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# *Endothelial Glycocalyx as an Additional Barrier Determining Extravasation of 6% Hydroxyethyl Starch or 5% Albumin Solutions in the Coronary Vascular Bed*

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***Background:*** **The impact on the endothelial glycocalyx for the extravasation of colloidal infusion solutions has not been investigated sufficiently.**

***Methods:*** **Isolated guinea pig hearts were perfused with Krebs-Henseleit buffer in a Langendorff mode. Solutions of 0.9% saline, 5% albumin (70 kd), or 6% hydroxyethyl starch (200 kd) were infused into the coronary system for 20 min at a rate of one third of the coronary flow, also during reperfusion after 15 min of ischemia, and after enzymatic digestion of the endothelial glycocalyx by heparinase. Net coronary fluid filtration was assessed directly by measuring the formation of transudate on the epicardial surface, and solute extravasation was assessed by measuring albumin and hydroxyethyl starch in the coronary effluent and transudate. Hearts were perfusion fixed to visualize the endothelial glycocalyx using transmission electron microscopy.**

***Results:*** **Only infusion of hydroxyethyl starch, not infusion of albumin, significantly decreased net coronary fluid filtration. Heparinase application without ischemia increased coronary leak by 25% but did not accelerate the passage of colloids. Ischemia alone did not alter permeability. However, there was a large (approximately +200%), transient (approximately 4 min) increase in permeability for water, albumin, and hydroxyethyl starch after ischemia with heparinase application. Also, histamine ($10^{-6}$ M) only increased permeability after pretreatment of the hearts with heparinase. The thickness of the glycocalyx after colloid administration was 0.2–0.3 $\mu$m. No glycocalyx could be detected after application of heparinase.**

***Conclusion:*** **The endothelial glycocalyx acts as a competent barrier for water and colloids. Only after its destruction do changes in endothelial morphology (postischemic reperfusion or histamine application) become effective determinants of coronary extravasation.**

A HEALTHY vascular endothelium is coated by the glycocalyx. Based on electron microscopic investigations, this structure was primarily regarded as a layer of membrane-bound proteoglycans and glycoproteins having a thickness of only a few tens of nanometers.[1–3] The results of more recent direct and indirect estimations, however, showed that this layer is much thicker.[4–7] Especially intravital microscopic studies have revealed that there is a difference between the anatomic width of a microvessel and the width of the space available for circulating erythrocytes and that there is an exclusion zone for erythrocytes adjacent to the endothelial surface in which plasma motion is significantly retarded. This zone, also termed the *plasma layer* or the *endothelial surface layer*, has a thickness of approximately 0.4–0.5 $\mu$m and contains fluid in dynamic equilibrium with the flowing plasma.[6–9] Furthermore, it was shown that not only enzymatic digestion with heparinase, pronase, or hyaluronidase[4,8,10] but also oxidized lipoproteines[11,12] or tumor necrosis factor $\alpha$ can reduce the thickness of this layer.[13] Diminution of the endothelial glycocalyx, however, does not only have an impact on the movement of erythrocytes through capillaries[14]; it can also lead to an increase in thrombocyte aggregation,[11] leukocyte adhesion,[13,15] and probably an increase in endothelial permeability to water and solutes[8] leading to tissue edema.[16]

Based on a meta-analysis of hemodilution studies and supplementary experimental data, Pries *et al.*[7] concluded that the dilution of blood with artificial media in form of infusion solutions can induce a dissolution of the absorbed layer of plasma proteins into the flowing blood, leading to a reduction in flow resistance and possibly to an increase in vascular permeability. As we have recently demonstrated in surgical patients by means of double-label measurements of blood volume (*i.e.*, the simultaneous measurement of plasma volume and erythrocyte volume), approximately 60% of the infused volume left the intravascular space within 30 min during volume loading with 20 ml/kg (one third of the initial blood volume) of 5% albumin (HA) or 6% hydroxyethyl starch (HES) solutions.[17] Not only the infused fluid but also considerable amounts of the infused solutes, albumin or HES, extravasated quickly. The ensuing, greater decrease in the large vessel hematocrit in relation to the whole body hematocrit (HKwb = erythrocyte volume/blood volume) led to the suspicion that a considerable decrease in the volume of the endothelial surface layer occurred during volume loading with colloids.[17] Possibly, this washout of the plasma originally bound in this layer facilitated the extravasation of the infused colloids by altering the properties of the glycocalyx. The current study evaluates, for the first time, the impact of the endothelial glycocalyx on the extravasa-

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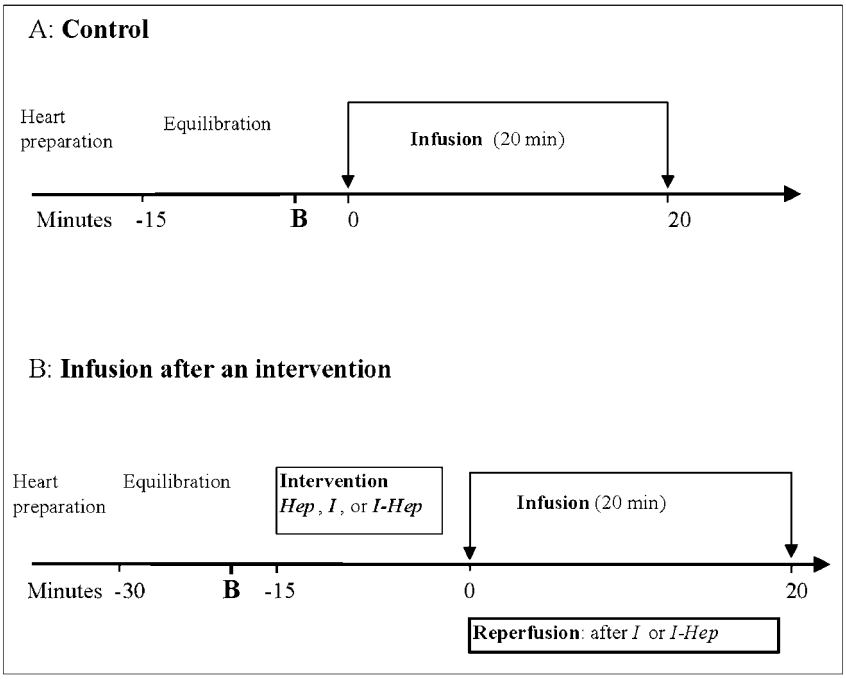


**Fig. 1. Experimental protocols. Infusions: 0.9% saline, 5% human albumin, or 6% hydroxyethyl starch solution, each at a rate constantly adjusted to one third of the actual coronary flow. Interventions: *Hep* = heparinase application within 15 min (without ischemia); *I* = 15 min of ischemia without heparinase application; *I-Hep* = heparinase application during 15 min of ischemia. In two series of control and *Hep* hearts, histamine ($10^{-6}$ M) or atrial natriuretic peptide ($10^{-9}$ M) was applied during the infusion of HES. Transudate and effluent samples were taken at baseline (B) and 1, 2, 3, 4, 6, 8, 10, 15, and 20 min after the start of the infusions.**

tion of HA and HES solutions, two commonly used colloidal infusion solutions, and provides functional data for the important function of this structure in preventing tissue (myocardial) edema.[16] The investigations are conducted in an intact vascular bed, namely, the coronary system of the isolated and perfused heart (guinea pig Langendorff heart preparations), using precisely those infusion solutions used in clinical volume loading.[17] Enzymatic degradation of the glycocalyx (heparinase) and perturbation of the morphology of the vascular endothelial cells (postischemic reperfusion or histamine application) are combined with measurements of net fluid filtration (vascular leak), colloid passage, and electron microscopic visualization of the glycocalyx.

## Materials and Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals*.[18] Licensure of the investigator from the Government of Upper Bavaria (file No. 209.1/211-2531.3-2/99) was obtained.

### *Heart Preparation*

Guinea pig hearts were isolated and perfused in a Langendorff mode as previously described.[19] In brief, animals (male; weight, 200–250 g) were stunned by neck dislocation using a specially designed instrument, and immediately after opening of the thorax, the hearts were arrested with cold isotonic saline. Quickly, the aorta was cannulated and retrogradely perfused at constant aortic pressure (80 cm $H_2O$) with a modified Krebs-Henseleit buffer (116 mM NaCl, 23 mM $NaHCO_3$, 3.6 mM KCl, 1.16 mM $KH_2PO_4$, 1.2 mM $CaCl_2$, 0.58 mM $MgSO_4$, 5.4 mM glucose, 0.3 mM pyruvate, and 2.8 U/l insulin, gassed with 94.5% $O_2$ and 5.5% $CO_2$) at 37°C, pH 7.40 ± 0.05. Hearts were removed from the thorax, and the pulmonary, caval, and azygos veins were ligated. Coronary venous effluent passed from the coronary sinus and right atrium into the right ventricle and left the heart through the pulmonary artery. Interstitial fluid/lymphatics formed by net fluid filtration appeared at the epicardial surface.[20] The rate of coronary flow was measured by means of a small animal flowmeter (T 106; Transonic Systems Inc., Ithaca, NY) and registered online. Epicardial fluid (transudate) was collected from the apex of the heart, and coronary effluent was collected from the pulmonary artery. Both were weighed by means of a precision scale and immediately frozen. To administer the different infusion solutions, a small catheter, connected with an infusion pump (Perfusor Secura®; Braun Melsungen AG, Melsungen, Germany), was inserted into the aortic feed line of the hearts. In all experiments with ischemia, a second infusion line was inserted into the aortic line to infuse 1 ml saline containing 154 mM NaCl and 1 mM $CaCl_2$, with or without heparinase enzyme, during the 15 min of ischemia.

### *Experimental Protocols*

Figure 1 illustrates the experimental protocols. In the first set of experiments, the impact of the different infusion solutions on transudate formation was studied under control conditions (fig. 1A). After an equilibration interval of 15 min, either 0.9% saline solution (NaCl; Braun Melsungen AG), 5% human albumin (HA; Centeon Pharma GmbH, Marburg, Germany), or 6% HES solution (molecular weight, 200,000 ± 25,000; degree of substi-

tution, 0.5; Fresenius AG, Bad Homburg, Germany) was infused continuously for 20 min at a rate constantly adjusted to one third of the actual coronary flow. Samples of the transudate and effluent were collected before infusion (baseline) and at minutes 1, 2, 3, 4, 6, 8, 10, 15, and 20 after the start of the infusion. The dynamic viscosities of the 2:1 mixtures of perfusate with NaCl, HA, or HES were 0.77, 0.83, and 1.07 centipoise, respectively. The colloid osmotic pressures of these mixtures of perfusate with HA or HES amounted to approximately 7 and 12 mmHg, respectively (values provided by the manufacturers). In two groups of hearts, the infusion of HES was combined with the simultaneous application of $10^{-6}$ M histamine (histamine dihydrochloride; Sigma-Aldrich, Taufkirchen, Germany) or $10^{-9}$ M atrial natriuretic peptide (Sigma-Aldrich) in the perfusate *via* an additional small catheter inserted into the aortic feed line.

In a second set of experiments (fig. 1B), the three infusion solutions were administered after different interventions, before which baseline samples of transudate and effluent were taken. Intervention *Hep* was the application of 10 U enzyme (heparinase I; Sigma-Aldrich) in 1 ml NaCl into the arterial feed line of the hearts within 15 min. Immediately after the enzyme application, HES together with or without histamine ($10^{-6}$ M) was infused for 20 min at a rate of one third of the actual coronary flow. Transudate samples were taken again at 1, 2, 3, 4, 6, 8, 10, 15, and 20 min after the start of the infusion. In the experiments termed *I*, the different colloids (HA or HES) were administered after 15 min of global, stopped flow ischemia (35°–37°C; fig. 1B). Ischemia was performed by clamping the aortic feed line. Samples of effluent and transudate were taken at the same measuring points as in the experiments above. In intervention *I-Hep*, we applied heparinase (5 U in 1 ml saline) during the 15 min of global ischemia *via* an infusion cannula inserted just below the aortic clamp. Afterward, we infused NaCl, HA, or HES solutions. The rest of the experiment was conducted as described for *I* (see above). Neither after-intervention *I* nor after-intervention *I-Hep* histamine or atrial natriuretic peptide were applied together with the colloid infusions. No perfusion protocol took more than 50 min.

### *Determination of Albumin and Hydroxyethyl Starch Concentrations*

Human albumin was quantified by colorimetry of the bromocresol complex[21] (variation coefficient $< 2\%$) in the samples of coronary effluent and transudate. The concentration of hydroxyethyl starch (cHES) was determined using a modification of a method described by Förster *et al.*[22]: 0.5 ml of the sample was transferred into a screw-topped tube containing 0.25 ml potassium hydroxide (35%) and placed into a boiling water bath for 45 min. After cooling and adding 7.5 ml ethanol (100%), the suspension was put into a refrigerator at 4°C for 12 h before being centrifuged (3,500 rpm, 0°C, 60 min). The supernatant was discarded, and the remaining fluid was mixed with 2.5 ml hydrochloric acid, 2 M, and then again placed into a boiling water bath for 120 min. After a second cooling procedure, the suspension was transferred into a 10-ml tube. Sodium hydroxide (2.5 ml, 2 M) was added, and the tube was filled with water to the 10-ml mark. The same procedure was performed with a hetastarch standard dilution sample (1.2 g/dl hetastarch in water). Aliquots (0.5 ml) of the hydrolyzed sample or the hydrolyzed standard sample were transferred into a cuvette, 2 ml suspension 1 (buffer–adenosine triphosphate–nicotinamide adenine dinucleotide phospate) of the gluco-quant® Testkombination (Boehringer, Mannheim, Germany) was added, and then the first optical absorption value (E1) was measured (340 nm, spectrophotometer, Cary 100 Bio; Varian, Melbourne, Australia). Afterward, 0.04 ml gluco-quant® suspension 2 (hexokinase-glucose-6-phosphate-dehydrogenase) was added and, after 10 min, the second light absorption (E2) was determined. The difference (dE) between both values (E2 − E1) was proportional to cHES, which was derived according to the formula $cHES = c_{standard} \times dE_{sample}/dE_{standard}$, with $c_{standard}$ being 1.2 g/dl. The mean difference and SD of 200 *in vitro* measurements with different known cHES values were −0.016 and ± 0.106 g/dl, respectively, in comparison with the predicted (known) cHES values.[17]

### *Electron Microscopy*

Electron microscopy was performed in modification of a method described by Vogel *et al.*[23] At the end of the perfusion protocol or in hearts flushed free of blood for less than 1 min *in situ*, the aorta was perfused with a fixation solution containing 2% glutaraldehyde, 2% sucrose, 0.1 M sodium cacodylate phosphate, and 2% lanthanum nitrate. Lanthanum is a trivalent cation and binds to negatively charged glycoprotein moieties.[23] When the contractions of the hearts stopped (usually within 1 min), the left ventricular wall was diced, and the pieces were immersed in fixation solution for 2 h at 20°C. Afterward, the tissue samples were washed with a solution containing 2% $H_2O_2$, 2% sucrose, 0.1 M sodium cacodylate phosphate, and 2% lanthanum nitrate for 12 h at 20°C. Thereafter, the pieces of myocardium were washed again in a solution mixed from 18 ml NaOH, 0.1 N; 27 ml $H_2O$; and 9 ml sucrose, 12%. After contrast enhancing with a solution containing 2% osmium tetroxide and 2% lanthanum nitrate in distilled water, the glycocalyx was photographed using an electron microscope (Philips CM 10; Aachen, Germany). Black-and-white images of approximately 50 capillaries of at least two hearts each of control experiments and the different

**Table 1. Basal Values for Control Experiments and Experiments with Different Interventions**

| Infusion Solution | Intervention | Effluent, ml/min | Transudate, ml/min |
|---|---|---|---|
| NaCl | Control (n = 6) | 5.3 ± 0.4 | 0.171 ± 0.024 |
| NaCl | *I-Hep* (n = 5) | 6.4 ± 1.2 | 0.200 ± 0.032 |
| HA | Control (n = 5) | 5.8 ± 0.4 | 0.188 ± 0.020 |
| HA | *I* (n = 7) | 5.9 ± 0.4 | 0.194 ± 0.020 |
| HA | *I-Hep* (n = 7) | 6.1 ± 0.3 | 0.203 ± 0.033 |
| HES | Control (n = 6) | 6.8 ± 0.8 | 0.225 ± 0.038 |
| HES | *Hep* (n = 4) | 5.9 ± 0.5 | 0.141 ± 0.042 |
| HES | *I* (n = 7) | 7.2 ± 0.6 | 0.208 ± 0.027 |
| HES | *I-Hep* (n = 7) | 6.7 ± 0.4 | 0.215 ± 0.036 |

Data are presented as mean ± SEM.

Control = control experiment without ischemia or enzyme application; HA = 5% albumin; *Hep* = heparinase application without ischemia; HES = 6% hydroxyethyl starch; *I* = ischemia; *I-Hep* = heparinase application during ischemia; NaCl = 0.9% saline.

interventions were compiled. Collapsed vessels were excluded from investigation.

*Statistical Analysis*

Because most of the measured data were distributed normally (tested by Kolmogorov-Smirnov tests), these are presented as mean ± SEM, with n indicating the number of experiments. For normally distributed data, comparisons were made using the Student *t* test or, for multiple comparisons, analysis of variance with the Bonferroni correction. For data that were not normally distributed, comparisons were made using the Mann–Whitney rank sum test or analysis of variance on ranks for multiple comparisons. *Post hoc* testing was performed using the Student-Newman-Keuls method for multiple comparisons. A *P* value less than 0.05 was considered to be significant.

## Results

*Experiments without Heparinase*

Transudate formation, *i.e.*, the direct measure of net fluid filtration in the intact coronary bed of isolated guinea pig hearts, amounted to approximately 2–3% of coronary flow (table 1). The baseline values (*B* in figs. 1A and B) for transudate formation or coronary effluent flow of the control experiments did not differ significantly between the various experimental groups (table 1). Accordingly, to minimize scatter, values at later times were normalized to the basal value for each individual heart. Figure 2 illustrates transudate formation and coronary effluent flow for the control experiments. Replacing approximately one third of the volume of Krebs-Henseleit perfusate by infusion of 0.9% NaCl, corresponding to a vehicle and "time control" condition, was associated with a slight increase in transudate formation with respect to baseline, amounting to approximately +50% after 20 min. In contrast, transudate formation did not

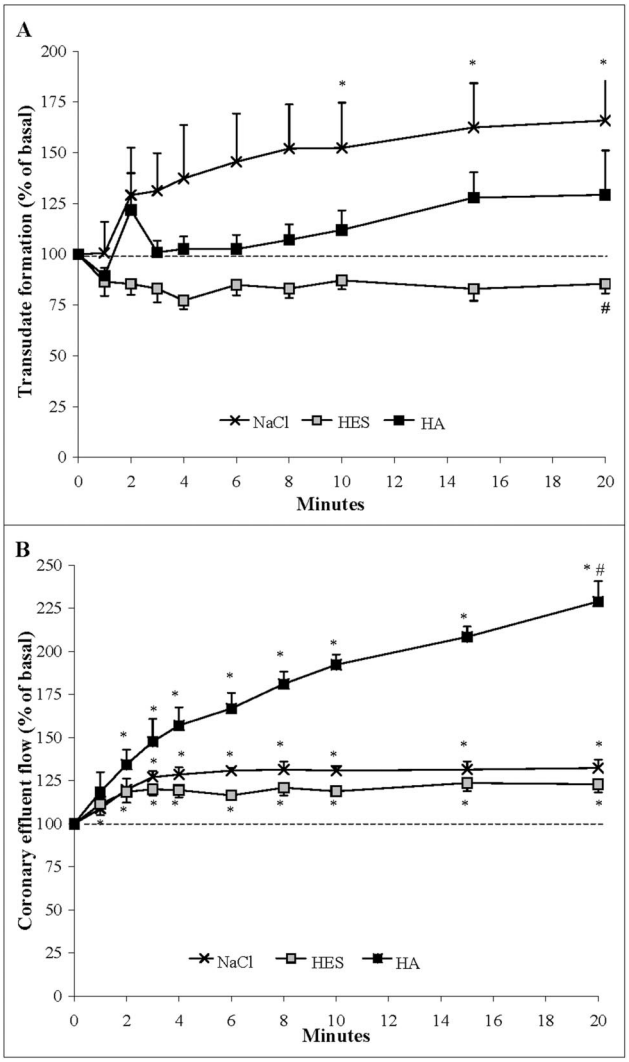


**Fig. 2. (*A*) Effects on transudate formation and (*B*) coronary effluent flow in control experiments; infusion of 0.9% saline (NaCl; n = 6), 5% albumin (HA; n = 5), and 6% hydroxyethyl starch (HES; n = 6). The absolute values corresponding to 100% are given in table 1. * *P* < 0.05, intragroup difference with respect to basal (minute 0). # *P* < 0.05, difference at minute 20 with respect to NaCl infusion.**

change significantly *versus* basal during infusion of either HA or HES. In fact, transudate formation after 20 min of HES infusion was significantly lower than in the NaCl control. Coronary effluent flow increased moderately with respect to baseline during the infusions of NaCl or HES (approximately +32% and +23%, respectively) but increased extensively during the infusion of HA (+129%; fig. 2B).

Time courses for the appearance of colloids in transudate and effluent are presented in figure 3. Figure 3A shows that, in experiments without heparinase or ischemia, there was no significant difference between the concentrations of HA and HES in effluent or transudate, respectively. Moreover, there was no difference in colloid concentration between the two fluid compartments after 20 min of infusion (fig. 3A). However, for both

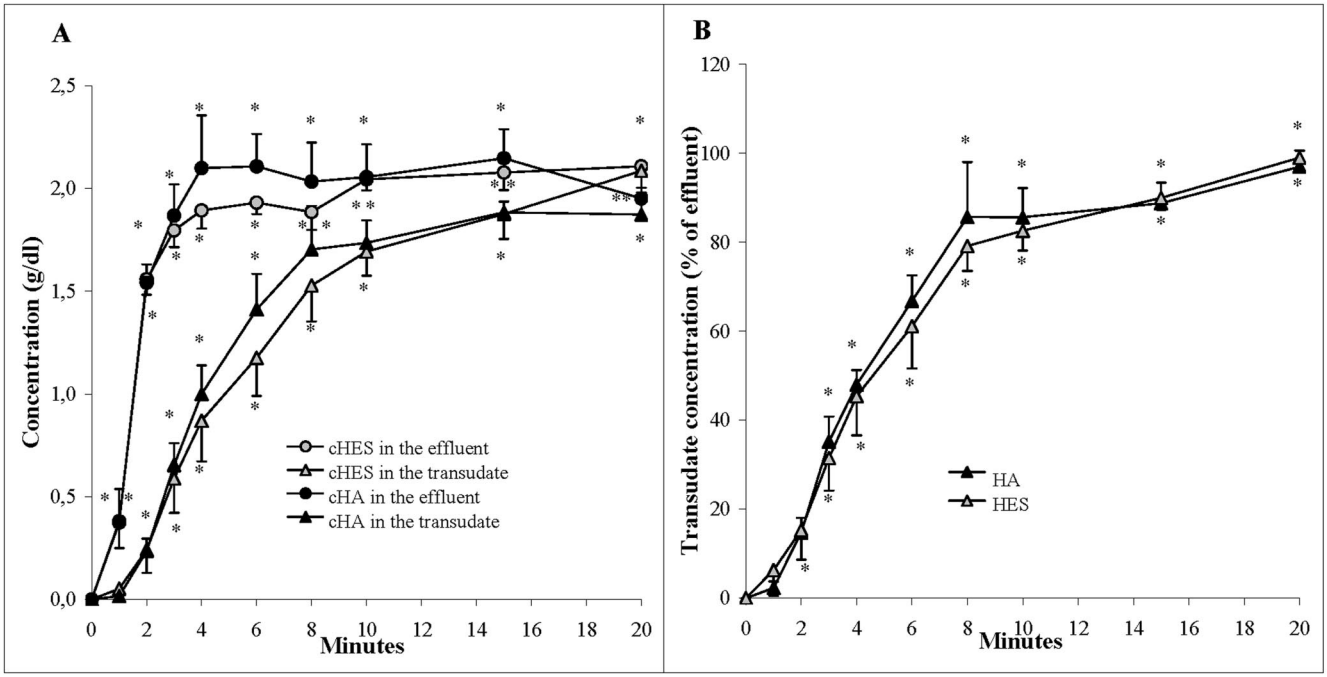


**Fig. 3. Concentrations of albumin (cHA) and hydroxyethyl starch (cHES) in the effluent and transudate in control experiments: infusion of 5% albumin (HA; n = 5) and 6% hydroxyethyl starch (HES; n = 6) without ischemia or enzyme application. (*A*) Absolute concentrations (g/dl); (*B*) concentration in the transudate related to the coinciding concentration in the coronary effluent (%). * $P < 0.05$, intragroup difference with respect to basal (minute 0).**

substances, the increase of concentration in transudate lagged behind that in coronary effluent: Within 2 min, the concentration of albumin (cHA) and cHES in the effluent increased to approximately 1.5 g/dl and reached a stable value of approximately 2.0 g/dl after another 2 min. The respective concentrations in the transudate were approximately 0.2 g/dl after 2 min and approximately 0.9 g/dl after 4 min of infusion (fig. 3A). The time to reach half maximum values in the transudate ($t_{1/2}$) was 6.2 ± 0.9 min for cHES and 4.8 ± 0.4 min for cHA ($P > 0.1$). Figure 3B illustrates that there was no difference between the increase in cHES and cHA in the transudate when related to the respective concentration in the effluent.

*Experiments with Heparinase Alone*

Application of 10 U heparinase over the course of 15 min (intervention *Hep*) had no discernible effect on transudate or coronary flow (not shown). Table 2 presents transudate and effluent flow during HES infusion after the application of heparinase. Initially, there was no difference from control; however, transudate formation after 20 min of HES infusion was slightly but significantly higher (+25%) than without heparinase pretreatment

**Table 2. Infusion of Hydroxyethyl Starch after Application of 10 U Heparinase (over 15 min; intervention *Hep*).**

| | | | | Hydroxyethyl Starch Concentration, g/dl | |
|---|---|---|---|---|---|
| Min | Transudate, ml/min | Transudate, % of Basal | Effluent, ml/min | In Effluent | In Transudate |
| 0 | 0.141 ± 0.042 | 100 | 5.9 ± 0.5 | 0 | 0 |
| 1 | 0.121 ± 0.053 | 77.4 ± 9.9 | 5.9 ± 1.3 | 1.13* ± 0.26 | 0.07 ± 0.04 |
| 2 | 0.144 ± 0.057 | 92.7 ± 11.8 | 6.9 ± 1.2 | 1.43* ± 0.20 | 0.25 ± 0.08 |
| 3 | 0.120 ± 0.045 | 80.4 ± 5.5 | 6.4 ± 1.0 | 2.00* ± 0.13 | 0.53* ± 0.12 |
| 4 | 0.130 ± 0.044 | 86.9 ± 7.5 | 6.4 ± 1.0 | 1.95* ± 0.05 | 0.82* ± 0.17 |
| 6 | 0.141 ± 0.056 | 92.8 ± 11.2 | 6.4 ± 0.9 | 2.04* ± 0.13 | 1.24* ± 0.15 |
| 8 | 0.151 ± 0.057 | 101.2 ± 10.6 | 6.2 ± 0.7 | 2.04* ± 0.09 | 1.61* ± 0.16 |
| 10 | 0.159 ± 0.057 | 108.1 ± 11.3 | 6.1 ± 0.8 | 2.06* ± 0.11 | 1.79* ± 0.13 |
| 15 | 0.156 ± 0.056 | 105.6 ± 9.5 | 6.1 ± 0.7 | 2.08* ± 0.10 | 2.00* ± 0.1 |
| 20 | 0.165 ± 0.061 | 110.5† ± 9.4 | 6.1 ± 0.7 | 2.10* ± 0.07 | 2.14* ± 0.08 |

Data are presented as mean ± SEM (n = 4).

* $P < 0.05$ intragroup difference with respect to the basal value (0 min). † $P < 0.05$ difference at minute 20 with respect to control (see fig. 2: hydroxyethyl starch infusion without enzyme application).

*Hep* = heparinase application without ischemia.

(110% compared with 85% *vs.* basal; *cf.* table 2 and fig. 2). Surprisingly, there was no change in $t_{1/2}$ for the appearance of HES in transudate (6.0 ± 0.8 min). This shows a selective increase in fluid filtration.

*Experiments with Ischemia*

Ischemia leads to alterations in endothelial cell shape and, therefore, should modulate the classic vascular barrier. Moreover, application of heparinase into the coronary bed during a short period of attenuated coronary perfusion (ischemia) enhances exposure of the glycocalyx to enzymatic degradation. The effects of 15 min of global warm ischemia without and combined with exposure to 5 U/ml heparinase (interventions *I* and *I-Hep*) on net fluid filtration and coronary flow are presented in figure 4. Surprisingly, transudate formation was not significantly increased during reperfusion after intervention *I*, irrespective of whether HES or HA was infused. However, after intervention *I-Hep*, there was a significant early increase in transudate formation during the infusion of either HES or HA (approximately +200% in the second minute of reperfusion). This manifestation of enhanced coronary leak was only transitory and had subsided again by approximately the fourth minute of reperfusion (fig. 4A). In the absence of colloid (infusion of NaCl), only a slight and transient increase in coronary leak (+71% in the second minute of reperfusion; $P = 0.07$) was noted after heparinase, as opposed to a sustained increase in coronary flow. The former result suggests that the endothelial glycocalyx does not markedly control fluid extravasation in the coronary system when no colloids are present in the perfusate. Moreover, the expected postischemic perturbation of endothelial cell contacts is, deviously, only of brief duration.

After ischemia, there was the expected reactive hyperemia (fig. 4B). This subsided with time in the case of the infusion of NaCl or HES, but with HA, it passed on into the increase in coronary flow already seen under control application of HA (*cf.* figs. 2B and 4B). Heparinase had no effect on the responses.

After intervention *I* and intervention *I-Hep*, cHES and cHA in coronary effluent increased to the steady state value of almost 2 g/dl within 4 min in the same manner as already seen in the control experiments. However, as can be seen in figure 5A, cHES was significantly higher in transudate 2 min after intervention *I-Hep* in comparison to both control and ischemia alone. This accelerated extravasation was reflected by the $t_{1/2}$ values of cHES in transudate: 3.6 ± 1.4 min after *I-Hep* was significantly lower than the respective control value of 6.2 ± 0.9 min. After intervention *I*, $_{1/2}$ of cHES in transudate was 4.8 ± 0.7 min, not significantly different from control. In the case of albumin (fig. 5B), $t_{1/2}$ in transudate did not differ significantly between *I*, *I-Hep*, and control experiments (4.9 ± 0.9, 3.9 ± 0.7, and 4.8 ± 0.5 min, respectively). To illustrate the change in colloid permselectivity induced by pretreatment with heparinase in combination with ischemia, we calculated the amount of extravasated solute as: extravasated colloid = (rate of transudate formation) × (colloid concentration in transudate).

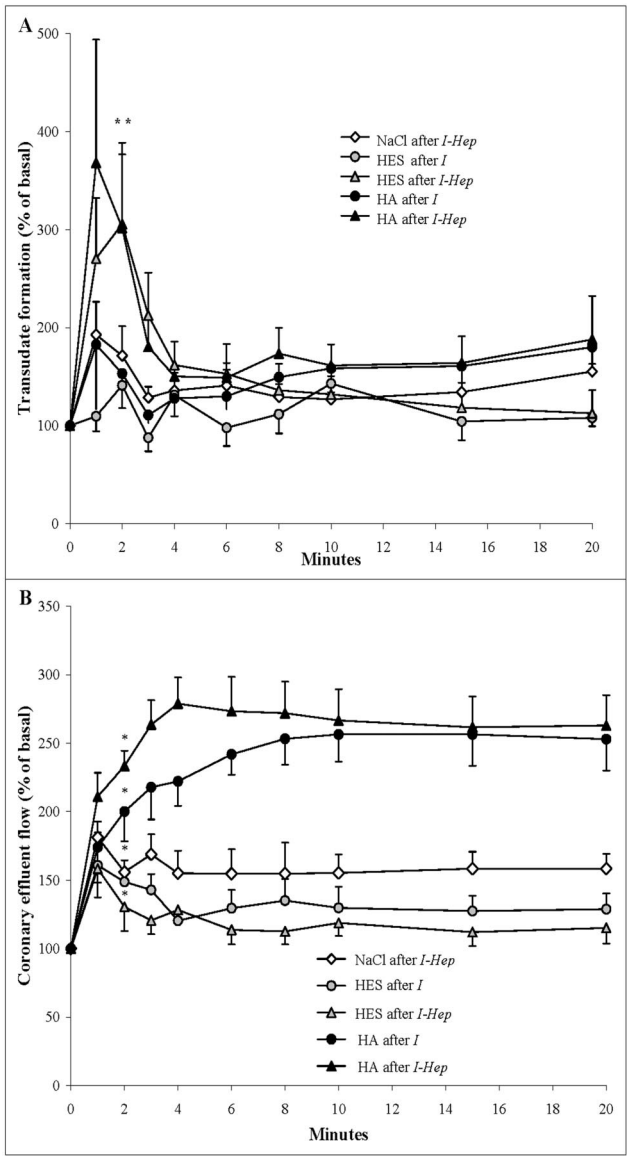


**Fig. 4. Infusion of saline 0.9%, (n = 7), 5% albumin (HA; n = 7), or 6% hydroxyethyl starch (HES; n = 7) after ischemia without heparinase (intervention *I*) or after ischemia and heparinase application (intervention *I-Hep*). (*A*) Effects on transudate formation and (*B*) effects on coronary effluent flow. * $P < 0.05$, intragroup difference between basal (minute 0) and minute 2.**

The comparison of the mean values 2 min after reperfusion after interventions *I* and *I-Hep* shows a 5.3-fold increase in HES and a 2.1-fold increase in albumin extravasation due to heparinase application.

*Experiments with Histamine and Atrial Natriuretic Peptide*

Histamine application alters the integrity of the classic vascular barrier by modulating endothelial cell gaps. Fur-

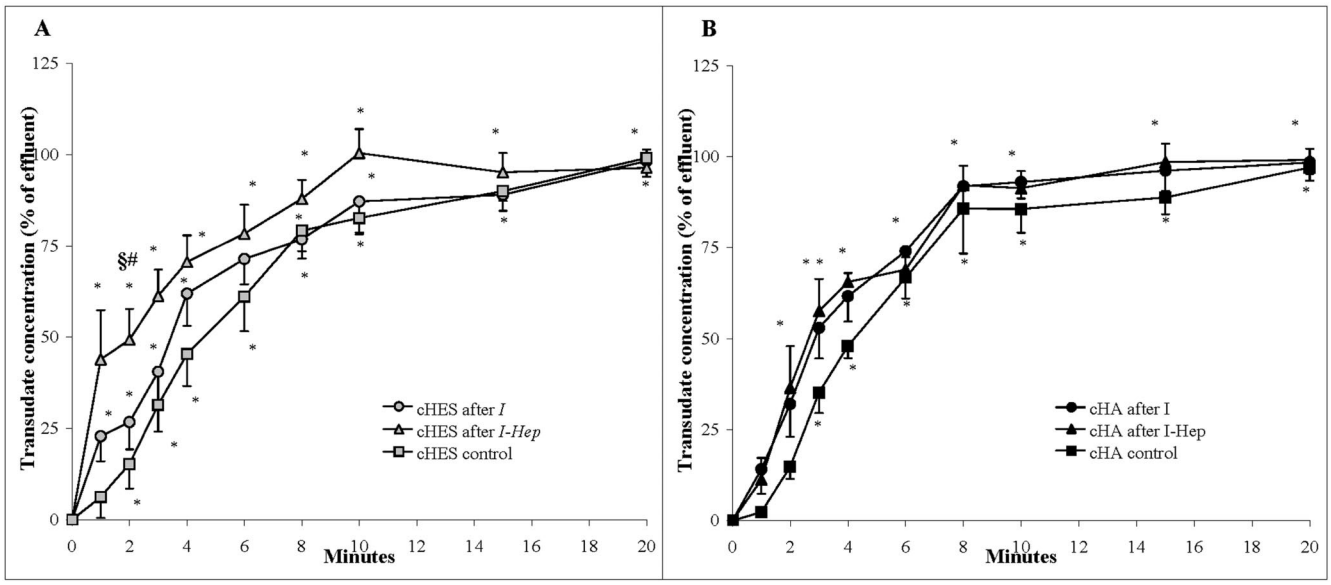


**Fig. 5. Concentrations of (*A*) hydroxyethyl starch (cHES) and (*B*) albumin (cHA) in the transudate related to the coinciding concentration in the coronary effluent (%) during the infusion of 6% hydroxyethyl starch (HES; n = 7) or 5% albumin (HA; n = 7) in control experiments, after ischemia without heparinase (intervention *I*), or after ischemia with heparinase application (intervention *I-Hep*). * $P < 0.05$, intragroup difference with respect to basal (minute 0). # $P < 0.05$, difference (at minute 2) between the colloid concentration after intervention *I-Hep* with respect to the colloid concentration in the control experiments. § $P < 0.05$, difference (at minute 2) between the colloid concentration after intervention *I-Hep* with respect to the colloid concentration after intervention *I*.**

thermore, histamine is a vasodilatator and increased flow to 142 ± 10% of the basal value when applied $10^{-6}$ M together with HES (n = 5). However, this application of histamine did not lead to any change in transudate flow *versus* the control infusion of HES (91.5 ± 3.3% of basal at 20 min). After pretreatment with heparinase (n = 6; intervention *Hep*) transudate formation increased significantly when histamine was applied together with HES (148.1 ± 10.5% of basal at 20 min; $P < 0.05$ *vs.* heparinase alone [table 2] and *vs.* histamine alone). Preliminary experiments were also performed with infusion of $10^{-9}$ M atrial natriuretic peptide together with 6% HES. Without any significant change in coronary flow, an increase in transudate flow was observed independently of whether pretreatment with heparinase (10 U, 15 min) was conducted or not (increase *vs.* basal transudate, 35 ± 7 and 27 ± 9%, respectively; n = 6 each; $P < 0.05$).

*Description of the Glycocalyx*

Electron microscopic photographs illustrating the endothelial glycocalyx of the coronary vessels are depicted in figure 6. An endothelial glycocalyx with a thickness of approximately 0.2–0.3 μm is readily apparent in hearts after the infusion of HA or HES in the control experiments (figs. 6A and B), as well as in the experiments with intervention *I* (figs. 6C and D). This same glycocalyx was visible in vessels of perfused hearts without any infusion of a colloid (not shown). After heparinase application (intervention *Hep* or intervention *I-Hep*), no glycocalyx could be visualized by electron microscopy (figs. 6E and F).

## Discussion

Starling's hypothesis[24] about the forces governing vascular permeability was born without any knowledge of the existence of an endothelial glycocalyx. Indeed, our results partly contradict expectations based on the Starling forces. Especially the finding that, despite the presence of equal concentrations of colloidal HES in effluent and transudate fluid at the end of a 20-min equilibration phase, net fluid filtration was lower than in the absence of colloid (fig. 2) seem to be illogical. Furthermore, net fluid filtration was not significantly suppressed *versus* NaCl or HES when albumin was applied, although the molar concentration of this colloid in the steady state (2 g/dl) was approximately three times that of HES (molecular weight ratio approximately 70 to 200 kd). These conflicting results leave no doubt that something is missing in Starling's concept of microvascular fluid filtration.

One of the first attempts to elucidate the role of the endothelial glycocalyx for vascular permeability was made by Curry and Michel in 1980.[25] Its possible importance for vascular barrier function was considered more seriously since its extraordinary thickness was discovered approximately 15 yr ago.[26–29] Vink and Duling[30] demonstrated that large anionic or neutral dextran molecules (> 70 kd) remain fully excluded by the glycocalyx (for > 3 h), whereas smaller anionic dye molecules, smaller dextran molecules, fibrinogen, or albumin penetrated the glycocalyx. There seems to be an important interaction of plasma proteins with the endothelial glycocalyx.[31–34] Binding of albumin to the glycocalyx of

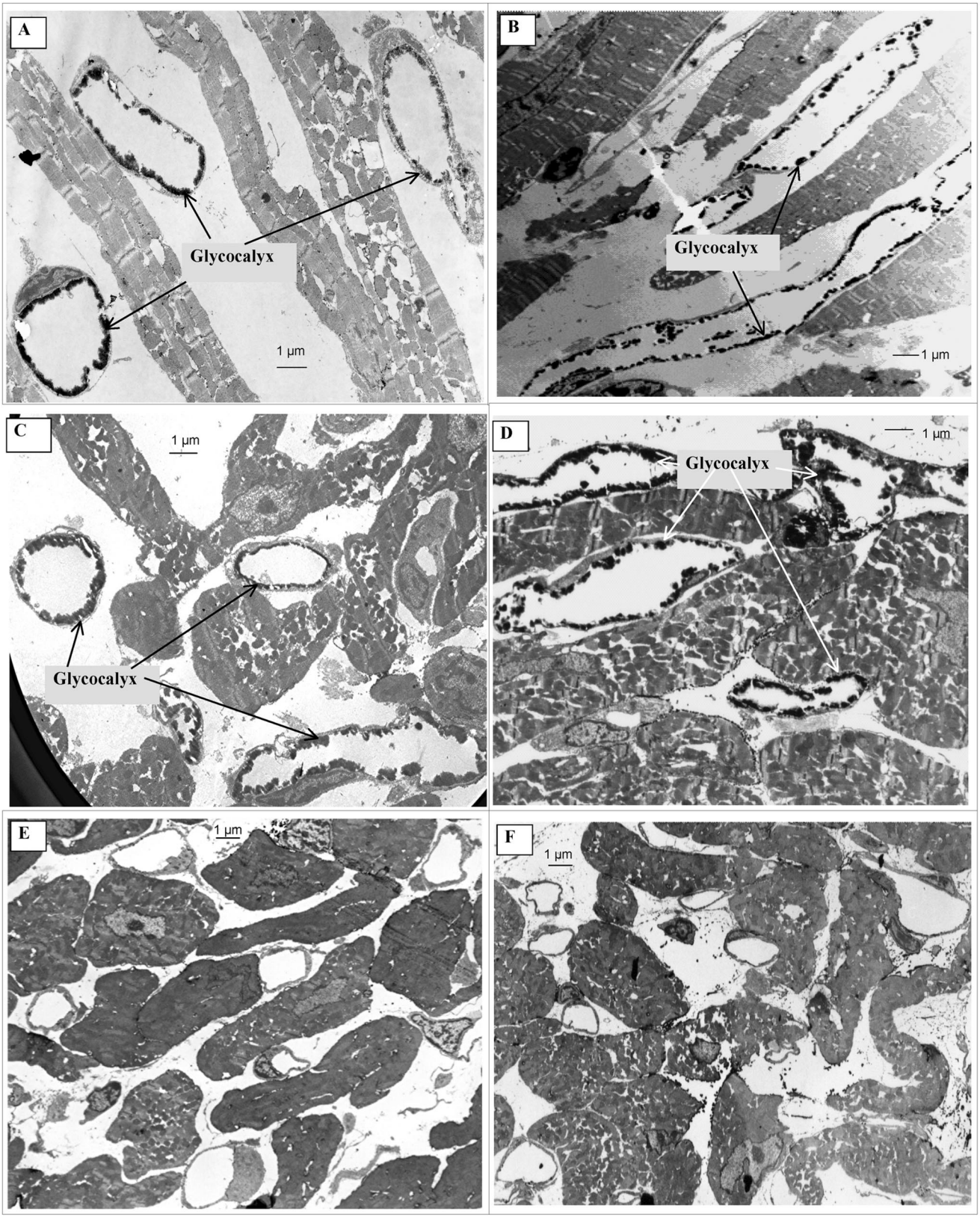


**Fig. 6. Electron microscopic views of hearts stained to reveal the glycocalyx (representative of two hearts each). (*A*) Control experiment, infusion of 5% albumin (HA); (*B*) control experiment, infusion of 6% hydroxyethyl starch (HES); (*C*) after ischemia (intervention *I*) and infusion of HA; (*D*) after ischemia (intervention *I*) and infusion of HES; (*E*) after heparinase application without ischemia (intervention *Hep*); (*F*) after heparinase application during ischemia (intervention *I-Hep*).**

adult microvessels served to decrease hydraulic conductivity.[35–37] Henry *et al.*[38] assumed that a retention of HES molecules within the glycocalyx might tighten the matrix and result in a decrease in vascular extravasation for small dextran molecules (40 kd). Recent theoretical and experimental investigations showed the local protein concentration and pressure immediately behind the *surface-matrix layer* (another term for the glycocalyx) to be much lower than the respective tissue concentration and pressure.[39,40] This results in an important difference between the global Starling forces across the entire endothelial layer and those across the glycocalyx. Accordingly, a large increase in the oncotic pressure within the vascular lumen after the infusion of HES with respect to a remaining small oncotic force directly behind the glycocalyx may explain the reduction in transudate formation seen especially during the infusion of HES in our study (fig. 2; HES *vs.* NaCl infusion). The failure of HA to act as strongly in this way supports the finding of a notable intrinsic permeability of the glycocalyx for this plasma protein.[30]

The change in vascular permeability after enzymatic digestion of the glycocalyx was first investigated by Adamson[41] using frog mesenteric microvessels. After pronase application, a 2.5-fold increase in hydraulic conductivity was found, and the authors assumed that at least 60% of the hydraulic resistance is associated with the glycocalyx. Pronase, however, is a combination of proteolytic enzymes and can digest the basement membrane in addition to the glycocalyx.[41,42] In contrast thereto, heparinase, an enzyme specific for the glycoprotein heparin sulfate, leads to digestion of the glycocalyx without major impact on endothelial cell integrity.[4,42] Therefore, it is not astonishing that in canine kidney MDCK-cell monolayers, the application of pronase resulted in a larger increase in mannitol permeability (of approximately 700%) than heparinase,[43] which, surprisingly, did not increase mannitol permeability in this experimental setting. Also, in cultures of bovine lung microvascular endothelial cells, heparinase I had no effect on barrier regulation.[44] In an isolated rat lung model,[45] heparinase application resulted only in a moderate increase in microvascular extravasation for albumin (+65%) and a small increase in total lung water (+9%). In contrast, heparinase application into isolated perfused rat kidneys and the following clamping of the vessels (for 30 min) to incubate the organ with enzyme (*i.e.*, ischemia) resulted in a substantial increase in permeability of the glomerular basement membranes to ferritin.[46] Using coronary arterioles isolated from pigs, heparinase treatment led to 4.8-fold and 2.3-fold increases in permeability for $\alpha$-lactalbumin and albumin, respectively.[42] On the basis of these investigations, one can state that the glycocalyx is important for maintaining Starling forces; however, a destruction of this layer can have large, moderate, or even no effects on vascular permeability. This perplexing variability is also reflected in the data of our current study.

The main functional findings of this study were as follows. First, net fluid filtration (transudate) increased only moderately (+25%) after heparinase application without ischemia (table 2), and the rate of appearance of HES in transudate was unchanged ($t_{1/2}$ for cHES, approximately 6 min). Second, heparinase application during ischemia (*I-Hep*; fig. 4) led to a large but transient increase in fluid extravasation (+200%) during early reperfusion. Not only more fluid but also more colloid passed the vascular barrier after intervention *I-Hep* (5.3-fold increase in HES and 2.1-fold increase in albumin extravasation). Third, ischemia without heparinase application (intervention *I*) did not lead to a significant increase in transudate formation or enhanced colloid passage. Fourth, similarly, histamine only enhanced transudate when hearts had been pretreated with heparinase.

Moreover, we were able to visualize the glycocalyx (thickness, 0.2–0.3 $\mu$m) by means of electron microscopy in saline-perfused hearts and after the infusion of HA and HES in the control experiments (fig. 6). Even after 15 min of ischemia (intervention *I*), a thick endothelial glycocalyx remained. This finding stands in contrast to other investigations in which longer periods of ischemia were imposed.[46–50] After the application of heparinase (with or without ischemia), there was no glycocalyx. These facts together with the functional data indicate that, in our experimental model, it was possible to investigate the properties of the endothelial glycocalyx.

In the following explanation, we present a model to explain not only all the results of the current study but probably also the results of many other investigators mentioned above. The key to this explanation is to regard the endothelial glycocalyx as an additional component to the barrier formed by the endothelial cells. Perturbation of only one of these components without alteration of the other should result in no increase or an only moderate increase in vascular permeability. Figure 7 presents a schematic illustration of the changes in the endothelial glycocalyx and the hypothetic changes in the volume and shape of endothelial cells after the different interventions. During the control experiments, there was a competent endothelial glycocalyx barrier and a competent endothelial cell barrier. After intervention *Hep*, there was no glycocalyx, but the endothelial cells were not affected. This cellular barrier was so tight that the changes in vascular fluid or colloid extravasation were only minimal. The electron microscopic pictures showed clearly that the short period of ischemia (intervention *I*) used in our study did not affect the glycocalyx. Because this competent barrier was not destroyed after ischemia, the respective increase in vascular permeability was minimal, independent of any postischemic changes in the second barrier, the endothelial cells.

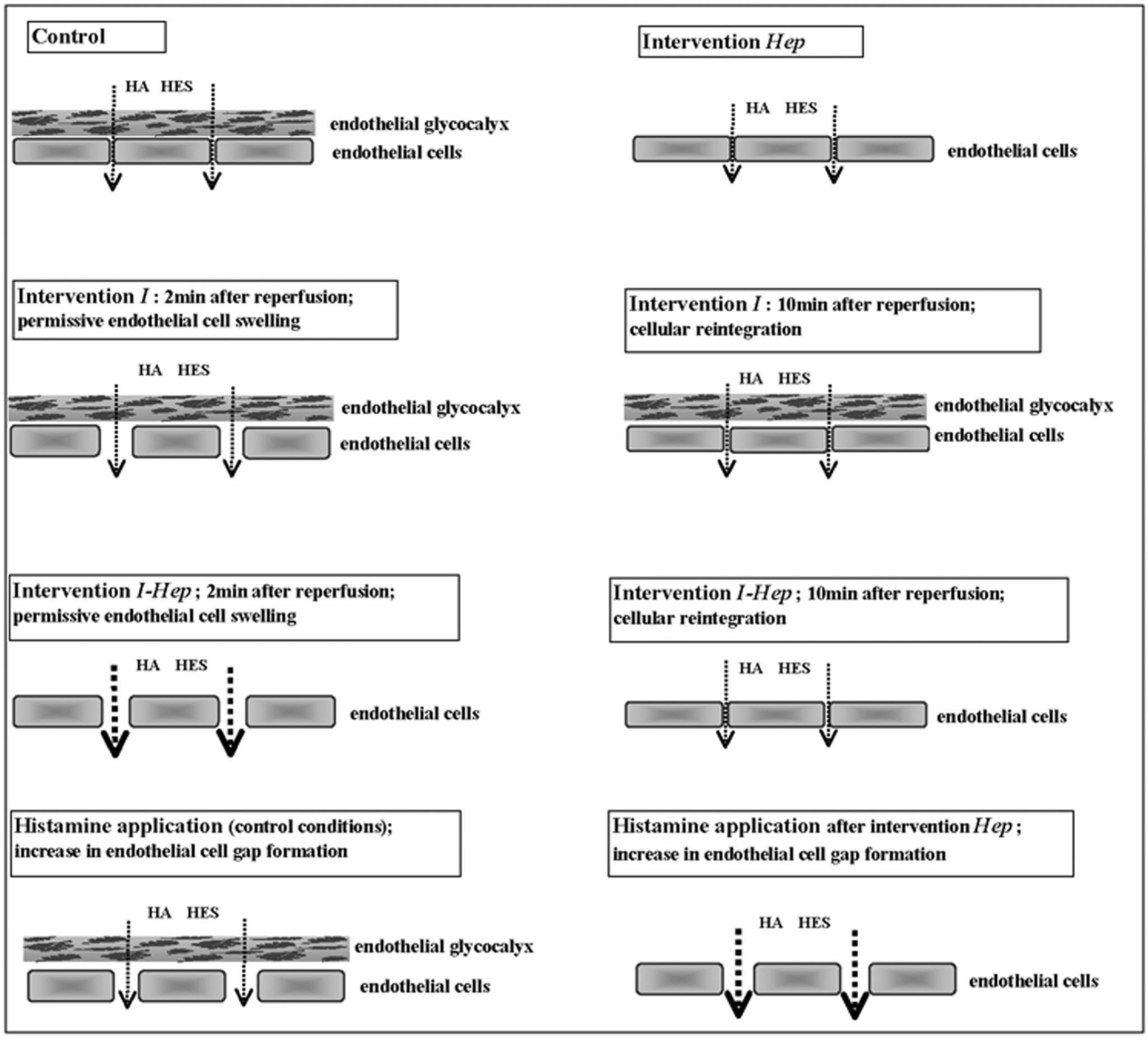


**Fig. 7. Schematic illustration indicating the changes of the glycocalyx and the hypothesized changes in endothelial cell volumes and intercellular clefts under various experimental conditions. Interventions: *Hep* = application of heparinase (without ischemia); *I* = ischemia (without enzyme application); *I-Hep* = application of heparinase during ischemia. HA = albumin; HES = hydroxyethyl starch. The thickness of the *arrows* indicates the amount of extravasation. See text for further explanation.**

What really happened to the second barrier after ischemia can be inferred from the experiments with intervention *I-Hep*. After the digestion of the glycocalyx by heparinase application during ischemia, the transient disturbance of the second barrier came to bear. Probably, this transient leakage was caused by a quickly reversible swelling and contraction of the endothelial cells.[51] Also, histamine induces a leak in the cellular barrier due to modulating endothelial cell gap formation. The exact mechanism for this phenomenon remains unclear, but active contraction of endothelial cells and passive recoil are some possible explanations.[27] However, treatment of hearts with histamine only increased coronary leak when the glycocalyx had been removed by the application of heparinase.

This double-barrier concept, *i.e.*, the concept of two competent barriers determining extravasation of colloids, can also reconcile seemingly incompatible results of other investigations of changes in permeability after enzymatic digestion of the glycocalyx. If the cellular barrier is only marginally affected, as in models using complete organs (without induction of ischemia), the destruction of the glycocalyx may result in only moderate effects on vascular permeability.[45] The additional alteration of the second competent barrier, however, leads to a major increase in vascular permeability. This

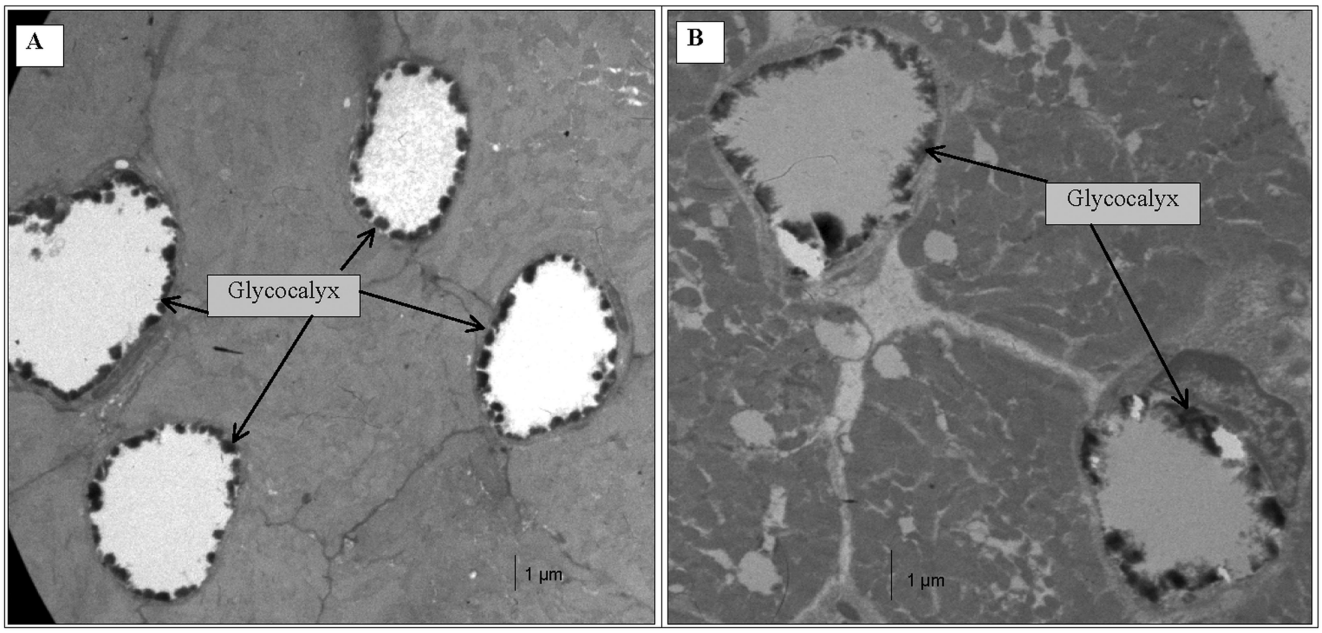


**Fig. 8. Electron microscopic views of control hearts stained to reveal the glycocalyx after minimal perfusion (< 1 min). (*A*) Heart without tissue edema; (*B*) heart with minimal tissue edema.**

can be assumed for experimental settings using prepared isolated vessels in artificial perfusion media[42] or models with complete organs in which enzyme application is associated with postischemic reperfusion.[46,47] On the other hand, endothelial cells in culture have a glycocalyx of questionable maturity and quality, so that heparinase application will not lead to any marked increase in permeability[43] or alteration in barrier regulation.[44] Besides this, confluent monolayers of cultured endothelial cells are usually 10–100 times more permeable to macromolecules than the endothelia of microvascular walls.[28] Therefore, such experimental settings suffer not only from an insufficient glycocalyx but also from an insufficient cellular barrier.

Two additional findings of the current study deserve mentioning. First, in each experimental setting, the infusion of human albumin resulted in a continuous increase in coronary effluent flow. Probably, this vasodilatation is due to contaminating mediators (*e.g.*, prekallikrein activator) in this special infusion solution.[52] As recently demonstrated, however, even a large increase in perfusion rate exerts only a minimal effect on net fluid exchange and on permeability of small vessels for macromolecules.[53] Therefore, failure of albumin to significantly attenuate vascular leak in our experiments cannot be ascribed to the enhanced coronary flow. This is supported by the findings with histamine (dilatation but no increase in transudate). *In vivo*, the vasodilating potential of infused HA is usually small,[17,54,55] presumably because of metabolism of contaminants or counterregulatory mechanisms of vasoconstriction or both. Second, it remains unclarified exactly by which route intravascularly applied colloids appear as rapidly as they did in the transudate fluid of the isolated perfused heart preparations. However, the same phenomenon has already been reported for macromolecules in other vascular beds.[28] Heparinase application can lead to a reduction in vascular resistance in blood perfused vessels.[7,23] In our experiments with heparinase application alone ($n = 10$) and the perfusion medium used (hematocrit = 0%), we did not see an increase in coronary effluent flow which could reflect a decrease in flow resistance.

The isolated heart model has some methodologic shortcomings. The preparations have tissue edema after a complete perfusion protocol (approximately 35 min), even under control conditions with intact glycocalyx (fig. 6). This is in contrast to investigations of van den Berg *et al.*,[16] who showed myocardial capillaries with intact glycocalyx without myocardial edema in nonbeating rat hearts after 3 min of perfusion. Figure 8 illustrates representative pictures of two control (guinea pig) hearts after immediate preparation for electron microscopy after only a very short perfusion period (< 1 min) to flush out erythrocytes and plasma proteins. In both hearts, vessels with an endothelial glycocalyx in tissue with minimal edema or even without edema could be visualized just as van den Berg *et al.*[16] demonstrated. Accordingly, the perfusion with artificial media for more than 30 min, using a constant perfusion pressure amounting to 80 mmHg, was the cause of tissue edema in all the hearts in the current study (fig. 6) but not an artifact introduced by our staining or electron microscopic techniques. The staining technique using lanthanum nitrate, simultaneously to the fixation procedure, was the reason that a much thicker endothelial glycocalyx could be visualized in comparison to the historic

pictures of other authors.[1–3] Also, it can be argued that there is inhomogeneity of coronary flow and that some vessels may be damaged. Recruitment of such vessels during reactive hyperemia could lead to enhanced surface area in leaky zones. However, leak never consistently paralleled coronary flow (*cf.* HA and HES infusion). Moreover, the prevalence of damaged vessels was obviously not great enough to obliterate differential effects of colloids and heparinase on fluid extravasation; such putative events at best would blunt the observed effects of a destroyed glycocalyx, *i.e.*, its impact *in vivo* should be even greater. It should also be noted that the coronary vascular bed, even *in vivo*, seems to be highly permeable toward albumin, with coronary lymphatic levels at 80–90% of the plasma value.[56] Therefore, the reflection coefficient for albumin taken over the entire coronary bed is much lower than that commonly assumed for single capillaries. In general, the changes seen in our model must be interpreted cautiously. At this early stage, they cannot be quantitatively generalized to all vascular systems or all organs other than the heart.

The extravasation of almost 60% of the infused colloids (HA or HES) shown by double-label blood volume measurements in surgical patients during preoperative volume loading can probably be explained by an alteration of both components of the vascular barrier.[17] In this special case, the release of atrial natriuretic peptide could play a key role for extravasation of fluids and macromolecules.[57,58] Preliminary results in the isolated heart showed that, contrary to histamine, the application of low-dose atrial natriuretic peptide ($10^{-9}$ M in the perfusate) increases transudate formation after 20 min of HES infusion independently of pretreatment with heparinase. This leads to the assumption that release of atrial natriuretic peptide can alter both components of the vascular barrier.

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