Changes of Glycosidase Activity of Frozen-Thawed Spermatozoa in Human

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ABSTRACT

To evaluate the effect of spermatozoa culture on glycosidase activity of frozen-thawed spermatozoa in human, the spermatozoa were treated experimentally and assayed for activities of α-L-fucosidase, α-D-mannosidase, β-D-galactosidase and N-acetyl-β-D-glucosaminidase (β-GlcNAc'ase). The β-GlcNAc'ase activity was at least two-folds higher than other glycosidases regardless of spermatozoa incubation ($p<0.05$). The spermatozoa motility was decreased with incubation periods, but no effects by different glycosidases on the changes of spermatozoa motility during the various periods of incubation. In all glycosidases, the spermatozoa-zona binding rates in spermatozoa without incubation were higher than in spermatozoa incubated for 2 h ($p<0.05$). β-GlcNAc'ase is present mainly in the plasma membrane of spermatozoa frozen-thawed in human. It was also shown that the glycosidase activity was increased in all glycosidases in spite of lower sperm-zona binding by spermatozoa incubation.

(Key words : Acrosome reaction, Glycosidase activity, *In vitro*, Human, Spermatozoa incubation)

INTRODUCTION

The involvement of glycoconjugates in cell-cell interaction has been widely analyzed leading to the description of animal lectins (O'Rand, 1988). Mammalian fertilization is the result of a complex set of molecular events that enable the spermatozoa to recognize and bind to the oocyte's extracellular coat, the zona pellucida (ZP). Sperm-oocyte binding is initiated when the spermatozoa first attaches to the zona-intact oocyte. The attachment and the species-specific binding of the spermatozoa to the oocyte zona pellucida is prerequisite for the penetration of the zona pellucida and finally for the successful fertilization of the oocyte (Hinrichsen-Kohane *et al.*, 1984). Evidence thus far available strongly suggests that in most species, the receptor molecule present on the space of the acrosome intact spermatozoa recognizes and binds to the glycan residue of the homologous ZP. Among the proposed zona binding proteins are a number of saccharide-binding proteins including fucose-binding protein (Alhadeff *et al.*, 1999), mannose-binding protein (Miller *et al.*, 1993) and galactose-binding protein (Miller *et al.*, 1993), as well as a number of glycosyltransferases and glycosidases including β-1,4-galactosyl-transferase (GalTase; Miller *et al.*, 1992), fucosyltransferase (Ram *et al.*, 1989), α-D-mannosidase (Tulsiani *et al.*, 1989) and β-glucuronidase (Brandelli *et al.*, 1996).

Miranda *et al.* (1997) reported that the aggregation of N-acetylglucosamine (GlcNAc) binding sites by p-aminophenyl-N-acetyl-β-D-glucosaminide-bovine serum albumin induces the acrosome reaction in human spermatozoa through a mechanism of action resembling that described for the ZP-induces acrosome reaction (Brandelli *et al.*, 1996). Additionally, GlcNAc-bovine serum albumin induced acrosome reaction correlates with *in vitro* fertilization outcome and appears to be related to sperm-zona binding and penetration (Brandelli *et al.*, 1995). The participation of terminal GlcNAc residues of the ZP in human sperm-ZP interaction may be inferred from a previous study, where oocyte treatment with a GlcNAc specific lectin completely blocked sperm bunding (Mori *et al.*, 1989). This result suggested the presence of complementary GlcNAc-binding sites on spermatozoa.

It is known that fresh spermatozoa have higher fertilization potential than frozen-thawed spermatozoa (Hunter, 1990), possibly due to a lower survival rate. In human IVF system, frozen-thawed spermatozoa are exposed to fertilization medium to induce capacitation before insemination and/or during culture with oocytes. In our previous study, significant advantages of preincubation of frozen-thawed spermatozoa with oviductal vesicles and epithelial cell monolayer for 1 and 2 h to maintain penetration potential without increased polyspermy rates during *in vitro* fertilization in the pig (Park and sirard, 1996). In recent publications, our group presen-

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ted that fertilization-promoting peptide may have a positive role in promoting sperm function and glycosidase activity in the pig. It was difficult, however, to determine whether glycosidase activity was to relate to the fertilizing ability *in vitro* of spermatozoa, because spermatozoa was not preincubated in fertilization medium and incubated with oocytes for a long period. In this study, we have investigated the effect of different preincubation periods on changes of glycosidase activity and fertilizing ability *in vitro* of frozen-thawed human spermatozoa.

MATERIALS AND METHODS

Semen Collection and Preparation

Semen from the sperm rich fraction of the ejaculate was collected from fertile men. The semen diluted 1:1 with BTS within 30 min after evaluating the concentration and motility. Aliquots containing 6×10^9 spermatozoa were centrifuged for 10 min at 1,500 rpm. The seminal plasma was removed and the spermatozoa were resuspended to 5 ml with the 1st cryodiluent. The diluted semen was cooled gradually to 4℃ for 1~2 h by placing the tubes in a 50 ml conical tube containing 30 ml water (room temperature); 5 ml of the 2nd cryodiluent containing cryoprotectants were added gradually for 1 h. The diluted semen samples equilibrated for 40 min at 4℃ and was packed into straws before being pre-frozen horizontally for 20 min on a thick paper rack, 5 cm (approximately −120℃) above LN_2 vapor in a styrofoam container. The pre-frozen semen was finally transferred directly into LN_2 for storage.

Treatment of Spermatozoa for Glycosidase Analyses

Frozen semen (2 straws, each with a 0.5 ml in volume) was thawed in a water bath at 37℃ for 30 sec. The spermatozoa were washed twice in Ham's-F10 medium containing 2 mM caffeine (Sigma Chemical Co., St Louis, MO, USA) by centrifugation at 833 ×g each for 10 min. The final sperm pellet was resuspended in the same medium supplemented with 2 mM caffeine to give a sperm concentration of 1×10^6 spermatozoa/ml. Multiple aliquots (1 ml) of the sperm suspension were introduced into 1.5 ml polypropylene microcentrifuge tubes. These aliquots were variously treated and incubated in the atmosphere of 5% CO_2 in air for various periods according to the different experiments. The sperm suspension was centrifuged at 833 ×g for 5 min after incubation. The supernatant was carefully removed using a micropipette and discarded, or transferred to another microcentrifuge tubes. The pellet of spermatozoa were detergent-extracted by adding 100 μl Ham's-F10 supplemented with 30 mM n-octylglucoside for 10 min at room temperature and resuspended after adding Ham's-F10 to give a final volume of 1 ml. Each sperm suspension was assayed for glycosidase activity.

Measurement of Glycosidase Activity

The activity of glycosidase was measured by the liberation of p-nitrophenyl-derivarized substrates (α-L-fucoside, β-D-galactoside, α-D- mannoside and N-acetyl-β-D-glucosaminide). After the addition of 100 μl of substrate dissolved in solution (pH 5.2) supplemented with 2 mM of each substrate to 100 μl of detergent-extracted sperm suspension, the mixture was kept at 39℃ in air for 2 h, finally stopping the re-action by the addition of 1 ml of 1 M Na_2CO_3. The substrate-dissolved solution was composed of citrate-phosphate buffer (60 mM trisodium citrate; 40 mM NaH_2PO_4; 1 mM $CaCl_2$; 1 mM $MgCl_2$; 1 mM KCl and 1 mg/ml BSA) supplemented with protease inhibitor cocktail (PIC; Miller *et al.*, 1992). Absorbance of the product (p-nitrophenol) was measured at a wave length of 405 nm using a spectrophotometer.

Chlortetracycline (CTC) Assessment of Spermatozoa

The functional state of the spermatozoa was assessed using a CTC fluorescence assay described by DasGupta *et al.* (DasGupta *et al.*, 1993). Briefly, 4 μl Hoechst solution, containing 100 μg/ml Hoechst bis-benzimide 33258 (Sigma) in Ham's-F10 medium for spermatozoa washing was added to 396 μl sperm suspension. After mixing and gently vortexing, the suspension was incubated for 3 min at room temperature in the dark, layered onto 4 ml of 3% (w/v) polyvinylpyrrolidone (PVP-40; Sigma) in PBS and centrifuged at 500 ×g for 6 min. The pelleted spermatozoa was resuspended in 50 μl of Ham's-F10 medium. From this suspension, 45 μl was added to 45 μl CTC solution, and mixed together with 8 μl of 12.5% (w/v) paraformaldehyde in 0.5 mM Tris-HCl (pH 7.4). The CTC solution was prepared on the day of use and contained 750 μM CTC (Sigma) in a buffer of 130 mM NaCl, 5 mM cysteine and 20 mM Tris-HCl, pH adjusted to 7.8. This solution was kept wrapped in foil at 4℃ until just before use. Slides were prepared by placing 10 μl of the stained, fixed suspension on a slide. One drop of 0.22 M 1,4-diazabicyclo(2.2.2) octane dissolved in glycerol : PBS (9:1) was carefully mixed to retard fading of the fluorescence. A cover slip was placed on top. The slide was compressed firmly between tissues to remove any excess fluid and to maximize the number of spermatozoa lying flat on the slide. The cover slip was then sealed with colorless nail varnish and refrigerated and wrapped in foil.

An assessment was carried out either on the same or on the following day using an Olympus BHS microscope equipped with phase-contrast and epifluorescent

optics. Cells were assessed for CTC staining using violet light. The exicitation beam passed through a 405 nm band pass filter and fluorescence emission was observed through a DM 455 dichroic mirror. In each sample, 600 live cells were assessed for CTC staining patterns. There were three main patterns of CTC fluorescence which could be identified : F, with uniform fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact cells; and AR, with a dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells. Bright fluorescence on the midpiece could be seen at all three stages.

Spermatozoa Zona-Binding Test

The activity of sperm binding to zona pellucida was evaluated through binding to salt-stored homologous zona pellucida. Mouse oocytes matured *in vivo* were treated with 0.1% hyaluronidase to dissociate the cumulus oophorus and equilibrated with 1.5 M magnesium chloride containing 1% dextran. The oocytes were stored at 4℃ for 3~4 days until examined. The oocytes were washed and re-equilibrated in Ham's-F10 medium with FCS for 1 hour prior to experimentation. Spermatozoa (1×10^6 sperm/ml) were incubated for 1 h with experimental design. The number of spermatozoa attached to the zona pellucida was then evaluated using an inverted microscope.

Experimental Design

In Experiment 1, to examine the effect of spermatozoa preincubation on glycosidase activity, the frozen-thawed spermatozoa were washed twice in Ham's-F10 medium with different glycosidases by centrifugation at 833 ×g each for 10 min. For enzyme assays, 100 μl sperm suspensions was added an equal volume 2 mM substrate. After 0 or 2 h of incubation at 37℃, the activity of glycosidases was measured. In another experiment, glycosidase activity was measured after sperm suspension was incubated for 4, 8, 12, 16, 20 and 24 h in a medium with different glycosidases.

In Experiment 2, the spermatozoa were washed by centrifugation before being incubated in 50 μl fertilization medium with different glycosidases for 0 or 2 h to determine the effect of spermatozoa incubation on acrosome reaction *in vitro*. The spermatozoa were examined for CTC patterns. The survival motility of spermatozoa was also measured at 0, 1, 2, 3 and 4 h of spermatozoa incubation in fertilization medium with different glycosidase.

In Experiment 3, the effects of spermatozoa incubation and glycosidases on spermatozoa binding to oocytes at various times after insemination were examined. To determine the ability of spermatozoa binding, the human spermatozoa-mouse oocytes were cultured in fertilization medium with different glycosidase for 0 and 2 h. The spermatozoa binding were observed at 30 min of culture with sperm-oocyte in medium with different glycosidases.

RESULTS

In Experiment 1, when frozen-thawed spermatozoa had been treated with different glycosidases during the incubation of 0 and 2 h, the glycosidase activity in all glycosidase were higher in medium with that than without spermatozoa incubation (Fig. 1), but no differences were observed. As shown in Fig. 2, activity of each of the evaluated glycosidase could be detected in spermatozoa incubated during the different periods. The activity of each glycosidase had a tendency to increase as the time of incubation was extended (4~24 h), and the glycosidase activity in medium with GlcNAc'ase was at least double of other glycosidase in spite of different periods of spermatozoa incubation.

In Experiment 2, when frozen-thawed spermatozoa had been treated with different glycosidases, there were more acrosome-reacted in spermatozoa incubated for 2 h (Fig. 3). The rates of acrosome reaction were significantly ($p<0.05$) higher in spermatozoa with that than without incubation in medium containing mannosidase. As shown in Fig. 4, the motility of spermatozoa incubated with different glycosidase was evaluated. The motility of spermatozoa had a tendency to decrease as the time of incubation was extended, but there were no significant differences among the medium with different glycosidases.

In Experiment 3, the mouse oocytes were cultured

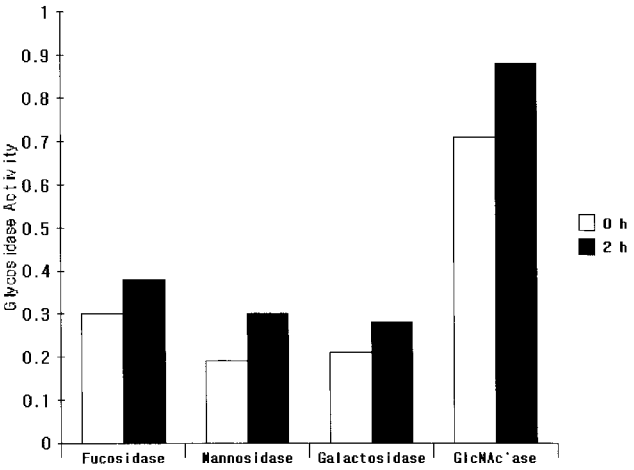

Fig. 1. Activity of glycosidase in frozen-thawed spermatozoa incubated for 0 and 2 h in human. There were no statistically differences between the treatment groups within same glycosidase.

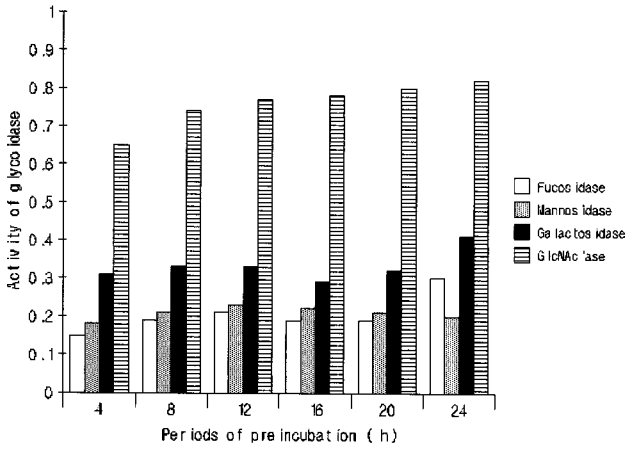

Fig. 2. Changes of glycosidase activity in frozen-thawed human spermatozoa incubated with different periods. No statistically significant differences within same glycosidase groups were found.

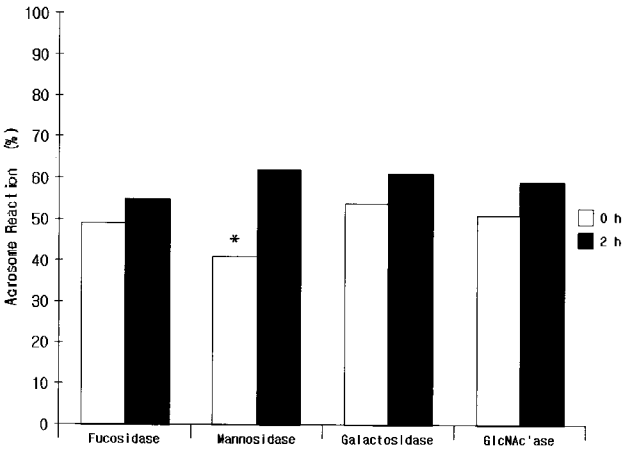

Fig. 3. Effects of glycosidase on acrosome reaction *in vitro* in human frozen-thawed spermatozoa with and without incubation. * $p<0.05$: Significant difference between treatment groups of 0 h and 2 h of spermatozoa incubation in medium with mannosidase.

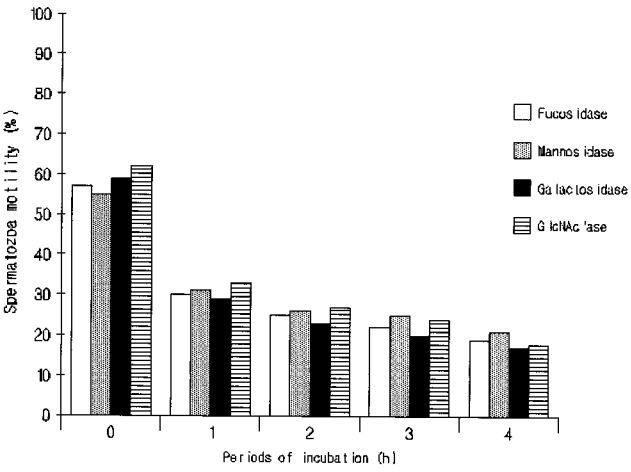

Fig. 4. Effect of glycosidases on *in vitro* motility of spermatozoa incubated with different periods. No statistically significant differences within same glycosidase groups were found.

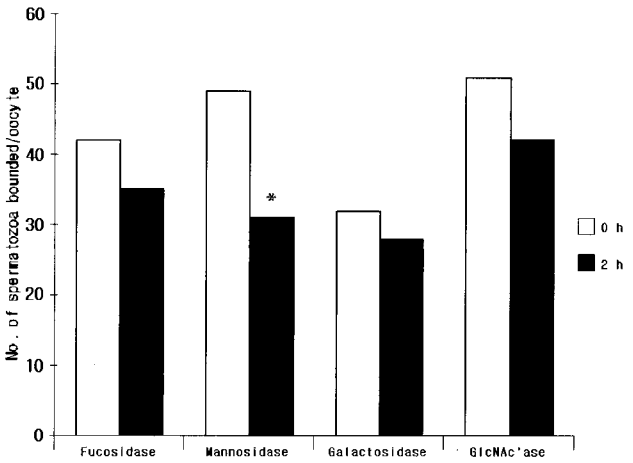

Fig. 5. Effect of glycosidases on zona pellucida binding to mouse oocytes of spermatozoa with and without incubation in human spermatozoa frozen-thawed. * $p<0.05$: Significant difference between treatment groups of 0 h and 2 h of spermatozoa incubation in medium with mannosidase.

for 30 min after insemination by human spermatozoa with or without incubation. As shown in Fig. 5, in all glycosidases, the spermatozoa-zona binding rates in spermatozoa without incubation were higher than in spermatozoa incubated for 2 h. The significant difference were obtained in spermatozoa treated with α-D-mannosidase ($p<0.05$).

DISCUSSION

The present results indicate that preincubation of frozen-thawed spermatozoa was helpful in medium with glycosidases on sperm ability and glycosidase activity. Several reports support the participation of fucose and mannose in human sperm-ZP binding (Chen *et al.*, 1995; Lucas *et al.*, 1994). Most studies analyzing the participation of sperm lectin-like proteins in sperm-ZP binding used sperm suspension shortly after the selection of motile cells, suggesting that capacitation may have not taken place (Chen *et al.*, 1995).

In the pig, Park and Sirard (1996) reported that preincubated frozen-thawed spermatozoa could not penetrate porcine oocytes matured in culture. In their study, the spermatozoa were preincubated for 4 h before insemination, which resulted in great reduction of sperm motility. The reduced motility of frozen ejaculated spermatozoa during the preincubation was also reported by Clark and Johnson (1987). In the present study, however, we showed that it was possible to maintain the rates of acrosome reaction with spermatozoa preincubated in medium with different glycosidases, and showed that spermatozoa preincubation can also maintain the penetration rates in spite of low rates of sperm mo-

tility and sperm binding to oocyte.

In recent investigation, we found boar sperm penetration was possible when oocytes were cultured after 4 h of insemination in fertilization medium with and without fertilization-promoting peptide (Park *et al.*, 2002). These results indicate that spermatozoa preincubation can be helpful for penetration at any time after insemination. Since a low proportion of spermatozoa incubated during the various periods were survival motility in medium with glycosidases, this additive may have not a role in maintain motility, at least under the experimental conditions of this study.

The results on the present study indicated that the β-GlcNAc'ase is present mainly in the plasma membrane of human spermatozoa. It was also shown that the glycosidase activity was increased in all glycosidases in spite of lower sperm-zona binding and penetration rates by spermatozoa incubation. The CTC assessment showed that different glycosidases did not affect induction acrosome reaction in spermatozoa incubated for 2 h.

Many studies have implied the macromolecules, such as lectin-like proteins and enzymes in the plasma membrane and acrosomes of spermatozoa were concerned with recognition of oligosaccharides of ZP glycoproteins. Among them, certain sperm glycosidases were implicated in sperm-oocyte interaction. It is believe to form stable enzyme-substrate complexes by binding with high specificity and affinity to the oligosaccharide residues of glycoproteins of the ZP without acting as a catalyzer (Macek and Shur, 1988). However, certain sperm-associated glycosidases have been localized in the acrosome (Bellve and O'Bren, 1983) and this acrosomal localization has led to the hypothesis that glycosidases released as a consequence of the acrosome reaction might act to degrade glycoconjugates of the investments of the oocyte and thus facilitate sperm penetration.

Although the spermatozoa may contain a variety of enzymes, the function of these enzymes is not understood during capacitation and fertilization *in vitro*. The present studies investigated the effect of incubation of frozen-thawed spermatozoa on fertilizing ability and glycosidase activity. The results of this study indicate that glycosidases such as α-L-fucosidase, α-D-mannosidase, β-D-galactosidase and β-GlcNAc'ase are all present in human spermatozoa. The β-GlcNAc'ase activity was present in sperm acrosomes at high levels, at least double that of other glycosidases. All of these glycosidases can be more reactively released from spermatozoa incubated. Since spermatozoa which were initially bound to the ZP had intact plasma membranes (Razeli *et al.*, 1977), any glycosidases involved in sperm binding would need to be present in the plasma membrane. In the present study, spermatozoa incubation induce the release of more β-GlcNAc'ase activity in spite of periods of different incubation. These results suggest that β-GlcNAc'ase in human spermatozoa may be present mainly in the plasma membrane. However, it is unclear why the acrosome was rather more sensitive to spermatozoa incubation on β-GlcNAc'ase activity than other glycosidases. This may result simply in a higher affinity of the acrosomal glycosidase for ZP glycoprotein substrates, relative to low molecular mass p-nitrophenyl substrates, so that more β-GlcNAc'ase would be required to inhibit the activity towards the glycoproteins. It could be presumed from these results that acrosomal β-GlcNAc'ase was required for efficient penetration through the ZP. In the present study, frozen-thawed spermatozoa had been preincubated for different periods, the activities of these glycosidases were higher in medium with than without preincubation. The most attractive hypothesis is that all glycosidases was released more reactively by spermatozoa incubation before initial sperm-oocyte binding.

In conclusion, β-GlcNAc'ase is present mainly in the plasma membrane of human spermatozoa. It was also shown that the glycosidase activity was increased in all glycosidases in spite of lower sperm-zona binding by incubation of frozen-thawed spermatozoa in human.

ACKNOWLEDGEMENTS

This work was supported by awards from the Research Project (ATIS No : PJ907008) in Rural Development Administration (RDA). The authors thank the Institute of Animal Resources at Kangwon National University for the technical assistance.

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(Received: 9 June 2011 Accepted: 25 June 2011)