Rhamnan Sulfate in a Proprietary Extract of *Monostroma Nitidum* Regenerates a Compromised Endothelial Glycocalyx Shed Caused by High Glucose

Study conducted by: State Key Laboratory of Bioengineering, Institute of Process Engineering, Chinese Academy of Sciences

Introduction

The endothelial glycocalyx (EGX) is a negatively charged network of proteoglycans, glycoproteins, and glycolipids barrier covering the luminal side of the endothelium throughout the human vascular system.[1] As a semipermeable barrier between the flowing blood and the endothelium, the EGX performs multiple essential functions. It modulates the passage of components in the blood, such as LDL, through the endothelium;[2] it inhibits the adhesion of platelets and leukocytes[3,4]; it houses the important antioxidant superoxide dismutase;[5] it also harbors coagulation regulatory factors;[6] and it triggers the endogenous production of nitric oxide.[7]

The EGX is inherently fragile and is susceptible to degradation in response to a number of factors, including excess glucose in the blood.[8] In recent years, increasing attention has been given to the role of this delicate structure in vascular health.

Current research on the EGX uses both *in vivo* and *in vitro* models. The *in vivo* measurement of endothelial glycocalyx requires sophisticated and costly technologies to deal with complex biological environments. This makes it difficult to obtain large sample sizes for study. On the other hand, *in vitro* cell culture is simple and easy to perform in most laboratories. However, traditional cell culture is generally performed under static conditions and is difficult to adapt to the dynamic flow conditions present in the lumen and required for the optimal growth of the EGX. It is known that EGX grown under static cell culture conditions can be significantly different from EGX observed *in vivo*. This limitation hampers research and consequently the development of products for innovative therapies.

Microfluidic chip or lab-on-a-chip (LOC),[9] comprises microchips of centimeter scale that include microchannels at micron levels. This technology integrates various chemical and biological stages and processes such as sample preparation, reaction, separation, detection, cell culture, cell manipulation, DNA purification, gene isolation, antigen-antibody interaction, drug delivery, biosensing, and diagnosis.

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Microfluidic chip technology is suited to cell biology research for a number of reasons. First, the size of microchannels on the microfluidic chip (10-100 μm) is similar to that of individual cells (10-20 μm), making cell manipulation easier. Secondly, the multi-dimensional network of microchannels on microfluidic chip provides a relatively isolated environment that mimics that of living cells under normal physiological conditions. Thirdly, microfluidic chips can satisfy the demand of high throughput analysis and acquire large volumes of biological information simultaneously. Finally, the flat configuration of microfluidic chips allows for easy observation. In sum, the microfluidic chip is arguably the optimum *in vitro* platform model for mammalian cell culture and manipulation.[10]

To further research on the EGX, use of a patented microfluidic chip model allowed high throughput screening of bioactive compounds that protects and regenerates glycocalyx, i.e., glycocalyx regenerating compounds (GRC).[9] Specifically, the method uses the fabricated chip as a model to study the dynamic changes and related biological functions of the EGX under different conditions. The model adequately simulates endothelial cell validity and endothelial permeation. It can be used to screen biologically active compounds and their effect on the EGX.

Marine polysaccharides have been demonstrated to possess a variety of biological and physiological actions that are relevant to human health. Rhamnan sulfate (RS) is a heteropolysaccharide with L-rhamnose that may be covalently bonded to sulfate providing the primary repeating monosaccharides on both the linear and branched chains. Rhamnan sulfate has been found to promote cardiovascular health.[11,12] An early study indicated RS enhanced the EGX.[13]

The data presented here used the new microfluidic chip model to evaluate the effect of rhamnan sulfate in MonitumRS™, a proprietary extract derived from *Monostroma nitidum*, on the EGX condition under high circulating glucose, which is known to damage the endothelial glycocalyx.[14]

Methods

A microfluidic chip was fabricated using PDMS (polydimethylsiloxane) slabs to culture endothelial cells capable of forming a healthy layer of the endothelial glycocalyx as described before.[9] Different cell culture experiments were performed using the microfluidic chip.

Human umbilical vein endothelial cells (HUVEC) were seeded and incubated inside the microchannels for 4 hours in a CO_2 cell culture incubator. The endothelial cells were then grown to 80% confluence with a low flow rate of pumped culture medium. Subsequently, the flow rate of culture medium was accelerated to a high flow rate to mimic blood flow shear stress *in vivo* (10-23 dyn/cm^2) for optimal growth and maintenance of endothelial glycocalyx on the endothelial cells. WGA-FITC (wheat germ agglutinin fluorescein isothiocyanate conjugate) was introduced in the flow to bind to polysaccharides in the glycocalyx. The thickness and integrity of the endothelial glycocalyx was then directly visualized and measured by 3-dimensional fluorescence intensity of WGA-FITC under a normal confocal microscope.

To challenge the EGX, HUVECs were maintained under a standard cell culture medium with additional glucose (hyperglycemic at 50 mM) at the high flow (shear) rate for 24 hours. In a separate experiment,

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heparan sulfate (HS), a major component of the EGX, was added into the circulating cell culture medium at 200 µg/mL after the glucose challenge for another 24 hours under the high flow rate. In yet another experiment, rhamnan sulfate from the proprietary extract was added into the media in the multiple channels at different levels (20-500 µg/mL), in parallel with HS for an additional 24 hours in the presence of high glucose. All experiments were conducted in quintuplicate. The t-test was performed on the means between treatment groups at a statistical significance of 0.05.

At the end of each experiment, endothelial cell morphology was also visualized and examined with or without the nuclear fluorescence stain, 4',6-diamidino-2-phenylindole (DAPI).

Results

HUVECs grown in the multiple channels of our microfluidic chip were examined for gross morphology. Figure 1 shows the morphology of live cells under a normal confocal microscope (left) and fluorescence staining of endothelial cell nucleus (blue) and the endothelial glycocalyx (green) in a microfluidic chip.

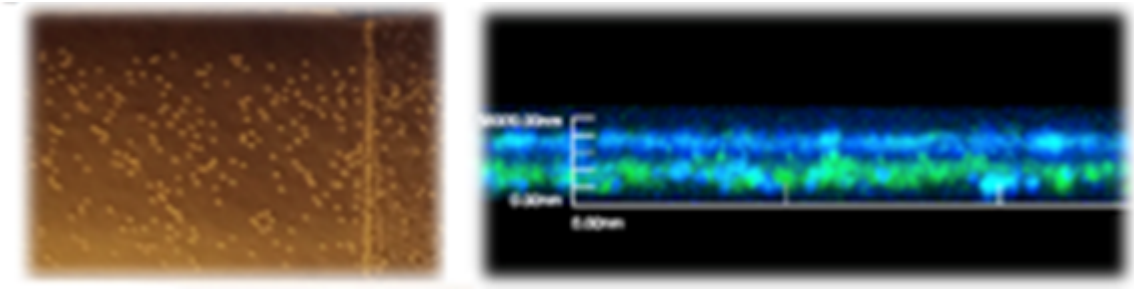

Figure 1. Left: Live HUVECs cultured in a microchannel of microfluidic chip. Right: Nucleus (blue) and the glycocalyx (green) of the HUVECs after fluorescence staining in the microfluidic chip (sideview).

When an excessive amount of glucose at 50 mM was added into the cell culture media under high shear, the mean fluorescence intensity (MFI) of the endothelial glycocalyx significantly decreased by over 30% after 24 hours compared to a control (Figure 2).

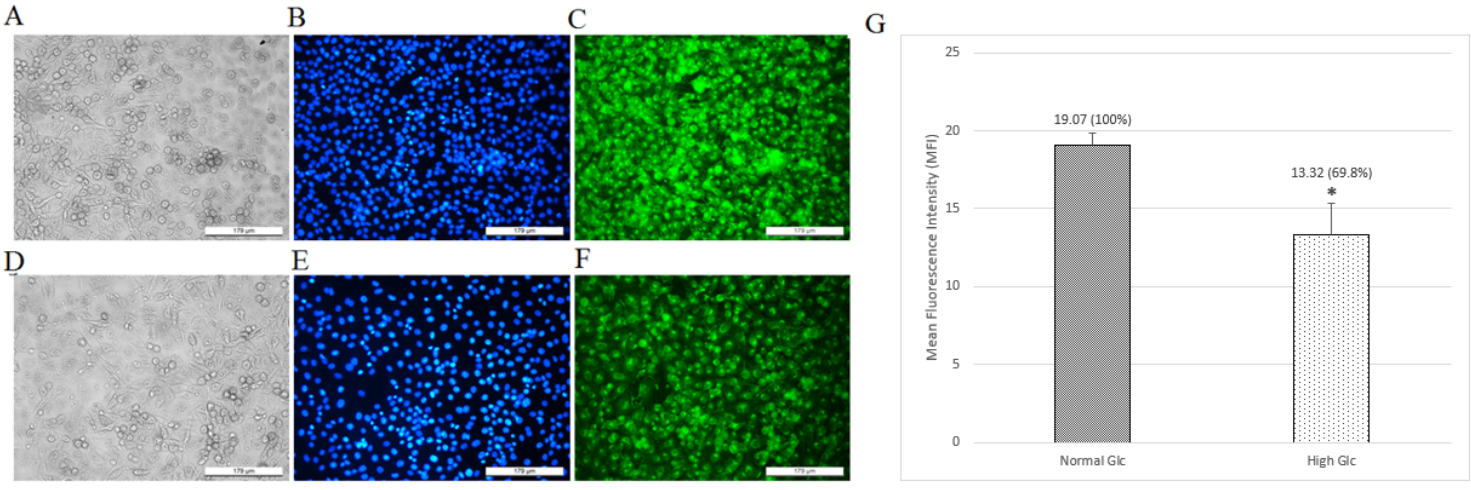

Figure 2. A, B, and C are endothelial cells, cell nucleus, and the glycocalyx of control (normal glucose level in cell culture media) after 24 hours under high shear. D, E, and F are endothelial cells, cell nucleus, and the glycocalyx cultured with a high glucose level (50 mM) in cell culture

media after 24 hours under high shear. G is a bar chart showing the MFI of the EGX after fluorescence staining. Experiment was conducted in quintuplicate. Glc: glucose.

In another experiment, after the initial 24-hour perfusion of HUVECs with high glucose under high shear, heparan sulfate was added to the cell culture media (200ug/mL) for an additional 24 hours. As shown in Figure 3, the control cells again had a statistically significant decrease of MFI of the EGX by about 30% from 18.21 to 12.78 after 48 hours with high glucose under high shear. Heparan sulfate not only brought the MFI back to the normal level but also caused a small increase of 14% from 20.82 compared to 18.21 of control at the end of the 24 hours. This is a statistically significant increase compared to the MFI of 12.78 when HUVECs were treated with high glucose for 48 hours.

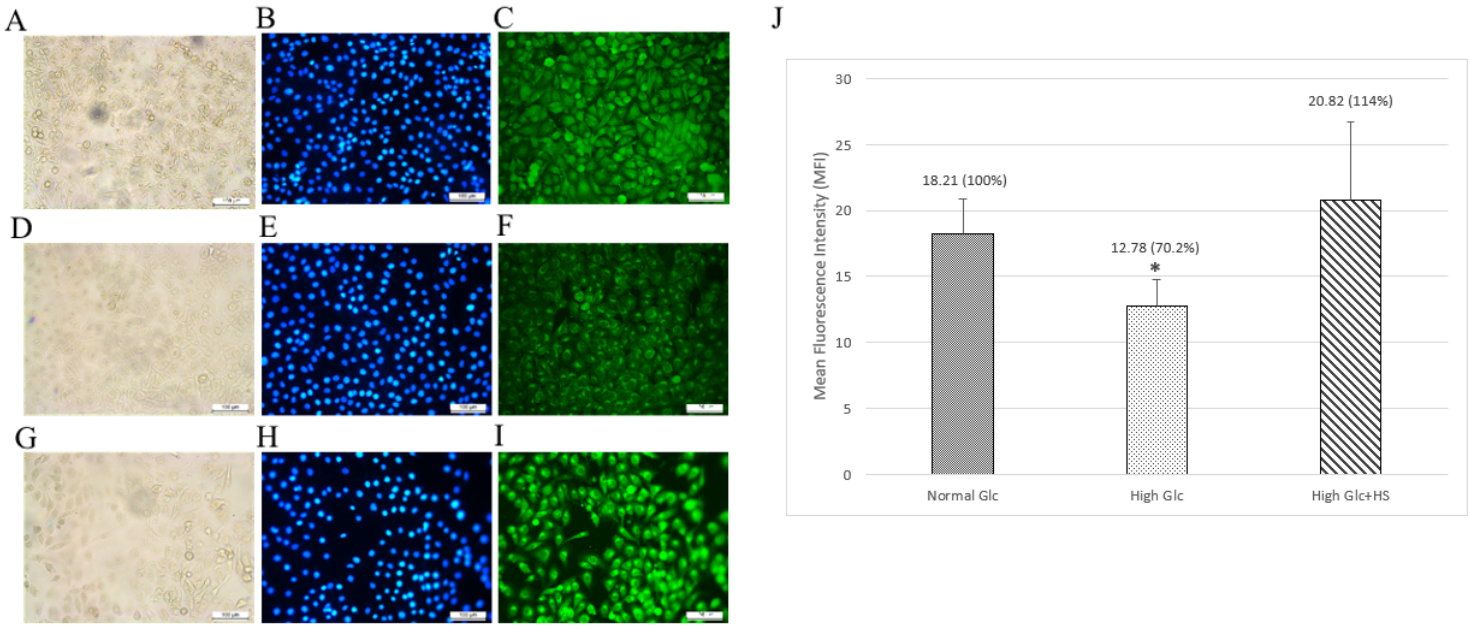

Figure 3. A, B, and C are endothelial cells, cell nucleus, and the glycocalyx of control (normal glucose level in cell culture media) after 48 hours under high shear. D, E, and F are endothelial cells, cell nucleus, and the glycocalyx cultured with a high glucose level (50 mM) in cell culture media after 48 hours under high shear. G, H, and I are endothelial cells, cell nucleus, and the glycocalyx cultured with a high glucose level (50 mM) in cell culture media after 48 hours under high shear while heparan sulfate was introduced into cell culture media at 200 µg/mL after the first 24 hours. J is a bar chart showing the MFI of the EGX after fluorescence staining at the end of the experiment. Experiment was conducted in quintuplicate. Glc: glucose. HS: heparan sulfate.

Researchers then tested varying levels of RS in MonitumRS™, a proprietary extract of *Monostroma nitidum*, alongside heparan sulfate using different microchannels in the microfluidic chip model. Beside 200 µg/mL of heparan sulfate, 20, 50, 100, 200, and 500 µg/mL of the rhamnan sulfate extract MonitumRS™ were added into different microchannels for 24 hours under high shear.

Figure 4 shows high glucose (50 mM) caused a significant decrease of MFI by about 20% at the end of the perfusion. Heparan sulfate at 200 µg/mL brought the MFI back to 102.9% of the control after 24 hours. MonitumRS™ extract from 20 to 500 µg/mL also negated the decrease of MFI caused by high glucose. All the increases of MFI by MonitumRS™ compared to the high glucose treated sample (negative control) are statistically significant. At 200 µg/mL, MonitumRS™ (103.8% to the control) was as effective as heparan sulfate (102.9% to the control) to prevent and repair the damage of the EGX induced by high glucose. All dosages tested in the experiment performed similarly, with no dose response observed for this range of MonitumRS™.

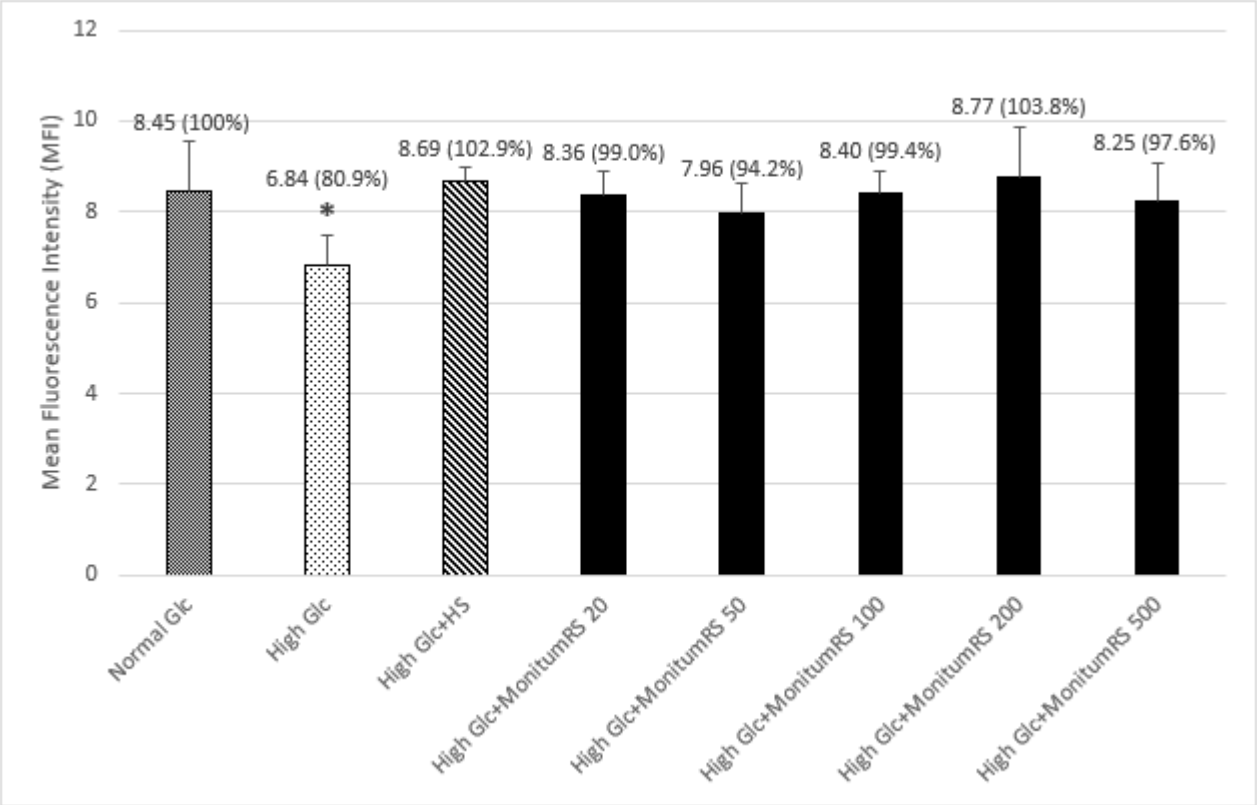

Figure 4. MFI of endothelial glycocalyx of HUVECs treated with normal glucose, high glucose (50 mM), and 200 µg/mL of heparan sulfate or 20, 50, 100, 200, and 500 µg/mL of MonitumRS™ with high glucose. Experiment was conducted in quintuplicate. Glc: glucose. HS: heparan sulfate.

Discussion

This study used a previously developed microfluidic chip model to grow human endothelial cells with a robust endothelial glycocalyx.[9] The model has also been used to simulate damage of the EGX caused by different mechanisms *in vivo* such as degradation enzymes of heparanase/hyaluronidase/chondroitinase (data not shown).

When HUVECs were treated with high circulating glucose (50 mM) under high shear for 24 hours, the EGX was damaged, as shown by significant reduction of MFI by 30%. Heparan sulfate at 200 µg/mL for an additional 24 hours under the high glucose completely negated the damage and brought the MFI above the control level. MonitumRS™ - a proprietary extract of RS from the rare green seaweed *Monostroma nitidum* - showed a similar protective and restorative effect on the EGX.

Specifically, MonitumRS™ rhamnan sulfate extract from 20 to 200 µg/mL significantly increased the MFI of the EGX when compared to that of the high glucose treated cells. In fact, the MFI was comparable to the control level without high glucose treatment. These data demonstrate RS in the proprietary MonitumRS™ extract prevented and repaired the damage of the EGX caused by high glucose. The rhamnan sulfate found in MonitumRS™ is an effective glycocalyx-regenerating compound.

RS from *Monostroma nitidum* may protect and regenerate the EGX via different mechanisms. First, RS repairs, regenerates and restores the EGX by direct replacement as a heparan sulfate mimetic.[15] Second, RS stimulates the synthesis of the endothelial glycocalyx.[14] Third, RS prevents the EGX damage caused by the degradation enzymes[13,16,17] Last but not the least, RS has been shown to inhibit TNF-α induced NF-κB activation in human endothelial cells which has an implication in the EGX damage in vivo. Given the limitations of *in vitro* experimentation, however, more research is warranted to explore the mechanisms underlying the biological activities of RS**.**

RS has been shown to have a broad range of cardiometabolic health benefits. It may exert its biological activities via protecting and regenerating the EGX as a heparan sulfate mimetic. This data provides strong evidence to support its role as an effective glycocalyx-regenerating compound.

Disclosures

Researchers had no conflict of interest to disclose.

The proprietary extract of rhamnan sulfate from *Monostroma nitidum* (MonitumRS™), the primary ingredient of the dietary supplement Arterosil HP® (US patent 11,135,238, China patent ZL2018800888226), was provided by Calroy Health Sciences, LLC, Scottsdale, Arizona.

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