane destabilization and acrosome reaction, increase in the number of sperm secondarily bound to and penetrating the ZP, suppression of ZP hardening following sperm-oocyte co-incubation and increase in the rate of polyspermic fertilization.

Key words: Bovine, Fertilization, Monosaccharides, Sperm, Zona pellucida

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Fertilization of oocytes comprises several interactions between the sperm and oocyte. Contact between the sperm and oocyte first occurs at the outer surface of the surrounding extracellular matrix of the egg, the zona pellucida (ZP). Binding of the lectin-like protein on the sperm head to distinct oligosaccharides of ZP glycoproteins leads the capacitated sperm to recognize the oocyte. Upon anchoring of the sperm to the oocyte through the ZP carbohydrates, a signaling cascade induces the acrosome reaction of the sperm, allowing the sperm to pass through the ZP. The acrosome-reacted, penetrated sperm fuses with the egg plasma membrane, which enables the egg to complete meiosis, and initiates mechanisms to prevent polyspermic fertilization by the exocytosis of cortical granules (CG) [1]. Oligosaccharides on the ZP include some monosaccharides, such as N-acetyl-D-glucosamine (GlcNAc), D-mannose and D-fucose [2], and these monosaccharides are involved in many sperm-oocyte interactions. For example, in sperm-oocyte recognition, N-acetyl-D-glucosaminidase (NAG) inhibits sperm binding to the ZP in the human [3, 4]. The mouse sperm has 20-fold more NAG compared with other enzymes [5], and recognition of GlcNAc on the ZP glycoprotein ZP3 by β 1-4-galactosyltransferase on the sperm acrosome membrane is dependent on the initial attachment of the sperm to the ZP [6, 7]. Monosaccharides are involved in sperm capacitation and the

acrosome reaction. During capacitation, NAG, a lectin-like protein that recognizes GlcNAc, is increased in the human sperm [8] and allows capacitated sperm to recognize a decrease in fucose compared with un-capacitated sperm, which lack this ability, in the bovine [9]. In addition, GlcNAc induces the sperm acrosome reaction, and NAG inhibits the sperm acrosome reaction caused by GlcNAc in the human [3, 4, 10]; however, GlcNAc suppresses the acrosome reaction in the hamster [11]. Miller *et al.* [12] reported that NAG is contained in the CG and affects carbohydrate chains on the ZP, which inactivates the sperm-binding site on the ZP in the mouse. Furthermore, enzymes that are contained in the CG and released following sperm-oocyte fusion target the monosaccharides in the ZP [13, 14]. These findings suggest that addition of monosaccharides to the sperm-oocyte co-incubation medium will affect sperm-oocyte interactions and alter the efficiency of sperm-oocyte interactions *in vitro*. In the bovine, addition of 50 mM GlcNAc, D-mannose or D-fucose to the sperm-oocyte co-incubation medium decreases the fertilization rate [15]; in the hamster, addition of 1 mM GlcNAc to the sperm-oocyte co-incubation medium decreases the number of sperm bound to and penetrated through the ZP [11]. Few studies, however, have examined the effect of adding monosaccharides to the bovine sperm-oocyte co-incubation medium at each step of the sperm-oocyte interaction. The primary aim of the present study was to examine the effect of supplementation of the sperm-oocyte incubation medium with monosaccharides (GlcNAc, D-mannose and D-fucose) at various

steps during sperm-ZP interaction, such as at sperm-ZP primary attachment, secondary (tight) binding and penetration and the effect on fertilization rate, sperm membrane integrity, sperm acrosome reaction and polyspermy block.

Materials and Methods

Experimental design

Experiments were conducted to examine sperm-oocyte interactions (Expts. 1–4), fertilization rate (Expts. 5, 6), sperm membrane integrity (Expt. 7), acrosome reaction state (Expt. 8) and polyspermy block (Expt. 9).

In Expts. 1 and 2, we first evaluated the effect of monosaccharides (GlcNAc, D-mannose or D-fucose) on primary sperm-ZP attachment after a short period (5 min) of co-incubation. In Expt. 1, each monosaccharide (5 or 25 mM) was added to an IVF medium in which sperm and ZPs were co-incubated, and the number of sperm attached to the ZP was assessed (see the sperm-oocyte interaction section). In Expt. 2, to examine whether GlcNAc affects the sperm or ZP, the ZP and sperm were separately pretreated with GlcNAc (5 mM) for 5 min before co-incubation (see ZP preparation), and the number of sperm attached to the ZP was compared between selective combinations, non-treated sperm and non-treated ZP (control group) vs. non-treated sperm and pretreated ZP or control group vs. pretreated sperm and non-treated ZP.

In Expts. 3 and 4, we examined the effect of either GlcNAc, D-mannose or D-fucose on the number of sperm that secondarily (tightly) binding to the ZP or penetrating through the ZP. Oocytes and sperm were co-incubated for 5 h with or without each monosaccharide at various concentrations (0, 5 or 25 mM; see sperm-ZP interaction section).

In Expts. 5 and 6, we examined the effect of GlcNAc (0 or 5 mM) on the fertilization rate. Either sperm and denuded oocytes (Expt. 5) or sperm and ZP-free denuded oocytes (ZP-free oocytes; Expt. 6) were co-incubated for 5 h, and the fertilization rate was assessed.

In Expts. 7 and 8, the membrane integrity (Expt. 7) and acrosome reaction (Expt. 8) were assessed by HOST and PNA/PI staining. Sperm were incubated in IVF medium containing GlcNAc (0 or 5 mM; see the HOST and PNA/PI staining sections).

In the final expt. (Expt. 9), we examined polyspermy block. Sperm and denuded oocytes or ZPs were co-incubated for 5 h, and the effect of GlcNAc on changes in the ZP during fertilization was assessed (as described in the ZP solubility assay; see Fig. 2).

Culture media and chemicals

Monosaccharides (GlcNAc, D-fucose and D-mannose) and all other chemicals were purchased from Nacalai Tesque (Kyoto, Japan), unless otherwise indicated. The medium used for oocytes and sperm treatment was modified synthetic oviductal fluid (SOF) [16] supplemented with 5 mM taurine and 1 mM glutamine. *In vitro* maturation (IVM) medium, comprising SOF supplemented with 10% fetal bovine serum (FBS; ICN, Costa, Mesa, CA, USA) and 5.56 mM glucose, was used for oocyte maturation. *In vitro* fertilization (IVF) medium, comprising SOF with 5 mg/ml bovine serum albumin and 10 IU/ml heparin (Sigma Aldrich, St. Louis,

MO, USA), was used for sperm-oocyte or sperm-ZP co-incubation. *In vitro* culture (IVC) medium, comprising SOF with 1% FBS and 1.5 mM glucose, was used for embryo culture.

Oocyte collection and maturation

Bovine ovaries were collected from carcasses at a slaughterhouse, preserved in physiologic saline at approximately 35 °C and transported to the laboratory within 4 h. Cumulus-oocyte complexes (COCs) were aspirated from small antral follicles (3–6 mm in diameter) using a 20-G needle connected to a 10-ml syringe. COCs surrounded by compact and thick cumulus cells were randomly selected and cultured in 100 μ l IVM medium (10 oocytes/drop) under paraffin oil at 39 °C in an atmosphere of 5% CO₂ in air with maximum humidity. After 21 h of incubation, COCs were treated with 0.2% hyaluronidase for 6 min and then vortexed for 6 min to denude the oocytes. To obtain ZP-free oocytes, denuded oocytes were incubated in acid Tyrode's solution and then washed with IVF medium.

ZP preparation

ZPs were collected from denuded oocytes with a narrow-pulled Pasteur pipette (diameter, 120 μ m). The ZPs were washed with IVF medium and used for the Expts. To prepare pretreated ZPs with GlcNAc (used in Expt. 2; see experimental design), ZPs were incubated in IVF medium containing GlcNAc (0 or 5 mM) for 5 min and washed 3 times with IVF medium.

Sperm preparation

Samples of frozen-thawed semen from three different bulls were combined into one test tube. For pretreatment of sperm with GlcNAc (Expt. 2), IVF medium containing 10 mM GlcNAc was added to an equal volume of semen suspension to obtain a final concentration of 5 mM GlcNAc, and the mixture was incubated for 5 min. Either frozen-thawed (non-treated sperm) or pretreated sperm were washed with a 30% to 60% discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient solution by centrifugation (700 $\times$ g, 10 min). After centrifugation, the semen was diluted with IVF medium and used for all Expts. at a final concentration of 6×10^5 cell/ml.

Assessment of sperm-ZP interactions

Sperm-ZP interactions were examined with regard to (1) primary sperm-ZP attachment after a short co-incubation period (5 min), (2) secondary sperm-ZP binding after a long co-incubation period (5 h) and (3) sperm penetration of ZP after a long co-incubation period (5 h; Fig. 1).

To evaluate the first step (Expt. 1), sperm and ZPs were co-incubated for 5 min in IVF medium containing a monosaccharide (GlcNAc, D-mannose or D-fucose) at various concentrations (0, 5 or 25 mM), and the ZPs were then washed with a fine-pulled Pasteur pipette (diameter, 200 μ m) to remove loosely attached sperm. The ZPs were mounted on glass slides and stained with Hoechst 33342, and the number of sperm attached to the ZPs was counted under a fluorescence microscope (Olympus, Tokyo, Japan). To assess the second step (Expt. 3), sperm and ZPs were co-incubated for 5 h in IVF medium with or without each monosaccharide at var-

ious concentrations (0, 5, 25 mM), and the ZPs were washed by maximum power vortexing for 5 sec. The ZPs were then mounted on glass slides and stained with Hoechst 33342, and the number of sperm attached to the ZPs was counted. To assess sperm penetra-

tion of the ZP (Expt. 4), sperm and denuded oocytes were co-incubated in IVF medium with or without each monosaccharide (0, 5, 25 mM) for 5 h. After co-incubation, denuded oocytes were washed by maximum power vortexing for 5 sec and then transferred to aceto-alcohol (1:3) to remove the ZP. These ZP-free oocytes were mounted on glass slides and stained with Hoechst 33342, and the number of sperm attached to the oolemma was counted. Ten ZPs or denuded oocytes were used for each replicate of each Expt. group. Experiments were conducted 3 times using a distinct oocytes series, and a total of either 30 ZPs or 30 denuded oocytes were used for each experimental group.

Fig. 1. Schematic explanation of the assessment of sperm-zona pellucida (ZP) interactions. 1) For evaluation of primary attachment of sperm-ZP in Expts. 1 and 2, sperm and ZPs were co-incubated with or without various monosaccharides for 5 min. The number of sperm attached to a ZP was assessed after washing by gentle pipetting. 2) For evaluation of sperm-ZP binding, sperm and ZPs were co-incubated with or without various monosaccharides for 5 h. The number of sperm that secondarily (tight) bound to a ZP was assessed after washing by strong vortexing. 3) To examine the penetration ability of sperm into a ZP, sperm and oocytes were co-incubated for 5 h, and then the number of sperm attached to the oolemma was assessed after washing by strong vortexing and dissolution of the ZP.

Assessment of fertilization rate

Denuded oocytes or ZP-free oocytes were washed with IVF medium and transferred to a 100- μ l sperm suspension (6×10^5 cell/ml) for fertilization (10 oocytes/drop) and then cultured with sperm for 5 h at 39 C in an atmosphere of 5% CO_2 in air with maximum humidity. After co-incubation, oocytes were gently washed with a finely pulled Pasteur pipette (diameter, 200 μ m) to remove loosely attached sperm and then cultured in 100 μ l IVC medium (10 zygotes/drop) for 16 h. For each replicate, 20 oocytes were fertilized and 15 randomly selected oocytes were used to assess the fertilization rate. The Expt. was repeated 4 times with a different series of oocytes. A total of 60 oocytes were used for each Expt. group. To assess the fertilization rate, oocytes were fixed in aceto-alcohol (1:3) and stained with orcein after 16 h of IVC.

Assessment of sperm membrane and acrosome state

Hypo-osmotic swelling test: Frozen-thawed and washed sperm were incubated in IVF medium containing GlcNAc (0 or 5 mM) for 0, 3 or 5 h. A hypo-osmotic swelling test (HOST) was conducted as previously described [17], with slight modifications. In brief,

Fig. 2. Solubility assay of the zona pellucida (ZP). a) Matured oocytes were co-incubated with sperm in the presence or absence of 5 mM GlcNAc for 5 h, and then ZPs were isolated from the ova. After washing, the isolated ZPs were transferred into 0.1% pronase solution, and the time of complete dissolution of the ZPs was measured. b) ZPs of matured oocytes were isolated from the ova and then co-incubated with sperm in the presence or absence of 5 mM GlcNAc for 5 h. The ZPs were washed and transferred into 0.1% pronase solution, and the time required for complete dissolution of the ZPs was measured.

Fig. 3. Schematic explanation of the effects of GlcNAc on sperm-oocyte interactions. 1) GlcNAc interacts with a part of the sperm receptor that binds to glycans, including GlcNAc, on the zona pellucida (ZP) and then interferes with normal attachment between sperm and the ZP. 2) The GlcNAc that interacts with the sperm receptor induces membrane destabilization. The membrane destabilization quickly induces the acrosome reaction. 3) GlcNAc also suppresses ZP hardening, probably via inhibition of the CG-derived enzyme activity that induces ZP hardening, resulting in an increase of the number of sperm binding to and penetrating the ZP. 4) Finally, addition of GlcNAc to an IVF medium induces polyspermic fertilization.

each incubated sperm sample was diluted with hypo-osmotic solution (sodium citrate-2H₂O, 0.49 g, and fructose, 0.9 g/100 ml), incubated for 45 min, and then stained with propidium iodide (PI, 12 mM, Sigma Aldrich). The number of sperm with coiled flagella and negative fluorescent heads was counted under a fluorescence microscope (Olympus, Tokyo, Japan). For each replicate, a total of 200 sperm were examined for each Expt. group. The Expt. was repeated 4 times.

Sperm PNA/PI staining: Frozen-thawed and washed sperm were incubated in the IVF medium containing GlcNAc (0 or 5 mM) for 0 to 5 h. The acrosome state of the sperm was examined by FITC-PNA/PI (Sigma Aldrich) staining. Sperm PNA/PI staining was conducted as previously described [18]. Briefly, incubated sperm were exposed to PNA (10 µg/ml) and PI (12 mM) for 5 min at 38.5 °C and fixed in 4% paraformaldehyde in 0.5 M Tris buffer (pH 7.4). Acrosome state was determined on the basis of the criteria described by Siciliano *et al.* [18]. The number of acrosome-reacted sperm was counted under a fluorescence microscope (Olympus, Tokyo, Japan). For each replicate, a total of 200 sperm were examined for each Expt. group. The Expt. was repeated 4 times.

Assessment of polyspermy block (ZP solubility assay)

The ZP digestion methods were based on a previous report described by Coy *et al.* [19] with a few modifications, and the methods are depicted in Fig. 2. Matured denuded oocytes or ZPs collected from matured oocytes were co-incubated for 5 h with or without sperm in IVF medium containing GlcNAc (0 or 5 mM). After treatment, both ZPs and denuded oocytes were washed to

Table 1. Effect of various monosaccharides on primary sperm-zona pellucida (sperm-ZP) attachment

Carbohydrates ^A	Concentration (mM)	No. of ZPs	No. of sperm/ZP ^B
—	—	30	56.9 ± 2.9 ^a
GlcNAc	5	30	43.5 ± 2.3 ^b
	25	30	45.3 ± 1.7 ^b
D-mannose	5	30	54.9 ± 3.7 ^a
	25	30	54.1 ± 3.1 ^a
D-fucose	5	30	52.3 ± 3.1 ^a
	25	30	52.2 ± 2.9 ^a

^A Sperm and ZPs were co-incubated for 5 min in a medium containing GlcNAc, D-mannose or D-fucose. ^B Mean ± SE

Table 2. Effect of pretreatment of sperm or zona pellucida (ZP) with GlcNAc on sperm-zona pellucida primary attachment

Treatment ^A	GlcNAc (5 mM)	No. of ZPs	No. of sperm/ZP ^B
Zona pellucida	—	30	56.6 ± 3.1 ^a
	+	30	49.1 ± 2.3 ^b
Sperm	—	30	57.2 ± 4.9 ^c
	+	30	13.9 ± 1.7 ^d

^A Sperm or ZPs were treated with or without GlcNAc for 5 min before co-incubation. ^B Mean ± SE of the number of sperm attached to the ZP. ^{a-b,c,d} Values with different superscripts differ significantly (P<0.01).

Table 3. Effect of various monosaccharides on secondary (tight) sperm-zona pellucida (sperm-ZP) binding

Carbohydrates ^A	Concentration (mM)	No. of ZPs	No. of sperm/ZP ^B
—	—	30	40.6 ± 2.8 ^a
GlcNAc	5	30	47.5 ± 1.8 ^b
	25	30	66.7 ± 2.1 ^c
D-mannose	5	30	38.3 ± 2.1 ^a
	25	30	48.9 ± 2.8 ^b
D-fucose	5	30	39.4 ± 1.8 ^a
	25	30	48.7 ± 1.6 ^b

^A Sperm and ZPs were co-incubated for 5 h in a medium containing GlcNAc, D-mannose or D-fucose. ^B Mean ± SE of the number of sperm attached to the ZP. ^{a-c} Values with different superscripts differ significantly ($P < 0.01$).

Table 4. Effect of monosaccharides on sperm penetration through the zona pellucida

Carbohydrates ^A	Concentration (mM)	No. of ZPs	No. of sperm/ZP ^B
—	—	30	5.0 ± 0.5 ^a
GlcNAc	5	30	7.9 ± 1.0 ^b
	25	30	10.0 ± 1.0 ^c
D-mannose	5	30	5.0 ± 0.5 ^a
	25	30	4.6 ± 0.5 ^a
D-fucose	5	30	5.2 ± 0.5 ^a
	25	30	4.9 ± 0.6 ^a

^A Sperm and ZPs were co-incubated for 5 h in a medium containing GlcNAc, D-mannose or D-fucose. ^B Mean ± SE of the number of sperm attached to the oolemma. ^{a-c} Values with different superscripts differ significantly ($P < 0.01$).

Table 5. Effect of GlcNAc on the fertilization rates of denuded oocytes

GlcNAc ^A (5 mM)	No. of oocytes	No. of replicates	Rate of fertilization (%) ^B		
			Norm.	Poly.	Non.
—	60	4	43.3 ± 2.8	18.7 ± 3.9 ^a	38.0 ± 7.1
+	60	4	34.6 ± 1.8	41.7 ± 3.1 ^b	24.7 ± 3.9

^A Sperm and ZPs were co-incubated for 5 h in a medium containing GlcNAc. ^B Fertilization was classified as normal, norm; polyspermy, poly; and non-fertilization, non. ^{a-b} Values with different superscripts differ significantly ($P < 0.01$).

remove sperm binding to the ZPs. The ZPs were then collected from the denuded oocytes and transferred into 0.1% pronase (Calbiochem, San Diego, CA, USA)/PBS. ZPs were continuously observed to confirm their dissolution under an inverted microscope at room temperature, and the time required for dissolution of the ZPs was measured. Ten ZPs were used for each replicate of each Expt. group. The Expt. was conducted 3 times, and a total of 30 ZPs were used for each Expt. group.

Statistical analysis

All data obtained were compared using one-way ANOVA followed by Fisher's protected least significant difference test. The fertilization rate was arcsine-transformed prior to analysis. A P value of less than 0.05 was considered to be significantly different.

Results

Experiments 1 & 2—Effect of monosaccharides on primary sperm-ZP attachment after 5 min co-incubation

The effect of monosaccharides on primary sperm-ZP attachment is shown in Table 1. When ZPs and sperm were co-incubated with 5 or 25 mM of various monosaccharides for 5 min, only the GlcNAc significantly reduced the number of sperm attached to the ZPs, but there was no difference among the examined concentrations of monosaccharides (Table 1). In Experiment 2, pretreatment of either sperm or ZPs with 5 mM GlcNAc also decreased the number of sperm attached to the ZPs, but pretreatment of sperm with 5 mM GlcNAc had a greater effect than pretreatment of ZPs (Table 2).

Table 6. Effect of GlcNAc on fertilization of zona pellucida (ZP)-free oocytes

GlcNAc ^A (5 mM)	No. of oocytes	No. of replicates	Rate of fertilization (%) ^B		
			Norm.	Poly.	Non.
–	60	4	45.3 ± 3.6	34.4 ± 5.4	20.3 ± 7.3
+	60	4	59.1 ± 6.1	26.8 ± 4.0	14.1 ± 3.3

^A Sperm and ZP-free denuded oocytes were co-incubated for 5 h in a medium containing GlcNAc. ^B Fertilization was classified as normal, norm; polyspermy, poly; and non-fertilization, non.

Table 7. Effect of GlcNAc on sperm membrane integrity determined by the hypo-osmotic swelling test (HOST)

GlcNAc ^A (5 mM)	Rate of positive sperm (%) ^B		
	0 h	3 h	5 h
–	83.5 ± 2.5	81.1 ± 1.2 ^b	74.1 ± 1.6 ^d
+	83.4 ± 2.6	76.7 ± 1.6 ^a	63.4 ± 1.3 ^c

^A Sperm was incubated in a medium containing GlcNAc at a concentration of 6×10^5 cells/ml for 5 h. ^B Rate of positive sperm (mean ± SE) determined by HOST. ^{a-d} Values with different superscripts differ significantly (^{a-b} $P < 0.05$, ^{c-d} $P < 0.01$).

Table 8. Effect of GlcNAc on sperm acrosome reaction determined by FITC-PNA/PI staining

GlcNAc ^A (5 mM)	Rate of acrosome reacted sperm (%) ^B					
	0 h	1 h	2 h	3 h	4 h	5 h
–	29.5 ± 1.8	34.6 ± 2.5	34.8 ± 1.9 ^a	38.5 ± 2.5 ^c	45.0 ± 3.1	48.1 ± 3.9
+	29.3 ± 1.9	38.8 ± 1.4	41.8 ± 1.1 ^b	46.3 ± 2.3 ^d	48.1 ± 3.8	52.5 ± 4.8

^A Sperm was incubated in medium containing GlcNAc at a concentration of 6×10^5 cells/ml for 5 h. ^B Rate of acrosome-reacted sperm (mean ± SE) determined by PNA-PI. ^{a-d} Values with different superscripts differ significantly (^{a-b} $P < 0.01$, ^{c-d} $P < 0.05$).

Table 9. Effect of GlcNAc on zona pellucida solubility after 5 h of co-incubation with sperm

Group ^A	GlcNAc (5 mM) ^B	No. of ZPs	Time required to dissolve (sec) ^C
A	–	30	437.9 ± 14.3 ^b
A	+	30	417.2 ± 18.4 ^{ab}
B	–	30	598.7 ± 23.9 ^c
B	+	30	382.1 ± 18.3 ^a

^A Group A: ZPs were collected from matured oocytes and co-incubated with sperm for 5 h in an IVF medium before measuring solubility. Group B: matured denuded oocytes were co-incubated with sperm for 5 h in IVF medium, and then ZP were collected from oocytes before measuring solubility. ^B GlcNAc was added to the IVF medium. ^C Time required for complete zona pellucida dissolution. ^{a-c} Values with different superscripts differ significantly ($P < 0.01$).

Experiments 3 & 4—Effect of monosaccharides on secondary (tightly) sperm-ZP binding and sperm penetration through the ZP after 5 h co-incubation

In Experiment 3, ZPs were co-incubated with sperm in medium containing 0, 5 or 25 mM monosaccharides for 5 h, and the number of sperm bound to the ZPs was counted. Supplementation with GlcNAc significantly increased the number of sperm bound to ZPs with the increase in GlcNAc concentration from 0 to 25 mM (Table 3). Co-incubation of ZPs and sperm for 5 h with 5 mM D-mannose or D-fucose did not affect the number of sperm bound to the ZPs, but incubation with 25 mM of D-mannose or D-fucose significantly increased the number of sperm bound to the ZPs (Table 3). The effects of monosaccharides on the number of sperm penetrating the

ZP were examined in Expt. 4 (Table 4). The number of sperm attached to the oolemma was significantly increased upon supplementation with GlcNAc, and the increase was concentration-related (Table 4). No effects of supplementation with the other monosaccharides (D-mannose and D-fucose), however, were observed.

Experiments 5 & 6—Effect of supplementation with GlcNAc on the fertilization rate of oocytes

When GlcNAc was added to the sperm and denuded oocyte co-incubation medium and culture was performed for 5 h, the rate of polyspermic fertilization increased significantly (polyspermy; 18.7 vs. 41.7%, Table 5). On the other hand, when GlcNAc was added

to the sperm and ZP-free oocyte co-incubation medium, there was no difference in the fertilization rate (Table 6).

Experiments 7 & 8—Effect of GlcNAc on sperm membrane integrity and acrosome reaction

In Expt. 7, when sperm was incubated with 5 mM GlcNAc, membrane integrity as determined by HOST was significantly decreased during the 5 h of incubation (Table 7). In Expt. 8, the proportion of acrosome-reacted sperm determined by PNA/PI staining was significantly increased after 2 or 3 h of incubation with 5 mM GlcNAc (Table 8).

Experiment 9—Effect of GlcNAc on ZP solubility after 5 h of co-incubation with sperm

The effect of GlcNAc on ZP solubility is shown in Table 9. Co-incubation of sperm and ZPs with or without 5 mM GlcNAc for 5 h had no significant effect on the time required for dissolution of ZPs in 0.1% pronase (GlcNAc: 0 mM, 437.9 ± 14.3 sec; 5 mM, 417.2 ± 18.4 sec). The time required for dissolution of ZPs after co-incubation of denuded oocytes and sperm with GlcNAc (382.1 ± 18.3 sec) was significantly shorter than that requires after co-incubation without GlcNAc (598.7 ± 23.9 sec; $P < 0.01$).

Discussion

We examined the effect of monosaccharides, especially GlcNAc, on each step of the sperm-oocyte interaction during fertilization. GlcNAc suppressed the primary sperm-ZP attachment, but increased secondary sperm-ZP binding and sperm penetration through the ZP as well as the rate of polyspermic fertilization. In addition, GlcNAc destabilized the sperm membrane and induced the acrosome reaction. Furthermore, GlcNAc suppressed ZP hardening following fertilization.

Primary attachment between the sperm and ZP is the first step of fertilization. Rath *et al.* (2005) [20] reported that, in the pig, the acrosome reaction is induced in many sperm 10 min after primary attachment to the ZP. In our preliminary Expt, the effect of monosaccharides on the number of sperm that attached to the ZP did not differ between two co-incubation periods (2 min and 5 min). In the present study, we used 5 min as the short period to assess primary sperm-ZP attachment. Supplementation of the sperm-ZP co-incubation medium with 5 or 25 mM GlcNAc for 5 min reduced sperm-ZP attachment, but other monosaccharides, D-mannose and D-fucose, had no effect. Furthermore, in a preliminary experiment, addition of either 10 or 20 mM of D-fucose or D-mannose did not affect primary attachment (data not shown). Pretreatment of either gamete with 5 mM GlcNAc significantly reduced the number of sperm that attached to the ZP. This effect was much stronger for sperm pretreatment than for ZP pretreatment, which is similar to the results of a previous report in the hamster [11] showing that pretreatment of sperm with 10 mM GlcNAc affects sperm-oocyte interactions but that pretreatment of oocytes does not. The fact that the number of sperm binding to the oocytes was reduced even though the GlcNAc-pretreated gametes were thoroughly washed before co-incubation may be explained by the following: 1) even a very low concentration of GlcNAc affects

the sperm-ZP interaction; 2) the ZP contained a small amount of GlcNAc, even after washing, that inhibited sperm-ZP binding; and 3) GlcNAc bound to the sperm strongly during pretreatment of the sperm with GlcNAc, and then the pretreated spermatozoa could not bind to the ZP. The lower limit of the GlcNAc concentration that affects sperm-ZP primary attachment remains to be determined. In bovine, the carbohydrate chain on the surface of the ZP is a high mannose-type glycan that contains GlcNAc [2], and binding between lectin and this specific glycan is mediated by hydrogen bond among these molecules, which impacts their three-dimensional structure [21]. In addition, when GlcNAc was added to the sperm incubation medium, sperm-sperm aggregation was increased just after GlcNAc supplementation (data not shown). Based on these results, the following are some possible causes of the reduction in sperm-ZP primary attachment: 1) GlcNAc may be involved in a key glycan sequence on the surface of the ZP, and GlcNAc in the medium may interact with a lectin-like protein on the sperm acrosome membrane, resulting in inhibition of primary sperm-ZP attachment; 2) GlcNAc increases sperm aggregation and this aggregation, may decrease the chance of sperm attaching to the ZP; or 3) GlcNAc changes the sperm state such that the membrane becomes destabilized (discussed below), which reduces the ability of sperm to attach to the ZP.

We then examined the effect of monosaccharides on secondary sperm-ZP binding and sperm penetration through the ZP. Sperm and ZPs were co-incubated for 5 h, which is the same period required for fertilization (Expts. 5 or 6), and then the number of sperm that were bound to the ZP and the number of sperm that penetrated the ZP (i.e., attached to the oolemma) were counted. Both the number of sperm bound to the ZP and number of sperm that penetrated the ZP were significantly increased by GlcNAc in a dose-related manner. In the mouse, GlcNAc is also suggested to be involved in the regulation of sperm-ZP binding [22]. Addition of 5 mM D-mannose or D-fucose, however, did not affect sperm binding and penetration of the ZP, but increasing the concentration to 25 mM slightly but significantly increased the number of sperm bound to the ZP. Furthermore, D-mannose and D-fucose did not affect the number of sperm that penetrated the ZP. Zitta *et al.* [11] reported that a low concentration of GlcNAc (1 mM) enhances sperm-ZP binding in the hamster. Furthermore, these authors suggested that the effect of a high concentration (50 mM) of D-fucose on gamete interaction may be dose-related. We also considered the effect of 25 mM D-mannose or D-fucose on sperm-ZP binding to be dose-related. The precise mechanism for the increase in the number of sperm secondarily binding to the ZP remains unclear, but the results of the present study suggest the following possibilities: 1) factors that facilitate sperm binding, such as membrane instability, induction of the acrosome reaction and inhibition of polyspermy block (discussed below), may concomitantly increase the number of secondary binding sperm, and 2) GlcNAc may be a key glycan involved in the interaction between the inner acrosome membrane of the sperm and the carbohydrates in the ZP.

To examine the effects of GlcNAc on sperm binding to and/or sperm penetration through the ZP, we prepared two groups of oocytes, denuded oocytes and ZP-free oocytes. Addition of GlcNAc to the IVF medium increased the polyspermic fertilization

rate in the denuded oocytes, but had no effect on the ZP-free oocytes. Whether GlcNAc affects the interaction between the oolemma and penetrating sperm was not examined, but these findings suggest that GlcNAc affects sperm-ZP interactions but not sperm-oolemma interactions.

Tanghe *et al.* [15] reported that addition of 50 mM GlcNAc to bovine sperm-COC co-incubation medium reduces the fertilization rate. In a preliminary experiment, we co-cultured sperm and cumulus cell in a medium with 5 mM GlcNAc, and GlcNAc reduced the number of sperm attached to cumulus cells (data not shown). Furthermore, sperm-somatic cell interaction depends on the carbohydrate chains [23]. In addition, 5 mM GlcNAc increases sperm binding to oviduct cells in bovine [24]. Therefore, the contradictory results might be due to differences in oocyte state (COCs vs. denuded oocytes) and/or GlcNAc concentrations (50 vs. 5 mM).

Sperm penetration of the ZP depends on sperm state, capacitation and induction of the acrosome reaction [22]. Only acrosome-reacted sperm can penetrate the ZP and fuse with the oolemma [25]. In the next experiment, we examined the effect of GlcNAc on sperm membrane integrity and the acrosome reaction. HOST is used to evaluate sperm membrane integrity [17]. When sperm was incubated in IVF medium containing 5 mM GlcNAc, the HOST index decreased compared with that incubated in IVF medium without GlcNAc. To assess the effect of GlcNAc on the sperm acrosome state, we performed PNA/PI staining of sperm. PNA is a lectin that binds to D-galactose and can detect the sperm acrosome reaction by fluorescent labeling [18]. The rate of acrosome reacted sperm was significantly increased after incubation for 2 and 3 h with 5 mM GlcNAc than in sperm incubated without GlcNAc, but not after culture for a longer duration. These results indicate that GlcNAc accelerates sperm membrane destabilization and may lead to a fast acrosome reaction. In the mouse, galactosyltransferase on the surface of the sperm acrosome recognizes GlcNAc on the ZP surface [7]. β -N-acetylglucosaminidase, which is contained in the sperm head in amounts 20-fold higher than those of other enzymes, recognizes GlcNAc on the ZP glycoprotein ZP3 and the inhibitor of this enzyme suppresses sperm penetration through the ZP [5]. In addition, GlcNAc is involved in inducing the human sperm acrosome reaction [3, 4, 10]. These findings suggest that the increase in sperm-ZP binding and sperm penetration of the ZP are due to the acrosome reaction induced by GlcNAc, which binds to a GlcNAc-affinity lectin-like protein on the sperm acrosome membrane. Zitta *et al.*, however, [11] reported that 1 mM GlcNAc suppresses hamster sperm penetration through the ZP and the acrosome reaction. We attribute this contradictory result to differences in the recognition mechanism of the sperm-ZP interactions among species, although the detailed mechanisms are still unclear.

ZP receptors for sperm as well as ZP properties are changed by CG exocytosis resulting from sperm fusion with the oolemma [13, 26]. The Functions and contents of CGs, however, are not well understood [1, 25, 27]. In the mouse CG, NAG has the highest glycosidase activity and inactivates a ligand on the ZP that binds to the sperm surface after CG exocytosis, thereby increasing polyspermy block [5]. Miller *et al.* [5] reported that inactivation of the enzymes in the CG by heat treatment enhances sperm-ZP penetration. Therefore, we hypothesized that supplementation with GlcNAc

interferes with the effect of CG enzymes and induces polyspermic fertilization. Enzymes released from the CG change the structure of the ZP, resulting in ZP hardening, which is detected as an increase in the time required to digest the ZP with a protease [19]. To assess whether GlcNAc prevents changes in the ZP during fertilization, we examined ZP dissolubility after 5 h of sperm-oocyte co-incubation with or without GlcNAc (Expt. 9). The time required for ZP dissolution was prolonged by 5 h of sperm-oocyte co-incubation, indicating that during the co-incubation period, the ZP was hardened, probably by CG exocytosis. Addition of 5 mM GlcNAc to the co-incubation medium decreased the time required to dissolve the ZP. Based on these findings, we suggest that GlcNAc may prevent the structural changes of ZP by CG enzymes mostly via the inhibition of NAG activity.

Finally, we examined how addition of GlcNAc promotes a high fertilization rate, even with a very low concentration of sperm. We decreased the sperm concentration to 2×10^5 or 5×10^4 cells/ml, which is 1/3 or 1/12 that of the normal sperm concentration for IVF, and observed no effect of GlcNAc on the fertilization rate (data not shown).

In conclusion, addition of monosaccharides to the sperm-oocyte co-incubation medium has many effects on sperm oocyte interactions. The effects of GlcNAc are summarized in Fig. 3. 1) GlcNAc interferes with the normal attachment between sperm and the ZP, probably via an interaction between GlcNAc and the sperm receptor against ZP glycan. 2) GlcNAc interacts with the sperm receptor and induces membrane destabilization. GlcNAc-induced membrane destabilization quickly induces the acrosome reaction. 3) GlcNAc suppresses ZP hardening, probably via inhibition of activities of enzymes released from CGs, resulting in an increase in the number of sperm binding to and penetrating the ZP. 4) Finally, all these facts lead to stimulation of sperm penetration and an increase of polyspermic fertilization.

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