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# Sulfation patterns in heparin and heparan sulfate: effects on the proliferation of bovine pulmonary artery smooth muscle cells

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**Abstract**

Heparin's (HP's) antiproliferative effect on smooth muscle cells is potentially important in defining new approaches to treat pulmonary hypertension. The commercially available HP and heparan sulfate (HS) are structurally heterogenous polymers. In order to examine which sulfonate groups are required for endogenous antiproliferative activity, we prepared the following six chemically modified porcine mucosal HP and HS, which fell into three groups. One group consisted of fully *O*-sulfonated-*N*-acetylated, the second group consisted of de-*N*-sulfonated and re-*N*-acetylated, and the third group consisted of 6-*O*-desulfonated HP and HS derivatives. These six preparations were assayed for their antiproliferative potency on bovine pulmonary artery smooth muscle cells. The results of this assay show that (a) over-*O*-sulfonation of both HP and HS increases antiproliferative activity, (b) substitution of hexosamine with *N*-acetyl diminishes antiproliferative activity in both HP and HS, and (c) 6-*O*-desulfonation of HP and HS diminishes antiproliferative potency. Surprisingly, the type of uronic acid residue present at a given level of sulfation is unimportant for antiproliferative potency. In conclusion, only the level of *O*- and *N*-sulfo group substitution correlates well with HP and HS antiproliferative activity.


*Keywords:* Heparin; Heparan sulfate; Smooth muscle cell; Proliferation; Post-column derivatization HPLC; $^{1}$H NMR

## 1. Introduction

Heparin (HP) (**1**, Fig. 1) and heparan sulfate (HS) (**2**, Fig. 1) are a family of macromolecules found in virtually all tissues in a wide variety of species. HP and HS are chemically similar except for the content of *N*- and *O*-sulfo groups, the content of *N*-acetyl groups, and ratio of the type of hexuronic acids [1,2]. These macromolecules are composed of repeating units of alternating glucosamine and hexuronic acid [either D-glucuronic acid (GlcA) or L-iduronic acid (IdoA)] residues with a (1→4) linkage. Sulfo substituents occur as *N*-sulfo [at *C*-2 of the D-glucosamine (GlcN) residues] or as *O*-sulfo groups (at *C*-6 of the GlcN or at *C*-2 of the IdoA units). Irregular regions of these polysaccharides can also contain other *O*-sulfo groups (at *C*-3 of the GlcN or at *C*-2 of the GlcA units) [3]. GlcA is more abundant in HS and IdoA in HP [4].

Sulfo groups in HP appear to play an important role in the inhibitory effect on smooth muscle cell (SMC) proliferation [5]. Removal of *N*-sulfo groups from HP reportedly negates its growth inhibitory effect on SMCs [6]. *N*-acetylation after *N*-desulfonation in HP reportedly does not restore the SMCs growth inhibitory potency [6]. Earlier studies [5,7–9] concerning the growth inhibitory effect of HP on bovine pulmonary artery (BPA) SMCs have shown that (a) an increase in the charge density of HP affects the antiproliferative activity; (b) the molecular size of the HP does not affect the potency of growth inhibition; (c) the protein core has no antiproliferative activity; (d) the glycosaminoglycan (GAG) chains of HP are responsible for the antiproliferative activity; (e) 3-*O*-sulfo group on the internal GlcN residue is not critical for native HP's antiproliferative activity; (f) *N*-sulfo groups in HP are not

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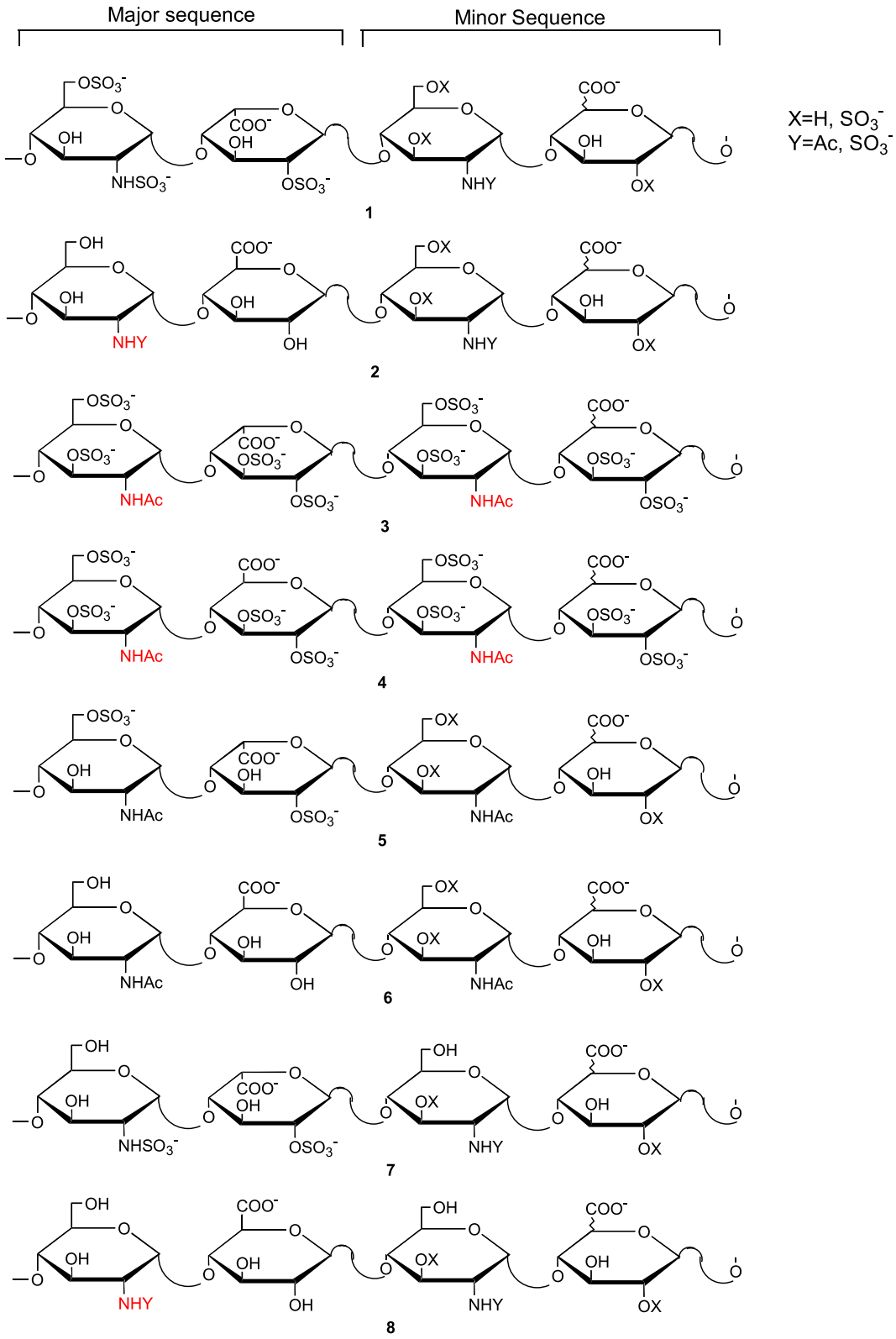


Fig. 1. Structural formulae of major and variable sequences of repeating disaccharide units of HP and HS preparations. **1**, heparin (HP); **2**, heparan sulfate (HS); **3**, fully *O*-sulfonated and re-*N*-acetylated HP; **4**, fully *O*-sulfonated and re-*N*-acetylated HS; **5**, de-*N*-sulfonated and re-*N*-acetylated HP; **6**, de-*N*-sulfonated and re-*N*-acetylated HS; **7**, 6-*O*-desulfonated HP; **8**, 6-*O*-desulfonated HS.

critical for antiproliferative activity; and (g) the GlcN basic sugar residues in HP are replaceable with galactosamine residues. The objective of the present study is to analyze further the structural determinants in HP/HS responsible for the effect on the growth of BPASMCs. We have investigated (a) the requirement for either IdoA or GlcA, (b) the significance of *N*-sulfo group of GlcN residues, and (c) the importance of 6-*O*-sulfo group of GlcN residues, for PASMCs proliferation.

## 2. Materials and methods

### 2.1. Materials

Fetal bovine serum and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Porcine mucosal HP and HS were purchased from Celsus Laboratories. Cell culture media, RPMI-1640 (Meditech, Washington, DC) contained streptomycin (Lilly, Indianapolis, IN), penicillin (Pfizer, New York, NY) and amphotericin B (GIBO, Grand Island, NY).

### 2.2. $^1$H-NMR conditions

$^1$H-NMR spectroscopy was performed using conditions described previously [10]. Briefly, a HP sample (approximately 2 mg) was dissolved in 0.5 ml of $D_2O$ (99.9%) and repeatedly freeze-dried to remove exchangeable protons. The sample was kept in a desiccator over phosphorus pentoxide in vacuo overnight at room temperature. The thoroughly dried sample was then dissolved in 0.5 ml of $D_2O$ (99.96%) and passed through 0.45-μm syringe filter and transferred to a NMR tube (5.0 mm o.d. × 25 cm; Wilmad Glass Co., Buena, NJ). 1D and 2D NMR experiments were performed on a JEOL GSX500A spectrometer equipped with 5-mm field gradient tunable probe with standard JEOL software at 303 K for NOE spectra or 333 K for other experiments on 500-μl samples. The HOD signal was suppressed by presaturation during 3 or 1.5 s for 1D or 2D spectra, respectively. To obtain 2D spectra, 512 experiments resulting to 1024 data points for a spectral width of 2000 Hz were measured, and the time domain data were multiplied after zero-filling (data matrix size, 1 × 1 K) with a shifted sine-bell window functions for 2D double quantum filtered (DQF)-COSY, NOESY or TOCSY experiments. An MLEV-17 mixing sequence of 100 ms was used for 2D TOCSY and NOESY experiments by using 150, 250 and 500 ms as the mixing times.

### 2.3. Enzymatic digestion of HP and HS

Complete depolymerization of HP and HS uses a mixture of HP lyases I, II and III [11]. Briefly, the sample ($\leq$1 mg in 100 μl of water) was placed in a 500-μl polypropylene centrifuge tube. Sodium phosphate buffer (10 μl, 500 mM, pH 7.5) was added to the sample, after which 5–10 mU each of heparin lyase I, II and III are added. The sample was digested for 8–12 h at 35 °C and analyzed immediately by the HPLC or stored frozen at −60 °C until analysis.

### 2.4. HPLC conditions

The established conditions for the determination of the sulfated unsaturated disaccharide from HP and HS were as follows: The HPLC system was assembled with gradient pumps [Jusco 880-PU, Intelligent HPLC pump (Tokyo, Japan)], a variable sample injector [VMD-350, Shimamura Instrument Co., Tokyo, Japan)], a double-plunger pump (PSU-2.5W, Shimamura Instrument Co.) for delivery of the post-column reagents, a dry reaction bath (Type DB-3, Shimamura Instrument Co.), a fluorescence spectrophotometer (Hitachi model F-1050, Hitachi Seisakusho, Tokyo, Japan) and a chromato-integrator (D-2500, Hitachi Seisakusho). Separation column, Asahipak $NH_2$P-50 (250 × 4.6 mm i.d.) was from Shodex Co. (Tokyo, Japan). A linear gradient elution program was started at 100% of eluent A (0.1 M sodium phosphate buffer, pH 10.0, containing 0.1 M sodium NaCl) to 100% of eluent B (0.1 M phosphate buffer, pH 10, containing 0.5 M NaCl) for 10 min, and maintained 100% of eluent B for 10 min, and then returned to 100% of the initial buffer. Flow rate was constant at 0.5 ml/min sodium hydroxide (0.5 M), and 50 mM guanidine was added to the eluate of the separation column at a flow rate of 0.25 ml/min using a double-plunger pump. The mixture was passed through a polytetrafluoroethylene (PTFE) reaction coil (10 m × 0.5 mm i.d.) set in a dry reaction bath thermostated at 110 °C and then through a PTFE cooling coil (2 m × 0.25 mm i.d.). The effluent was monitored by fluorescence with excitation at 320 nm and emission at 420 nm.

### 2.5. Fully O-sulfonated re-N-acetylated HP and HS (3,4)

Chemical *O*-sulfonation to obtain fully sulfated HP and HS was carried out under mild conditions with adducts of sulfur trioxide ($SO_3$) in aprotic solvents [12]. Fully *O*-sulfated HP and HS were prepared from the tributylamine (TBA) salt, obtained from 100 mg of each sodium salt by

Table 1
Properties of HP and HS derivatives (**1**–**8**)

| Compound | $SO_3^-$/$COO^-$ | % 2-*O*-$SO_3^-$ | % 6-*O*-$SO_3^-$ | % *N*-$SO_3^-$ | IdoA/GlcA |
|---|---|---|---|---|---|
| HP **1** | 2.68 | 86.2 | 89.7 | 90.4 | 92.3/7.7 |
| **3** | 3.88 | 100 | 100 | <0.5 | 92.3/7.7 |
| **5** | 1.74 | 86.2 | 89.7 | <0.5 | 92.3/7.7 |
| **7** | 1.66 | 86.2 | <0.5 | 90.4 | 92.3/7.7 |
| HS **2** | 0.25 | 1.6 | 6.4 | 17.5 | 26.7/73.3 |
| **4** | 3.92 | 100 | 100 | <0.5 | 26.7/73.3 |
| **6** | 0.10 | 1.6 | 6.4 | <0.5 | 26.7/73.3 |
| **8** | 0.19 | 1.6 | <0.5 | 17.1 | 26.7/73.3 |

strong cation-exchange chromatography, concentration by lyophilization. The resulting salt was dissolved in 0.8 ml of *N*,*N*-dimethylformamide (DMF) to which a required excess (15 mol/equivalent of available hydroxy group in HP or HS) of pyridine–sulfur trioxide complex had been added. After 1 h at 40 °C, the reaction was interrupted by addition of 1.6 ml of water and the raw product was precipitated with 3 volumes of cold ethanol saturated with anhydrous sodium acetate, and then collected by centrifugation. The resulting fully *O*-sulfonated HP and HS were dissolved in water, dialyzed to remove salts and lyophilized. Since partial loss of *N*-sulfo groups from GlcNS residues was

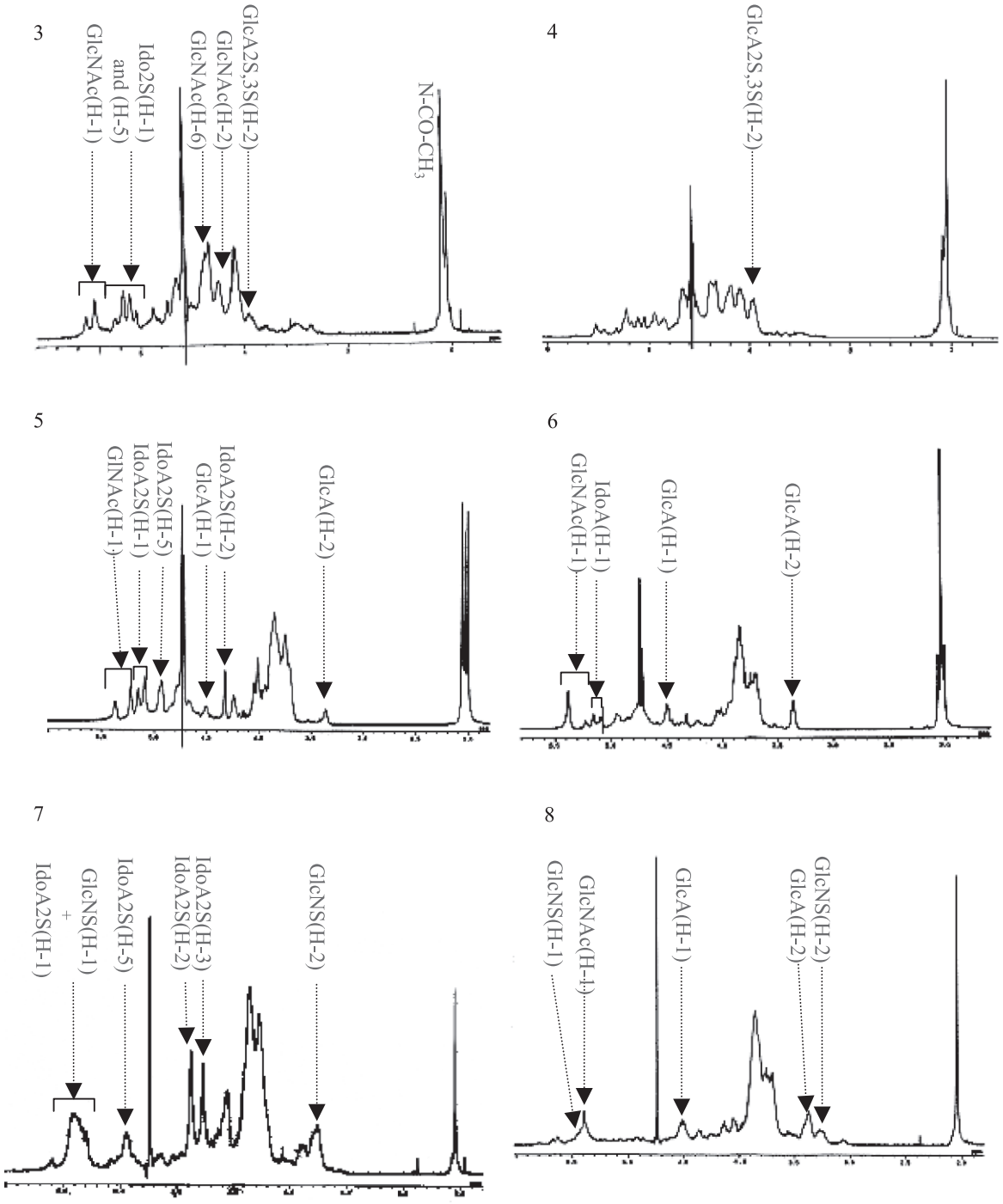


Fig. 2. $^{1}$H-NMR analysis of samples of chemically modified HP and HS samples. **3**, fully *O*-sulfonated and re-*N*-acetylated HP; **4**, fully *O*-sulfonated and re-*N*-acetylated HS; **5**, de-*N*-sulfonated and re-*N*-acetylated HP; **6**, de-*N*-sulfonated and re-*N*-acetylated HS; **7**, 6-*O*-desulfonated HP; **8**, 6-*O*-desulfonated HS.

observed after *O*-sulfonation of HP and HS, re-*N*-acetylation was performed according to previously described methods [13,14].

### 2.6. De-N-sulfonated re-N-acetylated HP and HS (5,6)

HP or HS sample (5 mg) was dissolved in 10 ml of water and passed through a Dowex 50WX8 ($H^+$ form, 200–400 mesh) column (0.8 cm i.d. × 10 cm) to exchange sodium to hydrogen ion. The acidic fraction was neutralized with pyridine and lyophilized. Pyridine complex of GAG was dissolved in 1.5 ml DMSO containing 5% (v/v) water and heated at 50 °C for 90 min to remove *N*-sulfo group completely. The resulting *N*-desulfonated HP or HS was dissolved in 3 ml of water, and 0.5 ml methanol and 90 μl acetic anhydride were added. The pH of the mixture was adjusted to 6.5 by the addition of 10% sodium hydrogen carbonate and was kept at 3–4 °C for 2 h. The mixture was dialyzed against water and lyophilized [14].

### 2.7. 6-O-desulfonated HP and HS (7,8)

Selective 6-*O*-desulfonation of intact HP and HS were achieved according to the method described previously by Matsuo et al. [15]. Briefly, an aqueous solution of the sodium salt of HP or HS (10 mg) was passed through a Dowex 50WX8 ($H^+$ form, 200–400 mesh) column (0.8 cm i.d. × 10 cm) at room temperature, and the eluate was neutralized with pyridine and lyophilized. The pyridinium salt of HP or HS was dissolved in 1.0 ml dry pyridine (Merck, Darmstadt) and 2 ml *N*,*O*-bis(trimethylsilyl)acetamide (BTSA) was added. The mixture was kept for 2 h at 70 °C to give a clear solution, and then 1.0 ml water was added to the mixture to decompose the excess reagent and silyl ester. The mixture was dialyzed against water and pH was adjusted to 7.0 with 0.1 M NaOH; the mixture was then lyophilized to obtain the 6-*O*-desulfonated HP or HS sodium salt (7.5 mg).

Characteristics of HP and HS derivatives are summarized in Table 1. $^1$H-NMR of HP and HS derivatives (**3**–**8**, Fig. 1) confirmed their structure (Fig. 2).

### 2.8. Cultured pulmonary artery smooth cells proliferation assay

Smooth muscle cell proliferation assays were performed as previously described [8,9]. Briefly, isolated BPASMC were seeded at $1.25 \times 10^4$ cells/well into six-well tissue culture plates, grown for 2 days, then growth was arrested for 48 h by reducing the serum concentration of the medium from 10% to 0.1%. Media was then changed for experimental samples to contain either standard medium (RPMI-1640 with 10% FBS), growth arrest media (0.1% FBS) or standard media containing HP or other GAG derivatives (10 μg/ml). All media contained streptomycin (10 mg/ml), penicillin (100 U/ml) and amphotericin B (1.25 μg/ml). After 4 days, the SMCs present in the cell culture wells were rinsed with Hank's balanced salt solution to remove the remaining cell culture medium and unattached dead cells. Trypan blue exclusion showed no dead SMC either in control or in the media treated with GAGs. After detachment of SMCs with trypsin/EDTA, the cell number was counted by Coulter counter.

The HSs and HPs were each dissolved (1 mg/ml) in distilled sterile water. This solution (140 μl) was added to culture medium (13.86 ml), and 2 ml of the resulting media was added to each well. The GAG preparations (**1**–**6**) were completely soluble as the culture medium was clear (no turbidity) after addition in the media.

The effective dose of HP previously has been identified as 1–10 μg/ml [7]. In the present study, 10 μg/ml was used for each of the GAGs.

Growth was measured at a time point corresponding to the logarithmic growth phase where there is no contact inhibition among cells [16]. Differences in growth reflected in the differences in thymidine incorporation was not done because the secretion of high amounts of endogenous thymidine in cell culture falsify the assessment of DNA synthesis with labeled thymidine [17].

The percent inhibition was calculated as: [1 − net cell growth in treated medium/net cell growth in standard medium]100, where net cell growth = cell growth in standard or treated medium minus cell growth in growth arrest media.

### 2.9. Statistics

Results are presented as mean ± S.E. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the stateview software package (SAS Institute Inc., Cary, NC) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using Fisher protected least significant difference (PSLD) test. In all cases, significance was set as $P < 0.0001$.

## 3. Results and discussion

Every cell types including vascular endothelial cell produces HP and HS [18]. All these preparations are potent inhibitors of SMC proliferation in vitro [19]. However, due to the limitations of supply from above sources, we have used in this study commercial porcine mucosal HP and HS. HP differs from HS primarily in its higher content of both *O*- and *N*-sulfo groups, and its higher content of iduronic acid, which is mainly sulfonated at its 2-position. These structural features lead to a slightly (but not significantly) higher level of activity at a concentration of 10 μg, on the percent inhibition of proliferation of bovine pulmonary artery SMCs (Fig. 3). Full *O*-sulfonation and re-*N*-acetylation of the amine groups afford HP derivative **3** and HS derivative **4**. When added individually to

cultured BPASMCs, both **3** and **4** show significantly increased inhibition of cell growth in comparison to unmodified HP **1** and HS **2**. Interestingly, the activity of HP derivatives **3** and HS derivative **4** were not significantly different from each other (HP **3**, 60.21 ± 4.0 and HS **4**, 58.76 ± 4.5% inhibition). These results suggest that while oversulfation enhances antiproliferative activity, differences in the GlcA/IdoA ratio in repeating disaccharide units have no effect on the antiprolifertive potency. Preliminary data suggests that inhibitory properties of modified HP/HS are reversible.

The importance of the *N*-acetyl/*N*-sulfo ratio in GlcN residues on antiproliferative potency is not well understood. Castellot et al. [20,21] have reported that *N*-sulfo groups on GlcN residues in HP can be replaced by *N*-acetyl groups without significant loss of antiproliferative activity. On the other hand, Tiozzo et al. [6] reported that *N*-reacetylation following *N*-desulfonation does not restore HP antiproliferative activity. However, a recent report has shown that HP antiproliferative activity on bovine pulmonary artery smooth muscle cells requires both *N*-acetylation and *N*-sulfonation on GlcN [22]. To further clarify the above discrepancy, HP derivative **5** and HS derivative **6** were assayed for their antiproliferative activity on BPASMCs. While both derivatives showed reduced activity when compared to HP **1** and HS **2**, derivative **5** (31.85 ± 6.0% inhibition) and derivative **6** (30.43 ± 5.9% inhibition) showed no significant difference in their antiproliferative potency. These results suggest that the *N*-acetyl content GlcN residues in either HP or HS are not critical for antiproliferative potency but rather that removal of *N*-sulfo groups in either HP or HS reduces overall negative charge and results in reduced antiproliferative activity.

In an attempt to determine the contribution of 6-*O*-sulfo groups of GlcN residues to the growth inhibition of BPASMCs, 6-*O*-desulfonated HP derivative **7** and HS derivative **8** were prepared. Both HP derivative **7** and HS derivative **8** show reduced antiproliferative activity compared to unmodified HP **1** and HS **2**. HP derivative **7** (35.15 ± 5.4% inhibition) was not significantly different from HS derivative **8** (33.75 ± 5.4% inhibition) in affecting growth inhibition of BPASMCs. These results show that 6-*O*-sulfo groups of GlcN residues are not essential for antiproliferative activity but again merely contribute to the overall level of sulfation. The antiproliferative effect of HP/HS might involve interaction of these GAGs with growth factors, growth factor receptors, chemokines and other proteins. There are many potential targets, and the structural requirements for GAG-binding are unclear for most of these. Furthermore, commercial HP and HS are heterogenous polymers composed of repeating uronic acid-GlcN disaccharide units that have undergone a variety of chemical modifications during maturation [16]. These modifications include *C*-5 epimerization of D-GlcUA to L-Ido, *O*-sulfonation of GlcN residues at *C*-6 and *C*-3 and *N*-sulfonation. These structural variations are important in determining the biological activity of a particular preparation. In a previous study, we have shown that 3-*O*-sulfonation is not critical in native HP [8]. The data obtained in our laboratory suggests that either or/and *N*- or *C*-6-sulfonate (s) in native HP may be important for antiproliferative activity.

In conclusion, although the structural requirements for antiproliferative activity are still not fully understood, we have demonstrated that (a) oversulfonation of both HP and HS increases antiproliferative activity, (b) the ratio of D-glucuronic acid/iduronic acid residues in the repeating disaccharide has no effect on antiproliferative potency, (c) *N*-sulfo and 6-*O*-sulfo present in the GlcN residues of HP and HS are and their loss reduces the overall sulfation level reducing antiproliferative potency.

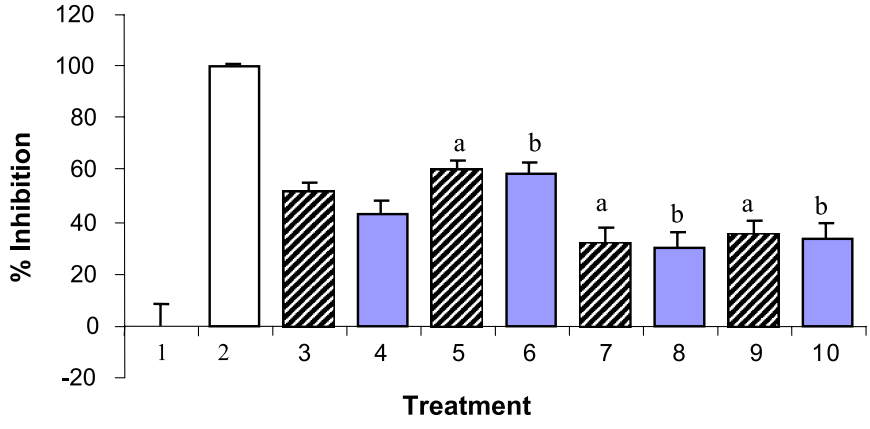


Fig. 3. Inhibition of PASMC proliferation by modified HP, HS, including starting HP and HS. Percent inhibition of bovine pulmonary artery smooth muscle cell grown in media containing 10% FBS without HP or HS as negative control ( − ), column 1; 0.1% FBS without HP or HS as positive control (+), column 2; 10% FBS plus porcine lung HP (**1**), column 3; 10% FBS plus porcine mucosa HS (**2**), column 4; containing 10% FBS plus fully *O*-sulfonated-re-*N*-acetylated HP (**3**), column 5; 10% FBS plus fully *O*-sulfonated-re-*N*-acetylated HS (**4**), column 6; 10% FBS plus de-*N*-sulfonated-re-*N*-acetylated HP (**5**), column 7; 10% FBS plus de-*N*-sulfonated-re-*N*-acetylated HS (**6**), column 8; 10% FBS plus 6-*O*-desulfonated HP (**7**), column 9; 10% FBS plus 6-*O*-desulfonated HS (**8**), column 10. "a" represents a significant inhibition in cell growth compared to HP; "b" represents a significant inhibition in cell growth compared to HS.

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