Master's Thesis

Effect of Glycosaminoglycan Treated Sperm on the *In Vitro* Developmental Capacity of Bovine Embryos

Department of Biology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

Eun Hyung Noh

2012. 2.

GAG 처리된 정자가 소 난자의 체외발달에 미치는 영향

지도교수 박 세 필

노 은 형

이 논문을 이학 석사학위 논문으로 제출함

2012 년 2 월

노은형의 이학 석사학위 논문을 인준함

심사위원장 _____류 기 중_____ ㊞

위 원 _____김 은 영_____ ㊞

위 원 _____박 세 필_____ ㊞

제주대학교 대학원

2012 년 2 월

Effect of Glycosaminoglycan Treated Sperm on the *In Vitro* Developmental Capacity of Bovine Embryos

Eun Hyung Noh

(Supervised by Professor Se Pill Park)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

February, 2012

This thesis has been examined and approved

Chairperson of the Committee

Professor Key Zung Riu, Ph.D., College of Applied Life Sciences, Jeju National University

Director Eun Young Kim, Ph.D., Mirae Biotech

Professor Se Pill Park, Ph.D., College of Applied Life Sciences, Jeju National University

Department of Biology

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

LIST OF FIGURES

LIST OF TABLES

Effect of Glycosaminoglycan Treated Sperm on the *In Vitro* Developmental Capacity of Bovine Embryos

Eun Hyung Noh

Department of Biotechnology
Graduate School of Jeju National University

(Directed by Professor Se Pill Park)

ABSTRACT

The glycosaminoglycan (GAG) presents in female reproductive tract fluid as promoting factor of sperm capacitation. When the same concentrations (10 μg/ml) of four different GAGs (Chondroitin sulfate, CS; Dermatan sulfate, DS; Hyaluronic acid, HA; Heparin, HP) were exposed to bovine sperm for 5 hour, the values of total motility, VSL and VCL were higher in HP or HA treatment group than in control and other GAG treatment groups (CS or DS). Also, HP or HA treatment effects on the increase of the rates of capacitated- and acrosome reacted sperm compared to other treatment groups.

In addition, HP or HA exposed sperm for 1 hour before IVF improved significantly the fertilizing ability including of 2 PN formation rates and cleavage

rates at day 2, and it subsequently effects on *in vitro* embryo development rate and embryo quality plus ICM and total cell number at day 8 after IVF ($P< 0.05$). In real-time PCR analysis, the relative mRNA expression levels of pluripotency (Oct 4), cell growth (Glut 5) and anti-apoptosis (Bax inhibitor) genes were significantly higher in HA or HP treatment groups than in control or other treatment groups, while the pro-apoptotic gene Caspase 3 expression was significantly low in all GAG treatment groups ($P< 0.05$). These results demonstrated that HP or HA exposed bovine sperm have positive effects on *in vitro* fertilizing ability, *in vitro* embryo developmental potential and their gene expression.

INTRODUCTION

Fertilization is a unique and accurately constructed cellular event between the two haploid cells, spermatozoon and egg, and result in the creation of a genetically special individual. For the fertilization success, freshly ejaculated sperm must undergo physiological changes called capacitation during their transit through the female genital tract (1). Capacitation is an important process in sperm maturation and is an obligatory step prior to fertilization (2). The mechanism of capacitation involves many biochemical changes including the removal of adsorbed components from the sperm surface, a change in membrane lipid composition, an increase in permeability to certain ions such as Ca^{2+}, a change in internal pH, increase in plasma membrane fluidity and a decrease in the membrane cholesterol, phospholipid ratio (3-4).

Many investigations reported the glycosaminoglycans (GAGs) are present in the oviduct and they have a major role in sperm capacitation, influence sperm motility and improve fertilizing ability in various species such as in bovine, pig, rat, ovine, and human (5-10). In bovine oviduct fluid, there are two main groups of GAGs, the sulfated GAGs comprising chondroitin sulphate, dermatan sulphate and heparin and the non-sulphated GAG hyaluronic acid (11). They are secreted by the cumulus and granulosa cells and the addition of GAGs to bovine sperm medium has effect on stimulating motility and capacitation of sperm through the directed change of intracellular environment of the sperm (12-14). However, there was no comparison report of the effect of four different GAGs exposed sperm on *in vitro* bovine embryo developmental potential and their gene expression.

The objective of the present study was to examine the effects of four different GAGs (heparin, HP; hyaluronic acid, HA; chondroitin sulphate, CS and dermatan sulphate, DS) on bovine sperm fertilizing ability and *in vitro* embryo developmental potential, i investigated the sequential effects of different individual GAGs treatment on i) bovine sperm motility using Sperm Analysis Imaging System program, ii) sperm capacitation status through chlorotetracycline (CTC) assay, iii) 2 pronuclear formation rate following IVF with different GAG exposed sperm, iv) *in vitro* embryo development rate, v) embryo cell number count by differential staining and vi) their relative gene expression level of candidate genes using real time RT-PCR.

MATERIALS AND METHODS

Unless otherwise stated, all chemicals and reagents were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO).

1. Preparation of Sperm

Sperm was prepared from frozen-thawed semen of Grade number one Korean Bull (Korean native cattle; *Bos Taurus coreanae,* #KPN685). For thawing, the straw containing frozen semen was immersed in water at 37^{o}C for 30 sec. Sperm preparation before GAG treatment was carried out by my registered two-step swim-up protocol (patent No: KR101064415). Briefly, for removal of egg yolk, thawed semen was slowly added in 3ml of 20% Triladyl® solution (Triladyl: D.W. = 1:4) and centrifuged at 2000 rpm for 1 min. After aspiration of the resultant supernatant, 1ml of 20% Triladyl solution was loaded onto sperm pellet and incubated for 1st swim-up at 38 ^{o}C for 15 min. And the upper part motile sperm was transferred into a new 15 ml conical tube (Falcon, #2095), added with 1ml SP-TALP and then pelleted by centrifugation at 2000 rpm for 1 min. After the supernatants were discarded, 2nd swim-up was tried using 1ml of SP-TALP for 15 min. Finally recovered motile sperm was counted using a haemocytometer, concentrated at a 2 x 10^{8} cells/ml and then applied for analysis of sperm motility, capacitation or *in vitro* fertilization.

2. Exposure of GAG

GAGs (HA, CS and DS) excluding HP were supplied from TCI-GR (Tokyo Chemical Industry Co., LTD.). To examine the effect of GAGs on the sperm motility, capacitation and *in vitro* fertilization, sperm was exposed with final concentration of 10 μg/ml GAGs, respectively, and then incubated in 38.5°C and 5% CO_2 incubator for 1 hour or 5 hour.

3. Sperm Analysis Imaging System (SAIS)

The sperm motility in each treatment group was assessed using the Sperm Analysis Imaging System (SAIS Plus; Medical Supply Co, Ltd. Korea). During at hourly intervals, sperm was placed in a 10 μm standard counting chamber. 5 fields were selected for each analysis. Sperm motility was assessed with respect to the following parameters: straight line velocity (VSL), meaning the average velocity measured in a straight line from the beginning to the end of the track (μm/s); the amplitude of the lateral head displacement (ALH), meaning width of the head oscillation as the cell swims (μm), velocity curvilinear (VCL), meaning point to point velocity (total distance traveled) per second were multiplied by two to give the full width and total motility.

4. Sperm Capacitation

Percentages of capacitated and acrosome reacted spermatozoa were determined by the chlortetracyclin epifluorescent technique (CTC) modified from Ken et al. (15). Briefly, the CTC solution was composed of 750 μM CTC, 5 mM

cysteine, 130 mM NaCl and 20 mM tris (hydroxymethyl) aminomethane (pH 7.8). The sperm suspension 45 μl was treated with the 45 μl CTC solutions for 30 sec and the subsequence was mixed with 12.5% paraformaldehyde in 0.5 mM Tris buffer (8 μl, pH 7.4). One drop of the sperm suspension was placed on a glass slide with one drop of 0.22 M 1, 4-diazabicyclo [2, 2, 2] octane dissolved in glycerol and PBS (9:1, v/v) was covered with a cover slip. Seventy cells were replicated three times for each preparation and examined by DIC (differential interference contract) equipped fluorescence microscopy (Olympus, Tokyo, Japan). Sperm was classified into the following three patterns. The F pattern indicated uniform fluorescence over the entire head (uncapacitated sperm). The B pattern indicated dark fluorescence in the postacrosome and relatively bright fluorescence in the intact acrosome (capacitating and capacitated sperm). The AR pattern indicated almost no fluorescence over the entire head except for a thin band of fluorescence in the equatorial segment (acrosome-reacted sperm) or dark fluorescence in the disintegrated acrosome (acrosome-reacting sperm). The percentage of capacitated and acrosome reacted spermatozoa was observed at zero time from incubation control and different GAGs treatment.

5. Collection of Oocyte and *In Vitro* Maturation (IVM)

Bovine ovaries were obtained within 2 hour at a local slaughterhouse and transported to my laboratory in phosphate buffered saline (PBS) containing 0.9% penicillin/ streptomycin at 37-38^{o}C. Transported bovine ovaries were washed with PBS twice and keep 37-38^{o}C in water bath. Cumulus-oocytes complexes (COCs)

were aspirated using an 18-G injection needle from a diameter of 3-6mm sized visible follicle. Selected COCs were washed two times in TL-HEPES supplemented with 0.1 g BSA, 1% Py-stock and 25 μg/mL gentamycin and matured in 50 μl drop of *in vitro* maturation (IVM) media containing TCM 199 (Gibco, Grand Island, NY, USA) supplemented with 10% Fetal bovine Serum (FBS, Gibco), 0.2 mM sodium pyruvate, 1 μg/mL follicle-stimulating hormone (FSH, Folltropin^{TM}, Bioniche Animal Health, Belleville, ON, Canada), 1 μg/mL estradiol-17β, 10 ng/mL Epidermal growth factor (EGF, Bio-Research Product, Inc, North Liberty, lowa) and 25 μg/mL gentamycin sulfate. COCs were matured at 38.8^{o}C for 22-24 hour in an atmosphere of saturated humidity and 5% CO_2, 5% O_2, and 90% N_2. Each drop of medium, containing 10 oocytes was under a layer of mineral oil to maturation.

6. *In Vitro* Fertilization (IVF) and *In Vitro* Culture (IVC)

In vitro fertilization (IVF) was performed with prepared by each GAG treatment sperm among control. In IVF, sperm was washed through Triladyl solution (patent No: KR101064415). After 24 hour *in vitro* maturation, COCs were washed three times with SP-TALP, and transferred to IVF medium. Fertilization plates consisted of 44 ul drops of IVF media without glucose in 60 mm dishes covered with 8 ml of mineral oil. 10 COCs were moved to each fertilization drop and co-cultured with GAGs treated and/or untreated sperm for 48 hour, inseminated with 2 ul of motile sperm ($1x10^6$ cells/ 0.5 ml drop of medium). 2 ul of heparin (50 ug/ml) and 2 ul of PHE (1 mM hypotaurine, 2 mM penicillamine and 250 mM epinephrine) stock.

After 2 days, embryos were stripped of cumulus cells by passing through a

narrow fire-polished pipette, washed once in TL-HEPES and once in CR1aa medium then transferred to 30ul drops of CR1aa medium until day 4 (supplemented with BSA-FAF). Embryos were cultured in 30 ul/ drop of medium under mineral oil at 38.5°C in an atmosphere of saturated humidity and 5% CO_2. The media was changed every 2 days, at day 4, and the embryos were cultured with CR1aa medium containing 10% FBS until 8 days (Day 0; day of fertilization). Day 8 embryos were analyzed by differential

7. Evaluation of Sperm Penetration into Oocytes *In Vitro*

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

8. Blastocyst Differential Staining

The blastomere, inner cell mass (ICM), and trophectoderm (TE) cell numbers in blastocysts were counted with the use of differential staining according to Thouas *et al*. (16). 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

seconds. 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 a slide glass and observed by fluorescence microscopy equipped with a UV filter. The PI- and bisbenzimide-labeled TE nuclei appeared pink or red. Bisbenzimide-labeled ICM nuclei appeared blue.

9. mRNA Extraction

For real-time RT-PCR analysis, mRNA was prepared from blastocysts using magnetic beads (Dynabeads mRNA purification kit; Dynal, Oslo, Norway) following the manufacturer's instructions. Briefly, in each treatment group, fifteen *in vitro* produced day 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, and 5 mM DTT) and vortexed at room temperature for 5 min to lyse the tissue. A 50 μl aliquot of an oligo (dT) 25 magnetic-bead suspension was added, and the samples were incubated at RT for 5 min. The hybridized mRNA and oligo (dT) beads were washed twice using wash buffer A (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl, 1 mM EDTA and 1% LiDS) and then washed once with wash buffer B (10 mM Tris-HCl, pH 7.5, 0.15 M LiCl and 1 mM EDTA). mRNA samples were eluted from beads in 15 μl of double-distilled DEPC-treated water.

10. Real-Time RT-PCR Quantification

mRNA was extracted as described above and standard cDNA was synthesized using an oligo (dT) primer and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time RT-PCR was performed using the primer sets shown in Table 5 in a Bio-Rad Chromo 4 real-time RT-PCR machine. In all experiments, histone H2a mRNA served as an internal standard. The threshold cycle (Ct) value represents the cycle number at which the sample fluorescence rises statistically above background noise. To monitor the reactions, I 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 sec, 50 or 56 °C for 1 min, and 72°C for 1 min. The reactions were subject to a single fluorescence measurement, a melting curve program of 65–95°C with a heating rate of 0.2°C/sec, and continuous fluorescence measurement. Samples were then cooled to 12°C. SYBR Green fluorescence was measured after the extension step. PCR products were then analyzed by generating a melting curve. Since the melting curve of a product is sequence-specific, it can be used to distinguish non-specific from specific PCR products. To do this, it is necessary to determine the crossing points (CP) for each transcript, which is defined as the point at which fluorescence rises appreciably above background noise. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method (17).

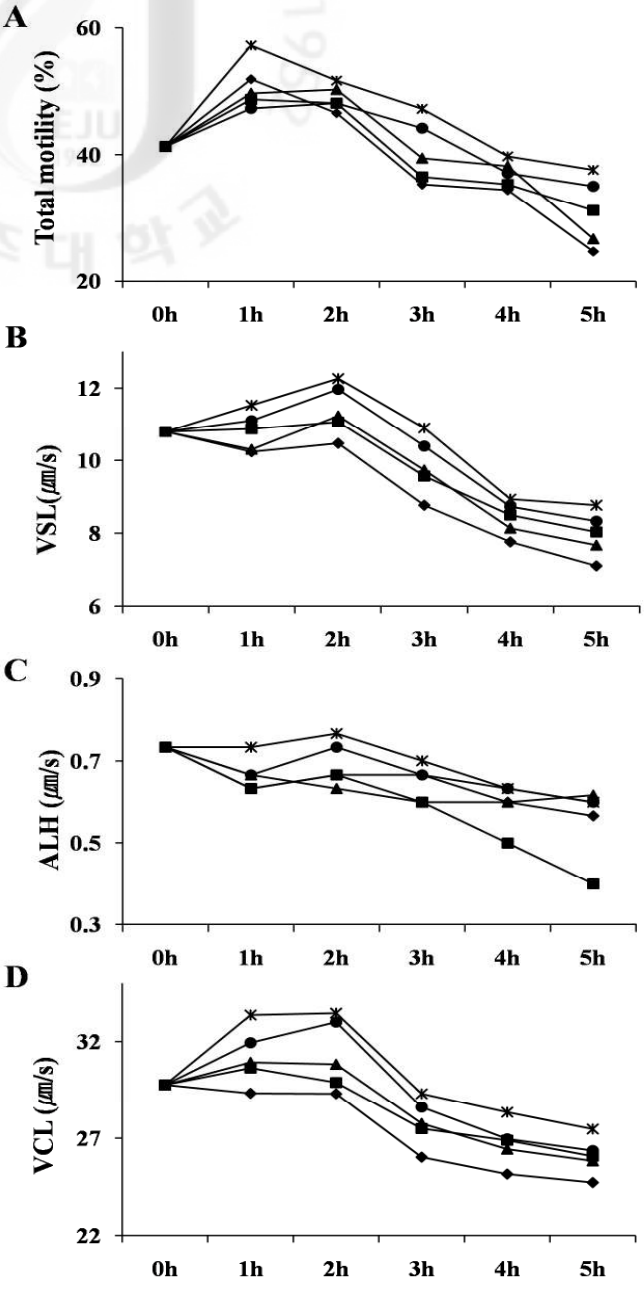
11. Statistical Analysis

In all experiments, the data were compiled from at least three independent replicates. The number of ICM and TE cell was expressed as the mean±SD. Account representing rates of embryo development, ICM and TE cell numbers and the relative gene expression levels were evaluated by analyses of variance with general linear model (PROC-GLM) in the SAS software program. P values ≤ 0.05 were considered significant.

RESULTS

1. Effect of GAG on Sperm Motility

When the sperm motility according to GAG exposure during 5 hour incubation was assessed through the SAIS plus (Fig. 1) program, the results of GAG exposure groups were better than that of control, and especially the value was the best in HP treatment group. In all treatment groups, the three parameters (total motility, VSL, and VCL) were seemed to be maintained until 2 hour post GAG exposure, but from 3 hour post GAG exposure the values were noticeably decreased, and nevertheless the most GAG exposure groups indicated a little improved result than control. In the result, at 1 hour post GAG exposure, the HP treated sperm was presented higher value in motility (57.2%), VSL (11.5 μm/s) and VCL (33.4 μm/s) than those of other groups (control: 51.9, 10.2 and 29.3 vs. DS: 48.7, 10.9 and 30.6 vs. CS: 49.7, 10.3 and 30.9 vs. HA: 47.3, 11.1 and 31.9), respectively. Also, at 3 hour post GAG exposure, the results of HP or HA treatment group were presented a little enhanced total motility (47.2%, 44.2%), VSL (10.9 μm/s, 10.4 μm/s) and VCL (29.3 μm/s, 28.6 μm/s) than control group (35.3%, 8.8 μm/s and 26.0 μm/s), DS treatment group (36.5 %, 9.6 μm/s and 27.5 μm/s) and CS treatment group (39.5 %, 9.7 μm/s and 27.8 μm/s), respectively. In addition, this pattern was maintained continually during 5 hour post GAGs exposure. However, ALH values among GAG groups and control group were not different during all exposure time.



	♦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

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

2. Effect of GAG Exposure on Sperm Capacitation

The CTC staining pattern according to the different GAG exposure of sperm was examined during 5 hour incubation (Fig. 2). When the fluorescent patterns were classified into three (pattern F: uncapacitation, pattern B: capacitation, pattern AR: acrosome reaction), frozen-thawed control sperm before GAG exposure was assessed into about 50% pattern F and 50% pattern B, respectively. In all treatment groups, as the incubation time was longer, the proportion of pattern F was gradually reduced (Fig. 2A) with conspicuous increase of the proportion of pattern AR (Fig. 2C), and those changes were accompanied with the increase and decrease of the proportion of pattern B around at 3 hour incubation time (Fig. 2B). GAG exposure surely has effect on the CTC pattern changes. Among GAG exposure groups, HA and HP treatment derive powerful effects on sperm capacitation than CS and DS treatment (DS<CS<HA<HP) from 1 hour incubation time. And among them, the most potent capacitation reagent was confirmed as HP, the effect was continually maintained during all incubation time.

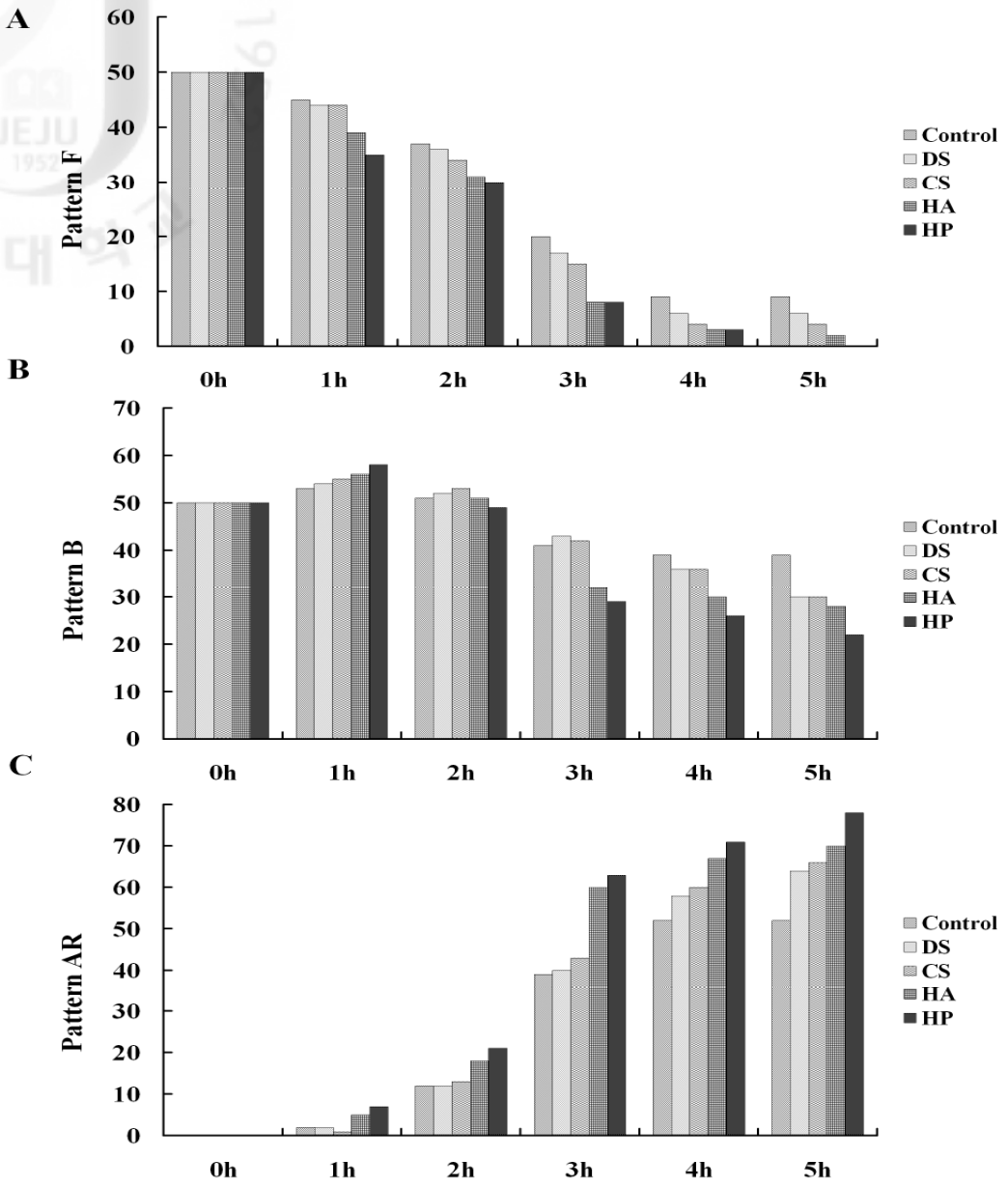


Fig. 2. Chlortetracycline (CTC) fluorescence pattern of various GAGs treated bovine sperm. The sperm was incubated in the absence or presence of each GAGs (DS; dermatan sulphate, CS; chondroitin sulphate, HA: hyaluronic acid or HP; heparin) for 5 hours and the binding sites were labelled using PSA-FITC. Every hour, the number of sperm exhibiting CTC pattern F (A, uncapacitated sperm), pattern B (B, capacitating and capacitated sperm) and pattern AR (C, acrosome-reacted sperm) was determined, respectively. Data are shown as mean ±S.E.M. ($P< 0.05$).

3. Effect of GAG Exposed Sperm on Pronuclear Formation and *In Vitro* Embryo Development

When the effect of GAG exposure of sperm on the 2PN formation and polyspermy rate at 18 hour after insemination was examined (Table 1), the rates of total penetration were not different control and GAG exposure groups (control, 76.6%; DS, 73.4%; CS, 70.3%; HA, 79.7%; HP, 87.6%). However, normal 2PN formation of HP exposure group (81.3%) was significantly higher than those of control (59.4%), DS (57.8%) or CS (62.5%) exposure groups except HA group (71.9%) ($P< 0.05$). In addition, the rates of abnormal fertilization by polyspermy (multi PN, 2PN+sperm) of HP (6.3%), HA (7.8%) or CS (7.8%) exposure groups were presented very low compared to those of control (17.2%) or DS (15.6%) exposure group.

Also, when the effect of different GAG exposed sperm on the developmental capacity of bovine IVF embryos was examined (Table 2), day 2 cleavage rate of HP group (87.3 %) was significantly higher cleavage rate than control (75.2 %) and other treatment group (DS,73.6 %; CS, 74.5 %), except HA group (81.8%) like as 2PN formation rates ($P< 0.05$). However, day 8 blastocyst rate was significantly higher values obtained in HP (54.1%) or HA (53.0%) groups than in control (34.1%), DS (35.8%) or CS (43.9%) groups ($P< 0.05$). In cell number count of day 8 blastocyst, there were effects on increase of total cell number and ICM cell number, those values were significantly higher in HP group (total, 137.6 ± 14.6 and ICM, 45.6 ± 13.2) and slightly higher in other GAG 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)] than those of control

group (116.6 ± 13.3 and 31.2 ± 12.9) ($P< 0.05$). Thus, ICM proportion was also higher in most of GAG exposure groups (HP, 33.1%; HA, 30.8%; CS, 29.3%) except DS group (25.7%) than control (26.8 %). These differences were also shown in Fig. 3 A-E, HP or HA exposed groups presented better embryo developmental morphology, blastocoel cavity expansion and embryo quality were more superior to control.

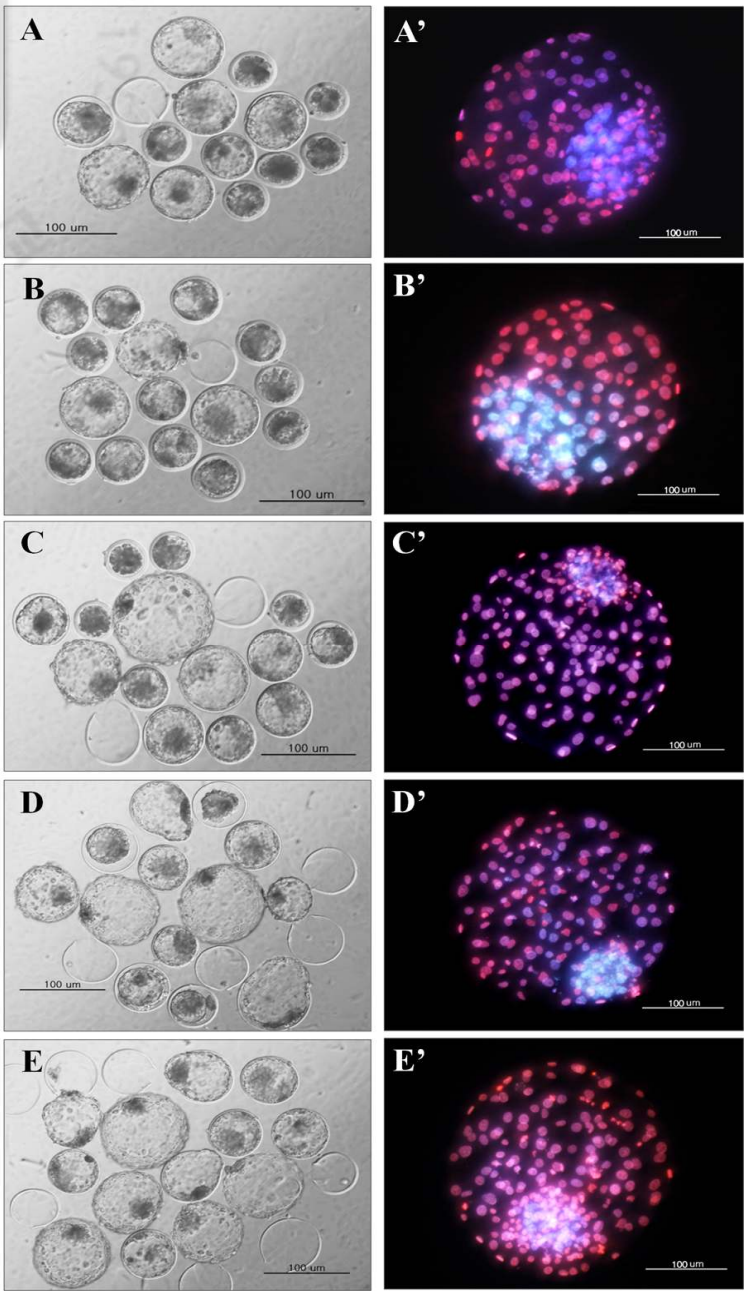


Fig. 3. Developmental morphology of bovine day 8 blastocysts produced *in vitro* in various GAGs treatment groups. For *in vitro* fertilization, sperm was incubated in the absence or presence of each GAGs (DS; dermatan sulphate, CS; chondroitin sulphate, HA: hyaluronic acid or HP; heparin) for 1 hour before insemination. The cell number was examined by differential labeling of various GAGs treatment groups, respectively. No treatment: A- A'. DS: B-B'. CS: C- C'. HA: D-D'. HP: E-E'.

Table 1. Pronuclear status of bovine zygotes at 18 hour after IVF using GAG treated sperm

Treatment*	No. of 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 sulphate, CS: chondroitin sulphate, HA: hyaluronic acid, and HP: heparin

** ($P< 0.05$) [a-b]

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

Treatment*	No. of examined	No.(%)** of embryos developed		Cell Number Total (ICM) M ± SEM**	ICM Proportion %
		Day 2 ≥ 2-4 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 sulphate, CS: chondroitin sulphate, HA: hyaluronic acid, and HP: heparin

** ($P< 0.05$) $^{a-b}$

4. Relative mRNA Expression in Bovine IVF Embryos Produced using Different GAG Exposed Sperm

The relative mRNA 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) were analyzed. As shown in Fig. 4E, the mRNA expression level for the core pluripotency marker gene Oct 4 was significantly higher in all GAG exposure groups compared to control ($P<0.05$). Also, apoptotic Caspase 3 mRNA expression was significantly low in all GAG treatment groups than control (Fig. 4C), anti-apoptotic Bax inhibitor mRNA expression was higher in HA and HP groups than other GAG or control groups (Fig. 4B) ($P<0.05$). In addition, the mRNA expression of Glut 5 was also in significantly higher in HA and HP groups than other treatment groups (Fig. 4D) ($P<0.05$). However, there were no differences in the expression levels of Bax (Fig. 4A) and FGF4 (Fig. 4F) mRNA among control and GAG treatment groups.

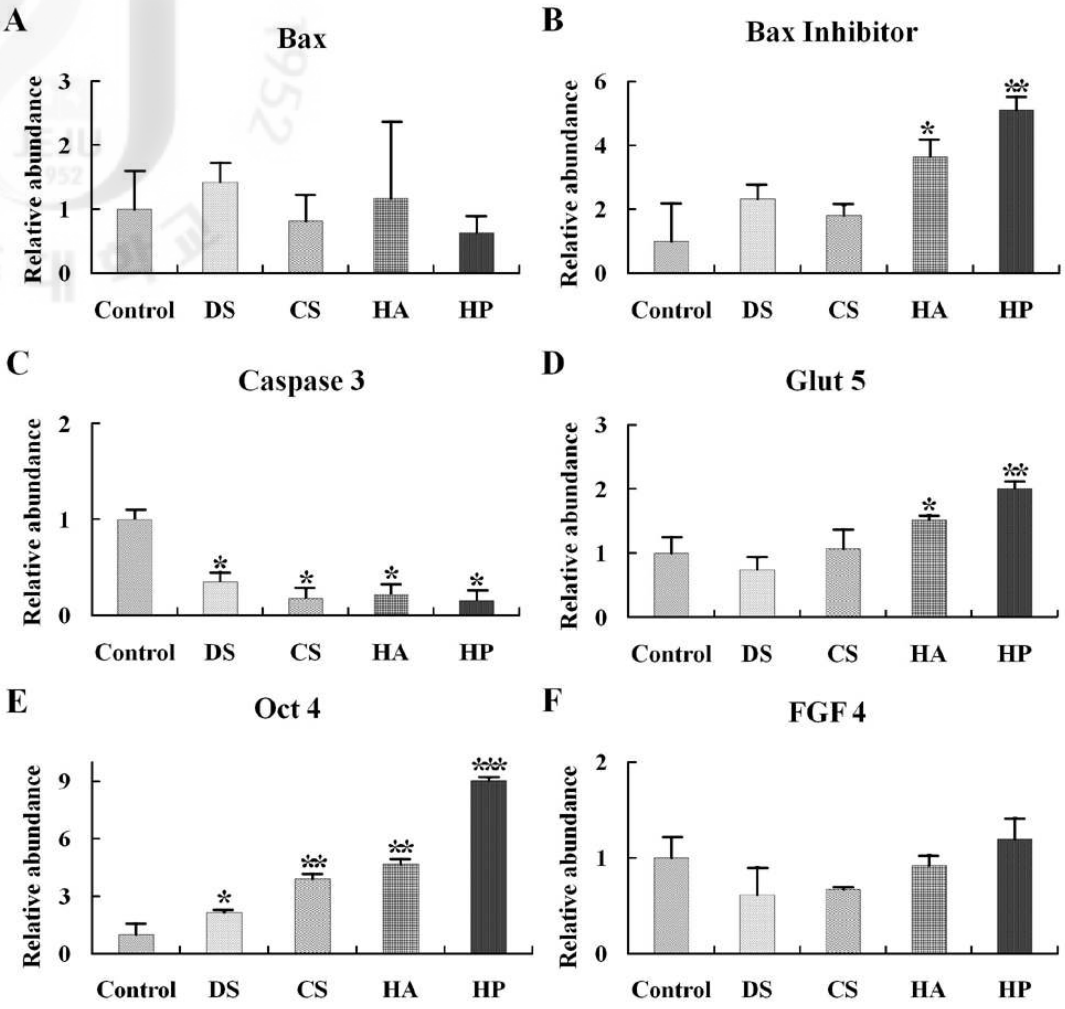


Fig. 4. Relative mRNA expression of candidate genes of bovine day 8 blastocysts produced in various GAGs treatment groups was examined in apoptosis (Bax, Bax inhibitor and Caspase 3), Growth (Glut 5), pluripotency (Oct 4) and implantation (FGF 4). The bars with different superscripts within a panel differ significantly from each other ($P< 0.05$). Error bars indicate the standard deviation.

Table 3. Primer sequences to detect variable genes

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	111bp	House keeping	NM-173979

DISCUSSION

This study demonstrated that exposing bovine sperm to GAGs affected the sperm fertilizing ability and the *in vitro* embryo developmental potential, as well as gene expression of embryos derived from bovine follicular oocytes. HP was the most potent GAG for enhancing sperm motility and inducing the acrosome reaction. Thus, HP exposed sperm exhibited improved total sperm penetration, which resulted in significantly higher 2 PN formation, cleavage rate, blastocyst formation rate and embryo cell numbers than 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, up), apoptosis (Bax inhibitor, up; Caspase 3, down), and cell growth (Glut5, up), 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 expression levels compared to the control. CS was less potent, and DS least potent, in eliciting changes of the assessed parameters. This is the first report to determine the effect of four different GAGs on i) bovine sperm motility, ii) capacitation, iii) acrosome reaction, iv) sperm penetration, v) 2 PN formation, vi) cleavage rate at day 2, vii) blastocyst formation rate at day 8, viii) total cell number and ICM cell number, and ix) expression levels of candidate genes in blastocysts.

In this study, I introduced my registered, novel sperm preparation method, the "two-step swim-up protocol", which increased the sperm viability post-preparation (> 5 hour) relative to the Percoll gradient preparation method (< 1 hour). Using my

protocol, a greater number of viable sperm were recovered and examined after different GAG treatments. For comparison of the effects of four different GAGs, frozen-thawed semen of a grade number 1 Korean bull was used, and the GAG effects on sperm capacitation were examined using 5 hour incubation. For *in vitro* embryo production, sperm were incubated with the GAG for 1 hour before IVF. The design was suggested by a previous report that found that a maximum sperm penetration rate was obtained at 5 h for bovine IVF (18).

In mammals, sperm attached to the zona pellucida of an egg immediately undergo the acrosome reaction. Thus, in order for sperm to subsequently penetrate into zona pellucida, the acrosome reaction must be completed (4). 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 (19-21). GAGs are present in components of the female reproductive tract including follicular fluid and uterine fluid (22). GAGs effectively capacitate bull sperm and lead to acrosome reactions *in vitro* (23). GAGs restrict hardening of oocytes and promote capacitation, easy penetration of sperm, and nuclear decondensation and formation of the male pronucleus (24-27). HP has been shown to cause functional changes in the cell membrane of bovine sperm and to activate cyclic AMP production (28). HA has been shown to induce capacitation of sperm by increasing the influx of Ca^{2+} in human sperm (29). DS also induces capacitation and CS also promotes sperm motility and capacitation in bovine sperm (30-31). However, most GAG tests have focused on HP treatment (32-34). Handrow et al., (6) 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 is partly responsible for promoting the acrosome reaction, which is consistent with the non-sulfated HA being the least effective. In previous studies, bovine epididymal sperm were used, but this study used frozen-thawed ejaculated sperm. There have been a few reports that HA might be an effective GAG in bovine; in this study, I confirmed that 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, producing effects similar to HP. According to my results, HP was clearly the best GAG treatment for enhancement of sperm function. HA was moderately effective and the remaining GAGs tested, DS and CS, were not effective (HP>HA>DS=CS).

By examining motility using the SAIS program and capacitation using a CTC staining assay, the effectiveness of four different GAGs was clear. The SAIS program enables an objective assessment of different characteristics of cell movement, velocity, and morphology. When the sperm motility pattern was measured over 5 hour incubation in each different GAG, HP showed the highest percentage of total motility (57.2%), VSL (11.5%) and VCL (33.4%) compared to the control or other GAG treatment groups. However, sperm motility declined sharply by 3 hour, and the HP or HA treatments were most effective in reducing the 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 my results showed that HP exposed sperm exhibited the best VCL. VSL has also been used as a predictor of sperm function, similar to VCL. One of the most useful methods for determination of the capacitation status is the CTC assay (14). This fluorescent antibiotic exhibit enhanced fluorescence over segments of the membrane where Ca^{2+}

accumulates (35-37). 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 the uncapacitated sperm pattern F decreased after 1 hour, concomitant with increases in the capacitated sperm pattern B and acrosome-reacted sperm pattern AR, as previously reported (38-40). After 5 h in the presence of GAGs, the proportions of pattern B and AR in the HP or HA exposure groups were 25-30% vs. 70-75%, respectively. 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 ICM cells in the HP treatment group were significantly higher than those in the control or other GAGs treatment groups (DS or CS) ($P< 0.05$).

To examine the effects of sperm exposure to different GAGs on *in vitro* embryo production, the relative 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. The level of Oct-4 expression may regulate cell lineage commitments in that a critical level of expression is required to maintain pluripotency (41). It is interesting that embryos derived from all GAG treated sperm showed significantly higher expression levels of Oct4 than control embryos ($P< 0.05$). Additionally, the

expression levels of the pro-apoptotic gene caspase 3 were significantly lower in all GAG treatment groups than in 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 other GAG treatment groups (DS or CS) ($P< 0.05$). Furthermore, the expression levels of Oct4, Bax inhibitor and Glut 5 genes differed between the HP and HA treatment groups ($P< 0.05$). Rizos *et al*. (42) suggested that differences in expression patterns are related to the quality of the bovine blastocysts produced under different culture conditions. From this viewpoint, significantly high pluripotent (Oct4, up regulated) and growth (Glut 5, up regulated), and anti-apoptotic (Bax inhibitor, up regulated; Caspase 3, down regulated) gene expression levels were correlated with the high-quality embryos produced from HP- or HA-treated sperm.

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

REFERENCES

1. Tienthai P, Johannisson A and Rodriguez MH. Sperm capacitation in the porcine oviduct. Anim Reprod Sci 80:131-146 (2004)
2. Lane ME, Therien I, Moreau R and Manjunath P. Heparin and high-density lipoprotein mediate bovine sperm capacitation by different mechanisms. Biol Reprod; 60:169-175 (1999)
3. Tienthai P, Johannisson A and Rodriguez MH. Sperm capacitation in the porcine oviduct. Anim Reprod Sci 80:131-146. (2004)
4. Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD (eds.), The Physiology of Reproduction. New York: Raven Press; 189-317 (1994)
5. Isabelle T, Annick B, Daniel B and Puttaswamy M. Isolation and characterization of glycosaminolycans from bovine follicular fluid and their effect on sperm capacitation. Mol Reprod Dev 71: 97-106 (2005)
6. Handrow RR, Lenz RW and Ax RL. Structural comparisons among glycosaminoglycans to promote an acrosome reaction in bovine spermatozoa. Biochem biophys Res Commun 107:1326-1332 (1982)
7. Dora GD, Patricia EM and Marcelo OC. Effect of heparin on *in vitro* capacitation of boar sperm. Biol Res 39:631-639 (2006)
8. Borg N and Holland M. The effect of glycosaminoglycans on rat gametes *in vitro* and the associated signal pathway. Reproduction 135:311-319 (2008)
9. Towhidi A, Meharabani YH, Daliri JM, Ranjbar A and Zhandi M. Effect of plasmin and heparin on *in vitro* ovine sperm-oocyte interaction. Afri J

Biotech 8:3677-3681(2009)

10. Hamamah S, Wittemer C, Barthelemy C, Richet C, Zerimech F. Royere D and Degand P. Identification of hyaluronic acid and chondroitin sulfates in human follicular fluid and their effects on human sperm motility and the outcome of *in vitro* fertilization. Reprod Nutr Dev 36:43-52 (1996)
11. Hileman RE, Fromm JR, Wiler JM and Linhardt RJ. Glycosaminoglycan-protein interactions: definition of nonsensus site in glycosaminoglycan binding proteins. Bioessays. 20:156-167 (1998)
12. Yanagichita M, Rodbard D and Hascall VC. Isolation and characterization of proteoglycans from porcine ovarian follicular fluid. J Biol Chem 254:911-920 (1979)
13. Ax RL and Ryan RJ. The porcine ovarian follicle. IV. Mucopolysaccharides at different stages of development. Biol Reprod 20:1123-32 (1979)
14. Bergqvist AS, Ballester J, Johannisson A., Lundeheim N and Rodriguez-Martinez H. Heparin and dermatan sulphate induced capacitation of frozen-thawed bull spermatozoa measured by merocyanine-540. Zygote 15:225-32 (2007)
15. Kuroda K, Fukushima M and Harayama H. Premature capacitation of frozen –thawed spermatozoa from subfertile Japanese black cattle. J Reprod Dev 53:1079-86 (2007)
16. Thouas GA, korfiatis NA, French AJ, Jones GM and Trounson AO. Simplified technique for differential staining of inner cell mass and trophectoderm cells of mouse and bovine blastocysts. Reprod Biomed Online 3:25-29 (2001)

17. Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) method. Methods 25:402-408 (2001)
18. Rehman N, Collins AR and Suh TK, Wright RW Jr. Effect of sperm exposure time on *in vitro* fertilization and embryo development of bovine oocytes matured *in vitro*. Theriogenology 41:1447-52 (1994)
19. Purdy PH and Graham JK. Effect of adding cholesterol to bull sperm membranes on sperm capacitation, the acrosome reaction, and fertility. Biol Reprod 71:522-7 (2004)
20. Therien I and Manjunath P. Effect of progesterone on bovine sperm capacitation and acrosome reaction. Biol Reprod 69:1408-15 (2003)
21. Marguez B and Suarez SS. Different signaling pathways in bovine sperm regulate capacitation and hyperactivation. Biol Repord 70:1626-33 (2004)
22. Kano K, Miyano T and Kato S. Effects of glycosaminoglycans on the development of *in vitro*-matured and fertilized porcine oocytes to the blastocyst stage *in vitro*. Biol Reprod 58:1226-32 (1998)
23. Berqqvist AS, Ballester J, johannisson A, Hemandez M, Lundeheim N and Rodriuez-Martinez H. *In vitro* capacitation of bull spermatozoa by oviductal fluid and its components. Zygote 14:259-73 (2006)
24. Grippo AA, Way AL and Killian GJ. Effect of bovine ampullary and isthmic oviductal fluid on motility, acrosome reaction and fertility of bull spermatozoa. J Reprod Fertil 105:57-64 (1995)
25. Anderson SH and Killian GJ. Effect of macromolecules from oviductal conditioned medium on bovine sperm motion and capacitation. Biol Reprod

48:537-544 (1997)

26. Kawakami E, Arai T and Oishi I. Induction of dog sperm capacitation by glycosaminoglycans and glycosaminoglycan amounts of oviductal and uterine fluids in bitches. J Vet Med Sci 62:65-68 (2000)
27. Mahmoud AI and Parrish JJ. Oviductal fluid and heparin induce similar surface changes in bovine sperm during capacitation: a flow cytometric study using lectins. Mol Reprod Dev 43:554-560 (1996)
28. Parrish JJ, Susko-Parrish J, Uguz C and First NL. Differences in the role of cyclic adenosine 3, 5-monophosphate during capacitation of bovine sperm by heparin or oviduct fluid. Biol Reprod 51:1099-1108 (1994)
29. Slotte H, Akerlof E and Pousette A. Separation of human spermatozoa with hyaluronic acid induces, and Percoll inhibits, the acrosome reaction. Int J Androl 16:349-354 (1993)
30. Parrish JJ, Susko-Parrish J, Handrow RR, Ax RL and First NL. Effect of sulfated glycoconjugates on capacitation and the acrosome reaction of bovine and hamster spermatozoa. Gamete Res 24:403-13(1989)
31. Lenz RW, Martin JL, Bellin ME and Ax RL. Predicting fertility of dairy bulls by inducing acrosome reactions in sperm with chondroitin sulfates. J Dairy Sci 71:1073-7 (1988)
32. Parrish JJ, Susko-Parrish J, Winer MA and First NL. Capacitation of bovine sperm by heparin. Biol Repord 38:1171-1180 (1988)
33. Miller DJ, Winer MA and Ax RL. Heparin-binding proteins from seminal plasma bind to bovine spermatozoa and modulate capacitation by heparin. Biol Reprod 42:899-915 (1990)

34. Lu KH and Seidel GE Jr. Effects of heparin and sperm concentration on cleavage and blastocyst development rates of bovine oocytes inseminated with flow cytometrically- sorted sperm. Theriogenology 62:819-830 (2004)
35. Dasgupta S, Mills CL and Fraser LR. Ca2+ related changes in the capacitation state of human spermatozoa assessed by a chlortetracycline fluorescence assay. J Reprod Fertil 99:135-43 (1993)
36. Fraser LR, Abeydera LR and Niwa K. Ca2+ regulating mechanisms that modulate bull sperm capacitation and acrosomal exocytosis as determined by chlortetracycline analysis. Mol Reprod Dev 40:233-41 (1995)
37. Ward CR and Storey BT. Determination of the time course of capacitation in mouse spermatozoa using a chlortetracycline assay. Dev Biol 104:287-96 (1984)
38. Sostaric E, Chris HA, Colenbrander B and Gadella BM. Dynamics of carbohydrate affinities at the cell surface of capacitating bovine sperm cells. Biol Reprod 72:346-357 (2005)
39. Rodriguez MH, Cuadras M, Anchondo A, Romo-Garcia S and Sanchez BE, Jimenez JA, Alarcon-Rojo AD. Heparin level effect on sperm capacitation of fresh and frozen-thawed bovine semen. Ani Sci 56 (2005)
40. Chamberland A, Fournier VS, Tardif MA and Sirad R. The effect of heparin on motility parameters and protein phosphorylation during bovine sperm capacitation. Theriogenology 55:823-835 (2001)
41. Kurosaka S, Eckardt S and Mclaughlin KJ. Pluripotent lineage definition in bovine embryo by Oct 4 transcript localization. Biol Reprod 71:1578-1582 (2004)

42. Rizos D, Gutierrez AA, Perez GS, De La Fuente J, Boland MP and Lonergan P. Bovine embryo culture in the presence or absence of serum: Implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 68:236-43 (2003)

GAG 처리된 정자가 소 난자의 체외발달에 미치는 영향

노 은 형

제주대학교 대학원

생명공학부

(지도교수 박 세 필)

국문초록

Glycosaminoglycan (GAG)은 자성생식수관액에 존재하며 정자의 수정능 획득을 촉진하는 물질이다. 본 실험에서는 4 개의 서로 다른 GAG 들 (Chondroitin sulfate, CS; Dermatan sulfate, DS; Hyaluronic acid, HA; Heparin, HP)을 10 ug/ml 의 같은 농도로 소 정자에 5 시간 동안 노출시켰을 때, HP 나 HA 를 처리한 군에서 정자의 total motility, VSL 그리고 VCL 수치가 대조 군이나 다른 GAG 처리군 (DS 혹은 CS) 에 비하여 높게 나타났다. 또한 HP 혹은 HA 처리 군의 정자 수정능 획득과 첨체반응율이 다른 처리 군에 비하여 증가하였다. 게다가 체외수정 전 1 시간 동안 HP 혹은 HA 에 노출된 정자는 유의하게 높은 수정 능력을

나타내어, 전핵 형성율과 수정 후 2 일 째 난할율, 그 이후 배반포 발달율을 증가시키는 효과를 보였으며 그와 더불어 내부세포괴를 포함한 전체적인 세포수 증가를 유도하였다 ($P< 0.05$). 그리고 HP 와 HA 를 처리한 군에서 발달된 배아를 Real-time PCR 을 시행하였던 바, pluripotency 유전자 Oct 4, cell growth 유전자 Glut 5 그리고 anti-apoptosis 유전자 Bax inhibitor 발현이 대조군이나 다른 GAG 처리군에 비하여 유의하게 높게 발현되며, pro-apoptotic 유전자인 Caspase 3 는 GAG 를 처리한 모든 군에서 현저히 낮음을 확인하였다 ($P< 0.05$). 따라서, 본 연구는 소 정자에 HP 또는 HA 처리가 체외 수정능력과 난자의 체외 발달능을 높이고 유전자 발현에 효과적으로 작용할 수 있음을 나타낸다.

ACKNOWLEDGEMENT

This study was supported by a grant (308008-5) of the Technology Development Program for Agriculture and Forestry, Ministry for Agriculture, Forestry and Fisheries, Republic of Korea.

이 논문을 완성 하기까지 도움을 주신 많은 분들께 감사의 뜻을 전하고자 합니다.

복학 후 전공에 대한 어려움을 느끼고 있을 때 제주대학교와 새로운 인연을 맺으신 교수님의 수업을 듣게 되었습니다. 첫 강의 때 교수님께서 “나무 하나를 자세히 설명하기 보다는 큰 숲을 볼 수 있는 강의를 하겠다” 라고 하신 말씀에 큰 힘을 얻은 것이 기억이 납니다. 지금 까지도 저에게 큰 숲을 볼 수 있는 가르침을 주시는 박세필 교수님께 가장먼저 깊은 감사의 뜻을 표합니다. 또한 저의 부족한 논문 발표에도 고생했다 격려해 주시고 진학에 대한 부분을 포함해 여러 면에서 아낌없는 조언을 해 주신 류기중 교수님께도 진심으로 감사 드립니다. 실험을 위해 장시간 글로브를 착용해 손등에 물집이 잡힐지언정 흔들림 없이 진정한 연구원의 모습과 프로페셔널의 의미를 몸소 실천하며 보여주신 김은영 소장님께도 고개 숙여 감사 드립니다. 같은 여성으로서 감히 쉽게 표현하지 못할 존경을 표합니다.

제주대학교 줄기세포 연구센터에서 만나게 된 여러 선생님들, 특히 연구원으로서나 박사의 모습으로서나 처음으로 닮고 싶다고 느끼게 해준 박효영 선생님 (저 때문에 고생 많으시죠? 많은 가르침 감사합니다. 앞으로도 잘 부탁 드려요. 쌤 파이팅! 빨리 결혼하세요!!), 한때 동거동락도 하며 세상에 존재하는 새로운 문화를 접할 수 있는 기회를 준 박민지 선생님, 언니이자 동료라는 정말 어려운 관계를 잘 유지해준 동기 노은지 선생님, 매 학기 방학마다 연수를 와 힘든 내색 없이 정말 열심히 배우고 생활하는 귀여운 (?) 막내 좌익전, 손여진, 아직은 어색한 귀염이와 경훈이에게도 고마움을 전합니다.

새로운 배움의 기회를 주셨던 국립 축산과학원의 모든 박사님들 특히 양병철 박사님을 비롯하여 이휘철 박사님께 감사 드리며 소중한 인연이 되어준 현미, 진희, 규희에게도 고마움을 표합니다.

시간 맞추기 힘든 친구이지만 끝까지 믿어주고 자랑스럽게 여겨주는 나의 친구들 박진영, 유하나, 송수현, 조서연 (결혼 축하해!), 전은순, 김소연. 얘들아 정말 고마워. 또 지금은 서로 다른 분야에서 생활하고 있지만 언제나 저를 응원해주고 힘을 준 제주대학교의 선배들과 동기들에게도 고마움을 표합니다. 송이언니, 고마워.

마지막으로 무한한 사랑과 믿음으로 너무나 지치고 힘든 순간에도 다시 일어날 수 있는 힘을 주신 아버지, 어머니. 제주도에 있을 때보다 훨씬 가까워 졌음에도 자주 찾아 뵙지 못하는 불효 손녀들에게 너희 일이 우선이니 걱정 말고 건강 꼭 챙기라고 해주시는 할머니. 언니 또는 동기 라는 누구보다 힘든 위치에서 묵묵히 힘이 되어주며 노력해준 노은지 (고맙고 미안해. 너에게 많은 것을 배웠어. 자랑스럽다). 누구보다 든든한 멘토가 되어 주고 날이 갈수록 성숙해지는 막내 노현진을 포함하여 항상 걱정해 주시는 모든 가족 분들께 진심으로 감사 드리며 저의 힘의 원동력임을 알기에 앞으로도 손녀, 딸, 언니, 누나로써 더욱 노력하는 모습 보여드리겠습니다.

감사합니다.