Effects of Mono- and Polysaccharides on *In Vitro* Fertility of Boar Spermatozoa

In-Sun Hwang[1], Hee-Tae Cheong[2], Boo-Keun Yang[1], Choung-Ik Kim[1] and Choon-Keun Park[1,†]

[1]*College of Animal Life Sciences and* [2]*School of Veterinary Medicine, Kangwon National University, Chuncheon 200-701, Korea*

ABSTRACT

This study was conducted to examine the effect of several saccharides on the induction of capacitation and acrosome reaction (AR) and to examine the effects of mono and polysaccharides on the penetration activity of boar spermatozoa. Spermatozoa were inseminated in medium with fucose, galactose and mannose as monosaccharide, and fucoidan, galactan and mannan as polysaccharide. The penetration rates were significantly ($p<0.05$) lower in medium with galactose (40.6%), mannose (38.1%), fucose (41.6%) and fucoidan (36.6%) compared with control (56.7%). The rates of AR were increased (40.7 to 59.8%) by the preincubation periods prolonged from 0 to 4 hr ($p<0.05$). Similar tendencies were observed in AR when spermatozoa were treated with monosaccharides, but not significantly differ among the groups treated with different time of preincubation with some exception of galactose. When spermatozoa were treated with polysaccharides, the rates of AR were significantly ($p<0.05$) increased by preincubation time prolonged from 0 to 4 hr with an exception of fucoidan. In conclusion, the present study suggests that penetration rate of spermatozoa is higher in presence of polysaccharides than monosaccharides. Also, it may resume that the comparing to control, the all saccharides (L-fucose, D-galactose, D-mannose, fucoidan, galactan and mannan)-treated groups slightly increase the AR pattern as preincubation time prolonged.

(Key words : Acrosome reaction, *In vitro* fertility, Mono/polysaccharides, Pigs)

INTRODUCTION

Sperm-oocyte interaction is the species-specific cell recognition and binding event, and a necessary prerequisite for the fertilization. For successful fertilization, spermatozoa should recognize and bind to the oocyte's extracellular coat, the zona pellucida (ZP). There is evidence that the sperm-zona interaction is a carbohydrate-mediated event (Tulsiani *et al.,* 1997; Wassarman, 2005), which triggers a signal transduction pathway that results in the penetration and fusion of the sperm plasma membrane and the outer acrosomal membrane [acrosome reaction (AR)]. This exposes and sequentially releases the acrosomal contents at the site of sperm-zona binding. The AR is believed to be a prerequisite event that enables spermatozoa to penetrate the ZP (Florman and Storey, 1982). In mouse, one of the best understood examples of mammalian fertilization, initial sperm-oocyte recognition is mediated by the binding of sperm surface GalTase (N-acetylglucosamine galactosyltransferase) to terminal N-acetylglucosamine residues on ZP3, one of three glycoproteins found in the egg ZP (Miller *et al.,* 1992; Shur, 1993). Pig ZP is composed of two glycoproteins: ZP2 and ZP3 (Nakano *et al.,* 1987). Fertilization begins when a capacitated pig spermatozoon binds the ZP, activating the AR. ZP3 acts as the primary sperm receptor, mediating both initial binding of the spermatozoa to the oocyte and activation of the AR (Bleil and Wassarman, 1990). In most species, sperm penetrate several oocyte coats before reaching the oocyte plasma membrane. The most particular important one is the ZP, which is a coat of proteins laid down by the oocyte before ovulation. The interaction of the sperm and ZP or other oocyte coat triggers the AR, during which the sperm releases the contents of its acrosome. Enzymes from the acrosome help the sperm to lyse its way to the oocyte cell property. Sperm adhere to the oocyte surface in a species-specific way, by means of matching molecules on the sperm head and the ZP of an oocyte from the same species. Binding has been found to be mediated by specific carbohydrate ligands in various species. Sialic acid is involved in mediating sperm-oviductal epithelium binding in hamster (DeMott *et al.,* 1995). In the horse, galactose is involved to sperm-oocyte binding (Lefebvre *et al.,* 1995). In the case of cattle, there is the evidence that a molecule on the surface of sperm binds to a fucose-containing ligand on the epithelium. Fucose blocks binding of bull sperm to bovine oviductal epi-

* This work was supported by the Research on the Production of Bio-organ, Ministry of Agriculture and Forestry, Republic of Korea.

† Corresponding author : Phone: +82-33-250-8627, E-mail: parkck@kangwon.ac.kr

thelium *in vitro* (Lefebvre *et al.*, 1997). Fucose-specific lectins have been used to demonstrate that fucose is densely distributed on the surface of bovine oviductal epithelium. Furthermore, pretreatment of oviductal epithelium with fucosidase reduces sperm binding (Lefebvre *et al.*, 1997). This evidence strongly suggests that fucose is involved in bovine sperm binding to oviductal epithelium. However, the effect of these saccharides in porcine oocyte after in fertilization on *in vitro* embryonic development and induction of capacitation or AR are not yet clear.

In this study, it was examined the effects of several saccharides on the induction of capacitation or AR, and the effects of mono and polysaccharides on penetration *in vitro* of boar spermatozoa.

MATERIALS AND METHODS

Oocytes Preparation

Porcine ovaries were obtained from a local slaughterhouse and transported to the laboratory at 30~35℃. Cumulus-oocyte complexes were aspirated from 2 to 6 mm follicles with a 10 ml syringe with an 18 gauge needle. The collected oocytes were washed three times in Hepes-buffered Tyrode's medium (TLH) and once in maturation medium. Oocytes with a compact and complete cumulus were introduced into droplets of maturation medium (10 oocytes/50-μl droplet), covered with mineral oil and cultured under an atmosphere of 5% CO_2 in air at 39℃ for 40~44 hr. The maturation medium consisted of TCM-199 with Earle's salt (Gibco-BRL, Grand Island NY, USA) supplemented with 3.05 mM glucose, 0.32 mM Ca-lactate, 2.5 mM Hepes, 10% FCS, 0.2 mM Na-pyruvate, 50 μl/ml gentamicin, 1 μg /ml follicle stimulating hormone, 5 μg/ml lutenizing hormone, 1 μg/ml estradiol 17β , and 10% (v/v) porcine follicular fluid (PFF). The PFF was aspirated from follicles (3 to 6 mm in diameter) of prepubertal gilts, and centrifuged at 3,850 × g for 15 min. The supernatant fluid was frozen at −20℃ until used. During the culture, the hormones were removed from maturation medium for a second period of 21~22 hr.

Treatment of Saccharides and *In Vitro* Fertilization

Fresh semen was obtained from different boars at AI center. Semen were diluted and equilibrated in air-tight tubes at 16℃. After equilibration, the 2 ml of semen were centrifuged twice of 400 × g for 10 min and resuspended in fertilization medium. After the final wash, the concentration of motile spermatozoa was adjusted to 25×10^6 cells/ml in fertilization medium. Spermatozoa were preincubated in the fertilization medium for 0 to 4 hr before insemination with 5 cumulus-removed oocytes in each insemination drops (50 μl). The fertilization medium was TCM-199 supplemented with 3 mM glucose, 3 mM Ca-lactate, 0.2 mM Na-pyruvate, 2 mM caffeine and 10% FCS, and with or without (control group) monosacharides (100 mM L-fucose, 100 mM D-galactose and 100 mM D-mannose) and polysaccharides (2 mg/ml fucoidan, 4 mg/ml galactan and 4 mg/ml mannan). Sacchaarides were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise. At the end point of preincubation, some spermatozoa were used for the CTC fluorescence assay. The final concentration of spermatozoa was adjusted to 1×10^6 cells/ml motile sperm cells during fertilization *in vitro*. At 22~24 hr after insemination, the oocytes were examined for spermatozoa penetration *in vitro*.

Chlortetracycline (CTC) Assessment of Spermatozoa

The functional state of the spermatozoa was assessed using the CTC fluorescence assay method described by DasGupta *et al.* (1993). 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, 20 mM Tris-HCl· the pH was adjusted to 7.8. This solution was kept wrapped in foil at 4℃ until just before use. Hoechst-treated sperm suspension (45 μl) was added to 45 μl of CTC solution at room temperature in a foil-wrapped centrifuge tube and mixed thoroughly. Spermatozoa were then fixed by adding 8 μl 12.5% (w/v) paraformaldehyde in 0.5 M Tris-HCl (pH 7.4). 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 mixed in carefully to retard fading of the fluorescence. A coverslip was placed on the top of slide. 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 coverslip was then sealed with colorless nail varnish and stored wrapped in foil on the cold (n=3).

An assessment was carried out on either the same or the following day using a phase-contrast microscope with an epifluorescent optic. Cells were assessed for CTC staining using violet light. The excitation beam was passed through a 405 nm bandpass filter and fluorescence emission was observed through a DM 455 dichroic mirror. There are three main patterns of CTC fluorescence which can be identified (DasGupta *et al.*, 1993) : 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 dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells. At all three stages bright fluorescence on the midpiece could be seen.

Evaluation of Sperm Penetration

At the end point of insemination, the oocytes were mounted, fixed (acetic acid : ethanol = 1 : 3) for 2 to 3 days and stained with 1% aceto-orcein in 40% acetic acid water solution. The penetration was examined with a light microscope at ×200 and ×400 magnifications. Oocytes were considered to be penetrated when spermatozoa with a swollen head or two pronuclei were found in the vitellus.

Statistical Analysis

Data were subjected to a Generalized Linear Model procedure (PROC-GLM) of the Statistical Analysis System (SAS Institute, Cary, NC, USA). Differences among treatment means were determined by using the Tukey's studentized range (HSD) test. A probability of $p<0.05$ was considered statistically significant.

RESULTS

Table 1 shows that the effects of mono- and polysaccharides on *in vitro* fertilization of boar spermatozoa. The penetration rates were significantly ($p<0.05$) lower in medium with galactose (40.6%), mannose (38.1%), fucose (41.6%) and fucoidan (36.6%) compared with control (56.7%). The penetration rates in medium with galactan (51.5%) and mannan (48.1%) were not different from control group.

Under the same conditions, the effects of a various saccharides on *in vitro* capacitation and AR of boar spermatozoa were examined after preincubation for 0 (control), 1, 2, 3 and 4 hrs. Table 2 shows that the change of spontaneous AR by different preincubation periods without saccharides. The rates of capacitated spermatozoa were decreased (58.5 to 40.2%) and the rates of AR (40.7 to 59.8%) were increased by the preincubation periods prolonged from 0 to 4 hr ($p<0.05$).

Fig. 1 shows that the status of capacitation and AR in boar spermatozoa treated with each saccharide without incubation (0 hr groups). It was indicated that the rates of AR in spermatozoa treated with saccharides were higher than that of control group ($p<0.05$). Especially, the proportion of AR of spermatozoa treated with galactose (55%) was significantly higher than that of control ($p<0.05$).

Fig. 2 shows that the changes of capacitation and AR pattern of spermatozoa preincubated for 1 hr with

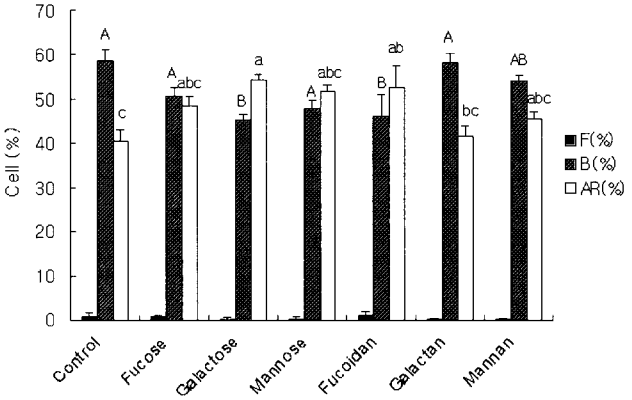

Fig. 1. Effects of mono- and polysaccharides on CTC patterns of boar spermatozoa without incubation. F, with uniform fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact cells; AR, with dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells. A, B and a~c: $p<0.05$, respectively.

Table 1. Sperm penetration *in vitro* of saccharides-treated porcine oocytes

Saccharides	No. of oocytes examined	No. of oocyte penetrated with*			No. (%) of polyspermic oocytes
		Total (%)	ESH	BPN	
Control	127	72(56.7)^{a}	63	9	15(20.8)
Galactose	133	54(40.6)^{bc}	53	1	12(22.2)
Mannose	126	48(38.1)^{c}	45	3	8(16.7)
Fucose	137	57(41.6)^{bc}	57	0	12(21.1)
Galactan	130	67(51.5)^{ab}	60	7	13(19.4)
Mannan	131	63(48.1)^{ab}	54	9	9(14.3)
Fucoidan	123	45(36.6)^{c}	43	2	8(17.8)

* ESH, enlarged sperm head; BPN, both pronuclei.

$^{a\sim c}$ Values with different superscripts differ ($p<0.05$).

Table 2. Changes of CTC patterns in spermatozoa preincubated during different periods*

Periods of preincubation (hr)	F (%)	B (%)	AR (%)	Total
0	5(0.8)	351(58.5)^{a}	244(40.7)^{c}	600
1	0(0)	337(56.2)^{ab}	263(43.8)^{bc}	600
2	0(0)	311(51.8)^{ab}	289(48.2)^{bc}	600
3	0(0)	293(48.8)^{b}	307(51.2)^{b}	600
4	0(0)	241(40.2)^{c}	359(59.8)^{a}	600

$^{a\sim c}$ Values with different superscripts in same column differ ($p<0.05$).

* 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; AR, with dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells.

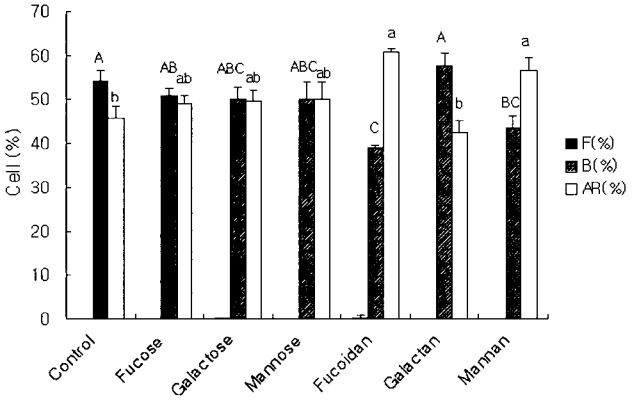

Fig. 2. Effects of mono- and polysaccharides on CTC patterns of boar at 1 hr after preincubation. F, with uniform fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact cells; AR, with dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells. A~C and a,b: $p<0.05$, respectively.

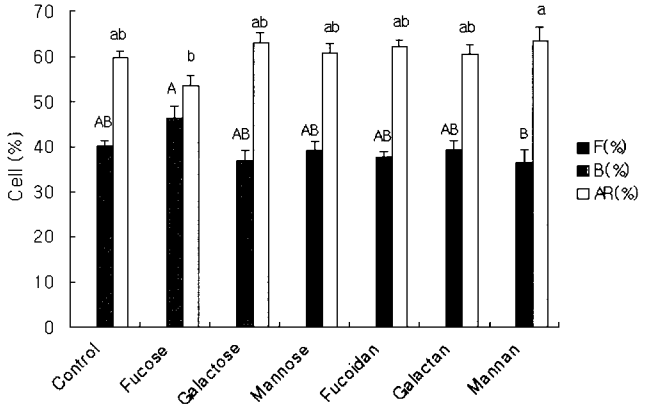

Fig. 3. Effects of mono- and polysaccharides on CTC patterns of boar spermatozoa at 4 hr after preincubation. F, with uniform fluorescence over the entire head, characteristic of uncapacitated, acrosome-intact cells; B, with a fluorescence-free band in the post-acrosomal region, characteristic of capacitated, acrosome-intact cells; AR, with dull or absent fluorescence over the sperm head, characteristic of capacitated, acrosome-reacted cells. A,B and a,b: $p<0.05$, respectively.

saccharides. The rate of AR of spermatozoa treated with fucoidan and mannan was higher than that of another treatment groups ($p<0.05$), but, there were no striking differences in capacitation and AR in spermatozoa treated with mono-saccharides.

Fig. 3 shows that changes of CTC pattern in spermatozoa incubated for 4 hr with saccharides. The capacitation rate in spermatozoa treated with fucose was significantly ($p<0.05$) higher than those of mannan-treated group. Moreover, there were significant difference in AR rates between fucose- and mannan-treated groups in spermatozoa incubated for 4 hr ($p<0.05$).

DISCUSSION

The results on the present study indicated that penetration rate of spermatozoa was higher in presence of polysaccharides than monosaccharides. Also, it may resume that the comparing to control, the all saccharides (L-fucose, D-galactose, D-mannose, fucoidan, galactan and mannan)- treated groups increased slowly AR pattern with preincubation time increased. The CTC assessment showed that the change of spontaneous AR by different preincubation periods without saccharides. The rates of AR of spermatozoa treated with galactose were higher than of control group. However, the rate of AR pattern of spermatozoa preincubated for 1 hr with fucoidan was higher than that of control group. These results suggested that the different activated mechanisms were presented between mono- and poly saccharides on sperm fertility.

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 believed 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 (Shur, 1993). However, certain sperm-associated glycosidases have been localized in the acrosome 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.

In the present study, the rates of capacitated spermatozoa were decreased and the rates of AR were increased preincubation periods prolonged from 0 to 4 hr. The ability of spermatozoa to undergo the AR develops over a period of time after ejaculation. During residence in the female genital tract, spermatozoa undergo biochemical and functional changes collectively referred to as capacitation (Yanagimachi, 1994). Ejaculated or epididymal spermatozoa can also be capacitated *in vitro* by incubating in a chemically defined medium (Toyoda *et al.,* 1971). *In vivo/in vitro* capacitation is a result of multiple molecular changes in sperm plasma membrane proteins/glycoproteins and lipid components, allowing transflux of ions that are important in initiating capacitation, hyperactivation, and the AR (Suarez, 1987; Suarez et al., 1989; Bedford, 1991).

The carbohydrate portion of several glycoproteins is known to mediate cell-cell adhesion, including sperm-oviduct adhesion (DeMott *et al.,* 1995), sperm-oocyte interaction (Tulsiani *et al.,* 1997), and implantation of the embryo (Lee *et al.,* 1983; Whyte and Allen, 1985). It is generally accepted that interaction of the opposite gametes is a carbohydrate-mediated species-specific event. Several sperm-surface proteins from various species have been proposed to function as receptor molecules on spermatozoa (Wassarman, 2005). Extensive studies in the mouse have resulted in the identification of several receptors on the male gamete and several glycan (ligand) residues on homologous ZP. The terminal sugar residues suggested to be recognized by the capacitated spermatozoa include mannosyl (Cornwall and Tulsiani, 1991), fucosaminyl (Lambert, 1984), glucosaminyl (Miller *et al.,* 1992), and a-galactosyl (Bleil and Wassarman, 1988). Although a terminal fucosyl residue has not been implicated in sperm binding, its presence appears to be obligatory for an oligosaccharide to bind spermatozoa with high affinity (Johnston *et al.,* 1998). In the present study, the fertilization were strongly inhibited by mannos and fucoidan, but did not inhibited AR by CTC assessment. Since fucoidan could inhibit acrosin amidase activity, it may consider that acrosin amidase activity was related to sperm-oocyte fusion.

On the other hands, Loeser and Tulsiani (1999) confirmed these studies using millimolar concentrations of monosaccharides, amino sugars, and polymannose in their assay conditions. Loeser and Tulsiani (1999) demonstrated that three neoglycoproteins (mannose-BSA, N-acetylglucosamine-BSA, and N-acetylgalactosamine-BSA) induced the AR in a dose-dependent manner. However, two other neoglycoproteins (galactose-BSA and glucose-BSA) had no effect on the induction of the AR. These results conclude that specific sugar residues covalently linked to a protein backbone can induce the AR. In addition, they determined that the sugars as mannose, glucosamine, galactosamine, and mannan, even at high concentrations, did not prevent the neoglycoprotein induced AR. This result was surprising since inclusion of some of the monosaccharides and oligosaccharides in an *in vitro* sperm-egg binding assay inhibits binding of the gametes in the mouse (Lambert, 1984; Cornwall and Tulsiani, 1991), rat (Shalgi *et al.,* 1986; Yoshida-Komiya *et al.,* 1997) and human (Mori *et al.,* 1989, 1993). A likely explanation could be that the sugars do compete with the neoglycoproteins for the complementary binding site on sperm plasma membrane overlying the sperm head. However, a small amount of the added neoglycoproteins that bound to the sperm receptors in spite of the competition is sufficient to induce the AR.

It is hypothesized that the primary molecules in the plasma membrane were implicated in low affinity attachment of spermatozoa, while ZP kept sufficient strength to resist lateral displacement forces generated by the motile tail. This initial attachment immediately triggers the acrosome reaction and thereby acrosomal contents including proacrosin and acrosin begin to disperse. At this stage, a second phase of sperm binding with high affinity takes place. Thus, proacrosin and acrosin are considered as secondary molecules implicated in sperm-oocyte binding. In summary, the present results suggest that penetration rate of spermatozoa were significantly higher at presence of polysaccharides than monosaccharides. Also, the results indicate that the every saccharide processing groups increase slowly the spontaneous AR pattern along with time goes compared with control. In connection with, further studies are required to understanding of the molecular mechanisms underlying the AR.

ACKNOWLEDGEMENT

The authors thank Institute of Animal Resources, Kangwon National University, Korea for the analysis of

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(Received: 12 May 2007 / Accepted: 17 June 2007)