

## The contribution of skin glycosaminoglycans to the regulation of sodium homeostasis in rats

(Short title : Skin interstitial glycosaminoglycans – sodium homeostasis)

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### Summary

The glycosaminoglycan (GAG) molecules are a group of high molecular weight, negatively charged polysaccharides present abundantly in the mammalian organism. By their virtue of ion and water binding capacity, they may affect the redistribution of body fluids and ultimately the blood pressure. Data from the literature suggests that the mitogens Vascular Endothelial Growth Factor (VEGF)-A and VEGF-C are able to regulate the amount and charge density of GAGs and their detachment from the cell surface.

Based on these findings we investigated the relationship between the level of dietary sodium intake, the expression levels of VEGF-A and VEGF-C, and the amount of the skin GAGs hyaluronic acid and chondroitin sulphate in an in vivo rat model. Significant correlation between dietary sodium intake, skin sodium levels and GAG content was found. We confirmed the GAG synthesizing role of VEGF-C but failed to prove that GAGs are degraded by VEGF-A. No significant difference in blood pressure was registered between the different dietary groups. A quotient calculated from the ion and water content of the skin tissue samples suggests that - in contrast to previous findings – the osmotically inactive ions and bound water fractions are proportional.

### Key Words

Glycosaminoglycans, Sodium, Body fluids, Blood pressure, Vascular Endothelial Growth Factor

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### **Introduction**

Recent experimental data have suggested that in addition to the kidney local tissue regulation of sodium and volume by the skeletal muscle, the bone and the skin is of great importance as these tissues are able to store and release significant amounts of sodium. A number of previous studies (Heer *et al.* 2000, Palacios *et al.* 2004, Titze *et al.* 2002a.) reported on the short term changes in volume of body fluid compartments following Na<sup>+</sup> ingestion : increment in total body mass as well as body fluid content were observed.

(Heer *et al.* 2000.) were the first to demonstrate that the rise in total body sodium content is not necessarily paralleled by commensurate water retention. The same conclusion was drawn by other research groups (Palacios *et al.* 2004, Titze *et al.* 2002b.) It has also been proved that the excretion of sodium through urine, stool and sweat does not match the amount of the ingested sodium, suggesting a water free storage of the excess sodium (Heer *et al.* 2000, Palacios *et al.* 2004.). Seeliger and his colleagues (Seeliger *et al.* 2006.) criticized these results, stating that water-free sodium storage is more likely to be an intracellular mechanism. Data from further examinations (Heer *et al.* 2009, Schafflhuber *et al.* 2007.) have shown that the mechanism in question accounts only for the 22% of Na<sup>+</sup> to remain in the body. The rest (78%) is presumably stored in an osmotically inactive manner in the EC compartment .

Based on these experimental data and clinical observations it became evident that there is not a close link between the total sodium content of the body and the volume of different water compartments in the long term. Given that the above mentioned intracellular Na<sup>+</sup>/K<sup>+</sup> exchange only partially accounts for the storage of excess sodium and that the sodium concentration of the serum remained in the normal physiological range during salt overload, it is presumable that considerable sodium reservoirs exist in the mammalian organism. Animal experiments conducted on salt sensitive and salt resistant rat strains supported the sodium buffering role of the hypothesized sodium reservoirs (Titze *et al.* 2002a.). Further animal experiments (Titze *et al.* 2002a, Titze *et al.* 2003.) determined the location of these osmotically inactive reservoirs: the majority of the excess sodium is

stored in the skin, and only a smaller fraction is located in the bone, the cartilage and the skeletal muscle (Sulyok 2006.) The presence of osmotically inactive sodium storages have also been confirmed applying  $^{23}\text{Na}$  and  $^1\text{H}$  MR techniques in humans *in vivo* (Dahlmann *et al.* 2015, Kopp *et al.* 2013, Kopp *et al.* 2012, Linz *et al.* 2015.).

Sodium in the skin is bound to negatively charged macromolecules, predominantly to glycosaminoglycans (GAGs). GAGs are nitrogen containing molecules made up of repeated dimers. The most common GAGs of the body are hyaluronic acid, heparan sulphate, chondroitin sulphate and dermatan sulphate . It has also been shown that the total content, charge density and polymerisation of GAGs correlates with the extremes in dietary sodium intake (Farber *et al.* 1957, Machnik *et al.* 2009, Titze *et al.* 2004.). Titze *et al.* proposed a regulating cascade in which increased subcutaneous sodium modulates lymphangiogenesis and blood pressure (Machnik *et al.* 2009.). Macrophages accumulating in the subcutaneous compartment sense hypertonicity and facilitate lymph capillary hyperplasia via Vascular Endothelial Growth Factor-C (VEGF-C) secretion. Increased lymph capillaries temporarily store the interstitial fluid and blunt blood pressure response to high salt.

This concept does not provide information about the mechanism by which this uniquely bound sodium is released and drained back to the circulation. (Foster *et al.* 2013.) provided evidences that two isoforms of Vascular Endothelial Growth Factor, VEGF-A and VEGF-C regulate the amount and charge density of the glycocalyx of conditionally immortalized glomerular endothelial cells of the kidney podocytes. Based on these results, we attempted to define the role of skin GAGs in the regulation of body sodium homeostasis. We wanted to investigate, whether this regulated synthesis and degradation of GAG molecules is also present in the skin, and whether this phenomenon plays significant role in the regulation of skin sodium levels.

## **Materials and Methods**

### **Animals**

8-week-old female Wistar rats (weight 180–200 g) were purchased from Toxi-Coop Ltd. (Dunakeszi, Hungary). All experimental protocols were approved by the Committee on the Care and Use of Laboratory Animals of the Council on Animal Care at the Semmelweis University of Budapest, Hungary (TUKÉB 380/2013)

### **Treatment groups**

In all experiments two types of dietary regimes were applied: low salt diet (LSD) consisted of a virtually sodium free ( $\text{NaCl} < 0.03 \text{ m/m } \%$ ) rat chow (E 15430-24; Ssniff GmbH, Soest, Germany) and tap water to drink. High salt diet (HSD) consisted of a sodium rich ( $\text{NaCl} = 8\% \text{ m/m}$ ) rat chow and 0.9% saline as drinking water.

In experiment 1, 24 rats after one week of standard diet were randomly assigned to three groups. Group LSD (n=8) received a low salt diet for 4 weeks. Group HSD (n=8) received a high salt diet for 4 weeks. Group HSD/LSD (n=8) received a high salt diet for 4 weeks followed by a low salt diet for another 4 weeks.

In experiment 2, 24 female Wistar rats after one week of standard diet were randomly assigned to two groups. Group LSD (n=12) received a low salt diet for 4 weeks. Group HSD (n=12) received a high salt diet for 4 weeks.

In experiment 3, 12 female Wistar rats after one week of standard diet were kept on a high salt diet for four weeks followed by a 72 hour period of low salt diet. The animals were kept under standard laboratory conditions with free access to the respective rodent chow and drinking water.

### **Body weight measurement**

Body weight of the animals was measured every week during the whole experimental period.

### **Blood pressure measurement**

In experiments 2 and 3 arterial blood pressure (systolic, diastolic, mean (MAP)) was measured with Coda (Kent Scientific Corporation, Torrington, USA) non-invasive digital blood pressure monitor. Blood pressure was measured on the tail arteries of conscious animals. Measurements were performed once every week during the whole experimental period.

### **Blood, urine and skin sampling**

At the end of the experiments the animals were anesthetized using intraperitoneal Ketamin-Xylazine injection, and blood samples (5 ml) were taken from the right cardiac ventricle before the animals were killed. We obtained urine samples by bladder puncture. The carcasses were skinned to provide tissue samples for biochemical and chemical analyses and for the determination of gene expression levels.

### **Determination of ions, creatinine and urea in serum and urine**

The  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cl}^-$ , creatinine and urea content of the blood and the urine samples was analysed with a Beckman Coulter AU480 Chemistry System.

### **Determination of skin water content**

In experiment 2 the skin samples were weighed (wet weight) and then desiccated at 90°C for 72 h (dry weight). Because further drying left weights unchanged, the difference between wet weight and dry weight was considered as tissue water content.

### **Determination of skin Na<sup>+</sup> and K<sup>+</sup> content**

In experiment 1 and experiment 2 skin samples were frozen by liquid nitrogen and pulverized mechanically with a commercially available coffee grinder. Thereafter 1 gram of the raw (experiment 1) or the desiccated (experiment 2) skin samples were dissolved in 5 ml HNO<sub>3</sub> overnight followed by adding 3 ml H<sub>2</sub>O<sub>2</sub> (30%) to it. Samples were digested using microwave heated teflon bombs. Homogenized samples were diluted to a final volume of 25 ml and filtered through a 0.45 µm pore size syringe filter. Na<sup>+</sup> and K<sup>+</sup> concentrations in the solutions were measured with a PFP7 type flame photometer (Buck Scientific, Norwalk, USA). In order to eliminate eventual disturbances which ionization may cause, separate series of dilution were made to calibrate Na<sup>+</sup> and K<sup>+</sup> content. Every time, to the standard series of dilution the standard of the other alkali metal was added - in a concentration as the sample to be measured it required (Chen *et al.* 2005.) (Dipietro *et al.* 1988.).

### **Glycosaminoglycan disaccharide analysis**

We cut 0.5 g of skin, taken from the left scapula angulus inferior from rats into small slices and digested with 7.0 mg proteinase K (Sigma-Aldrich, Taufkirchen, Germany) in 10 ml 20 mM Tris-buffer (pH 7.0) at 55 °C for 16 hours. The reaction was blocked by boiling the solution for 5 minutes. After centrifugation, we added 100 ml acetone to the supernatant and stored the tubes at -20 °C for 16 h. After precipitation, the tubes were centrifuged at 4000 rpm for 5 minutes and the precipitate was dried at 90 °C overnight. After lyophilization, we dissolved the purified skin GAGs in 1.5 ml deionized water for HPLC analysis. We treated 10 µl of samples with 7 mU of chondroitin ABC lyase (Sigma Aldrich, Taufkirchen, Germany) in 50 µl of 100 mM Tris/150 mM sodium acetate buffer pH 8.0 at 37 °C for 16 hours. The reaction was blocked by boiling the solution for 1 minute. We analyzed the unsaturated disaccharides generated from hyaluronan (HA; ΔdiHA) and dermatan/chondroitin sulphate (DS/CS; Δdi4s and Δdi6s) after enzymatic treatment of the purified GAGs using HPLC-MS in selective ion mode according to previous literature data (Gill *et al.* 2012.). HPLC analysis was performed by an Agilent 1260 Infinity LC system in conjunction with an Agilent 6460 triple-quadrupole mass spectrometer (Waldbronn,

Germany). Chromatography was carried out using a porous graphitized carbon column (Hypercarb, Hypercarb Column, 5  $\mu$ m, 100 mm  $\times$  4.6 mm) purchased from Thermo Scientific (Waltham, MA). For the separation gradient elution program was used: Solvent A was 0.1% formic acid adjusted to pH 5.5 by ammonia and solvent B was solvent A in 90% acetonitrile. The gradient used was 0-15% B in 4 min and 15% B over 50 min. Standards of Hyaluronic acid (H9649-1MG) and Chondroitin 4,6 sulphate (CD004) were purchased from Sigma Aldrich (USA) and Iduron (Manchester, UK) respectively.

### **Quantitative Real-Time PCR**

Total RNA was isolated from the skin tissue samples by Geneaid Total RNA Mini Kit (Geneaid, New Taipei, Taiwan), according to the manufacturer's instructions. 100  $\mu$ g of total RNA was reverse-transcribed and VEGF A and VEGF C mRNA expressions were determined by real-time polymerase chain reaction (PCR) on LightCycler480 (Roche Diagnostics, Mannheim, Germany).

For quantitative PCR the following primer pairs were applied : VEGF-A forward: 5'-GTG CAC TGG ACC CTG GCT TTA C- 3'; VEGF-A reverse: 5'-TTT TCT GGC TTT GTT CTA TCT TTC- 3'; VEGF-C forward:

5'-TCC ACC ATC AAA CAT GCA GC- 3' VEGF-C reverse:5'-TCA GTC GAT TTG TAC ATG GTC GT- 3'; GAPDH forward: 5'-GGT GAA GGT CGG AGT CAA CG- 3' GAPDH reverse: 5'-CAA AGT TGT CAT GGA TGA CC- 3'. The specificity of the PCR products was verified by the analysis of melting curves and by electrophoretic separation of the products on 2.5% agarose gel followed by staining with ethidium bromide. The  $2^{-\Delta\Delta CT}$  value (relative fold change) was calculated

## **Results**

### **Skin sodium concentration and dietary NaCl intake**

Skin sodium content in each group reflected the levels of dietary sodium intake (Fig 1 1.).

In experiment 1  $Na^+$  content of the skin in group HSD ( $1.334 \pm 0.022$  mg/g = 116%) showed an elevated level compared to group LSD ( $1.148 \pm 0.075$  mg/g = 100 %,  $p=0.05$ ), whereas group HSD/LSD showed an intermediate value ( $1.192 \pm 0.051$  mg/g = 104%,  $p>0.05$ ) The same tendencies were observed in experiment 2: (LSD:  $0.974 \pm 0.069$  mg/g = 100% vs. HSD:  $1.158 \pm 0.084$  mg/g = 118%;  $p=0.0001$ ).

Since the homeostasis of  $Na^+$  and  $K^+$  are closely related, we investigated the  $K^+$  content of skin samples. The levels of skin  $K^+$  changed in the opposite direction compared to skin  $Na^+$  . LSD  $1.205 \pm 0.197$  mg/g (100%), HSD

1.001±0.155 mg/g (83 %), HSD/LSD 1.092±0.020 mg/g (90,5%). Although the absolute amount of K<sup>+</sup> showed differences between groups, intergroup comparison failed to reach significance : HSD vs. LSD p>0.05; HSD/LSD vs. HSD p>0.05)

We obtained similar, although statistically not significant (p<0,07) result in experiment 2 (LSD: 1.059±0.133 mg/g (100%) vs. HSD 0.975±0.071 mg/g (92%). Parallely with increased sodium intake, differences in sodium excreted in urine were detected in experiment 1: LSD = 97.6±62.78 mmol/l (100%), HSD = 172.75±32.15 mmol/l (172%), HSD/LSD = 40.1±12.029 mmol/l (41%); (HSD vs. LSD p>0.05; HSD vs. HSD/LSD p<0.001). In experiment 2: HSD = 69.0±29.8 mmol/l (100%), LSD = 21.3±7.5 mmol/l (30.86 %) (p<0.01)

In order to rule out the possibility that Na<sup>+</sup> overload in the skin is a consequence of kidney malfunction, we monitored the blood creatinine and urea levels at the end of each experiment. Since blood creatinine and urea levels as well as blood sodium levels in all experiments remained in the normal physiologic range for rats (creatinine : 20.5 – 40.6 µmol/ml; urea : 4.5 – 8.96 µmol/ml; sodium 120-145 mmol/l) (data not shown) we concluded that the extremes in skin sodium content are the direct consequence of dietary manipulations.

#### **Excess Na<sup>+</sup>, K<sup>+</sup> and water storage in the skin**

In experiment 2 5.6 % difference in skin water content (m/m) between HSD and LSD was measured (HSD = 60.4±4.043 % vs. LSD = 54.79±4.055 %; p=0.0025) The difference between skin ion and water content holds important information about the level of osmotically inactive sodium storage. Therefore, from the Na<sup>+</sup>, K<sup>+</sup>, and water content of the skin a  $([Na^+ + K^+]/water)$  quotient was calculated:

$$[Na^+] (mmol, dry skin) + [K^+] (mmol, dry skin) / mls of water in wet skin$$

The  $([Na^+ + K^+]/water)$  quotient calculated from the respective data shows no significant difference between the two dietary groups : (LSD = 0.126±0,001 mmol/ml vs. HSD = 0.124±0.006 mmol/ml; p=0.51)

The changes in the Na<sup>+</sup> content of the skin were consistently counterbalanced by changes in skin K<sup>+</sup> content leaving the  $([Na^+ + K^+]/water)$  quotient between the two dietary groups unchanged. No difference was found in body weight between the two dietary groups in experiment 2 (LSD 107.89 % vs HSD 107.84 %, p=0.98.

#### **Relationship between skin Na<sup>+</sup> and skin GAG levels**

Measurement of skin GAG content showed significant differences between dietary groups. Experiment 1: hyaluronic acid : HSD = 100%, LSD = 76.9% ( $p < 0.01$ ), HSD/LSD = 87.5%; ( $p < 0.01$ ; HSD vs. HSD/LSD  $p = 0.096$ ); chondroitin 4,6-sulphate : HSD = 100%, LSD = 82.7%, ( $p < 0.01$ ), HSD/LSD 87.4% ( $p < 0.053$ ) (data expressed as means of groups; arbitrary units)

The same tendencies were observed in experiment 2: hyaluronic acid: HSD = 100%, LSD = 64.5 % ( $p < 0.0001$ ); chondroitin 4,6-sulphate : HSD = 100%, LSD = 75.6% ( $p < 0.0001$ ) ( Figure 3). We observed parallel changes in skin  $\text{Na}^+$  and GAG content : the levels of both GAGs raised in the high salt group, decreased in the low salt group, and showed an intermediate level in the group switched back from high salt to low salt diet. A coefficient, calculated from the respective data shows significant positive correlation between skin GAGs and skin  $\text{Na}^+$  content :  $\text{Na}^+$  - hyaluronic acid ( $r = 0.85$ ,  $p = 0.03$ );  $\text{Na}^+$  - chondroitin 4,6-sulphate ( $r = 0.82$ ,  $p = 0.046$ ).

### **Relationship of skin GAG content to VEGF-C and VEGF-A**

In experiment 1 parallelly with a 30% increase in hyaluronic acid and 20% in chondroitin 4,6 sulphate content, mRNA levels of VEGF-C were also higher in group HSD (138.9%) compared to group LSD (100%) suggesting the GAG synthesizing role of VEGF-C. (HSD vs. LSD  $p = 0.492$ ).

Similar tendencies were observed in experiment 2: hyaluronic acid LSD = 100 %, hyaluronic acid HSD = 152.9%; ( $p < 0.0001$ ) chondroitin 4,6 sulphate LSD = 100%, chondroitin 4,6 sulphate HSD = 132.2 % ( $p < 0.0001$ ); VEGF-C mRNA expressions LSD=100%, HSD = 151.2% ( $p = 0.07$ ). Interestingly, no significant differences in the expression levels of VEGF-A were found in groups with different sodium intake. It slightly increased in both the LSD (HSD = 100%, LSD = 112%  $p > 0.05$ ) and the HSD/LSD groups (122%  $p > 0.05$ ); HSD vs. HSD/LSD  $p > 0.05$ ).

Since changes in mRNA levels happen over a shorter time course it was reasonable to examine VEGF-A mRNA levels at an earlier time point (i.e. 72 hours after the change in sodium intake). Similarly to experiment 1., when comparing the VEGF-A mRNA levels of group HSD/LSD (in experiment 3.) to the mRNA level of group HSD (experiment 2.) no significant difference was observed (HSD 2. = 100% vs. HSD/LSD 3. = 84%;  $p = 0.65$ ).

### **Discussion**

In the present study the impact of dietary sodium intake on the skin sodium and GAG content (hyaluronic acid, chondroitin-sulphate), as well as on the expression of VEGF-A, VEGF-C mRNA was investigated in a rat model. Significant positive relationship was found between sodium intake, skin sodium and skin GAG content.



Moreover, experimental evidences were provided for the involvement of VEGF-C in GAG metabolism, but no association was documented between VEGF-A and GAG. It is suggested that some of the excess sodium is bound to GAGs and stored in osmotically inactive form in the skin but the regulatory role of VEGFs in this process remained to be explored.

Earlier studies have pointed out the Na<sup>+</sup>-binding ability of skin interstitial GAGs and the change in their amount followed by different levels of dietary sodium intake. Sulyok and colleagues (Sulyok 2013.) revealed that ECM –bound water consists of fractions (Ling 1992.) with different mobility and composition (tightly bound, loosely bound and free), although they could not establish a clear relationship between GAG (hyaluronan) and bound water fraction. The pioneering experiments of Titze et al. on the hyperplasia of the lymph capillary network induced by dietary sodium overload explains the mechanism by which excess interstitial fluid is temporarily stored and drained back to the circulation (Marvar et al. 2009.).

Since the sodium and volume buffering capacity of this system is supposed to be limited, a regulating mechanism is essential in order to liberate excess interstitial fluid from GAGs and drain it back to the circulation for clearance by the kidney.

Despite intensive research in this area, mechanism governing polymerization and depolymerisation of GAGs is still elusive. For the phenomenon of regulated synthesis and degradation of GAGs, we found evidence in a 2013 issue of American Journal of Pathology. (Foster *et al.* 2013.) and his research group provided evidences that the two isoforms of Vascular Endothelial Growth Factor, VEGFA and VEGFC influences the amount and charge density of the glycocalyx of conditionally immortalized glomerular endothelial cells of the kidney podocytes. In the kidney these cells are constituents of the glomerular filtration barrier and are presumedly involved in the fine tuning of the filtering function of kidney podocytes. In their experimental setting they proved that cultured cells can synthesise GAG molecules and are able to secrete them to the cell surface. They observed, that VEGF C significantly increased the negative charge density of the glycocalyx of GENC cells. VEGF C was also able to intensify the production and secretion of negatively charged hyaluronic acid molecules. They also observed that VEGF A does not affect the charge properties of negatively charged GAGs but instead promotes their detachment into the culture media.

Our attempt in establishing causal relationship between sodium intake, skin GAG content and the two isoforms of VEGF has brought only partial success: GAG concentration in the skin showed strong and significant correlation with the amount of dietary Na<sup>+</sup> intake. The decrease in GAG content after switch from high salt diet to low salt diet renders the regulated degradation of GAGs possible. The tendencies in the levels of VEGF-C also fits in this conception: increased VEGF-C levels are likely to reflect the organism's need for extra sodium binding capacity. Although not reaching the desired level of significance, there is a tendency pointing toward the possible role of VEGF-C in the synthesis of skin interstitial GAGs. Selective blockade of VEGF-C in salt overloaded animals would give further evidence for the causal role of VEGF-C in GAG homeostasis.

Based on the documented GAG-degrading effect (Foster *et al.* 2013.) of VEGF-A, we expected elevated levels of VEGF-A in group HSD/LSD. Contrary to our expectations, no significant differences in the expression levels of VEGF-A were found in group HSD/LSD: HSD =100%, LSD = 112%, HSD/LSD = 122% (HSD vs. LSD  $p>0.05$ ; HSD vs. HSD/LSD  $p>0.05$ ). Since changes in mRNA levels happen over a shorter time course it was reasonable to examine VEGF-A mRNA levels at an earlier time point (i.e. 72 hours after the change in sodium intake). Similarly to experiment 1., when comparing the VEGF-A mRNA levels of group HSD/LSD (in experiment 3.) to the mRNA level of group HSD (experiment 2.) no significant difference was observed (HSD 2. = 100% vs. HSD/LSD 3. = 84%;  $p=0.65$ )

Having used normotensive, salt resistant Wistar rats, the lack of difference in blood pressure between HSD and LSD groups can be attributed to the properly functioning regulating mechanisms including the kidney the cardiovascular system and – presumably – the skin. However, since we did not block the regulating mechanism of the skin (unlike in the experiment of Titze *et al.*), the absence of the rise in blood pressure can only be considered as an indirect evidence of its role played in blood pressure regulation.

As in our study the regulatory role of VEGFs in the control of GAGs-sodium interaction remained inconclusive further experiments are to be conducted to explore: a.) the time course of the response pattern of VEGF-A/VEGF-C mRNA and protein expression to manipulation of sodium intake, b.) the effect of anti-VEGF therapy to the sodium-GAG-VEGF interplay and c.) the possible contribution of hormones/hormone-like factors that are known to be involved in the synthesis and degradation of GAGs in various tissues needs also to be assessed.

## Conflict of Interest

There is no conflict of interest.

## Acknowledgements

This study was supported by the **OTKA K108688 grant**.

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Table 1. : Description of treatment groups

	Experiment 1.			Experiment 2.		Experiment 3.
Species, strain	Rattus norvegicus, Wistar					
Gender; age	female; 8-week old					
Treatment groups	LSD	HSD	HSD/LSD	LSD	HSD	HSD/LSD
Sample size (n)	8	8	8	12	12	12
Time span of the experiment	after 1 week of standard diet					
	4 weeks	4 weeks	4 + 4 weeks	4 weeks	4 weeks	4 weeks + 72 hours

Abbreviations : LSD : low salt diet; HSD: high salt diet; HSD/LSD : high salt diet followed by low salt diet

Table 2. Changes in skin electrolyte and water content, skin GAG content and the mRNA expression levels of VEGF-A and VEGF-C in animals receiving different levels of dietary sodium intake. LSD = low salt diet (4weeks), HSD = high salt diet (4 weeks) , HSD/LSD = high salt diet (4 weeks) followed by low salt diet (4 weeks)

	LSD	HSD	HSD/LSD	LSD	HSD
	Experiment 1.			Experiment 2.	
Skin Na <sup>-</sup> [mg/g]	1.148 ± 0.075	1.334 ± 0.022 * <sup>^</sup>	1.192 ± 0.051	0.974 ± 0.069	1.158 ± 0.084 *
Skin K <sup>-</sup> [mg/g]	1.205 ± 0.197	1.001 ± 0.155	1.092 ± 0.020	1.059 ± 0.133	0.975 ± 0.071
Skin water content [m/m%]				54.799 ± 4.055	60.440 ± 4.043 *
Skin (Na <sup>+</sup> +K <sup>+</sup> )/water content [mmol/ml]				0.126 ± 0.011	0.124 ± 0.006
Skin HA content (arbitrary units)	1104451 ± 170395	1435126 ± 119425 *	1256268 ± 117728	287552±57567	187598±38753*
Skin CS content (arbitrary units)	2326461 ± 165196	2787854 ± 249106 *	2459153 ± 241661	500518±40921	371565±55539*
Skin VEGF-A mRNA content (arbitrary units)	1.123 ± 1.072	0.999 ± 1.008	1.224 ± 0.639	1.098 ± 0.577	0.879 ± 0.408
Skin VEGF-C mRNA content (arbitrary units)	0.72 ± 0.45	1.001 ± 0.421	0.85 ± 0.53	0.542 ± 0.381	0.820 ± 0.467
Urine Na <sup>-</sup> [mmol/l]	97.625 ± 62.781	172.75 ± 32.15 <sup>^</sup>	40.125 ± 12.029	21.3 ± 7.5	69.0 ± 29.8 *
Body mass [%]				107.89 ± 5.95	107.84 ± 6.39
MAP (Week 4.) [Hgmm]				102 ± 19	98 ± 13

\* = p<0.05 vs. LSD; <sup>^</sup> = p<0.05 vs. HSD/LSD

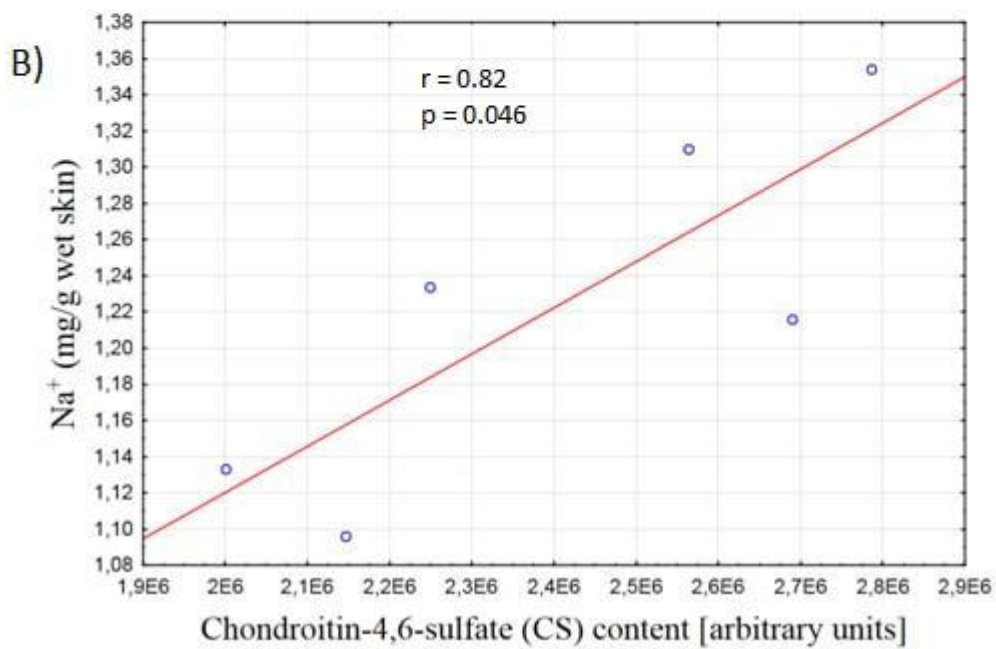
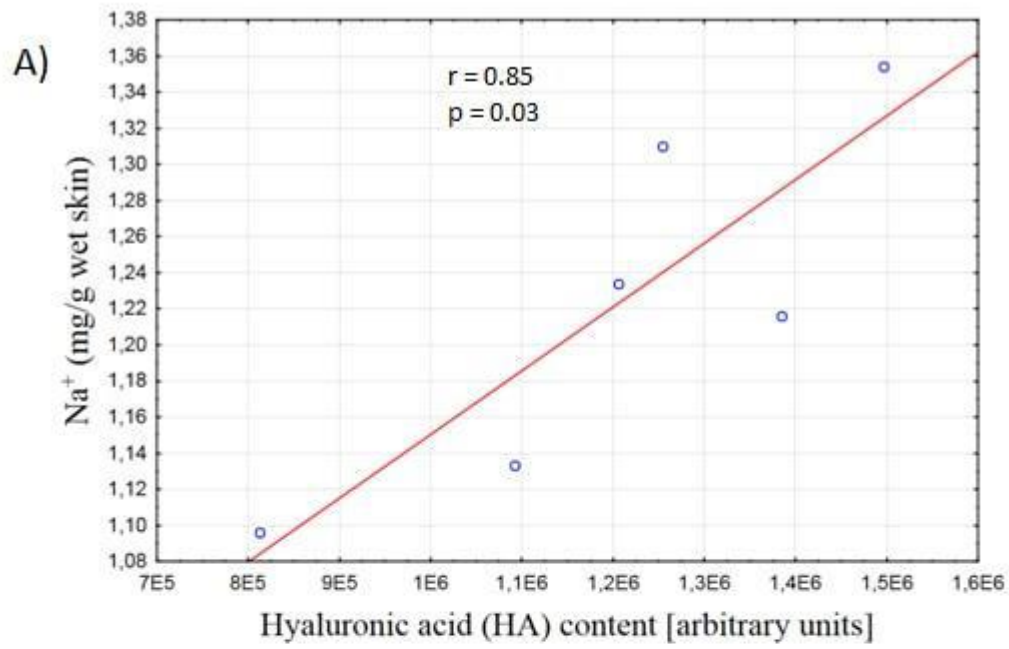


Figure 1. Correlation graphs showing significant and strong correlation between skin Na<sup>+</sup> and skin HA (Figure 1.A) and skin Na<sup>+</sup> and skin CS (Figure 1.B) content in animals with different levels of dietary sodium intake (Experiment 1., n=6)