Published in final edited form as:
J Cardiovasc Transl Res. 2008 ; 1(3): 236–240. doi:10.1007/s12265-008-9047-8.

Alterations in Heparan Sulfate in the Vessel in Response to Vascular Injury in the Mouse

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Abstract

Heparan sulfate (HS) is ubiquitous throughout the human body. The backbone of HS is composed of many types of sugars. HS serves as a docking site for a vast array of protein ligands. Recent evidence suggests a unique diversity in HS structure that alters protein binding and protein function. This diversity in HS structure has been overlooked till now. The goal of this study was to determine whether femoral artery wire injury modified HS structure. Femoral artery wire injury was performed in 16-week-old male C57BL6 mice. Transcript levels of a panel of enzymes that regulate HS fine structure, including *N*-deacetylase-*N*-sulfotransferases (Ndst) 1 and 2, exostoses (Ext) 1 and 2, C5 epimerase, and 2-*O* and 6-*O* sulfotransferases, were quantified with real-time quantitative polymerase chain reaction at 7 and 14 days post injury. All enzymes showed significant alterations in messenger RNA expression in response to injury. Ndst1, the most prevalent isoform, exhibited a 20-fold increase in response to injury. Injury induced significant alterations in fine structure specially increases in N-sulfated disaccharides at 14 days post injury. Vascular injury invokes transcriptional regulation of the enzymes that regulate HS structure, as well as changes in the pattern of HS chains in the vessel wall 14 days post injury. These findings may be important as the foundation of altered growth factor and chemokine binding in the process of vascular remodeling.

Keywords

Heparan Sulfate; Vascular Injury; *N*-Deacetylase-*N*-Sulfotransferase; Exostoses; *O*-Sulfotransferase

Introduction

Heparan sulfate (HS) is synthesized by nearly all cells in the human body and is localized to both the extracellular matrix and the cell surface. HS serves as a docking site for protein ligands [1], yet its function is often overlooked. Recent evidence suggests a unique diversity in HS structure that alters protein binding and contributes to modifications in protein function [2,3]. Unraveling HS structure has led to several landmark findings in growth factor signaling and our understanding of morphogen gradients.

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HS chains are made up of 50–400 monosaccharide units. These chains are composed of repeating units of alternating D-glucuronic acid and *N*-acetyl glucosamine residues (Fig. 1). The sugar moieties are modified through several enzymatic steps to generate the unique diversity in HS structure.

In brief, HS biosynthesis is a linear and orderly process in which each enzyme provides the substrate for the next enzyme in the pathway. Following polymerization, the HS chains are post-translationally modified by a series of reactions carried out by four classes of sulfotransferases and an epimerase. N-sulfation by a family of *N*-deacetylase-*N*-sulfotransferases (Ndst)1–4 enzymes is an early event following chain polymerization and is required for subsequent O-linked sulfation reactions [1,4].

These modifications produce distinct domains of sulfation along a single HS chain, where regions of high sulfation are interspersed with segments of low and mixed levels of sulfation (Fig. 1), and a great deal of this is controlled by the pattern and extent of sulfation. The role of HS in vascular remodeling, however, has remained largely unexplored. Hence, the overall goal of this investigation was to study the alterations in HS in response to vascular injury.

Materials and Methods

Surgical Intervention to Induce Vascular Lesion

Briefly, a straight wire (0.38 mm in diameter) was inserted into the left femoral artery via a small muscular branch as described by Sata et al. [5]. Mice were C57BL6 wild types, maintained on a normal chow diet. The wire was left in the lumen for 1 min to denude and dilate the artery. After removing the wire, the small branch was tied off, and blood flow was restored in the injured vessel. The animals were anaesthetized during the process. All protocols were in accordance with Institutional Animal Care and Use Committee guidelines.

High-Performance Liquid Chromatography Analysis

Profiling of HS disaccharides in mouse femoral arteries from wild type collected at 0 (uninjured vessels=4) and 14 days (vessels=4) was done according to the protocol described by Toyoda et al. [4]. Each sample included two vessels pooled from two animals. In brief, glycosaminoglycans were isolated from each femoral artery and enzymatically digested with a heparan lyase mixture into disaccharides. The disaccharides were then separated by reverse-phase ion-pairing high-performance liquid chromatography (HPLC), which allows for the quantification of six distinct disaccharide species. These include one unsulfated disaccharide, $\Delta^{4,5}$-uronic acid-*N*-acetylglucosamine (D0A0); two monosulfated disaccharides, $\Delta^{4,5}$-uronic acid-*N*-sulfated glucosamine (D0S0) and $\Delta^{4,5}$-uronic acid-*N*-acetylglucosamine-6-*O*-sulfate (D0A6); two disulfated disaccharides, $\Delta^{4,5}$-uronic acid-*N*-sulfoglucosamine-6-*O*-sulfate (D0S6) and 2-*O*-sulfated $\Delta^{4,5}$-uronic acid-N-sulfoglucosamine (D2S0); and one trisulfated disaccharide, 2-O-sulfated $\Delta^{4,5}$-uronic acid-*N*-sulfoglucosamine-6-*O*-sulfate (D2S6). The nomenclature for unsaturated disaccharides is described in Lawrence et al. [6].

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (RT-PCR) was performed as we described [7]. Briefly, RNA was extracted from vessels of wild type mice at 0 day (uninjured, *n*=4), 7 days (*n*=8) and 14 days (*n*=8) post injury using Rneasy kit (Qiagen) and reverse transcribed to complimentary DNA (Advantage RT-PCR, BD Biosciences). Each sample included two vessels pooled from two animals. RT-PCR with SYBR green protocol was performed using Cyclophilin A as an internal control (Roche Diagonistics). Primer pair sequences used are given in Table 1. Reactions were run at annealing temperature of 60°C for 40 cycles for all the primer pairs.

Data were analyzed using RelQuant software from Roche. This software allows the comparison of efficiency-corrected relative concentration for target and reference gene of each sample.

Statistical Analysis

All values are represented as mean±SE. Statistical significance compared to wild type control values was analyzed by one-way analysis of variance. A value of $p<0.05$ was considered statistically significant.

Results

To study the alterations in HS structure in vascular remodeling, a well-characterized wire injury was performed in wild type mice [5]. In a C57BL6 strain maintained on a normal chow diet, injury results in formation of an intimal lesion comprising mainly vascular smooth muscle cells [5,8,9]. The first goal of the study was to determine if injury altered transcription of the enzymes involved in HS chain structure. HS chains consist of repeating units of D-glucuronic acid and *N*-acetyl glucosamine. Exostoses (Ext) 1 and 2 function as copolymerases that catalyze chain elongation (see Fig. 1). In response to vascular injury, both the polymerases (Ext1 and Ext2) showed temporal change with significant up-regulation by fivefold at 7 days which decreased significantly by day 14 post injury (Fig. 2a).

Post translational modifications of the chains start by N-sulfation of *N*-acetyl glucosamine residues catalyzed by Ndst1–4. Ndst1 expression was significantly up-regulated 20- and 40-fold at 7 and 14 days, respectively (Fig. 2b). Ndst2 up-regulation was significant but minimal compared to Ndst1 (Fig. 2c). The C5 epimerase converts glucuronic acid residues to iduronic acid downstream of N-sulfated residues. Following epimerization, selected iduronic acid or glucuronic acid residues are 2-O-sulfated by the 2-*O* sulfotransferase. In the present study, the C5 epimerase expression did not change in response to injury (0 day, 0.07±0.01; 7 days, 0.11 ±0.5; 14 days, 0.11±0.6; p=ns). However, 2-*O* sulfotransferase exhibited a modest but significant two- to fourfold increase in expression over time (Fig. 2d). The 6-*O* sulfotransferase adds sulfates at the 6-*O* position of N-sulfated glucosamine or *N*-acetyl glucosamine residues. In the present study, 6-*O* sulfotransferase exhibited a significant threefold up-regulation at 14 days post injury (Fig. 2e).

The HS content, disaccharide composition, and the overall degree of N- and O-sulfation and the domain organization of the HS chains are characteristic for each individual mouse tissue [10]. In the present study, we analyzed HS disaccharide composition of femoral artery before and after injury using a state-of-the-art HPLC. The HPLC was able to separate the composition into six species of variously sulfated disaccharides after enzymatic dissociation of HS chains (see “Materials and Methods” section [4]). The six species of disaccharides shown in Table 2 are all represented in the uninjured vessel. The breakdown of disaccharides in the vessel wall was similar to the “average” composition seen in other tissues like liver and kidney [10]. Injury induced a significant increase in the mono-N-sulfated (D0S0) and a significant decrease in the D0S6 (bi-sulfated) and D2S6 (trisulfated) disaccharides 14 days post injury (Table 2).

Discussion

The relative importance of the diversity in HS structure for health and disease is just beginning to be recognized. The importance of HS structure in biology has been underscored by recent work showing that deletion of many of the enzymes that regulate HS structure have resulted in severe phenotypes or embryonic lethality [11]. Our understanding of the HS structure and regulation in the vessel wall is surprisingly limiting. In the present paper, we provide the first evidence for regulation of HS structure in response to vascular injury. A major finding of this

study was that vascular injury induces transcriptional regulation of many of the enzymes that catalyze HS fine structure. A second major finding of this study was that vascular injury induced changes in HS fine structure resulting in a significant but a modest increase in the mono-N-sulfated disaccharides at 14 days post injury.

We identified a significant 20- to 40-fold increase in Ndst1, one of the four isoforms that initiate post-translational N-sulfation of HS chains. Ndst1 is the major player in HS biosynthesis [12]. 2-*O* and 6-*O* sulfotransferases showed moderate but significant up-regulation in messenger RNA (mRNA) expression at 7 and 14 days post injury. Without N-sulfation, no 2-O-sulfation or epimerization of glucuronic acid into iduronic acid is thought to occur [1]. To understand the biological meaning of this finding, we have initiated a study in mice lacking Ndst1 in vascular smooth muscle.

This is the first report of a disaccharide analysis in the vessel wall. The breakdown of disaccharides in the vessel wall was similar to the average composition seen in other tissues like liver and kidney [10]. All six disaccharide species were detected by HPLC in the femoral artery as have been seen in most of the tissues analyzed previously [10]. HS content in the femoral arteries had more 2-O-sulfated disaccharides than the 6-O-sulfated disaccharides. This is similar to HS patterns from liver, kidney and spleen [10]. HPLC analysis of femoral arteries 14 days post injury revealed changes in three major disaccharide species: an increase in D0 S0 and decreases in D0S6 and D2S6. The reason for these changes is not entirely clear. The vessel included many cell types, and it is unclear from this type of analysis (which includes the entire vessel) which cell type (s) are being altered. Furthermore, the disaccharide analysis does not permit us to determine the domain arrangement of the chains. To our knowledge, a technique to do this is not readily available. It is noteworthy that the transcriptional changes in the enzymes (significant increases in the transcript of 2-*O* and 6-*O* sulfotransferase) do not line up with the decreases seen in the 2-O- and 6-O-sulfated residues (D0S6 and D2S6). There are several possibilities for this. Transcriptional regulation of these enzymes has largely remained unexplored. Grobe et al. [13] demonstrated that Ndsts undergo complicated transcriptional as well as translational and post-translational regulation. Certainly, protein expression and enzymatic activity do not always correspond to mRNA levels. Furthermore, a limitation of this analysis is that we have not fully explored all of the enzymes that regulate HS fine structure including the sulfate. Finally, the enzymatic digestion of HS followed by disaccharide compositional analysis by HPLC reveals only part of the information about the HS structure. A limitation of this technique is that the domain arrangement of the chains cannot be analyzed by this method [14]. Binding of protein ligands appears to be dependent on the overall organization of the domains along the HS chains [14].

In summary, this is the first report to identify regulation of HS structure and transcriptional control of the enzymes that catalyze these reactions within the vessel wall in response to injury. We hypothesize a working model in which vascular injury induces changes in HS fine structure that are critical for vascular remodeling. Specifically, changes in HS fine structure may underlie chemokine and growth factor binding and function that may guide cell migration, proliferation, and apoptosis. We recognize that HS fine structure is only a partial piece of the overall picture in HS domain organization; however, these findings suggest that injury actively modifies HS, and these changes may be important to remodeling.

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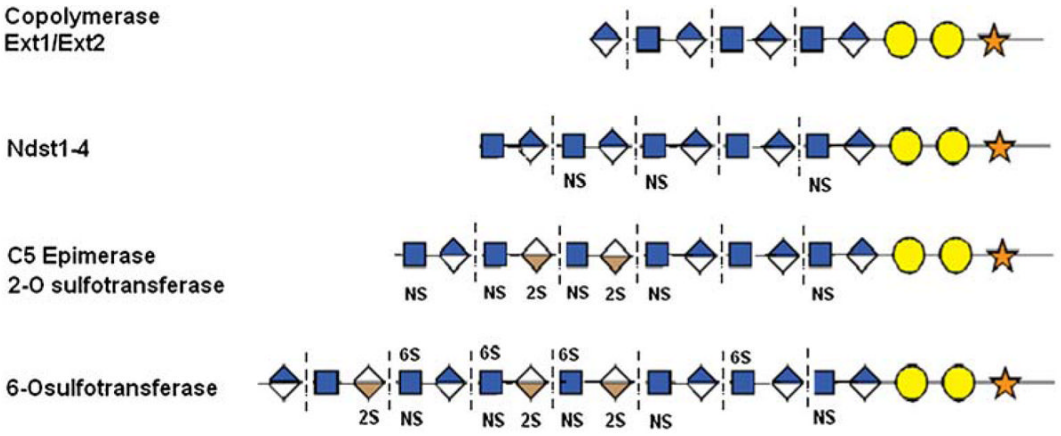


Fig. 1.

Schematic representation of biosynthesis and modification of HS chains. The HS chains are depicted using standard symbol nomenclature available at www.functionalglycomics.org. *Dashed lines* depict the disaccharide units that are generated by heparinases, which are then separated by HPLC according to the number and pattern of sulfate groups shown in Table 2

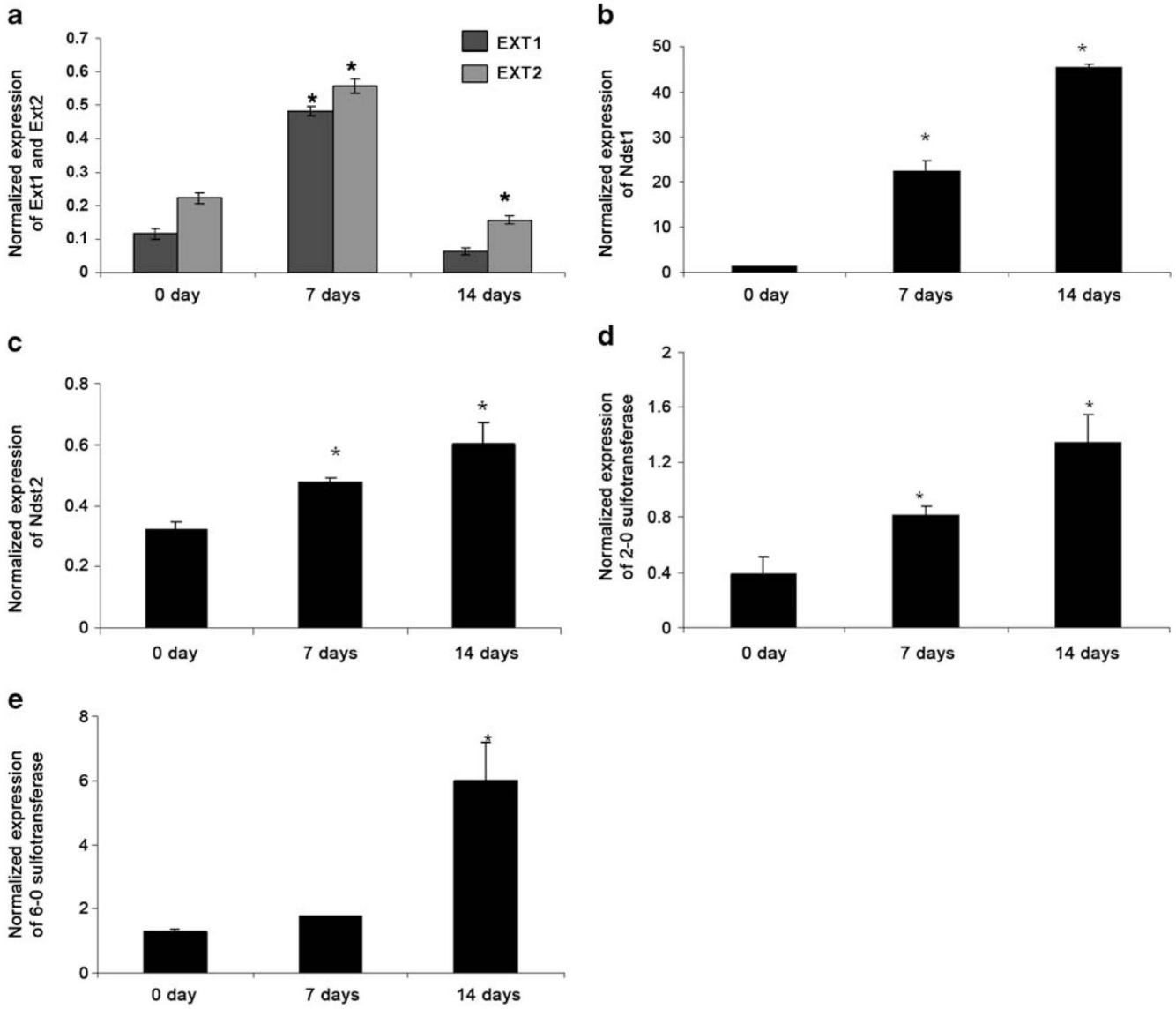


Fig. 2.

Expression of HS biosynthetic enzymes at 0, 7, and 14 days after injury. **a** Ext1 and 2, **b** Ndst1, **c** Ndst2, **d** 2-*O* sulfotransferase, and **e** 6-*O* sulfotransferase. RNA was extracted from femoral arteries of wild type mice harvested at 0 day (n=4), 7 days (n=8), and 14 days (n=8) post wire injury. Cyclophilin A was used as an internal control (n=4–8, p<0.05)

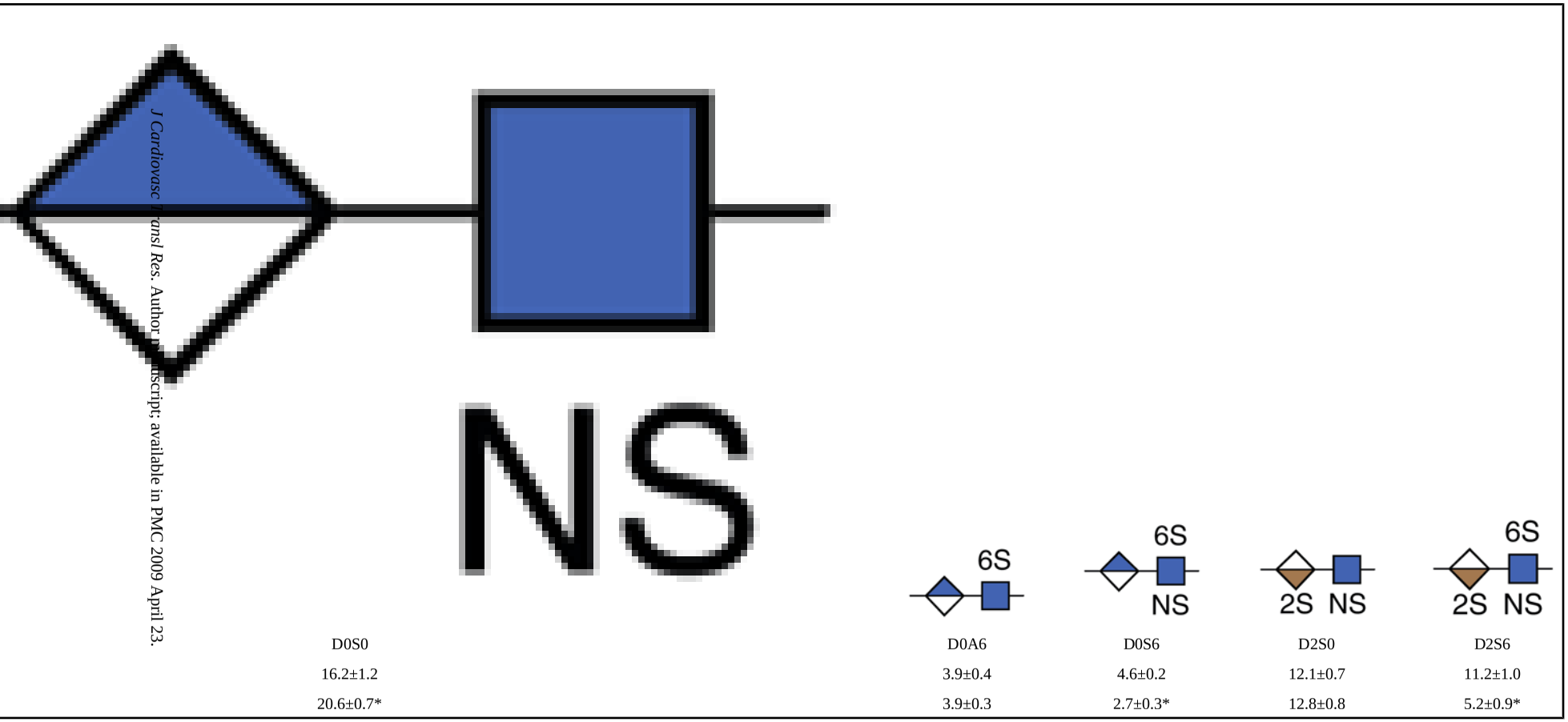
Table 1

List and sequences of primers used to perform RT-PCR in mouse femoral artery

Gene	Sequence
Ext1	5′GAGGACGTCTTGCTCCTCAC3′
	5′TCCGTTGCTGAGCATTACAG3′
Ext2	5′AGTCTGTCCAAGCTGCTGGT3′
	5′GCCTCTGTCTCGATTTCGTC3′
Ndst1	5′CCCAGTGGCCCTAAAGTACA3′
	5′AGGTTCTGTTCHSCAGTT3′
Ndst2	5′ACCAGCAAAAAGCCTGCTAA3′
	5′CCTCCCAGGTTCATAGGTCA3′
6-*O* Sulfotransferase	5′TCAAGTGCGGAATAGCTGTG3′
	5′TATCATCTGCCGTTCCATGA3
2-*O* Sulfotransferase	5′CTGTTGGAAAGTCGCTGTCA3′
	5′GGTCACGATCTGGGAGACAT3′
Epimerase	5′TGGGCACAGTCCATTCATTA3′
	5′GGAGTTGAAGGTGTGCCATT3′
Cyclophilin A	5′GTGGTCTTTGGGAAGGTGAA3′
	5′TTACAGGACATTGCHSCAG3′

Table 2

s from wild type mice harvested at 0 and 14 days post injury. HS chains were extracted and Disaccharides were analyzed by HPLC. Values are means ± SE and are represented as percent *p<0.05). Disaccharides analyzed are depicted using standard symbol nomenclature given at



D0S0	D0A6	D0S6	D2S0	D2S6
16.2±1.2	3.9±0.4	4.6±0.2	12.1±0.7	11.2±1.0
20.6±0.7*	3.9±0.3	2.7±0.3*	12.8±0.8	5.2±0.9*