Effect of acute hypernatremia induced by hypertonic saline administration on endothelial glycocalyx in rabbits

David Astapenko[a], Vlasta Dostalova[a], Vlasta Dostalova jr[a], Jaroslav Kraus[a],
Vera Radochova[b], Pavel Dostal[a], Alena Ticha[c], Radomir Hyspler[c], Christian Lehmann[d,e]
and Vladimir Cerny[a,d,f,g,*]

[a]*Department of Anaesthesiology and Intensive Care Medicine, Charles University in Prague,
Faculty of Medicine Hradec Kralove, University Hospital Hradec Kralove, Czech Republic*
[b]*University of Defence in Brno, Faculty of Military Health in Hradec Kralove, Czech Republic*
[c]*Department of Clinical Biochemistry and Diagnostics, University Hospital Hradec Kralove,
Czech Republic*
[d]*Departments of Anaesthesia, Pain Management and Perioperative Medicine, Dalhousie University,
Halifax, NS, Canada*
[e]*Department of Pharmacology, Microbiology and Immunology, Dalhousie University, Halifax, NS,
Canada*
[f]*Department of Anesthesiology, Perioperative Medicine and Intensive Care, J.E. Purkinje University,
Masaryk Hospital, Usti nad Labem, Czech Republic*
[g]*Department of Research and Development, University Hospital Hradec Kralove, Czech Republic*

Abstract.

BACKGROUND AND OBJECTIVE: The endothelial glycocalyx (EG) is fragile and sensitive to damage such as exposure to hypernatremia. Our aim was to describe the influence of hypernatremia on the EG in sublingual and brain microcirculation in rabbits.

METHODS: Hypernatremia was induced by intravenous administration of 10% NaCl solution. The sublingual and brain microcirculation were evaluated by the Side-stream Dark Field imaging before (T1) and 20 minutes after infusion of 10% saline (T2). Damage to the EG was quantified by automated analysis of Perfused Boundary Region (PBR) indicating the amount of penetration of red blood cells into the EG. Syndecan-1 levels were also measured.

RESULTS: Hypernatremia was reached in all 20 animals, the PBR values of the sublingual area raised from 1,98 (0,3) to 2,17 (0,18) μm ($p = 0,05$). The levels of syndecan-1 (1,23 (0,36); 1,31 (0,33) ng/l, $p = 0,3$) did not mirror PBR changes.

CONCLUSIONS: Hypernatremia increased the PBR within the sublingual microcirculation in our animal model, probably due to compression of the EG related to temporary intravascular hypervolemia and changes of the EG charge in RBC instead of direct damaging effect on EG, which has been excluded by rather unchanged levels of syndecan-1.

Keywords: Hypernatremia, endothelial glycocalyx, sublingual microcirculation, syndecan

1. Introduction

Hypernatremia represents the common clinical situation and has been associated with increased morbidity and mortality in general clinical medicine [1, 2]. On the other side, there is solid clinical and experimental evidence that use of hypertonic saline in therapy of intracranial hypertension related

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*Corresponding author: Vladimir Cerny. Tel.: 420 602 492 054; E-mail: cernyvla1960@gmail.com.

severe traumatic brain injury or aneurysmal subarachnoid haemorrhage may improve clinical outcome [3, 4].

The endothelial glycocalyx (EG) has been shown to have a central role in the regulation of microcirculation both in physiologic and pathophysiologic conditions [5–8]. Endothelial glycocalyx may be damaged by several mechanisms and conditions [9], among others, by hypernatremia [10]. Several experimental and clinical studies evaluated the effect of administration of hypertonic saline and subsequent hypernatremia on microcirculation [11–14], studies evaluating the effect of induced hypernatremia on glycocalyx have not been found.

The Perfused Boundary Region (PBR) is a novel parameter indirectly describing the degree of EG damage signifying the amount of penetration of red blood cells (RBC) into the EG [15]. It is calculated automatically by specialized software in a particular tissue area exposed to direct *in vivo* video microscopy.

As glycocalyx is a dynamic, highly hydrated gel-like structure, the hypothesis was established, that hypernatremia could damage endothelial glycocalyx due to osmotic and counter-ion effects, therefore the aim of the study was to describe the relationship between hypernatremia and EG damage measured by intravital microscopy in the brain and sublingual microcirculation in rabbit after hypernatremia induced by administration of hypertonic saline.

2. Methods

2.1. Animal preparation and anaesthesia

All experimental procedures were performed after the approval by the Animal Welfare Committee of the University of Defence, Faculty of Military Health Sciences in Hradec Kralove, Czech Republic (approval no. 50-37/2016-6848) in accordance with Czech legislation on the protection of animals that complies with the Directive 2010/63/EU of the European Parliament and Council. Twenty male and female rabbits (New Zealand white rabbit; weight: 2.5–3.0 kg; VELAZ 34081/2008-10001, CZ 21906828, Unetice, Czech Republic) were included in the study. The animals were housed in a standard cage at 21°C under a 12-h dark/12-h light cycle with access to laboratory chow and tap water *ad libitum*. After a 1-week acclimatization period, the rabbits were used for the study.

After overnight fasting with unrestricted access to tap water, the rabbits were anesthetized using an intramuscular induction dose of ketamine (40 mg/kg) and xylazine (4 mg/kg). The animals were placed in the supine position on an operating table. The sites for inserting intravenous access, electrocardiogram electrodes, and tracheostomy were shaved. Intravascular cannulas (Vasofix® Safety, B.Braun, Melsungen, Germany) were inserted in both marginal ear veins (G24) and the right central ear artery (G22) for continuous blood pressure monitoring, arterial blood gas analysis, and continuous infusion of a balanced crystalloid solution (Plasmalyte, Baxter SA, Belgium, 3 ml/kg/h), anaesthesia, and a muscle relaxant. Mean arterial blood pressure was maintained above 55 mmHg with norepinephrine infusion as necessary.

The animals were tracheotomised after they were hemodynamically stable. A cuffless tracheal tube with an outer diameter of 2.5 mm was inserted between the third and fourth tracheal rings. After verifying correct placement by auscultation, mechanical ventilation was initiated using an anaesthesia ventilator (Cirrus Trans2/Vent 2, Datex, Helsinki, Finland) with initial settings of pressure-controlled ventilation, a positive end-expiratory pressure of 3 cm H_2O (zero level was not allowed by the ventilator), respiratory rate of 40 breaths/min, and inspiratory pressure of 13–16 cm H_2O according to the weight of the rabbit which was adjusted according to the end-tidal carbon dioxide tension ($ETCO_2$) and first blood gas analysis results. Mean arterial blood pressure (MAP), heart rate, and rectal

temperature were recorded throughout the study. The temperature was maintained at 38.5–39.5°C using a heating plate and a thermal isolation blanket. Balanced anaesthesia was maintained by using isoflurane (0.6–1 volume percent of Forane, AbbVie Inc., Chicago, IL, USA) in a mixture of 1 L/min oxygen and 1.2 L/min air with an inspiratory oxygen fraction (FiO$_2$) of 50–55%, continuous intravenous infusion of fentanyl (0.4 μg/kg/min, Fentanyl Torrex, Chiesi Pharmaceuticals GmbH, Vienna, Austria), and the muscle relaxant pipecuronium bromide (0.6 mg/kg/h, Arduan, Gedeon Richter Plc., Budapest, Hungary).

Each animal was subsequently rotated into the prone position, and the right temporo-parieto-occipital area of the head was shaved. The skin and periosteum of the skull were incised and reflected; bleeding was contained by bipolar electrocoagulation. The margins of the exposed area were determined by the midline, the base of the right ear, the external occipital protuberance, and the right caudal supraorbital process. On the right side, a 3-mm hole was drilled through the exposed skull and was increased in size using a mosquito pean. The final size of the cranial window was obtained using a Kerrison rongeur. Bleeding from the diploe was prevented using bone wax. Dura mater was cut carefully around the edges of the cranial window using microscissors to minimize brain surface injury. The dimensions of the cranial window were approximately 12×8 mm, with intact arachnoid mater at the base of the window. A 15-min stabilization period was maintained after bleeding was contained. During this period, the wound was flushed frequently with sterile 37°C normal saline, hemodynamic data were recorded, a sample of arterial blood was sent for baseline laboratory examination (levels of blood gases, pH, bicarbonate, base excess, sodium, chloride, glucose, haemoglobin, haematocrit, and syndecan-1 were evaluated).

At the end of the experiment, the animals were sacrificed by a lethal dose of thiopental (50 mg/kg, VUAB Pharma a.s., Roztoky, Czech Republic).

2.2. Hypertonic saline administration

In our protocol, the animals received intravenous infusions (Syringe pump, B. Braun Medical Inc., 824 Twelfth Avenue Bethlehem, PA) of undiluted 10% NaCl solution (B. Braun Melsungen AG, Germany). The amount of infusion was 5 ml/kg injected over 20 minutes.

2.3. Assessment of the endothelial glycocalyx and microcirculatory imaging

In sublingual and brain area we performed 2 successive measurements. First one at the baseline (T1) and second one 20 minutes after 10%NaCl infusion (T2). The microcirculation videos were recorded by specialized hand-held Side-stream Dark Field imaging video microscope (KK camera, Research Technology Limited, Alliance Court, Honiton, UK) connected to a laptop computer with specialized recording and analysis software (GlycoCheck, Maastricht, the Netherlands) (Figs. 1, 2).

Perfused boundary region describes the extent of penetration of the flowing RBC in μm into the luminal surface of the EG by measuring the radial motion of RBC away from the central flow towards the endothelial cells. The more the EG is injured, the deeper RBC penetrate into the glycocalyx and the higher the PBR is. The software automatically measures PBR in vessels of diameter from 5 to 25 μm and the resulting number stands for an average of PBR that is corrected for the potential changes in the distribution of vessel diameters. A detailed description of PBR calculation has already been described elsewhere [15]. Briefly, the software identifies all available vessels and places 10 μm long vascular segments along them. The recording is stopped when 3000 segments in focus and without movement are acquired. Then the software selects segments with sufficient contrast with the background and counts the median RBC column width and its distribution from the intensity profile. From this intensity profile, the perfused diameter of the vessel is calculated by a linear regression analysis. The PBR stands for
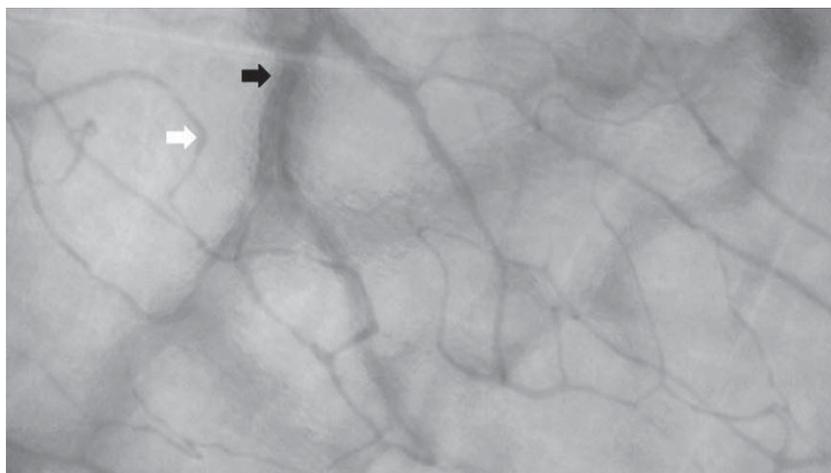
Fig. 1. The sublingual microcirculation of rabbit, tissue area 915 x 686 μm, magnification 325x. White arrow - capillary. Black arrow - postcapillary venule.
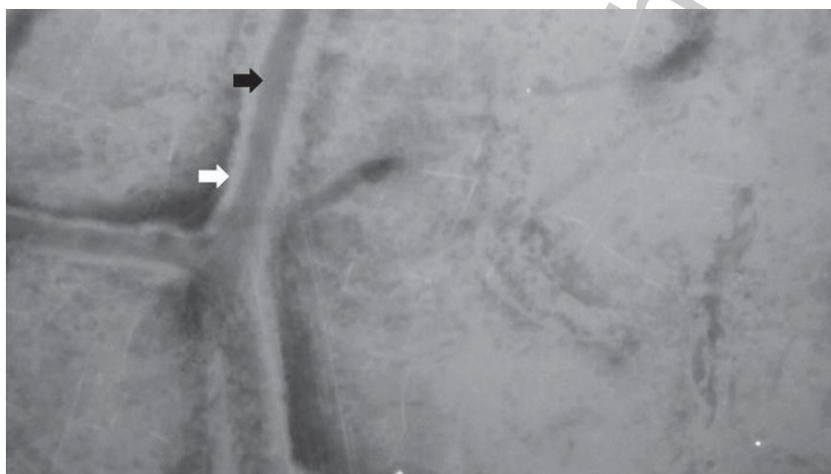

Fig. 2. The microcirculation of pia mater of the rabbit brain, tissue area 915 x 686 μm, magnification 325x. White arrow - blood brain barrier. Black arrow - capillary.

the distance between RBC column width and perfused diameter according to the equation: (Perfused diameter – median RBC column width)/2.

2.4. Biochemical analysis

Plasma and urine levels of syndecan were analyzed using the ELISA kit for Syndecan 1 (Rb SDC1/CD138, Blue gene, China) according to the manufacturer's instructions. The biochemical values were analyzed by blood gas analyzer (ABL 800FLEX, radiometer, Denmark) and Osmometer (Arkray, Japan).

131 *2.5. Statistical analysis*

132   For statistical analysis, we used Graph Pad Prism v6.0c (GraphPad Software, Inc., CA, USA). All
133 data were tested for normality prior testing by D'Agostino-Pearson omnibus normality test. Data are
134 expressed as a mean and standard deviation or median and interquartile range. One sample t-test was
135 used to assess baseline data and paired t-test and Wilcoxon test were used for comparison between
136 groups. Pearson's correlation coefficient was used to rate the relation between groups. A value of
137 $p \leq 0.05$ was considered as statistically significant.

138 ## 3. Results

139   We obtained data from 20 rabbits. There were no significant differences with regards to demographic
140 data, initial baseline hemodynamic and laboratory parameters among the animals (Table 1). There were
141 also no differences between animals in terms of blood loss during animal preparation or norepinephrine
142 dosage. The serum sodium level increased significantly after infusion of 10% NaCl: 140 (3,07); 157

Table 1
Baseline hemodynamic and laboratory data. Data are
shown as mean/median, standard deviation (SD)/interquartile
range (IQR), *p* values of one sample *t*-test and Wilcoxon test

	Mean/median	SD/IQR	*p* value
Weight [kg]	2.95	0.24	0.99
SBP [mm Hg]	87	13.07	0.84
DBP [mmHg]	66	12.57	0.95
MAP [mmHg]	75	12.43	0.93
HR [/min]	196	31.19	0.98
SpO_2 [%]	100	100–100	0.14
Et CO_2 [mmHg]	56	6.1	0.95
Osmo [mmol/l]	297	6.37	0.96
Na [mmol/l]	140	3.07	0.65
Cl [mmol/l]	103	4.6	0.8
Gly [mmol/l]	12.7	1.99	0.97
pH	7.44	0.06	0.98
pCO_2 [kPa]	5.4	0.61	0.95
pO_2 [kPa]	23.5	2.6	0.98
BE [mmol/l]	3.3	3.27	0.98
HCO_3^- [mmol/l]	27	2.96	0.59
Hb [g/l]	92	8.27	0.88
Htc	0.29	0.03	0.68

SBP - systolic blood pressure in, DBP - diastolic blood pressure,
MAP - mean arterial pressure, HR - heart rate, SpO_2 - peripheral
blood oxygen saturation, Et CO_2 - end expiratory concentration of
carbon dioxide, Osmo - osmolality, Na - serum concentration of
sodium, Cl - serum concentration of chloride, Gly - serum concen-
tration of glucose, pCO_2 - arterial pressure of carbon dioxide, pO_2
- arterial pressure of oxygen, BE - base excess, HCO_3^- - serum
concentration of bicarbonate, Hb - hemoglobin concentration, Htc -
hematocrit.

Table 2
Selected laboratory data at baseline (T1) and after infusion of 10% NaCl (T2).
Data are shown as mean (standard deviation), p values of the paired t-test

	T1	T2	p value
Osmo [mmol/l]	297 (6.37)	329 (5.3)	<0.0001
Na [mmol/l]	140 (3.07)	157 (2.57)	<0.0001
Cl [mmol/l]	103 (4.6)	122 (3.21)	<0.0001
Gly [mmol/l]	12.7 (1.99)	11.4 (2.5)	0.001
pH	7.44 (0.06)	7.37 (0.04)	<0.0001
pCO_2 [kPa]	5.4 (0.61)	5.8 (0.54)	0.0007
pO_2 [kPa]	23.5 (2.6)	22.9 (2.43)	0.25
BE [mmol/l]	3.3 (3.27)	$-$ 0.25 (2.63)	<0.0001
HCO_3^- [mmol/l]	27 (2.96)	25 (2.42)	<0.0001
Hb [g/l]	92 (8.27)	88 (8.92)	0.01
Htc	0.29 (0.03)	0.27 (0.027)	0.02

Osmo - osmolality, Na - serum concentration of sodium, Cl - serum concentration of chloride, Gly - serum concentration of glucose, pCO_2 - arterial pressure of carbon dioxide, pO_2 - arterial pressure of oxygen, BE - base excess, HCO_3^- - serum concentration of bicarbonate, Hb - hemoglobin concentration, Htc - hematocrit.
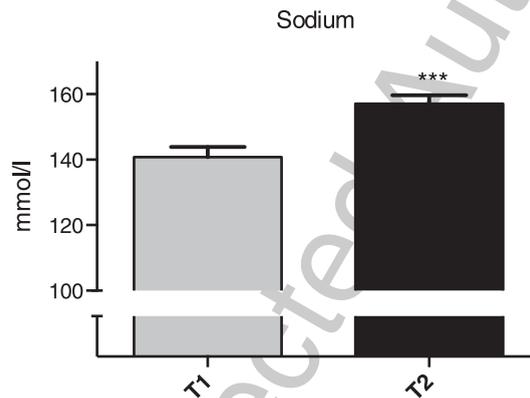

Fig. 3. Levels of serum sodium in millimoles per liter at baseline (T1) and after 10% NaCl infusion (T2).

(2,57) mmol/l ($p < 0,0001$) (Table 2, Fig. 3) as well as PBR in sublingual area: 1,98 (0,3); 2,17 (0,18) μm ($p = 0,05$) (Table 3, Fig. 4). There was weak correlation between hypernatremia and PBR changes in sublingual area ($r = 0,35$; 95% CI 0,08 to 0,58; $p = 0,013$). The level of serum syndecan-1 did not increased significantly: 1,23 (0,36); 1,31 (0,33) ng/l ($p = 0,3$) (Table 3, Fig. 5). The PBR in the brain was unaffected by the infusion of 10% NaCl: 1,87 (0,33); 1,85 (0,36) ($p = 0,55$). Selected hemodynamic, laboratory and microcirculatory data are shown on tables (Tables 2–4).

4. Discussion

The results of our study suggest that after inducing hypernatremia there is an immediate decrease of endothelial glycocalyx thickness, as expressed by increased PBR in sublingual microcirculation. However, plasma level of syndecan-1 was not significantly changed, therefore suggesting the absence

Table 3
Selected microcirculatory data at baseline (T1)
and after infusion of 10% NaCl (T2). Data are shown as mean
(standard deviation), *p* values of the paired *t*-test

	T1	T2	*p* value
PBR [μm]	1.98 (0.3)	2.17 (0.18)	0.05
SDC-1 [ng/l]	1.23 (0.36)	1.31 (0.33)	0.3
PBR brain [μm]	1.88 (0.33)	1.85 (0.36)	0.55

PBR - perfused boundary region in sublingual microcirculation,
SDC-1 - syndecan one, PBR brain - perfused boundary region in
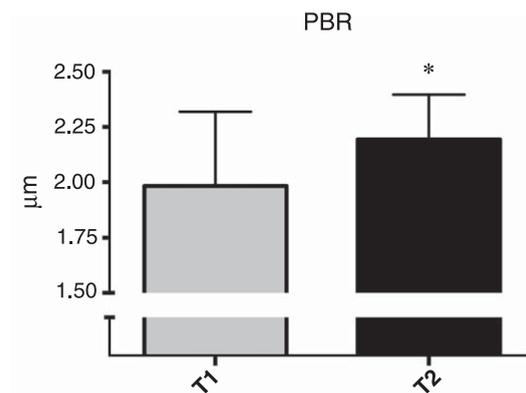pial microcirculation.

Fig. 4. Perfused boundary region (PBR) in the sublingual area at baseline (T1) and after 10% NaCl infusion (T2).
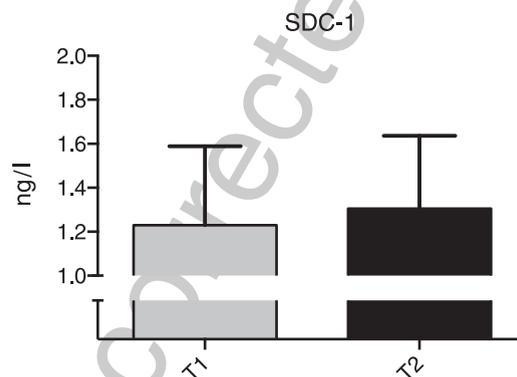

Fig. 5. Serum levels of syndecan-1 at baseline (T1) and after 10% NaCl infusion (T2).

of structural damaging effect on endothelial glycocalyx caused by sodium excess as stated by some authors [16, 17]. Perfused boundary region describes the extent of penetration of the flowing red blood cells into the luminal surface of the EG and the more the EG is injured, the deeper RBC penetrate into the glycocalyx and the higher the PBR is. It may be explained by increased number of "mini-ruptures" in contacted gel-like glycocalyx and increased RBC-endothelial adhesion [18]. Several experimental or clinical studies evaluated the effect of hypertonic saline on microcirculation and/or interaction between

Table 4
Selected hemodynamic data at baseline (T1) and after
infusion of 10% NaCl (T2). Data are shown as mean/median, standard
deviation/interquartile range, p values of paired t-test and Wilcoxon test

	T1	T2	p value
SBP [mm Hg]	87 (13.07)	83 (8)	0.19
DBP [mmHg]	66 (12.57)	64 (10.35)	0.45
MAP [mmHg]	75 (12.43)	72 (9.74)	0.38
HR [/min]	196 (31.19)	223 (20.55)	0.005
SpO$_2$ [%]	100 (100–100)	100 (100–100)	0.75
Et CO$_2$ [mmHg]	56 (6.1)	58 (6)	0.01

SBP - systolic blood pressure, DBP - diastolic blood pressure, MAP - mean
arterial pressure, HR - heart rate, SpO$_2$ - peripheral blood oxygen saturation,
Et CO$_2$ - end expiratory concentration of carbon dioxide.

leukocytes and endothelium [12, 19–21], only a few papers focused on the effect of sodium load on glycocalyx [10]. In the context of the only slightly changed level of syndecan-1 during hypernatremia, some non-structural, rather functional reasons for increased PBR must be assumed. Victorino and colleagues investigated the effect of hypertonic solutions on microvascular permeability and found that increased tonicity has a dose-dependent effect on hydraulic permeability as a measure of water flow across endothelial barrier in mesenteric vessels in rats [22]. Such increase of vascular permeability may be considered as an indirect sign of damage of EG. In presence of aldosterone, the increase of plasma sodium concentration from 135 to 150 mmol/l over five 5 consecutive days led the EG to shrink by approximately 50% [10]. Sodium plays an important role in the endothelial function and pathological sodium levels are associated with chronic diseases, such as hypertension [23, 24] and there is clear experimental evidence that increase of plasma sodium beyond 140 mmol/l leads to stiffening of endothelial cells with subsequent endothelial dysfunction [25]. Whether such chronic changes in sodium levels in terms of affecting endothelial functions are similar to those in the acute setting is unknown, but highly probable. Endothelial glycocalyx acts as an anionic biopolymer with specific ion binding properties [26] and ambient sodium overload alters the glycocalyx structure, probably due to a reduced heparan sulphate content leading to collapse of EG [10]. Shedding of EG expressed as an increased PBR in our experiment may be due also to transient intravascular hypervolemia and/or changes of EG charge in RBC instead of direct damage to EG by hypernatremia. Volume loading after hypertonic saline administration may release an excess of the atrial natriuretic factor, which has been associated experimentally and clinically with increased shedding of the endothelial glycocalyx [27–29]. Moreover, sodium flux into the endothelial cells has been also described as a trigger mechanism that initiates intracellular signalling and even small changes in plasma sodium concentration may influence endothelial function as long as intracellular aldosterone receptors are functional [30]. Shedding of glycocalyx allows sodium to enter into the endothelial cells and to subsequently disturb endothelial functions. Thus, high sodium levels weaken the protective sodium buffer barrier of EG. Increased osmolality after may also contribute to the glycocalyx shedding [31, 32]. Finally, infusion of hypertonic saline caused drop of the plasmatic pH toward to the hyperchloremic metabolic acidosis in the experimental animals that could, potentially, further aggravate the damage of EG [33].

Our experiment has some limitations. PBR analysis is not validated in animals (human use only). We think this is a major limitation of our results in terms of PBR validity and interpretation of results. Furthermore, it is problematic to extrapolate results from sublingual microcirculation to other vascular beds, especially to the brain microcirculation. Theoretically, we can speculate about the function of

the blood-brain barrier, the automated analysis of the software could have been influenced by the perivascular astroglial projections. Also, structure of "blood-brain barrier" endothelia including its glycocalyx is probably more resistant to changes in plasmatic natremia. Last but not the least, we cannot exclude effects of norepinephrine on EG, recently norepinephrine was found to be associated with EG damage [34].

Despite all study limitations, based on our findings, there is evidence that transient increase of plasma sodium level immediately affects endothelial glycocalyx thickness as measured by increased PBR. The results of our study are relevant in terms of e.g. safety considerations of the use of hypertonic saline as the therapeutic intervention in clinical medicine. Despite many apparent benefits of its use, especially in patients with refractory intracranial hypertension, there are emerging concerns about the safety of such interventions with regard to the endothelial glycocalyx. Further studies evaluating effect of selected treatments on EG glycocalyx may definitely broaden our view on safety of routinely used intervention in many fields of clinical medicine.

5. Conclusion

In conclusion, acute hypernatremia induced by administration of hypertonic saline was associated with an increased value of PBR in the sublingual microcirculation. Increased PBR values during hypernatremia were not accompanied by increased levels of plasma syndecan-1, suggesting transient and rather functional effects of hypernatremia on PBR in contrast to direct and structural EG damage.

Acknowledgments

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