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Effect of Glycosaminoglycans on *In vitro* Fertilizing Ability and *In vitro* Developmental Potential of Bovine Embryos

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ABSTRACT: The glycosaminoglycans (GAGs) present in the female reproductive tract promote sperm capacitation. When bovine sperm were exposed to 10 μg/ml of one of four GAGs (Chondroitin sulfate, CS; Dermatan sulfate, DS; Hyaluronic acid, HA; Heparin, HP) for 5 h, the total motility (TM), straight-line velocity (VSL), and curvilinear velocity (VCL) were higher in the HP- or HA-treated sperm, relative to control and CS- or DS-treated sperm. HP and HA treatments increased the levels of capacitated and acrosome-reacted sperm over time, compared to other treatment groups ($p<0.05$). In addition, sperm exposed to HP or HA for 1 h before IVF exhibited significantly improved fertilizing ability, as assessed by 2 pronucleus (PN) formation and cleavage rates at d 2. Exposure to these GAGs also enhanced *in vitro* embryo development rates and embryo quality, and increased the ICM and total blastocyst cell numbers at d 8 after IVF ($p<0.05$). A real-time PCR analysis showed that the expression levels of pluripotency (Oct 4), cell growth (Glut 5), and anti-apoptosis (Bax inhibitor) genes were significantly higher in embryos derived from HA- or HP-treated sperm than in control or other treatment groups, while pro-apoptotic gene expression (caspase-3) was significantly lower in all GAG treatment groups ($p<0.05$). These results demonstrated that exposure of bovine sperm to HP or HA positively correlates with *in vitro* fertilizing ability, *in vitro* embryo developmental potential, and embryonic gene expression. (**Key Words:** Glycosaminoglycan, Heparin, Capacitation, Embryo Development, Gene Expression)

INTRODUCTION

Fertilization is a unique and precisely controlled process between two haploid cells, the spermatozoon and the egg, that results in the creation of a genetically unique individual. For successful fertilization, freshly ejaculated sperm must undergo a series of physiological changes, called capacitation, during their transit through the female reproductive tract (Tienthai et al., 2004). Capacitation and the acrosome reaction are important processes in sperm maturation and are obligatory steps prior to fertilization (Lane et al., 1999). Capacitation involves many biochemical changes, including the removal of adsorbed components from the sperm surface; a change in plasma membrane lipid composition; an increase in permeability to ions such as Ca^{2+}; a change in internal pH; an increase in plasma membrane fluidity; and a decrease in the cholesterol to phospholipid ratio (Yanagimachi et al., 1994).

Many investigations have reported that glycosaminoglycans (GAGs) present in the oviduct play a major role in sperm capacitation, influence sperm motility, and improve the fertilizing ability of sperm in various species, including bovine, pig, rat, ovine, and human (Handrow et al., 1982; Hamamah et al., 1996; Dora et al., 2006; Borg et al., 2008; Towhidi et al., 2009). In bovine oviductal fluid, there are both sulfated GAGs, including chondroitin sulfate (CS), dermatan sulfate (DS) and heparin (HP), and the non-sulfated GAG hyaluronic acid (HA) (Hileman et al., 1998). GAGs are secreted by the cumulus and granulosa cells and the addition of GAGs to medium containing bovine sperm has the effect of stimulating the motility and capacitation of the sperm through direct changes in the intracellular environment of the sperm (Bergqvist et al., 2007). However, previous reports did not examine the effect of sperm exposure to these four different GAGs on post-fertilization events, such as *in vitro* embryo

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developmental potential and embryo gene expression.

The objective of the present study was to examine the effects of treating bovine sperm with four different GAGs (HP, HA, CS, and DS) by evaluating the fertilizing ability and *in vitro* embryo developmental potential. We investigated the effects of individual GAG treatments on i) bovine sperm motility using a Sperm Analysis Imaging System; ii) sperm capacitation or acrosome reactions using the chlorotetracycline (CTC) assay; iii) pronuclear formation rate post-IVF using Hoechst staining; iv) *in vitro* embryo development rate using microscopic examination; v) embryo cell numbers using differential staining; and vi) relative embryonic gene expression of candidate genes using real-time PCR.

MATERIALS AND METHODS

Chemicals and reagents

All chemicals and reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated.

Preparation of sperm

Sperm were prepared from frozen-thawed semen of a beef quality, index grade 1, Korean Proven bull (Korean native cattle; *Bos Taurus coreanae*, #KPN685). For thawing, a straw containing frozen semen was immersed in water at 37°C for 30 s. Sperm preparation before GAG treatment was carried out using our registered two-step swim-up protocol (patent No: KR101064415). Briefly, for the removal of egg yolk, thawed semen was slowly added to 3 ml of 20% Triladyl® solution (Triladyl:DW = 1:4) and centrifuged at 200 g (2,000 rpm) for 1 min. After aspiration of the resultant supernatant, 1 ml of 20% Triladyl solution was layered over the sperm pellet and the sample was incubated at 38°C for 15 min for the 1st swim-up step. The upper part of the solution, containing motile sperm, was transferred into a new 15 ml conical tube (Falcon, #2095), 1 ml of SP-TALP was added, and the solution was then pelleted by centrifugation at 200 g (2,000 rpm) for 1 min. After the supernatants were discarded, 1 ml of SP-TALP was added for the 2nd swim-up step for 15 min. Finally, the recovered motile sperm were counted using a hemocytometer, concentrated to 2×10^8 cells/ml, and used for the analysis of sperm motility, capacitation, or *in vitro* fertilization.

Exposure to GAGs

HA, CS, and DS were supplied by TCI-GR (Tokyo Chemical Industry Co., LTD). HP was supplied by Sigma. To examine the effect of GAGs on sperm motility, capacitation, and *in vitro* fertilization, sperm were exposed to a final concentration of 10 μg/ml of the GAG (Rodriguez-Almeida et al., 2005) and incubated at 38.5°C in a 5% CO_2 atmosphere for 1 h or 5 h.

Sperm analysis imaging system

The sperm motility in each treatment group was assessed using the Sperm Analysis Imaging System (SAIS Plus; Medical Supply Co, Ltd., Korea) described by Choi et al. (2011). At hourly intervals, aliquots of sperm were placed in a 10 μm standard counting chamber. Five fields of view were selected for each analysis. Sperm motility was assessed with respect to the following parameters: the straight-line velocity (VSL), which is the average velocity (μm/s) measured along a straight line from the position of the head in an initial image to the position of the head in the final image; the amplitude of the lateral head displacement (ALH), which is the width of the head oscillation, in μm, as the sperm swims; curvilinear velocity (VCL), which is the point to point velocity (total distance traveled) per second multiplied by two to give the full width; and total motility (TM), which is the percentage of motile sperm in the population. Three replicates were conducted for each experiment.

Sperm capacitation

Percentages of capacitated and acrosome-reacted spermatozoa were determined by the chlortetracycline (CTC) fluorescence assay described by Kuroda et al. (2007). After an incubation in CTC, a drop of the sperm suspension was placed on a glass slide with a drop of 0.22 M 1, 4-diazabicyclo [2, 2, 2] octane dissolved in glycerol and PBS (9:1, v/v) and covered with a cover slip. Sperm were scored in each of three independent experiments for each GAG treatment (Figure 2). Sperm were examined by differential interference contract (DIC) and fluorescence microscopy (Olympus, Tokyo, Japan). Sperm were classified into three patterns, as follows. The F pattern, uniform fluorescence over the entire head, is indicative of uncapacitated sperm. The B pattern, dim fluorescence in the postacrosomal region and relatively bright fluorescence in the acrosomal region, is indicative of capacitating and capacitated sperm. The AR pattern, dim fluorescence in the acrosomal region or only a thin band of fluorescence in the equatorial segment, is indicative of acrosome-reacting sperm or acrosome-reacted sperm, respectively. The percentage of capacitated and acrosome-reacted spermatozoa was also quantified prior to GAG treatment for all samples.

In vitro maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC)

Bovine ovaries were collected from a slaughterhouse and transported to the laboratory within 2 h in 0.9% saline at 35°C. Cumulus oocyte complexes (COCs) were aspirated from visible follicles (2 to 6 mm in size) into HEPES-buffered Tyrode's medium (TL-HEPES) using an 18 gauge

needle attached to a 10 ml disposable syringe. Groups of ten COCs were cultured in 50 μl droplets of maturation medium (TCM199 (Gibco) supplemented with 10% FBS, 0.2 mM sodium pyruvate, 1 μg/ml follicle-stimulating hormone (Folltropin^{TM}, Bioniche Animal Health, Belleville, On, Canada), 1 μg/ml estradiol-17β, and 1 mM EGF) under mineral oil at 38.8°C in an incubator with a 5% CO_2 atmosphere for 22 to 24 h. For IVF, COCs were transferred into TL-STOCK medium and inseminated with 2 μl of highly motile sperm (2.5×10^7 spermatozoa/ml) that had been recovered from the two-step swim-up protocol and then treated with 10 μg/ml of GAGs for 1 h. HP (2 μl, final concentration 10 ng/ml) and PHE stock (final concentrations: 18.2 μM penicillamine, 9.1 μM hypotaurine, 1.8 μM epinephrine) were also added to the 44 μl IVF droplet. For IVC, after 44±2 h of incubation, cleaved embryos were incubated in CR1aa medium containing 3 mg/ml FAF-BSA until d 4 at 38.8°C in a 5% CO_2 incubator. Embryos were then cultured in CR1aa medium containing 10% FBS until d 8.

Evaluation of sperm penetration of oocytes *in vitro*

Sperm penetration was defined as the presence of two pronuclei (2 PN) and/or the sperm head in the oocyte at 18 h after IVF (Figure 3). To remove cumulus cells, IVF embryos were treated with 0.1% hyaluronidase in TL-HEPES. Denuded embryos were washed with TL-HEPES and then fixed for 2 to 3 min in 2% formaldehyde. Fixed embryos were stained with 25 μg/ml bisbenzimide (Hoechst 33258) for 10 min, washed three times, loaded onto slide glass, and then observed by fluorescence microscopy at a magnification of ×200.

Blastocyst differential staining

The numbers of cells in the inner cell mass (ICM) and in the trophectoderm (TE) of blastocysts were counted using differential staining according to Thouas et al. (2001). Zona-intact blastocysts were incubated in 500 μl of Solution 1 (TL-HEPES containing 1% Triton X-100 and 100 μg/ml propidium iodide (PI)) for 30 s. Blastocysts were then immediately transferred into 500 μl of Solution 2 (100% ethanol with 25 μg/ml bisbenzimide; Hoechst 33258) and stored at 4°C overnight. The blastocysts were then mounted onto slides and observed by fluorescence microscopy. The PI- and Hoechst-labeled TE nuclei appeared pink or red. The Hoechst-labeled ICM nuclei appeared blue.

***mRNA* extraction**

For real-time PCR analysis, mRNA was prepared from blastocysts using magnetic beads (Dynabeads mRNA purification kit; Dynal, Oslo, Norway), according to the manufacturer's instructions. Briefly, for each treatment group, fifteen *in vitro*-produced d 8 blastocysts were resuspended in 100 μl lysis/binding buffer (100 mM Tris-HCl, pH 7.5, 500 mM LiCl, 10 mM EDTA, pH 8.0, 1% LiDS, 5 mM DTT) and vortexed at room temperature for 5 min to lyse the tissue. A 50 μl aliquot of an oligo (dT25) magnetic-bead suspension was added, and the samples were incubated at room temperature for 5 min. The hybridized mRNA and oligo (dT) beads were washed twice in wash buffer A (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA and 1% LiDS) and once in wash buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl and 1 mM EDTA). The mRNA samples were eluted from the beads in 15 μl of double-distilled DEPC-treated water.

Real-time PCR quantification

Blastocyst mRNA was extracted as described above and first strand cDNA was synthesized using an oligo (dT) primer and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR was performed using the primer sets shown in Table 3 in a Bio-Rad Chromo4 real-time PCR instrument. In all experiments, β-actin mRNA served as an internal standard. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence rises statistically higher than the background. To monitor the reactions, we followed the protocol described in the DyNAmo SYBR green qPCR kit, which contains a modified Tbr DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, and a dNTP mix that includes dUTP (Finnzyme Oy, Espoo, Finland). For the PCR protocol, the cycling conditions were 95°C for 15 min, followed by amplification and quantification cycles that were repeated 40 times at 94°C for 30 s, 50 or 56°C for 1 min, and 72°C for 1 min. SYBR Green fluorescence was measured after the extension step. Finally, the PCR products were analyzed by generating a melting curve. The reactions were subject to a single fluorescence measurement and then a melting curve program of 65 to 95°C with a heating rate of 0.2°C/s, and continuous fluorescence measurement. Samples were then cooled to 12°C. Because the melting curve of the PCR product is sequence-specific, it can be used to distinguish non-specific from specific PCR products. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen. 2001).

Statistical analysis

In all experiments, the data were compiled from at least three independent experiments. The numbers of ICM and TE cells were expressed as mean±SD. Values for rates of sperm capacitation, embryo development, ICM and TE cell numbers, and the relative gene expression levels were evaluated using analysis of variance (ANOVA) with the

general linear model (PROC-GLM) in the SAS software program $p \leq 0.05$ was considered significant.

RESULTS

Effect of GAGs on sperm motility

Sperm motility parameters were enhanced as a result of GAG exposure relative to the control, and the HP treatment group showed the best motility. In all treatment groups, the three parameters, TM, VSL, and VCL, were maintained at high levels for 2 h in the presence of each of the GAGs but then noticeably decreased at longer times of incubation. Nevertheless, the GAG exposure groups exhibited improved motility relative to the controls. At 1 h of GAG exposure, the HP-treated sperm had higher values for TM (57.2%), VSL (11.5 μm/s), and VCL (33.4 μm/s) than the other groups (control: 51.9, 10.2, and 29.3; DS: 48.7, 10.9, and 30.6; CS: 49.7, 10.3, and 30.9; HA: 47.3, 11.1, and 31.9, for TM, VSL, and VCL, respectively). At 3 h of GAG exposure, the HP and HA treatment groups showed a small enhancement of TM (47.2%, 44.2%), VSL (10.9 μm/s, 10.4 μm/s), and VCL (29.3 μm/s, 28.6 μm/s) relative to the control (35.3%, 8.8 μm/s, and 26.0 μm/s), DS (36.5%, 9.6 μm/s, and 27.5 μm/s) and CS (39.5%, 9.7 μm/s, and 27.8 μm/s) groups. Additionally, this pattern was maintained for 5 h of GAG exposure. By contrast, the ALH values among the GAG and control groups were not different at any time during GAG exposure (Figure 1).

Effect of GAG exposure on sperm capacitation

According to the CTC pattern, frozen-thawed control sperm, prior to GAG exposure, exhibited approximately 50% pattern F and 50% pattern B. In all treatment groups, the proportion of pattern F sperm gradually decreased over time (Figure 2A), with a corresponding increase in the proportion of pattern AR sperm (Figure 2C). These changes were accompanied by an increase and subsequent decrease in the proportion of pattern B sperm, beginning at approximately 3 h of incubation time (Figure 2B). Among GAG exposure groups, the HA and HP treatments elicited the most dramatic effects on sperm capacitation, relative to the CS and DS treatments (DS<CS<HA<HP) at 3 h or longer incubation times ($p<0.05$). The most potent capacitation reagent was HP, and the effect was maintained for the entire incubation time.

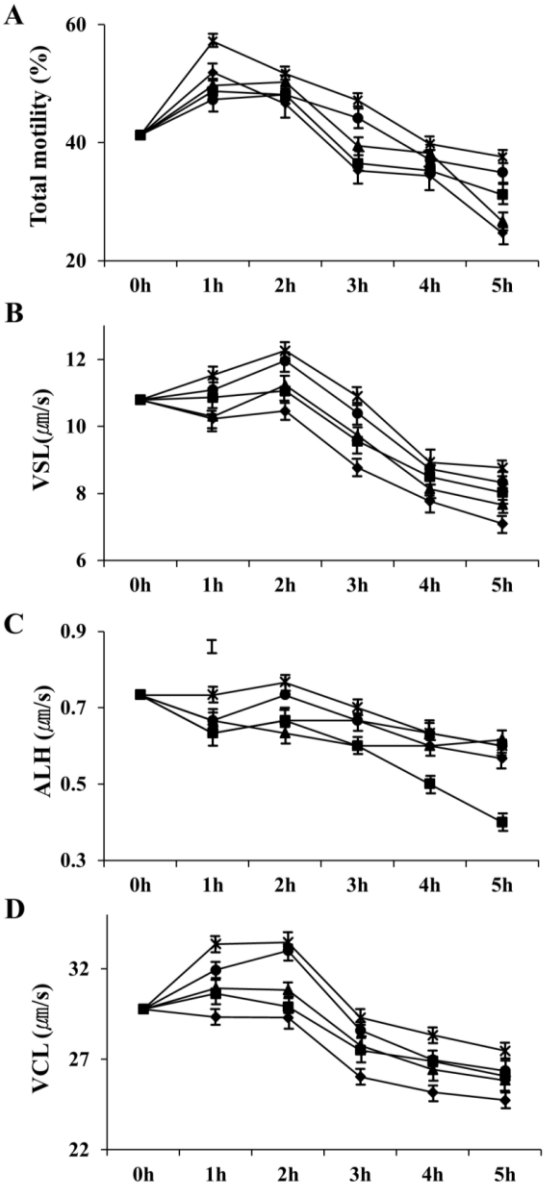
Effect of GAG exposed sperm on pronuclear formation and *in vitro* embryo development

The effects of exposing the sperm to GAGs on the rate of polyspermy and on formation of 2 pronuclei (2 PN) at 18 h after insemination was examined (Table 1). The rates of total penetration were not different between control and GAG exposure groups (control, 76.6%; DS, 73.4%; CS, 70.3%; HA, 79.7%; and HP, 87.6%). However, normal 2 PN formation was significantly higher in zygotes fertilized by HP exposed sperm (81.3%) than in control (59.4%), DS (57.8%), or CS (62.5%) exposed sperm ($p<0.05$). The HA-treated group (71.9%) produced intermediate levels of 2 PN (Figure 3A) zygotes. In addition, the rates of polyspermy (multi PN, 2PN+sperm; Figure 3B to C) in embryos derived from the HP (6.3%), HA (7.8%), or CS (7.8%) exposed sperm were very low compared to those of the control (17.2%) or DS (15.6%) exposed sperm.

When the developmental capacity of bovine IVF embryos resulting from fertilization by GAG exposed sperm was examined (Table 2), the d 2 cleavage rate of the HP group (87.3%) was significantly higher than control (75.2%), DS (73.6%), or CS (74.5%) treated sperm derived embryos ($p<0.05$). Embryos derived from the HA-treated sperm (81.8%) exhibited an intermediate rate of cleavage. However, by day 8, the blastocyst formation rate was significantly higher in the HP (54.1%) or HA (53.0%) treated groups compared to the control (34.1%), DS (35.8%), or CS (43.9%) groups ($p<0.05$). Cell counts of day 8 blastocysts revealed that there were significant increases in total cell number and in ICM cell number in the HP group (total, 137.6±14.6 and ICM, 45.6±13.2) and slightly higher numbers in the other GAG treated groups (DS (126.2±16.2 and 32.4±8.1), CS (128.2±14.6 and 37.5±11.3), HA (130.0±18.3 and 40.0±10.8)) relative to the control group (116.6±13.3 and 31.2±12.9) ($p<0.05$). The fraction of cells in the ICM of the blastocyst was also higher in most of the GAG exposure groups (HP, 33.1%; HA, 30.8%; and CS, 29.3%) relative to the control (26.8%). The DS group (25.7%) was not different from the control group. These differences are also shown in Figure 4A to E, where the HP or HA exposed groups exhibited embryo developmental morphology, blastocoel cavity expansion, and embryo quality that were superior to the control.

Relative mRNA expression in bovine IVF embryos produced using sperm exposed to different GAGs

The relative expression levels of genes related to apoptosis (Bax, Caspase 3), anti-apoptosis (Bax inhibitor), cell growth (Glut 5), pluripotency (Oct 4), and implantation (FGF 4) in embryos derived from GAG treated sperm were analyzed. As shown in Figure 5E, the mRNA levels for the core pluripotency marker gene Oct4 were significantly higher in all GAG exposure groups compared to the control ($p<0.05$). Expression of the pro-apoptotic gene Caspase 3 was significantly lower in all the GAG treatment groups relative to the control (Figure 5C), and expression of the anti-apoptotic Bax inhibitor was higher in the HA and HP groups relative to the other GAG treated or control groups (Figure 5B) ($p<0.05$). In addition, the expression of Glut 5 was significantly higher in the HA and HP groups relative



	♦CON.	■ DS	▲CS	● HA	* HP
0h	41.3	41.3	41.3	41.3	41.3
1h	51.9	48.7	49.7	47.3	57.2
2h	46.6	48.1	50.3	48.1	51.7
3h	35.3	36.5	39.5	44.2	47.2
4h	34.4	35.3	38.2	37.1	39.8
5h	24.7	31.2	26.7	35.0	37.6

	♦CON.	■ DS	▲CS	● HA	* HP
0h	10.8	10.8	10.8	10.8	10.8
1h	10.2	10.9	10.3	11.1	11.5
2h	10.5	11.1	11.2	12.0	12.3
3h	8.8	9.6	9.7	10.4	10.9
4h	7.8	8.5	8.1	8.7	8.9
5h	7.1	8.0	7.7	8.3	8.8

	♦CON.	■ DS	▲CS	● HA	* HP
0h	0.7	0.7	0.7	0.7	0.7
1h	0.6	0.6	0.7	0.7	0.7
2h	0.7	0.7	0.6	0.7	0.8
3h	0.7	0.6	0.6	0.7	0.7
4h	0.6	0.5	0.6	0.6	0.6
5h	0.6	0.4	0.6	0.6	0.6

	♦CON.	■ DS	▲CS	● HA	* HP
0h	29.8	29.8	29.8	29.8	29.8
1h	29.3	30.6	30.9	31.9	33.4
2h	29.3	29.9	30.8	33.0	33.5
3h	26.0	27.5	27.8	28.6	29.3
4h	25.2	26.9	26.4	27.0	28.3
5h	24.7	26.1	25.8	26.4	27.5

Figure 1. The effect of various glycosaminoglycan (GAGs) on bovine sperm motility. Sperm were incubated in the absence or presence of each GAG (dermatan sulfate, DS; chondroitin sulfate, CS; hyaluronic acid, HA; and heparin, HP) for 5 h, at 38.8°C, in a 5% CO_2 atmosphere. Every hour, the total motility (A), straight-line velocity (VSL, B), lateral head displacement (ALH, C), and curvilinear velocity (VCL, D) were assessed using a computer-assisted sperm motility analysis system.

to the other treatment groups (Figure 5D) ($p<0.05$). There were no differences in the expression levels of Bax (Figure 5A) and FGF4 (Figure 5F) between control and GAG treatment groups.

DISCUSSION

This study demonstrated that bovine sperm exposure to GAGs positively affected sperm fertilizing ability, *in vitro* embryo developmental potential, and embryonic gene expression. This is the first report to determine concurrently the effects of four different GAGs (HP, HA, CD, and DS). HP was the most potent GAG for enhancing sperm motility and inducing the acrosome reaction. HP exposed sperm exhibited improved 2 PN formation, cleavage rate, blastocyst formation rate, and embryo cell numbers relative to the control ($p<0.05$). Additionally, in embryos developing from fertilization with HP-treated sperm significant changes in gene expression were detected in genes involved in pluripotency (Oct4, upregulated), apoptosis (Bax inhibitor, upregulated; Caspase 3, downregulated), and cell growth (Glut5, upregulated), relative to control embryo gene expression ($p<0.05$). Sperm exposure to HA resulted in intermediate levels of changes, and, similar to HP, HA treatment of sperm resulted in significantly better *in vitro* embryo development and gene

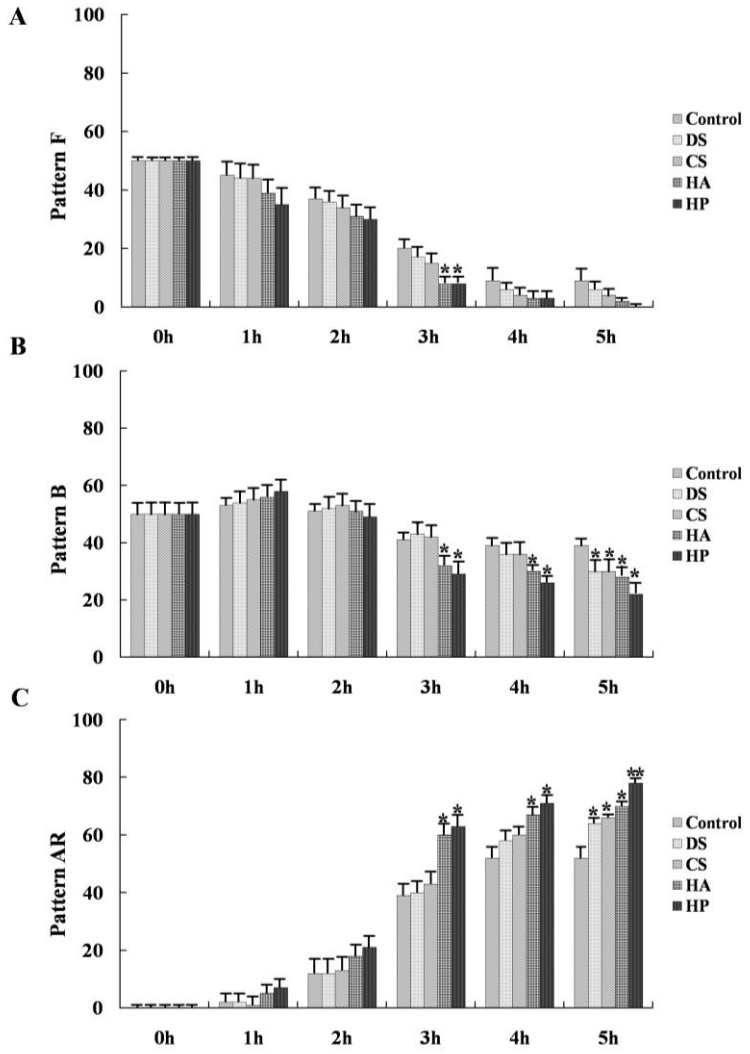


Figure 2. CTC fluorescence patterns of GAG-treated bovine sperm. Sperm were incubated in the absence or presence of each GAG for 5 h and labeled with CTC. The number of sperm exhibiting CTC pattern F (A, incapacitated sperm), pattern B (B, capacitating and capacitated sperm), and pattern AR (C, acrosome-reacted sperm) was determined at 1 h intervals. Results represent the mean±SEM of three independent experiments performed in duplicate; 100 sperm were assayed per sample ($p<0.05$)*.

expression levels compared to the control. CS was less potent, and DS least potent, in eliciting changes of the assessed parameters.

In this study, our registered (patent No: KR101064415) novel sperm preparation method, the "two-step swim-up protocol", was used. This method increased the sperm viability post-preparation (>5 h) relative to Percoll gradient preparation methods (<1 h). In addition, a greater number of viable sperm were recovered. To reduce sperm variation, high-quality frozen-thawed semen of a beef quality, index

Table 1. Pronuclear status of bovine zygotes 18 h after IVF using GAG-treated sperm

Treatment*	Number examined	Fertilization (%)				
		2 PN (%)	Polyspermy			Total
			Multi PN	2 PN+sperm	SUM	
Control	64	38 (59.4)[a]	6 (9.4)	5 (7.8)	11 (17.2)	49 (76.6)
DS	64	37 (57.8)[a]	8 (12.5)	2 (3.1)	10 (15.6)	47 (73.4)
CS	64	40 (62.5)[a]	3 (4.7)	2 (3.1)	5 (7.8)	45 (70.3)
HA	64	46 (71.9)[ab]	5 (7.8)	-	5 (7.8)	51 (79.7)
HP	64	52 (81.3)[b]	4 (6.3)	-	4 (6.3)	56 (87.6)

* DS: dermatan sulfate; CS = Chondroitin sulfate; HA = Hyaluronic acid; HP = Heparin.
[a-b] Means with different letters are significantly different from each other ($p<0.05$).

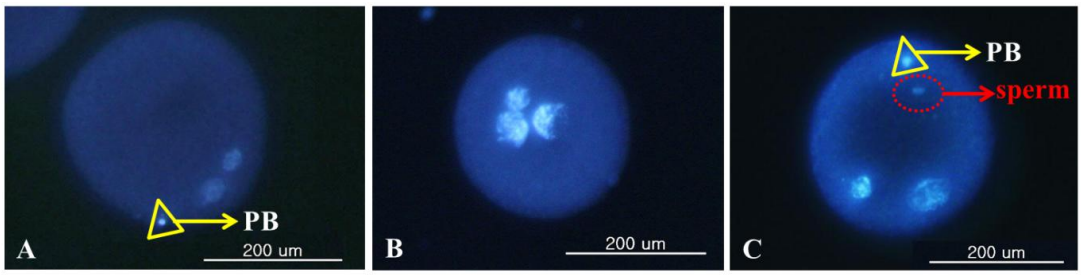


Figure 3. Nuclear status of bovine embryos at 18 h post IVF. Embryos classified as normal 2PN (A), or polyspermic: 3PN (B) or 2PN plus decondensed sperm (C).

grade number 1, Korean proven bull, was selected, and the GAG effects on sperm capacitation were examined using a 5 h incubation. For *in vitro* embryo production, sperm were preincubated with the GAG for 1 h before IVF. This design was derived from study of Rehman et al. (1994) that indicated that a maximum sperm penetration rate was obtained at 5 h for bovine IVF.

In mammals, sperm attached to the zona pellucida of an egg immediately undergo the acrosome reaction. The acrosome reaction must be completed for sperm to subsequently penetrate into the zona pellucida. A number of capacitation promoting compounds have been reported, including cholesterol, progesterone, caffeine, and seminal plasma protein; nevertheless, GAGs are among the best known inducers of sperm capacitation (Therien et al., 2003; Purdy et al., 2004). GAGs are present in components of the female reproductive tract including follicular fluid and uterine fluid (Kano et al., 1998). GAGs effectively capacitate bull sperm and lead to acrosome reactions *in vitro* (Berqqvist et al., 2006). GAGs restrict hardening of oocytes and promote capacitation, oocyte penetration by sperm, and nuclear decondensation and formation of the male pronucleus (Mahmoud et al., 1996; Anderson et al., 1997). HP causes functional changes in the cell membrane of bovine sperm and activates cyclic AMP production (Parrish et al., 1994). HA induces capacitation of human sperm by increasing the influx of Ca^{2+} (Slotte et al., 1993). DS induces capacitation and CS promotes sperm motility and capacitation in bovine sperm (Lenz et al., 1988; Parrish et al., 1989). However, most GAG tests have focused on HP treatment (Parrish et al., 1988; Miller et al., 1990; Lu et al., 2004). Handrow et al. (1982) suggested that HP, which is a highly sulfated GAG (30% sulfation), is the best inducer of the acrosome reaction. Thus, the degree of sulfation of a GAG may be partly responsible for promoting the acrosome reaction, which is consistent with the non-sulfated HA being the least effective in this study. In previous studies (Parrish et al., 1985; Therien et al., 1997), bovine epididymal sperm were used, but in this study, we used frozen-thawed ejaculated sperm. Previous reports suggest that HA might be an effective GAG for bovine sperm (Parrish et al. 1988; Sostaric et al., 2005; Bergqvist et al., 2007) and, in this study, HA treatment of sperm elicited intermediate levels of enhancement of bovine sperm motility, capacitation, 2 PN formation, *in vitro* development rate, and changes in gene expression. However, HP was clearly the best GAG treatment for enhancement of sperm function. HA was moderately effective and DS and CS were not effective (HP>HA>DS = CS).

By examining motility using the SAIS program and

Table 2. Effect of GAG-treated sperm on *in vitro* developmental capacity of bovine embryos

Treatment*	Number examined	No. (%) of embryos developed		Cell number total (ICM) mean±SEM	ICM proportion (%)
		Day 2 ≥2 to 4 cell	Day 8 ≥blastocyst		
Control	109	82 (75.2)^{a}	28 (34.1)^{a}	116.6±13.3^{a} (31.2±12.9)^{a}	26.8
DS	110	81 (73.6)^{a}	29 (35.8)^{a}	126.2±16.2^{ab} (32.4±8.1)^{a}	25.7
CS	110	82 (74.5)^{a}	36 (43.9)^{ab}	128.2±14.6^{ab} (37.5±11.3)^{ab}	29.3
HA	110	90 (81.8)^{ab}	44 (53.0)^{b}	130.0±18.3^{ab} (40.0±10.8)^{ab}	30.8
HP	110	95 (87.3)^{b}	46 (54.1)^{b}	137.6±14.6^{b} (45.6±13.3)^{b}	33.1

* DS = Dermatan sulfate; CS = Chondroitin sulfate; HA = Hyaluronic acid; HP = Heparin.
$^{a-b}$ Means with different letters are significantly different from each other ($p<0.05$).

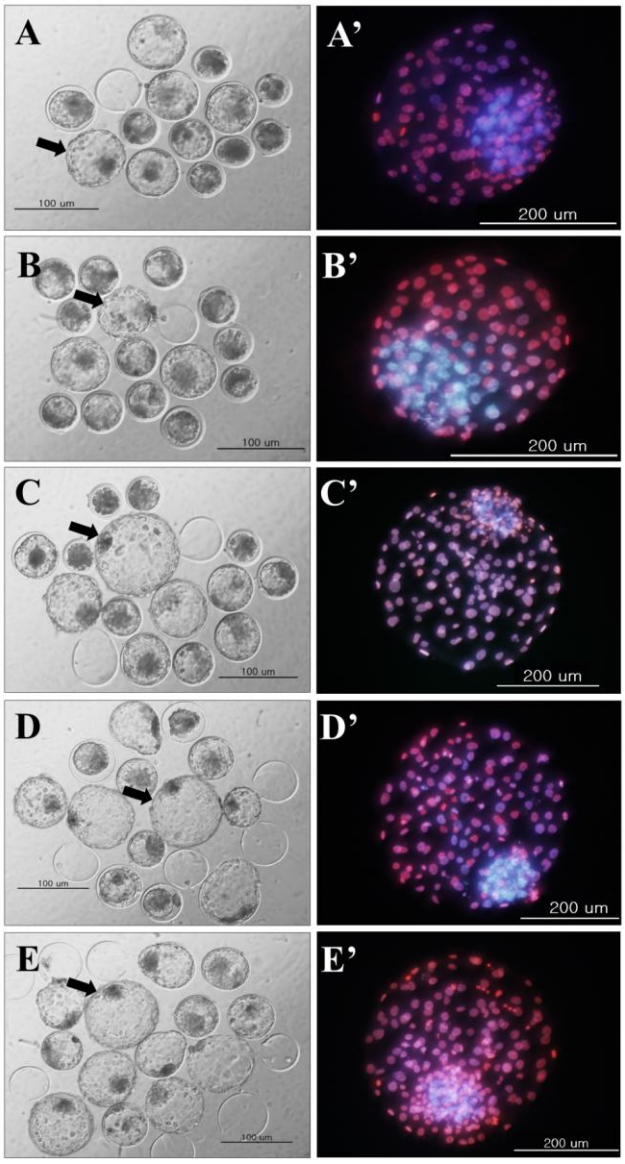


Figure 4. Developmental morphology of d 8 bovine blastocysts produced *in vitro* from sperm treated with different GAGs. For *in vitro* fertilization, sperm were incubated in the absence or presence of each GAG for 1 h before insemination. The cell number was examined by differential labeling of the embryos. No treatment: A-A'; DS: B-B'; CS: C-C'; HA: D-D'; HP: E-E'. The arrows indicate the differentially stained hatched blastocysts shown in A'-E'.

capacitation using a CTC staining assay, the effectiveness of the four different GAGs was clear. The SAIS program enables an objective assessment of different characteristics of cell movement, velocity, and morphology. When sperm motility was measured during a 5 h incubation in each different GAG, HP showed the highest percentage of TM (57.2%), VSL (11.5%), and VCL (33.4%) compared to the control or other GAG treatment groups. Sperm motility declined sharply by 3 h, and the HP or HA treatments were most effective in reducing this decrease in motility over time. Among sperm motility parameters, VCL has been correlated with fertilization success. The VCL value increases when sperm are capacitated and our results showed that HP exposed sperm exhibited the best VCL. VSL has also been used as a predictor of sperm function, similar to VCL (Choi et al., 2011).

One of the most useful methods for determination of the capacitation status is the CTC assay (Bergqvist et al., 2007). This fluorescent antibiotic exhibits enhanced fluorescence over segments of the membrane where Ca^{2+} accumulates (Dasgupta et al., 1993; Fraser et al., 1995). After HP or HA exposure, the proportion of capacitated and acrosome-reacted sperm clearly increased compared to control or other GAG exposure groups. Overall, the level of uncapacitated sperm (pattern F) decreased after 1 h, concomitant with increases in capacitated sperm (pattern B) and acrosome-reacted sperm (pattern AR), as previously reported (Ward et al., 1984). After 5 h in the presence of HP or HA, the proportions of pattern B and AR sperm were 25 to 30% and 70 to 75%, respectively.

GAG treatment concentration and capacitation time are important factors influencing *in vitro* fertilization and cleavage rates (Fukui et al., 1990). Previous reports suggest that treatment of sperm with certain reagents during an IVF procedure affects later development of the embryo (Way and Killian, 2006; Kato and Anagao, 2009; Gonçalves et al., 2010), and, additionally, that the pronuclear pattern of the zygote is closely related to blastocyst formation and quality (Ballaban et al., 2001). The higher percent of capacitated or acrosome-reacted sperm in the HP or HA exposed sperm could improve sperm penetration and pronuclear formation. Furthermore, *in vitro* fertilization and embryo development were also affected in embryos fertilized by sperm exposed to HP or HA, and thus a significantly higher developmental capacity was obtained in the HP or HA treatment groups relative to control or other GAG treatments ($p<0.05$). When the embryo quality was assessed by differential staining, the total cell numbers and the number of cells in the ICM in the HP treatment group was significantly higher than the control or other GAG treatment groups (DS or CS) ($p< 0.05$).

To examine the effects of sperm exposure to different GAGs on *in vitro* embryo production, the expression levels of apoptosis pluripotency, implantation, and growth related genes were evaluated using semi-quantitative RT-PCR. The octamer-binding transcription factor Oct-4 is a master regulator that is expressed at the beginning of mammalian embryogenesis, and is found in ICM and trophectoderm cells. Oct-4 expression may regulate cell lineage commitments in that a critical level of expression is required to maintain pluripotency (Kurosaka et al., 2004). Embryos derived from all GAG treated sperm showed significantly higher expression levels of Oct4 than control

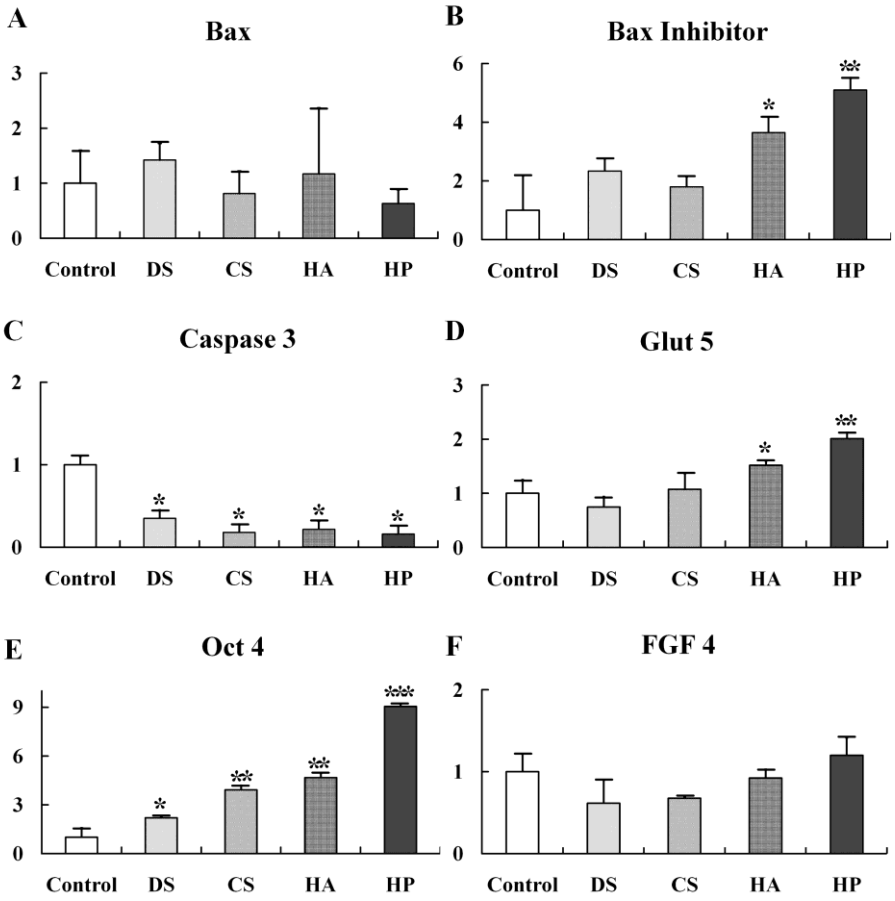


Figure 5. Relative expression of candidate genes in d 8 bovine blastocysts produced from sperm treated with different GAGs. Genes involved in apoptosis (Bax, Bax inhibitor, and Caspase-3), growth (Glut-5), pluripotency (Oct4), and implantation (bFGF) were examined. Bars with different superscripts within a panel differ significantly from each other ($p<0.05$). Error bars, SD.

embryos ($p<0.05$). Additionally, the expression levels of the pro-apoptotic gene caspase 3 were significantly lower in all GAG treatment groups relative to control ($p<0.05$), while levels of Bax expression were not different among the treatment groups. In addition, the relative expression levels of the anti-apoptotic gene Bax inhibitor and the cell growth gene Glut 5 were significantly different in the embryos derived from HP- or HA-treated sperm compared to control or DS or CS treatment groups ($p<0.05$). Furthermore, the expression levels of the Oct4, Bax inhibitor, and Glut 5 genes differed between the HP and HA treatment groups ($p<0.05$). Rizos et al. (2003) suggested that differences in

Table 3. Primer sequences used in this study

Gene	Primer sequence (5′-3′)	Fragment size	Function	Gene bank accession No.
bBax	GCTCTGAGCAGATCAAG AGCCGCTCTCGAAGGAAGTC	400 bp	Apoptotic	XM_001253643.2
bBax inhibitor	GCTCTGGACTTGTGCATT GCCAAGATCATCATGAGC	374 bp	Anti-apoptotic	BT026337.1
bCaspase-3	CGATCTGGTACAGACGTG GCCATGTCATCCTCA	359 bp	Pro-Apoptotic	NM_001077840.1
bGlut-5	TTGGAGAGCCAGTGAACAGT TGCTGATAACTGTCTGCGCT	292 bp	Growth	AF 308830.1
bOct-4	CTCTTTGGAAAGGTGTTCAG GTCTCTGCCTTGCATATCTC	155 bp	Pluripotency	AY490804.1
bFGF-4	GAGTGCAGGTTCAGAGAGAT GAGGAAGTGGGTGACCTT	621 bp	Implantation	NM_001040605
bβ-actin	GTCATCACCATCGGCAATGA GGATGTCGACGTCACACTTC	111 bp	House keeping	NM_173979

expression patterns are related to the quality of the bovine blastocysts produced under different culture conditions. From this viewpoint, significantly high pluripotent (Oct4, upregulated), growth (Glut 5, upregulated), and anti-apoptotic (Bax inhibitor, upregulated; Caspase 3, downregulated) gene expression levels were correlated with the high-quality embryos produced from HP- or HA-treated sperm. However, the activation of Caspase 3 and the Bax inhibitor protein would need to be examined to clarify the relationship between GAG exposure and apoptosis in embryos.

This study demonstrated that exposure of sperm to appropriate concentrations (10 μg/ml) of HP or HA before IVF had positive effects on *in vitro* fertilizing ability, as well as enhancement of 2 PN formation rates, cleavage rates at d 2, *in vitro* embryo development rates and embryo quality. In addition, exposure increased the ICM and total cell numbers at d 8 after IVF, and affected the expression level of key developmentally regulated genes ($p<0.05$). This protocol will be useful for *in vitro* production of high-quality embryos.

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