The Biomechanical Effects of Resuscitation Colloids on the Compromised Lung Endothelial Glycocalyx

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BACKGROUND: The endothelial glycocalyx is an important component of the vascular permeability barrier, forming a scaffold that allows serum proteins to create a gel-like layer on the endothelial surface and transmitting mechanosensing and mechanotransduction information that influences permeability. During acute inflammation, the glycocalyx is degraded, changing how it interacts with serum proteins and colloids used during resuscitation and altering its barrier properties and biomechanical characteristics. We quantified changes in the biomechanical properties of lung endothelial glycocalyx during control conditions and after degradation by hyaluronidase using biophysical techniques that can probe mechanics at (1) the aqueous/glycocalyx interface and (2) inside the glycocalyx. Our goal was to discern the location-specific effects of albumin and hydroxyethyl starch (HES) on glycocalyx function.

METHODS: The effects of albumin and HES on the mechanical properties of bovine lung endothelial glycocalyx were studied using a combination of atomic force microscopy and reflectance interference contrast microscopy. Logistic regression was used to determine the odds ratios for comparing the effects of varying concentrations of albumin and HES on the glycocalyx with and without hyaluronidase.

RESULTS: Atomic force microscopy measurements demonstrated that both 0.1% and 4% albumin increased the thickness and reduced the stiffness of glycocalyx when compared with 1% albumin. The effect of HES on glycocalyx thickness was similar to albumin, with thickness increasing significantly between 0.1% and 1% HES and a trend toward a softer glycocalyx at 4% HES. Reflectance interference contrast microscopy revealed a concentration-dependent softening of the glycocalyx in the presence of albumin, but a concentration-dependent increase in stiffness with HES. After glycocalyx degradation with hyaluronidase, stiffness was increased only at 4% albumin and 1% HES.

CONCLUSIONS: Albumin and HES induced markedly different effects on glycocalyx mechanics and had notably different effects after glycocalyx degradation by hyaluronidase. We conclude that HES is not comparable with albumin for studies of vascular permeability and glycocalyx-dependent signaling. Characterizing the molecular and biomechanical effects of resuscitation colloids on the glycocalyx should clarify their indicated uses and permit a better understanding of how HES and albumin affect vascular function. (Anesth Analg 2016;123:382–93)

The endothelial glycocalyx is believed to play an important role in vascular permeability by functioning as a passive molecular filter overlying the endothelial cell–cell junction[1–3] and acting as a signaling platform that regulates barrier function. The glycocalyx generates a passive permeability barrier by creating a scaffolding on which serum proteins absorb and form a gel-like layer on the vascular wall. Historically, this layer has been referred to as "the immobile plasma layer" and limits the flux of water and plasma proteins into the intercellular junction. The combination of the immobile plasma layer and the glycocalyx scaffold has been termed "the endothelial surface layer" by Pries et al[3] (Figure 1). Components of the glycocalyx function as mechanosensor(s) and mechanotransducer(s) that respond to pressure[1,4] and shear stress[5–7] and activate downstream signaling pathways associated with increased vascular permeability and tissue edema.[8,9]

Changes in plasma protein concentration or colloid composition may affect endothelial function and permeability[2] through passive barrier properties of the glycocalyx and by modulating mechanosensing and mechanotransduction.[7] Because the composition of plasma expanders, such as albumin and hydroxyethyl starch (HES), may affect endothelial cell responsiveness to hemodynamic changes,[10] the choice of resuscitation colloid can influence physiologic nitric oxide (NO) production and vascular reactivity. We previously reported that NO release evoked by acutely increased hydrostatic pressure[1] is responsible for changes in endothelial permeability in the isolated perfused lung preparation.[4] The difference between albumin and HES on glycocalyx-dependent NO production is dependent on their molecular interactions with the glycocalyx and subsequent effect(s) on biomechanical properties[10–12] that influence mechanosensitive signaling.

The glycocalyx is degraded during the acute inflammation of surgery,[13–15] trauma,[16,17] respiratory failure,[18] myocardial infarction/cardiac arrest,[19,20] sepsis,[21] and the Hemolysis Elevate Liver Enzymes Low Platelet Counts (HELLP) syndrome.[22] This degradation is mediated by the actions of plasma

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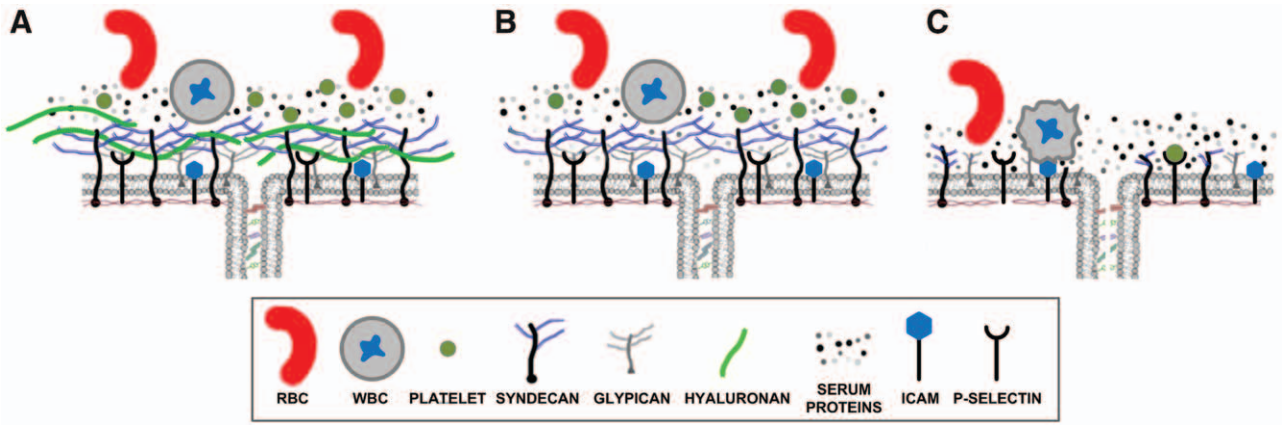

Figure 1. Structure of the glycocalyx. A, Intact glycocalyx demonstrating barrier properties of the glycocalyx in limiting penetration of serum proteins to the junction preventing the interaction of blood cells with cell surface receptors. B, Removal of hyaluronan increases penetration of glycocalyx by serum proteins. C, Complete degradation of glycocalyx during intense inflammation eliminates barrier function and allows platelets and white blood cell (WBC) to interact with their cell surface receptors, thus propagating the inflammatory response. ICAM indicates intra-cellular adhesion molecule; RBC, red blood cell.

proteases and endothelial metalloproteases.[23] The extent of glycocalyx degradation and the loss of specific constituents influence how resuscitation colloids bind to the endothelial surface[12,24,25] and the ability of specific colloids to maintain or restore barrier characteristics[12,26] (Figure 1, B and C). The debate regarding crystalloid versus colloids, and what type of colloid is more clinically appropriate, cannot be mechanistically addressed until we have a more complete understanding of how specific colloids influence the biological functions of the glycocalyx. The observation that resuscitation colloids have oncotic-independent effects through their binding to the glycocalyx suggests that better understanding of the functional attributes of colloids on glycocalyx-dependent processes may better inform clinical choices.[27]

In this study, we started with the hypothesis that the acute inflammatory response provoked by surgery and associated tissue trauma will degrade the endothelial glycocalyx and compromise how natural serum proteins like albumin, or resuscitation colloids like HES, interact with the intact glycocalyx and cell surface remnants that persist after degradation. To mimic the effect of glycocalyx degradation on albumin and HES interactions with glycocalyx remnants, we reassessed the effects of clinically relevant concentrations of albumin versus HES on glycocalyx biomechanics after degradation with hyaluronidase.

Our laboratories have established 2 complementary techniques to probe the micromechanics of the endothelial glycocalyx: (1) reflectance interference contrast microscopy (RICM) and (2) atomic force microscopy (AFM). Both techniques use a nanoscaled spherical probe to impart small loading forces onto the glycocalyx. The applied forces differ in these 2 techniques: because of very low loading forces, RICM probes the outermost region of the glycocalyx (<10 nm indentation), whereas AFM applies increasing forces that indent progressively deeper into the glycocalyx, over a range of 50 to 700 nm. These techniques were used independently to determine biomechanical parameters associated with the glycocalyx, including effective stiffness of the outermost region of the glycocalyx, and a pointwise elastic modulus (E) as a function of indentation depth. The later function was fitted to a simple 2-layer composite compliance model to find (a) the mean glycocalyx thickness, (b) the elastic moduli of the glycocalyx, and (c) the mechanics of underlying cell structure.

METHODS

Cell Culture

Bovine lung microvascular endothelial cells (BLMVECs; VEC Technologies; Rensselaer, NY; passage 5–13) were plated at a density of 1.25×10^5 cells/cm^2 and grown to confluence on ethanol-washed, autoclaved, 25-mm round glass coverslips pretreated with 0.4% gelatin (Sigma-Aldrich, Milwaukee, WI) and 100 μg/cm^2 fibronectin (Sigma-Aldrich), for 1 hour each. Growth media was MCDB-131 Complete Medium (VEC Technologies) or a 50/50 mixture of this media with MCDB 131 Medium (Sigma, M8537) supplemented with penicillin/streptomycin, 10% fetal bovine serum, and 15 mM sodium bicarbonate at pH 7.4. BLMVEC monolayers were maintained at 37°C and 5% CO_2 until use on days 7 to 12.[11]

Enzymatic Digestion of Hyaluronan

BLMVEC monolayers were incubated with 50 U/mL hyaluronidase (EC 4.2.2.1); hyaluronidase from *Streptomyces hyalurolyticus* (Sigma) was supplemented with 1% bovine serum albumin (BSA) and 25 mM HEPES at pH 7.4 for 1 hour before experiments in MCDB-131. Incubation was done at 37°C and 5% CO_2.

Colloid Addition

Before experiments, monolayers were rinsed with unsupplemented MIII (1:3 mixture of MCDB-131 supplemented with 25 mM HEPES at pH 7.4 and lactated Ringer's solution (pH 7.4). Stiffness measurements were performed in MIII in the presence of 0.1%, 1%, or 4% weight volume (w/v) of either Fraction V BSA (Proliant Biologicals, Boone, IA; molecular weight [MW] = 66.5 kDa) or high-molecular-weight hetastarch (HES) routinely used as a plasma expander (HEXTEND; weight average MW = 600 kDa, 0.75° of substitution).

Atomic Force Microscopy

The AFM experiments used to measure the elastic modulus, E, of BLMVECs have been previously described[28]

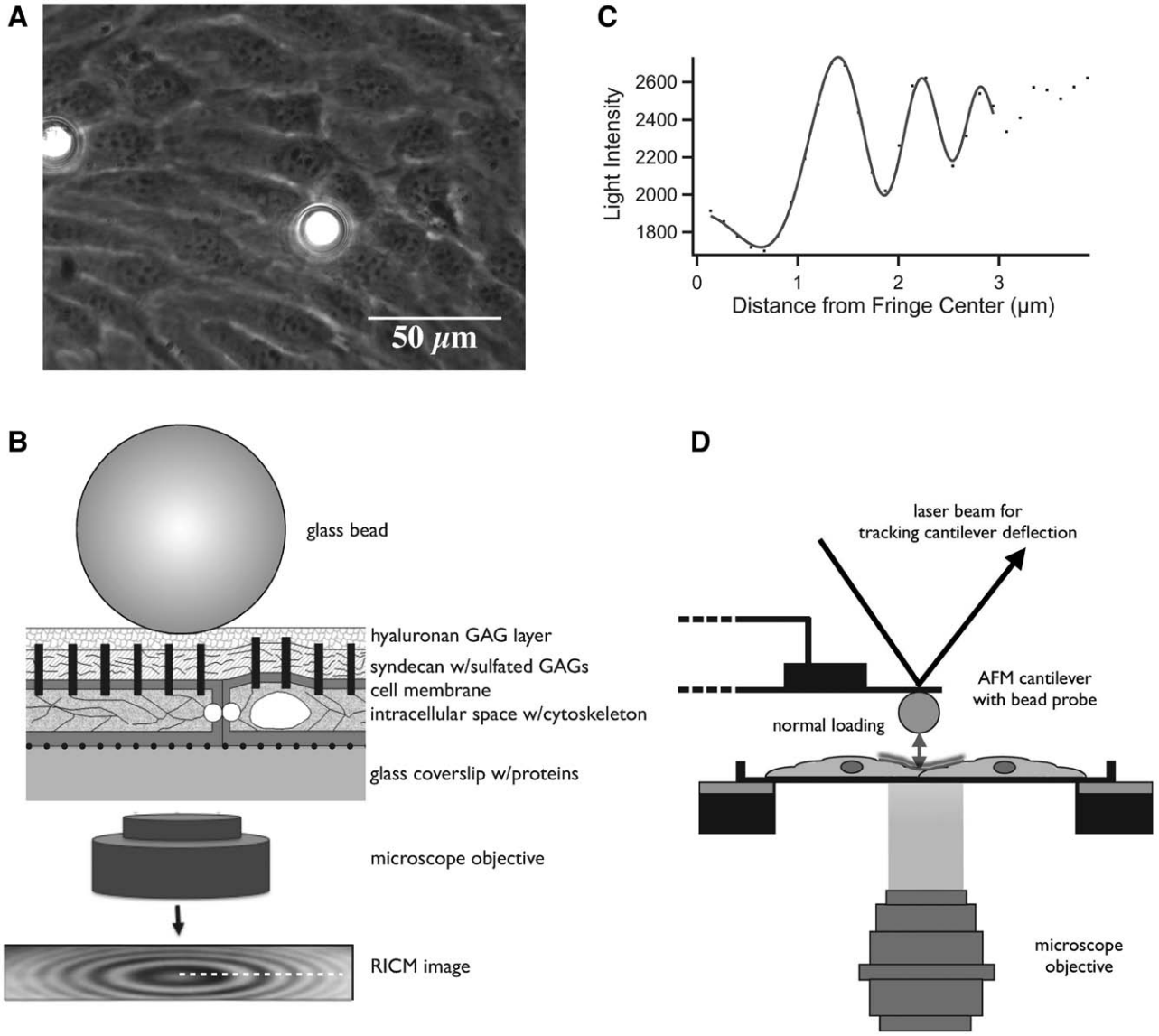

Figure 2. Biophysical techniques to measure mechanics of the glycocalyx. A, Phase contrast image of 2 glass beads on a bovine lung microvascular endothelial cell (BLMVEC) monolayer to be used in reflectance interference contrast microscopy (RICM) measurements. B, Schematic of RICM bead probe placed on the top of BLMVEC glycocalyx (not drawn to scale). Monochromatic light reflected from bead and coverslip surface constructively interferes to create an interference pattern in the RICM image. C, Intensity profile of the interference pattern in the RICM image. This interference pattern profile changes when the distance between the bead and the coverslip fluctuates. GAG = glycosaminoglycan. The bead fluctuations are used to derive mechanical properties of the glycocalyx. D, Schematic of atomic force microscopy (AFM) bead probe indentation measurements of BLMVEC glycocalyx stiffness. Glass bead, glued to AFM cantilever, is gently pushed into the surface layer overlying a cell–cell junction to compress the BLMVEC glycocalyx. The upward deflection of the cantilever is tracked by a laser beam and position-sensitive diode and is converted into a loading force. The loading forces and resulting indentation depths are then used to compute the glycocalyx elastic modulus.

(Figure 2A). An AFM cantilever was modified by attaching a borosilicate glass sphere, with a diameter of approximately 18 μm, which was then used to indent the glycocalyx and cell membrane. An increasing loading force, from 0 to 5 to 10 nN, was applied at a rate of 10 μm/s. Indentation locations were assigned based on the AFM topography map obtained at the end of the indentation experiments. Only cell–cell junction locations were selected for analysis. The AFM indentation data were analyzed pointwise to obtain the elastic modulus, E, as a function of the indentation depth, δ. The resulting $E(\delta)$ curves were analyzed using a 2-layer composite compliance model to obtain the mean elastic moduli of the glycocalyx and the underlying cellular structures[28,29]:

$$\frac{1}{E(\delta)} = \frac{1}{E_{\text{glycocalyx}}}\left(e^{-\frac{\alpha\delta}{\delta_g}}\right) + \frac{1}{E_{\text{cell}}}\left(1 - e^{-\frac{\alpha\delta}{\delta_g}}\right), \quad (1)$$

where E represent the elastic modulus of the glycocalyx ($E_{\text{glycocalyx}}$) or the cell (E_{cell}), the parameter α is a measure of mechanical interlayer interaction, and δ_g is the thickness of the glycocalyx. For such analysis to be conclusive, the $E(\delta)$ curves must display a sigmoidal shape; in the absence of such

shape, the glycocalyx modulus, $E_{glycocalyx}$, could be estimated from a pointwise modulus at 100-nm indentation depth, E_{100}.

The effect of the colloid concentrations on glycocalyx modulus and thickness was assessed by subtracting the $E(\delta)$ data measured at a given albumin (or HES) concentration from the $E(\delta)$ data measured at a colloid concentration of 1%.

$$\Delta E(\delta) = E(\delta)(\text{at x\%}) - E(\delta)(\text{at1\%}). \quad (2)$$

All data fitting was performed using IgorPro (WaveMetrics, Portland, OR).

Reflectance Interference Contrast Microscopy

Effective stiffness of the glycocalyx was measured using RICM as previously described[11] (Figure 2B). RICM is an interferometric technique that measures the fluctuations of a glass sphere in the vertical position when placed on the surface of a confluent monolayer of BLMVECs. The glass sphere (18-μm diameter) serves as a force probe that exerts very small loads of approximately 50 pN and indents the glycocalyx several nanometers. The sphere–cell equilibrium is modeled as a particle at a potential energy minimum, where gravitational and restoring elastic forces balance each other.[11,30] The vertical fluctuations follow the Boltzmann law,[11,30] so that the profile of the potential energy, U, around the minimum can be calculated as a function of the vertical position, h. Thus, the second derivative of $U(h)$ provides a measure of effective stiffness for sphere–cell interactions.[11] The effective stiffness is then attributed to the glycocalyx, because loading forces are small and the bead itself is rigid compared with underlying structures like the cell membrane and submembranous cytoskeleton. For analysis of effective glycocalyx stiffness, only sphere locations from cell–cell junction were used. RICM is estimated to have a spatial resolution of approximately 300 nm[31] and a vertical resolution that is subnanometer.[11]

Statistical Analyses

All references to sample size throughout the article (Methods, Results, and figure captions) refer to independent measurements made from a specific point within a confluent endothelial monolayer. For AFM, this point is where the AFM cantilever was used to indent the endothelial glycocalyx. For RICM, each sample represents an independent borosilicate bead placed on the cell surface (see Methods for greater detail) and used to measure glycocalyx mechanics. In most cases, all data for a specific group (eg, albumin 1%, $n = 51$) were obtained from 1 to 2 coverslips that contained a confluent monolayer of endothelial cells. The coverslips for each group were plated at the same time, and all measurements were taken on the same day.

Statistical analyses of RICM data (effective stiffness, kT/nm^2 units) and AFM data (elastic moduli, kPa units) were performed using Stata 13 (Stata Corp LP, College Station, TX). We performed logistic regression, with a binary dependent variable being the "group" to determine the odds ratios, and then compared the groups of all concentrations of albumin or HES with and without hyaluronidase during RICM and AFM treatment with each other, in all tested combinations. Differences were considered significant at $P < 0.0167$ after adjusting for familywise error rate from 3 sets of pairwise comparisons using Bonferroni correction.

RESULTS

AFM Measurement of Glycocalyx Mechanics

Figure 3 shows the elastic modulus, $E(\delta)$, measured using AFM on a confluent BLMVEC monolayer incubated in media containing 1% albumin or 1% HES. Each data set was fitted to a 2-layer composite compliance model (Equation 1); the modeled $E(\delta)$ curves are shown by the dashed lines in Figure 3, and the fitted parameters are presented in Table 3. One percent HES produced a larger elastic modulus at deeper indentation depths compared with albumin—values suggesting that, overall, HES made the glycocalyx stiffer. However, the 2-layer composite compliance model predicted that the difference in stiffness between 1% albumin vs 1% HES was predominantly because of an increased glycocalyx thickness, because the elastic moduli for both the glycocalyx $E(\square)$ and whole cell (E_{cell}) were similar between the 2 colloids (Tables 1 and 2). A consistent trend that was observed in AFM studies of differing colloid composition and concentrations was that a thicker glycocalyx was softer and a thinner glycocalyx was stiffer.

Figure 4 shows the mean $E(\delta)$ differences, ie, $\Delta E(\delta) = \Delta E(\delta)$ (at x%)$-\Delta E(\delta)$ at (1%), where x% is the value at a specific colloid%, for all 3 concentrations of albumin and HES, whereas Figure 5 shows the results of $\Delta E(\delta)$ after

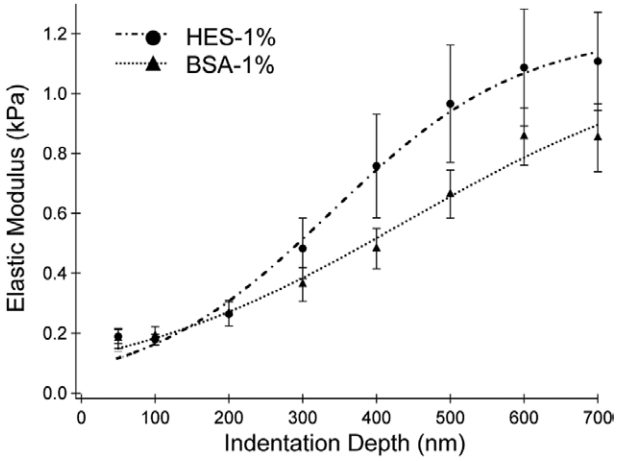

Figure 3. Elastic moduli versus indentation depth. Mean elastic moduli at cell–cell junctions of bovine lung microvascular endothelial cell monolayers as a function of indentation depth for samples with 1% hydroxyethyl starch (HES; $n = 20$) and 1% bovine serum albumin (BSA; $n = 37$) supplementation. Higher modulus values denote stiffer structures. The vertical bars represent the SEM.

Table 1. Two-Layer Composite Compliance Model Parameters Obtained for $E(\delta)$ Data at 1% BSA or 1% HES Concentrations

Parameter	1% HES	1% BSA
$E_{glycocalyx}$ (kPa)	0.08 ± 0.02	0.12 ± 0.03
E_{cell} (kPa)	1.21 ± 0.07	1.15 ± 0.23
A	2.46	2.59
Glycocalyx thickness, δ_g (nm)	317 ± 38	532 ± 102
χ^2	0.0106	0.0097

The goodness of each fit is measured by χ^2 parameter shown. Data presented as mean ± SD for AFM parameters obtained using the 2-layer compliance model (see Methods) at 1% HES and 1% BSA. Goodness of fit for each parameter, χ^2, was compared between colloid condition and $\chi^2 < 0.05$ was considered significantly different.

Abbreviations: BSA, bovine serum albumin; HES, hydroxyethyl starch.

Table 2. Two-Layer Composite Compliance Model Parameters Obtained From Fitting Δαδ Data Shown in Figures 2 to 4 at 3 Colloid Concentrations

Parameter	0.1% BSA	1% BSA	4% BSA
$E_{glycocalyx}$ (kPa)	0.14 ± 0.02	0.12 ± 0.03	0.11 ± 0.03
E_{cell} (kPa)	1.15 ± 0.40	1.15 ± 0.23	1.16 ± 0.23
α	2.59	2.59	2.59
Glycocalyx thickness, δ_g (nm)	1267 ± 68	532 ± 102	910 ± 49
χ^2	0.00003	0.0097	0.0122
	0% HES	**1% HES**	**4% HES**
$E_{glycocalyx}$ (kPa)	0.06 ± 0.01	0.08 ± 0.02	0.03 ± 0.01
E_{cell} (kPa)	1.20 ± 0.04	1.21 ± 0.07	1.23 ± 0.13
α	2.46	2.46	2.46
Glycocalyx thickness, δ_g (nm)	618 ± 13	317 ± 38	410 ± 6
χ^2	0.0029	0.0106	0.0045
	1% BSA	**1% BSA/HAase**	**4% BSA/HAase**
$E_{glycocalyx}$ (kPa)	0.12 ± 0.03	0.14 ± 0.02	0.04 ± 0.00
E_{cell} (kPa)	1.15 ± 0.23	1.10 ± 0.18	1.10 ± 0.18
α	2.59	2.59	2.59
Glycocalyx thickness, δ_g (nm)	531 ± 102	450 ± 4	322 ± 4
χ^2	0.0097	0.0005	0.0032
	1% HES	**1% HES/HAase**	**4% HES/HAase**
$E_{glycocalyx}$ (kPa)	0.08 ± 0.02	0.16 ± 0.05	0.10 ± 0.05
E_{cell} (kPa)	1.21 ± 0.07	1.21 ± 0.07	1.21 ± 0.07
α	2.46	2.46	2.46
Glycocalyx thickness, δ_g (nm)	317 ± 38	391 ± 13	378 ± 18
χ^2	0.0106	0.0037	0.0141

The goodness of each fit is measured by χ^2 parameter shown. Data presented as mean ± SD for AFM parameters (left column) obtained using the 2-layer compliance model (see Methods) for 10 unique conditions. Goodness of fit, χ^2 was considered significantly different when $\chi^2 < 0.05$.
Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; HES, hydroxyethyl starch; HAase, hyaluronidase.

Table 3. Comparison of the Treatment Groups for AFM Data Using Logistic Regression Analysis

Group 1 vs Group 2 (kPa)		*P* Value	98% Confidence Interval (kPa)
1% BSA (μ = 0.18; *n* = 37)	0.1% BSA (μ = 0.13; *n* = 39)	0.001	(0.236, 0.764)
1% BSA (μ = 0.18; *n* = 37)	4% BSA (μ = 0.14; *n* = 33)	0.012	(0.026, 0.650)
0.1% BSA (μ = 0.13; *n* = 39)	4% BSA (μ = 0.14; *n* = 33)	0.303	(−0.239, 0.421)
0% HES (μ = 0.13; *n* = 39)	1% HES (μ = 0.18; *n* = 20)	0.011	(0.018, 0.794)
0% HES (μ = 0.13; *n* = 39)	4% HES (μ = 0.16; *n* = 17)	0.082	(−0.147, 0.720)
1% HES (μ = 0.18; *n* = 20)	4% HES (μ = 0.16; *n* = 17)	0.325	(−0.290, 0.625)
1% BSA (μ = 0.18; *n* = 49)	1% BSA/HAase (μ = 0.21; *n* = 45)	0.140	(−0.208, 0.401)
4% BSA (μ = 0.14; *n* = 33)	4% BSA/HAase (μ = 0.22; *n* = 44)	0.012	(0.335, 0.753)
1% HES (μ = 0.19; *n* = 39)	1% HES/HAase (μ = 0.14; *n* = 41)	0.001	(0.208 0.662)
4% HES (μ = 0.16; *n* = 17)	4% HES/HAase (μ = 0.15; *n* = 36)	0.910	(−0.379, 0.366)

Groupwise comparison of AFM-derived elastic modulus (kPa) showing group condition (mean, sample size), Bonferroni-corrected *P* value and Bonferroni-corrected confidence interval. Data presented as mean for AFM parameters obtained using the 2-layer compliance model (see Methods) for 12 unique conditions. Based on Bonferroni correction, $P < 0.0167$ was considered significant.
Abbreviations: AFM, atomic force microscopy; BSA, bovine serum albumin; HES, hydroxyethyl starch; HAase, hyaluronidase.

digestion with hyaluronidase. Table 4 presents the results of fitting the experimental data to the elastic modulus difference model defined by Equations 1 and 2; the fitted parameter with the most significant change in response to different colloid concentrations was the glycocalyx thickness, δ_g. Although changes in fitted elastic modulus parameters were not as dramatic as the change in glycocalyx thickness, the changes in moduli with different colloid concentrations (Table 4) generally agreed with the elastic moduli measured at small indentation depth (eg, 100 nm). Figure 4 indicates that the elastic modulus (primarily influenced by the thickness parameter) is softer and thicker at 0.1% vs 4% BSA (Figure 4A) and 0% vs 4% HES (Figure 4B) compared with 1% BSA and 1% HES, respectively. Treatment of cells with hyaluronidase completely altered the effects of albumin concentration on the glycocalyx stiffness, causing the glycocalyx to be thinner and stiffer at 0.1% and 4% BSA compared with 1% BSA (Figure 5A). Hyaluronidase treatment significantly diminished the effect of HES on $E(\delta)$ as both 0% and 4% HES resulted in a thicker and softer glycocalyx compared with 1% HES when hyaluronidase was used (Figure 5).

Using point-wise analysis (Methods), the elastic modulus (E) at an indentation depth of 100nm was derived (E_{100}) for 3 concentrations of albumin and HES (Figure 6). The E_{100} relationship for both albumin and HES were very similar, demonstrating a significantly stiffer glycocalyx at 1% albumin compared to 0.1% ($P = 0.001$) and 4% albumin ($P = 0.012$). In media containing HES, E_{100} was significantly stiffer when comparing 1% HES vs 0% HES ($P = 0.011$); increasing HES from 1% to 4% had no further effect ($P = 0.325$) (Figure 6 and Table 3 for additional parameters).

Following removal of hyaluronan, albumin affected glycocalyx stiffness differently than HES. Treatment of endothelial cells with hyaluronidase had no effect on E_{100} in the presence of 1% BSA when compared with untreated cells in 1% BSA (Figure 7A, $P = 0.140$). E_{100} was significantly increased by

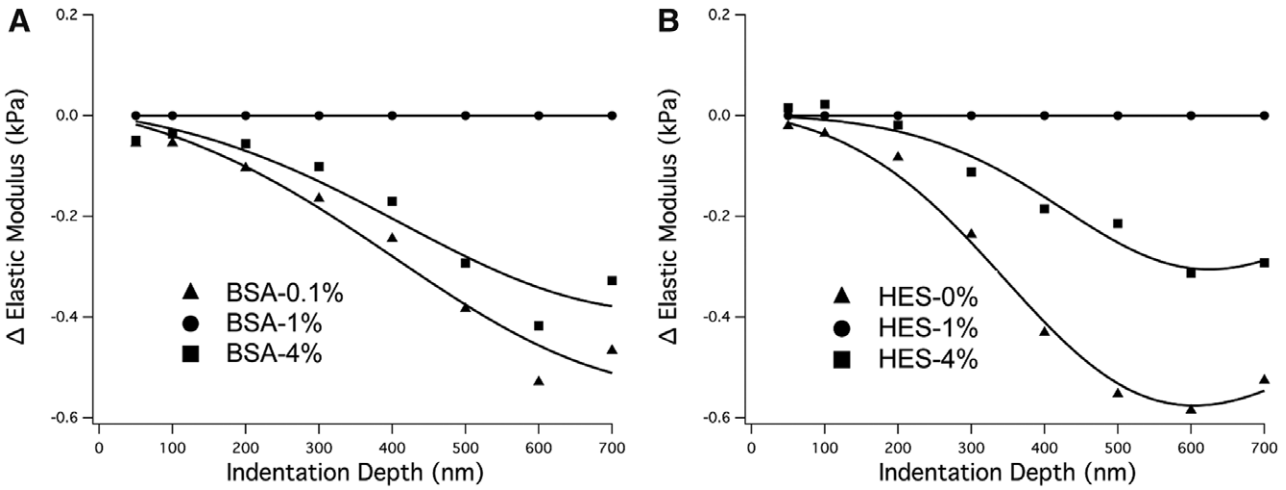

Figure 4. Change in elastic moduli versus indentation depth. Differences in mean elastic moduli shown as a function of indentation depth obtained by subtracting the $E(\delta)$ data from the respective 1% experiment controls. A, BSA, 0.1% ($n = 39$), 1% ($n = 37$), and 4% ($n = 33$); (B) HES, 0% ($n = 39$), 1% ($n = 20$), and 4% ($n = 17$). The modeled differences in $\Delta E(\delta)$ are shown by the solid lines. The $\Delta E(\delta)$ curves are negative indicating swelling of the glycocalyx compared with respective 1% experiment controls. BSA indicates bovine serum albumin; HES, hydroxyethyl starch.

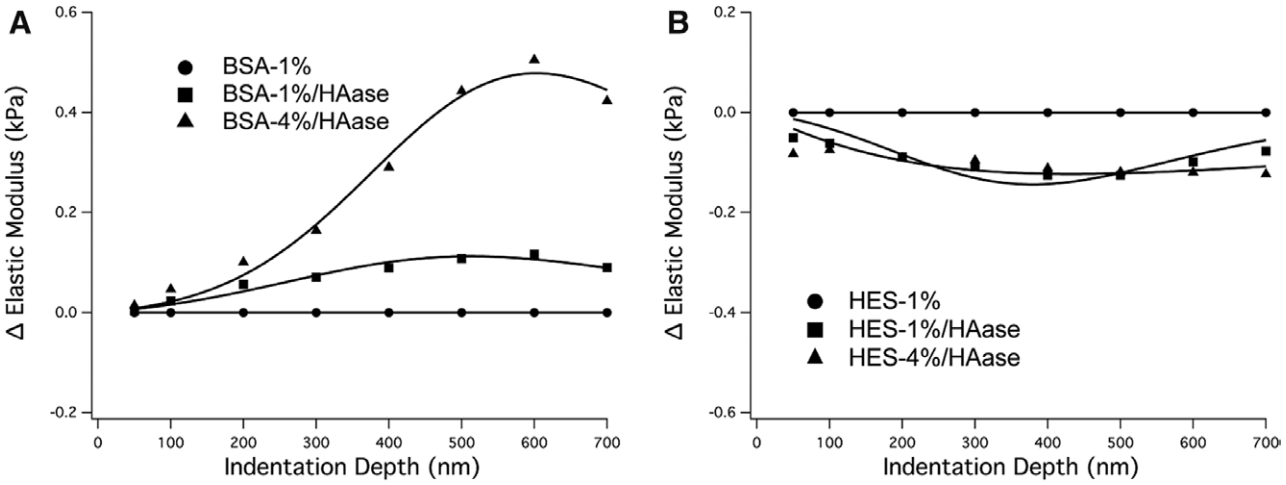

Figure 5. Change in elastic moduli versus indentation depth. Differences in mean elastic moduli after enzymatic digestion of glycocalyx hyaluronan (using 50 U/mL hyaluronidase [HAase]) shown as a function of indentation depth obtained by subtracting the respective the $E(\delta)$ data from 1% experiment controls. A, BSA, 1% control ($n = 35$), 1% after HAase ($n = 20$), and 4% after HAase ($n = 23$); (B) HES, 1% control ($n = 15$), 1% after HAase ($n = 42$), and 4% after HAase ($n = 9$). When the curves are higher than 0 (A), the fitted thickness parameter decreases compared with respective 1% experiment controls, whereas the opposite is true when the curves are negative (B). BSA indicates bovine serum albumin; HES, hydroxyethyl starch.

hyaluronidase treatment when the cells were in media containing BSA-4% (Figure 7A, $P = 0.012$). Thus, removal of hyaluronan in the presence of BSA-4% made the glycocalyx stiffer.

Figure 7B presents the effects of hyaluronidase on E_{100} in the presences of HES. Hyaluronidase significantly reduced E_{100}, resulting in a softer glycocalyx, when cells were incubated in media containing 1% HES ($P = 0.001$). Hyaluronidase had no effect on E_{100} in the presence of 4% HES ($P = 0.910$).

RICM: Mechanics at the Glycocalyx–Fluid Interface

The stiffness of the glycocalyx obtained from RICM experiments during incubation with albumin and HES are shown in Figure 8. Albumin induced a decrease in effective stiffness, resulting in a softer glycocalyx at 4% relative to 0.1% or 1% albumin (Figure 8A, $P = 0.005$). The effective stiffness of BSA = 0.1 and 1% BSA solution did not differ. Conversely, HES produced a concentration-dependent stiffening of the glycocalyx, as evidenced by an increase in effective stiffness at both 1% HES ($P = 0.014$) and 4% HES ($P = 0.004$; Figure 8B). Thus, albumin and HES have opposite effects on the stiffness of the outermost region of the glycocalyx (also see parameters in Table 2).

Hyaluronidase had no effect on the effective stiffness parameter for albumin at either 1% ($P = 0.063$) or 4% ($P = 0.170$; Figure 9A). Likewise, hyaluronidase had no effect on effective stiffness in the presences of 4% HES compared with untreated control cells (Figure 9B, $P = 0.468$).

DISCUSSION

Overview of Results

The results of this study demonstrate complex and anomalous effects of 2 different colloids (albumin versus HES) on biomechanical properties of the lung endothelial

Table 4. Comparison of Treatment Groups for RICM Data Using Logistic Regression Analysis

Group 1 vs. Group 2 (kT/nm²)		*P* Value	98% Confidence Interval (kT/nm²)
1% BSA (μ = 10.24; *n* = 35)	0.1% BSA (μ = 12.94; *n* = 14)	0.256	(−0.049, 0.144)
1% BSA (μ = 10.24; *n* = 35)	4% BSA (μ = 3.38; *n* = 25)	0.005	(0.396, 0.863)
0.1% BSA (μ = 12.94; *n* = 14)	4% BSA (μ = 3.38; *n* = 25)	0.005	(0.542, 0.978)
0.1% HES (μ = 7.88; *n* = 9)	1% HES (μ = 15.99; *n* = 9)	0.014	(0.126, 1.034)
0.1% HES (μ = 7.88; *n* = 9)	4% HES (μ = 24.54; *n* = 15)	0.004	(0.388, 1.049)
1% HES (μ = 15.99; *n* = 9)	4% HES (μ = 24.54; *n* = 15)	0.127	(−0.195, 0.891)
1% BSA (μ = 10.24; *n* = 35)	1% BSA/HAase (μ = 15.41; *n* = 20)	0.063	(−0.139, 0.636)
4% BSA (μ = 3.38; *n* = 25)	4% BSA/HAase (μ = 2.52; *n* = 23)	0.170	(−0.275, 0.522)
4% HES (μ = 24.54; *n* = 15)	4% HES/HAase (μ = 20.04; *n* = 9)	0.468	(−0.407, 0.866)

Groupwise comparison of RICM-derived effective stiffness (kT/nm²) showing group condition (μ = mean, *n* = sample size), Bonferoni-corrected *P* value, and Bonferroni-corrected confidence interval. Data presented as mean for RICM parameters obtained using the 2-layer compliance model (see Methods) for 8 unique groups. Based on Bonferroni correction, $P < 0.0167$ was considered significant.

Abbreviations: BSA, bovine serum albumin; HES, hydroxyethyl starch; HAase, hyaluronidase; RICM, reflectance interference contrast microscopy.

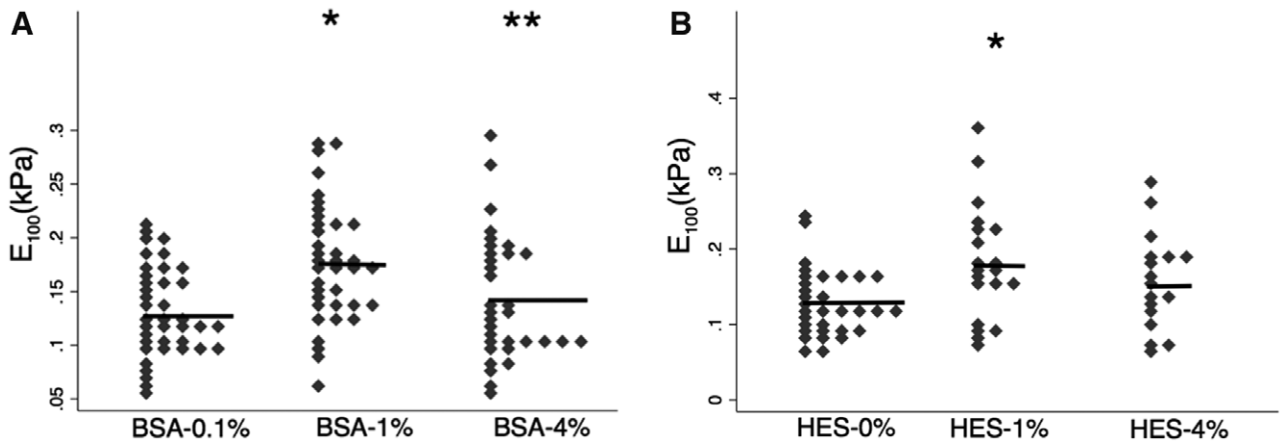

Figure 6. E_{100} measured in albumin or HES containing media. Both colloids demonstrated a biphasic effects on glycocalyx stiffness; the mean E_{100} value is indicated by the horizontal lines. A, E_{100} was significantly greater (stiffer) in 1% BSA ($n = 37$) versus 0.1% BSA ($n = 39$; $^*P = 0.001$). Increasing BSA from 1% to 4% ($n = 33$) resulted in smaller E_{100} (a softer glycocalyx); $^{**}P = 0.012$. B, The relationship of E_{100} versus HES concentration. 1% HES ($n = 20$) produced a larger E_{100} (stiffer glycocalyx) compared with 0% HES ($n = 39$; $^*P = 0.011$). E_{100} was reduced, but not significantly different, when HES concentration was increased from 1% to 4% ($n = 17$; $P = 0.325$). In the intact glycocalyx, albumin and HES have similar concentration-dependent effects on the interior stiffness. BSA indicates bovine serum albumin; HES, hydroxyethyl starch.

glycocalyx. These findings provide unique insight into the disparate site-dependent effects of albumin and starch on the interior of the glycocalyx (assessed by AFM) and at the glycocalyx—fluid interface (assessed by RICM). These variable, site-dependent effects of colloids may affect barrier function, mechanotransduction, and vascular permeability differently in the intact glycocalyx, leading to divergent clinical properties. We found that, during severe inflammatory states when clinicians are most likely to consider colloid use, the glycocalyx is compromised by proteases and other glycoprotein-degrading processes, and under such conditions, albumin has opposing effects versus HES on biomechanical properties. Collectively, these results suggest that HES is not comparable with albumin when considering their biomechanical effects on the glycocalyx or on the interactions of each colloid with the glycocalyx, per se, and therefore, the effects of colloids on glycocalyx-dependent processes that influence vascular physiology.

Atomic Force Microscopy

We previously established 2 complementary techniques to evaluate the soft-layer mechanics of the glycocalyx[11,32]: RICM and atomic force microscopy (AFM) each use an 18-μm diameter spherical probe to impart a loading force onto the glycocalyx. AFM measures mechanical properties with progressive indentation to approximately 700 nm into the glycocalyx, whereas RICM measures equilibrium mechanics at much shallower (<10 nm) depths and with very fast indentation rates. These techniques have allowed us to analyze glycocalyx mechanics and to quantify the mechanical effects of albumin and HES over a clinically relevant range. In addition, to simulate glycocalyx degradation as might occur during acute inflammation, we measured the mechanical effects of albumin and HES on the glycocalyx in the presence of hyaluronidase.[11,28]

In this study, we determined the elastic modulus, *E*, of BLMVEC monolayers. As evident in Figure 3, albumin was associated with a softer glycocalyx (lower *E*), whereas HES resulted in an increase in glycocalyx stiffness at indentations depths from 300 to 700 nm. This effect is, in part, attributed to a reduction in the thickness of the glycocalyx in the presence of HES (Table 3) because the AFM loading forces are more effectively transmitted to the cell cytoskeleton. By using depolymerization of actin with cytochalasin D in BMLVEC, we previously determined that, at indentations depths deeper than 200 to 300 nm, AFM measurements largely evaluate cytoskeleton mechanics. On the basis of those data, we conclude that the glycocalyx is

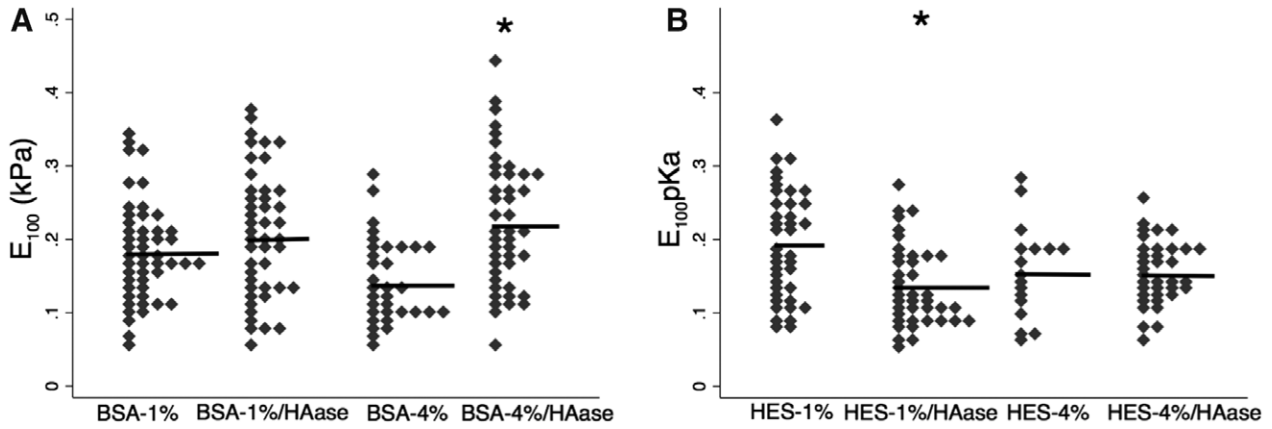

Figure 7. E_{100} measured after hyaluronidase (HAase) treatment. Mean E_{100} value is indicated by horizontal line. A, HAase had no effect on E_{100} in the presence of 1% BSA (n = 49; 1% BSA vs 1% BSA/HAase [n = 45]; P = 0.140). However, HAase significantly increased E_{100} (eg, stiffer glycocalyx) in the presence of 4% BSA (n = 33; 4% BSA vs 4% BSA/HAase [n = 44]; *P = 0.012). B, HAase significantly reduced E_{100} (softer glycocalyx) in the presence of 1% HES (n = 39; 1% HES vs 1% HES/HAase [n = 41]; *P = 0.001). In the presence of HES = 4% (n = 17), HAase had no significant effect on E_{100}. (4% HES vs 4% HES/HAase [n = 36]; P = 0.910). In summary, removal of hyaluronan from the glycocalyx has distinctly different effects on glycocalyx stiffness depending on the concentration of albumin or HES. BSA indicates bovine serum albumin; HES, hydroxyethyl starch.

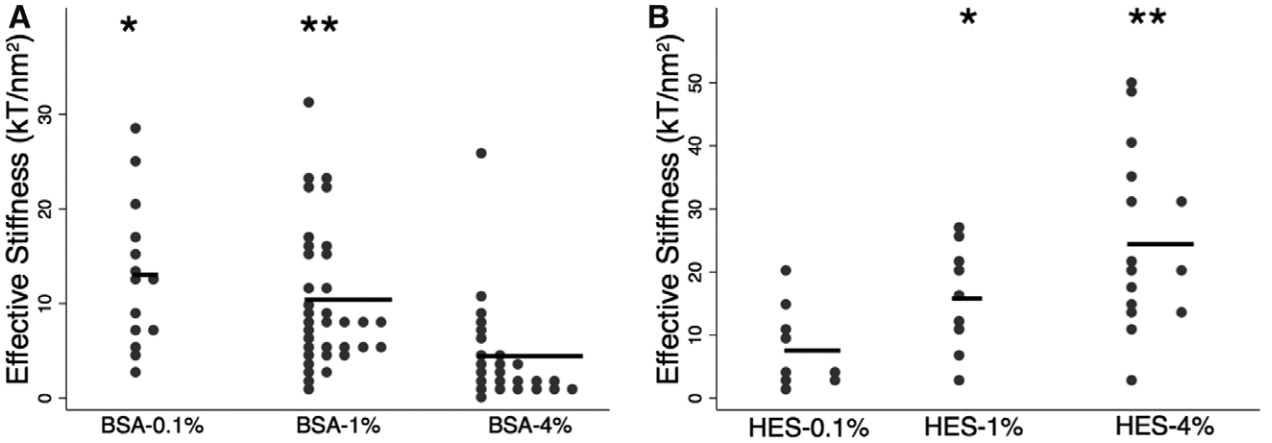

Figure 8. Effective stiffness of the outermost region of the glycocalyx. A, Albumin induces a concentration-dependent softening of the glycocalyx (lower effective stiffness). The glycocalyx was significantly stiffer at BSA = 0.1% (n = 14) vs 4% (n = 25) indicated by (*P = 0.005) and when comparing albumin concentrations of 1% (n = 35) and 4% (**P = 0.005). There was no difference in effective stiffness between albumin concentrations of 0.1% and 1% (P = 0.256). B, HES resulted in a concentration-dependent stiffening of the glycocalyx with significant differences occurring between HES = 0.1% (n = 9) and 1% (n = 9; *P = 0.014) and between concentrations of 0.1% and 4% (n = 23; **P = 0.004). Note that albumin and HES have opposite effects on glycocalyx stiffness at the glycocalyx–fluid interface. BSA indicates bovine serum albumin; HES, hydroxyethyl starch.

approximately 10 times softer than the underlying cellular structures. Given limitations in vertical AFM resolution, we assigned the glycocalyx modulus at the indentation depth of 100 nm (E_{100}) as a measure of glycocalyx stiffness (Figure 6). For each colloid, the 1% concentration resulted in the stiffest mean elastic modulus, and increasing or decreasing the albumin or HES concentration produced a softer glycocalyx.

Glycocalyx thickness (δ_g) was greater with albumin than with HES. In the presence of 1% albumin, δ_g = 532 ± 102 nm vs 317 ± 38 nm with 1% HES. These changes in the glycocalyx thickness were best revealed by analyzing $\Delta E(\delta)$ curves as shown in Figures 4 and 5. A $\Delta E(\delta) < 0$ (Figure 4, A and B) indicates glycocalyx thickening, and our data suggest that reducing albumin from 1% to 0.1% doubled glycocalyx thickness (at 0.1 %, δ_g = 1267 ± 68 nm vs at 1 %, δ_g = 532 ± 102 nm; Table 4 and Figure 4A). The 2-fold increase in glycocalyx thickness is most likely caused by a reduced number of albumin-dependent cross-links between HA and other glycocalyx components, allowing the glycosaminoglycan (GAG) chains to become unrestrained. Interestingly, an increase in albumin concentration from 1% to 4% also increased the glycocalyx thickness, from 532 ± 102 nm to 910 ± 49 nm, respectively (Table 4). This effect on glycocalyx thickness likely occurs as a result of excess albumin molecules within the glycocalyx and additional water and electrolytes that osmotically accompany albumin. The functional relationship that emerges between albumin concentration and glycocalyx thickness can be summarized as follows: at 1% albumin, the glycocalyx is in a cross-linked or restrained state. When albumin concentration is reduced to 0.1%, the albumin-dependent cross-linking is lost, and the GAG chains become unrestrained, increasing glycocalyx thickness. When albumin concentration is increased to 4%, the excess albumin, water, and electrolytes induce

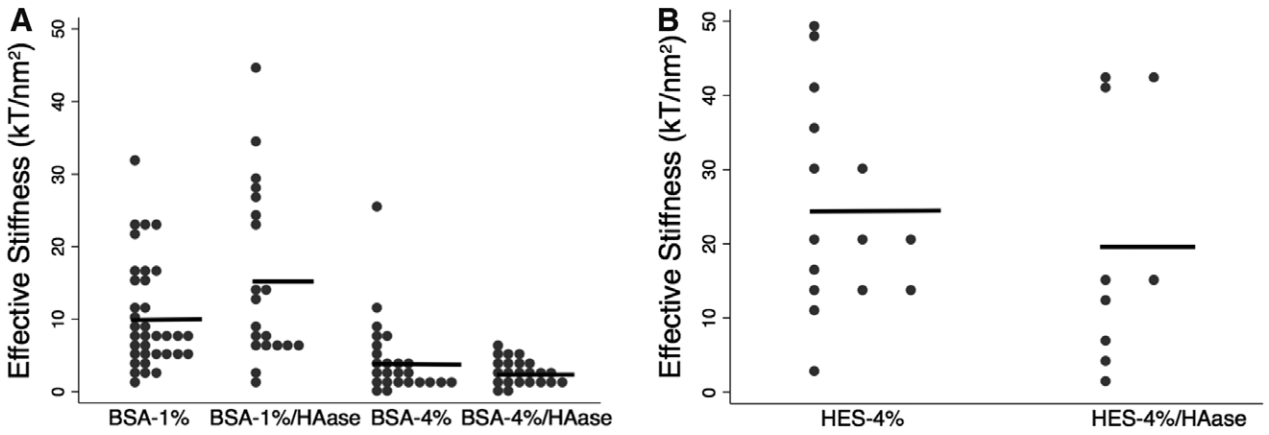

Figure 9. Comparison of the effective stiffness after hyaluronidase (HAase) treatment. The horizontal lines represent mean effective stiffness value. HAase had no effect on glycocalyx mechanics related to either albumin (A; $P = 0.063$ and $P = 0.170$) or HES concentrations (B; $P = 0.468$). These data suggest that hyaluronan is not a determinant of the effective stiffness at the glycocalyx–fluid interface. BSA indicates bovine serum albumin; HES, hydroxyethyl starch.

a swelling effect that also increases glycocalyx thickness. Thus, at both high and low albumin concentrations, the glycocalyx structure thickens, but by different mechanisms.

RICM Studies

RICM provided detailed assessment at the outermost regions of the glycocalyx, eg, at the aqueous–fiber interface. Albumin induced a concentration-dependent softening of the glycocalyx over a 0.1% to 4% range. Conversely, HES increased the effective stiffness over a 0% to 4% range. These results differ from measurements made by AFM from the interior of the glycocalyx. The easiest explanation for these findings is that outermost regions of the glycocalyx have different structural components and, therefore, interact differently with albumin and HES. However, these dichotomous findings might also have resulted from the difference in the speed with which loading forces were applied by AFM versus RICM. The AFM loading rate was quite low compared with the fast fluctuations in RICM. Because of these loading rate differences, the RICM-measured stiffness could have been influenced by viscous forces and related molecular rearrangements in a different way compared with the AFM modulus. Thus, the potential role of loading rates and viscous forces add an additional level of mechanical interactions that may influence endothelial signaling.

Are There Other Effects of Albumin and HES that May Explain These Data?

It is possible that increasing the colloid oncotic pressure altered cell physiology sufficiently to also alter cell surface biomechanics. Chiang et al[33] observed that polyethylene glycol, a supposedly inert neutral polymer, had a biphasic effect on endothelial permeability and actin organization, increasing transendothelial electrical resistance over the range of 0% to 8%, but inducing barrier dysfunction above 8%. The increase in electrical resistance was associated with a reduction of actin stress fiber and an increase in peripheral actin. The effect of polyethylene glycol on trans endothelial electrical resistance (TEER) and actin reorganization were complete in approximately 1 hour. In contrast, in our study, HES had a biphasic effect of glycocalyx stiffness over the range of 0% to 4%. Along with the increase in E_{100}, which should exclude a cytoskeletal mechanism, this finding suggests that HES has direct effects on glycocalyx mechanics. However, in Figure 4, 4% HES also affected cell mechanics at depths below 300 nm, which may indicate a cytoskeletal effect on the cytoskeleton. More work is needed to understand the effects on colloids on cytoskeletal mechanics.

Glycocalyx Degradation: The Effect on Biomechanics

During acute inflammation, glycocalyx constituents are shed by plasma proteases, neutrophil proteases, and endothelial membrane-associated metalloproteases.[23,34] For example, activated neutrophils release proteases and have membrane-bound proteases that participate in neutrophil transmigration out of the vascular system. Major constituents of the glycocalyx, such as syndecan, can be cleaved by several common proteases.[35] Plasma syndecan-1 has become a marker for the magnitude of vascular injury after trauma and hemorrhagic shock,[17,19] and plasma GAGs can be a signature for respiratory failure.[18]

On the basis of our previous biophysical characterization of BLMVEC glycocalyx, we concluded that HA is a major structural constituent and, on its removal from the glycocalyx, albumin dynamics within the glycocalyx was altered dramatically.[24] Likewise, others have demonstrated that HA removal significantly alters endothelial function.[36,37] Therefore, we used hyaluronidase to selectively remove HA to mimic the degradation of the glycocalyx that may occur during acute inflammation. The removal of HA altered both the physical and mechanical properties of glycocalyx. The fitted thickness parameter (Table 4) indicated that HA caused a reduction in the glycocalyx thickness from 532 ± 102 nm (1% albumin) to 450 ± 4 and 322 ± 4 nm, for 1% and 4% albumin, respectively. The changes in the fitted glycocalyx modulus ($E_{glycocalyx}$, Table 4) also agreed very well with the RICM-measured effective stiffness of the outermost part of the glycocalyx layer (Figure 8B); both parameters decreased significantly with 4% albumin in the absence of HA (compared with 1% albumin before or after removal of HA).

The changes in effective stiffness for 4% HES before and after removal of HA were statistically insignificant (Figure 9B),

as were changes in the fitted δ_g parameter at these conditions (Figure 5, B and C and Table 4). Overall, the mechanical parameters measured after HA removal from the glycocalyx support our previous observation that the HA chains are a major component of the glycocalyx and are responsible for interactions with albumin.[24] These results also support the conclusion that HA chains are inert toward HES. Finally, our results support the findings of Zeng et al,[25] who demonstrated that enzymatic removal of HA reduced the thickness of the cell surface albumin layer on bovine aortic endothelial cells.

Limitations

The primary limitation to our study is the necessity to conduct these experiments using an in vitro model. This methodology is both a strength and weakness, because RICM and AFM make precise measurements on a scale that is otherwise unattainable in vivo. However, there is controversy as to the composition and thickness of the glycocalyx of cultured cells versus its in vivo state. Measurements of the glycocalyx in vivo range from 0.4 to 5.0 μm,[38–40] depending on the vascular bed and species studied. The controversy regarding the use of cell culture models is also driven by the type of endothelial cells chosen for comparison. For example, based on electron microscopy and micro-velocimetry, human umbilical vein endothelial cells (HUVECs) have little, if any, glycocalyx.[41] This finding should not be surprising, because HUVECs are derived from a conduit vessel where permeability of the umbilical vein has little physiologic significance, limiting the value of HUVECs for studies on barrier properties.

We and others[6,24,25,40,42] have used a variety of methodologies, including fluorescence correlation spectroscopy (FCS), rapid freezing/freeze substitution transmission electron microscopy, and high-resolution confocal microscopy, to demonstrate that cultured endothelial cells from the bovine lung (BLMVEC), bovine aorta (bovine aortic endothelial cell, BAEC), and rat pad (rat fat pad endothelial cell, RFPEC) have a glycocalyx comparable in thickness with the in vivo state. By using rapid freezing/freeze substitution transmission electron microscopy, Ebong et al[40] reported a thickness of 11 μm for BAEC glycocalyx and 5 μm for rat fad pad endothelial cells. However, they used standard fixation techniques on these cells and observed a significant decrease in thickness of the glycocalyx. Thus, some forms of processing and fixation alter the measurable thickness. We used FCS, a biophysical modality that has submicron resolution, on live BLMVEC and measured a glycocalyx thickness of 1 to 3 μm. Using confocal microscopy and immunostaining for heparan sulfates, we observed a cell surface layer that was approximately 3.0-μm thick, providing good correlation with FCS. Tarbell and coworkers[6,25,42] have published a series of articles using high-resolution confocal microscopy of BAECs and RFPEC after immunostaining for syndecan, glypican, heparan sulfate, and albumin and consistently reported a glycocalyx thickness of 1.5 to 3.0 μm.

The resolution of standard confocal microscopy is approximately 0.5 to 1.0 μ, depending on the magnification and numerical aperture. In this study, AFM and our 2-layer composite model can derive changes of as little as 10 to 20 nm. The difference in glycocalyx thickness between albumin-treated and HES-treated cell was 200 nm, a difference that could not be measured with confocal imaging. This increased resolution highlights the value of biophysical techniques such as FCS (resolution, 0.1 μm), RICM (resolution subnanometer), and AFM (resolution, 10–20 nm) over other imaging modalities.

Clinical Relevance

The endothelial glycocalyx plays an important role in vascular barrier regulation,[4] white blood cell adhesion,[43] coagulation,[44,45] angiogenesis,[46] and tissue repair.[47] Nearly all these functions are relevant to perioperative clinicians. Moreover, understanding how resuscitation fluids like albumin and HES interact with the glycocalyx, and alter the structural and mechanical properties of the glycocalyx, has clinical implications in patient management and, potentially, in the development of novel resuscitation colloids.[48,49]

Safety concerns have been raised regarding the use of HES in specific clinical settings (eg, sepsis), because of increased risk of acute kidney injury. Low MW 130/0.4 HES has largely replaced the use of high MW 600/0.7 HES, but high MW HES is still available in the United States. Excluding sepsis and intensive care unit patients, the risk associated with the use of any HES solutions, especially in healthy patients, has not been adequately addressed in randomized controlled clinical trials.[50] At the time we began these studies, high MW HES was in routine use and we selected it as a prototype colloid that was structurally distinct from albumin. Clearly, the shift to low MW HES (130/0.4) may alter the results of our study. However, we would not anticipate significant differences between 600 and 130 MW HES on the biomechanical measurements, because both solutions are polydispersed, eg, have a wide range of polymer sizes. Predictably, solubility and absorbability (larger macromolecules tend to be less soluble but adsorb better) may differ with HES MW, but elastically the 2 HES fractions are similar. Overall, larger MW HES could adsorb more onto the glycocalyx, but produce a larger steric hindrance effect, whereas smaller MW starch solutions might have smaller adsorption, but allow better space usage (less steric hindrance). Ultimately, these 2 effects should compensate each other, meaning that their mechanical behavior as measured by AFM and RICM should remain unchanged.

Clinical choices for resuscitation fluids include crystalloid (most commonly lactated Ringer's solution or normal saline), human albumin solution, or HES. Although we chose to focus on the effects of colloids on the biomechanical properties of the intact and compromised glycocalyx, the effects of crystalloids that are clinically used much more often are equally important. Although there are no direct studies on the effect of crystalloids on glycocalyx biomechanics, a few points can be gleaned from our data. We assessed the effect of albumin over a concentration range from 0.1% to 4%; a plasma albumin concentration in this range would be considered hypoproteinemic by clinical standards but could easily result from hemorrhagic shock, where blood loss was replaced by a crystalloid solution. Reductions in plasma proteins on the interior mechanics of the glycocalyx are presented in Figure 6, demonstrating a biphasic effect on stiffness. In Figure 8, we observed a linear increase in glycocalyx stiffness as albumin concentration was reduced from 4% to 0.1%; these measurements were derived using RICM that measures the mechanics of the outmost structure of the glycocalyx. We can conclude that crystalloid resuscitation and the associated reduction in plasma protein would likely make the outer layer of the glycocalyx stiffer.

The ionic composition of resuscitation fluids, particularly hypertonic sodium solutions, and even 0.9% saline, will likely also affect the surface mechanics of the glycocalyx. Acute changes in extracellular sodium, >135 mEq/L, result in stiffening of endothelial cells and reduce NO production.[51] In these studies, however, the increase in cell stiffness occurred within minutes, but the change in NO production occurred over days. It is not known whether the acute change in cell stiffness is associated with rapid changes in NO production or glycocalyx shedding.

In addition to the composition of resuscitation fluid, the volume of fluid administered also affects the integrity of the glycocalyx. Acute hypervolemic volume loading with 6% HES solution (130/0.4) increased plasma levels of atrial natriuretic peptide (ANP), a protein that causes glycocalyx shedding.[52,53] Volume loading was associated with increased plasma syndecan-1, HA, and heparan sulfate concentrations, consistent with ANP-induced glycocalyx breakdown. The mechanism(s) by which ANP promotes widespread breakdown of the glycocalyx remain unknown. In contrast, acute normovolemic hemodilution with 6% HES did not increase plasma ANP and was not associated with changes in plasma markers of glycocalyx breakdown.

SUMMARY

Albumin and hetastarches differ in their effects on the biomechanical properties of the intact and partially degraded glycocalyx. These changes in colloid-dependent glycocalyx stiffness may have important implications in glycocalyx-dependent mechanotransduction and barrier function. It is clear that albumin and hetastarch are not comparable when used in studies of vascular function when factors other than oncotic pressure are operational. Whether a thicker or stiffer glycocalyx is better or worse is a complex question that cannot easily be answered given our current level of understanding. In terms of the glycocalyx as a passive barrier, thicker is considered better if other parameters (eg, porosity, composition) have not changed. Glycocalyx stiffness is related to signaling sensitivity in a biphasic manner; if the glycocalyx is too soft, it will be unable to transmit forces; if the glycocalyx is too stiff, it will not be stressed by the prevailing forces. In summary, understanding the molecular and biomechanical effects of resuscitation colloids should inform the practitioner about their indicated uses and provide the best possible clinical effects to enhance patient outcomes.

DISCLOSURES

Name: Kathleen M. Job, PhD.
Contribution: This author designed the studies, conducted the experiments, analyzed data, and assisted in manuscript preparation.
Name: Ryan O'Callaghan, MS.
Contribution: This author helped design the studies, conducted some of the experiments, and analyzed data.
Name: Vladimir Hlady, PhD.
Contribution: This author planned experiments, assisted in data interpretation, assisted in manuscript preparation.
Name: Alexandra Barabanova, MS.
Contribution: This author assisted in data interpretation and analysis and assisted in manuscript preparation.
Name: Randal O. Dull, MD, PhD.
Contribution: This author planned experiments, assisted in data interpretation, and assisted in manuscript preparation.
This manuscript was handled by: Avery Tung, MD.

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