FINAL REPORT

„Immunomodulatory effect of sodium: new mechanisms in the background of hypertension and organ damage”

National Research, Development and Innovation Office – NKFIH

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Background

According to the WHO, the average salt (NaCl) consumption in the general population is 8.75–10.5 g/day which far exceeds the recommended limit of 5 g/day. According to the classic view, excessive salt intake is a risk factor for several diseases, including hypertension, heart disease, or stroke. It has been shown that excess dietary salt intake leads to sodium (Na^+) accumulation in various tissues, including muscles and skin. Furthermore, it has also been shown that local Na^+ excess can induce pathological alterations, however, the underlying mechanisms need to be elucidated. Therefore, our research project aimed to better understand the molecular mechanisms that play a role in tissue Na^+ storage and its pathological effect on the target organ, including inflammation and tissue remodeling. In the following, we summarize the main results of the research and review articles which were supported by OTKA K125470.

Results

1. Cyclooxygenase-2 modulates the sodium binding glycosaminoglycan production in the skin during salt overload

Agócs et al. Frontiers in Physiology, 11, 561722. (2020)

In this study, we investigated the underlying cellular and molecular mechanisms of glycosaminoglycan (GAG) mediated dermal sodium (Na^+) homeostasis. Our *in vitro* results demonstrated that high salt environment activates the Cyclooxygenase-2 (COX-2) /prostaglandin E2 (PGE2) pathway in dermal fibroblasts (DFs) leading to increased production of GAG member hyaluronic acid (HA). Furthermore, pharmacological (Celecoxib) COX-2 inhibition decreases the synthesis of HA. Our in vivo results demonstrated that In the skin of rats receiving high-salt diet, the dermal Na^+ content and COX-2 expression were increased. In line with this data, the dermal HA content was also increased, however, COX-2 inhibition blocked its production. In conclusion, our results revealed a new DF-mediated regulation of GAG synthesis in the skin during salt overload.

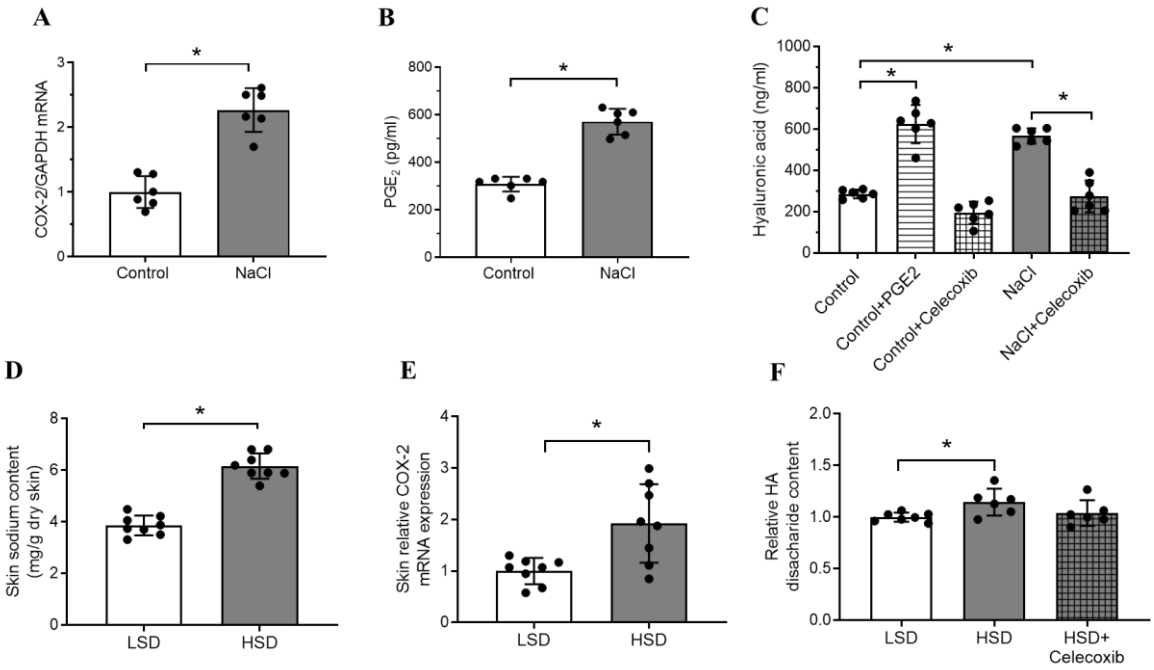

Figure 1. Molecular mechanisms of salt storage in the skin. Effect of high NaCl loading on COX-2 mRNA expression (A) and PGE2 production (B) of dermal fibroblasts. Effect of COX-2 inhibition on PGE2 and NaCl induced HA production (C) of dermal fibroblasts. High salt diet-induced alteration of skin Na^+ content (D), COX-2 mRNA expression (E), and the effect of COX-2 inhibition on dermal HA content (F).

A high salt diet impairs dermal tissue remodeling in a mouse model of IMQ-induced dermatitis

Pajtók et al. PloS one, 16(11), e0258502. (2021)

Recent animal studies and quantitative sodium MRI observations on humans demonstrated that remarkable amounts of sodium can be stored in the skin. It is also known that excess sodium in the tissues leads to inflammation in various organs, but its role in dermal pathologies has not been elucidated. Therefore, our aim was to study the effect of dietary salt loading on the inflammatory process and related extracellular matrix (ECM) remodeling in the skin. Our data demonstrated that excessive salt intake leads to increased dermal sodium content that influences the dermal inflammation and tissue remodeling. We described the pro-inflammatory effect of high salt loading, mediated by the dermal alteration of *IL-17*, *IL-13*, and *IL-10* expression. We demonstrated the inhibitory effect of high salt environment on the *IL-10*, *IL-13* and *PDGF-B* production of immune cells. Our results also showed that, under inflammatory conditions, high salt consumption contributes to decreased dermal fibroblast activation and ECM production, including α-SMA, Fibronectin, and Colla1. Finally, we also described the direct inhibitory role of high sodium environment on the migratory capacity dermal fibroblasts *in vitro*. Our observations raise the possibility that excess salt content in diet is a potential risk factor for skin pathology associated with inflammation and impaired or chronic wound healing.

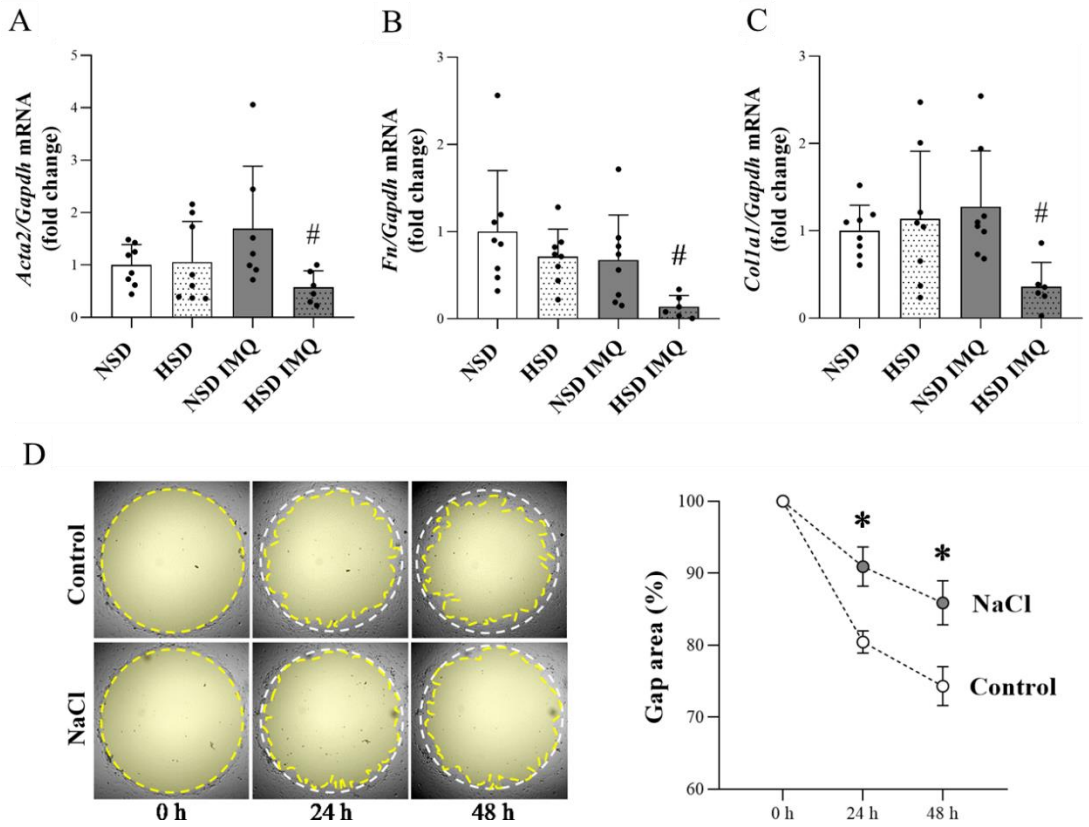

Figure 2. The role of high salt loading on the cellular and molecular mechanisms of dermal ECM remodeling. Effect of high salt intake on the mRNA expression of *Acta2* (A), *Fn,* and *Col1a1* (B, C). Impact of high salt environment on the migratory capacity of dermal fibroblast cells (D).

Transient Agarose Spot (TAS) assay: a new method to investigate cell migration – original research

Veres-Székely et al. International Journal of Molecular Sciences, 23(4), 2119. (2022)

Our above study, investigating the effect of dermal Na^+ excess on dermal pathologies suggested that high salt loading induces molecular changes that may affect the migratory capacity of dermal fibroblasts. Therefore, as we demonstrated in the manuscript above, we decided to investigate the effect of salt loading on the cell motility of dermal fibroblasts. The gold standard method to examine cell migration is the so-called scratch assay, which has numerous limitations. Indeed, during the scratch assay, the cell-free gap is manually generated by scratching the surface of a confluent cell monolayer with a pipette tip on 6 or 12-well plates. The main disadvantages of the scratch assay originate from the mechanical scratching itself, which causes the damage of the cells, and also that of the plate surface, which has a significant impact on cell motility. In addition, scratching with a pipette tip results in inconsistent initial gap sizes, which is reflected in the high intra- and inter-assay variability of the assay. Therefore, in the framework of the OTKA project, we decided to develop a more effective, 96-well plate-based method to examine the migration of dermal fibroblasts, as an alternative approach to the scratch assay. Our assay eliminated the disadvantages of the above-mentioned technique, using a simple, cost-effective, and high-throughput approach. Based on the advantages of our assay we decided to publish a detailed description of this method, with instructions for its routine application.

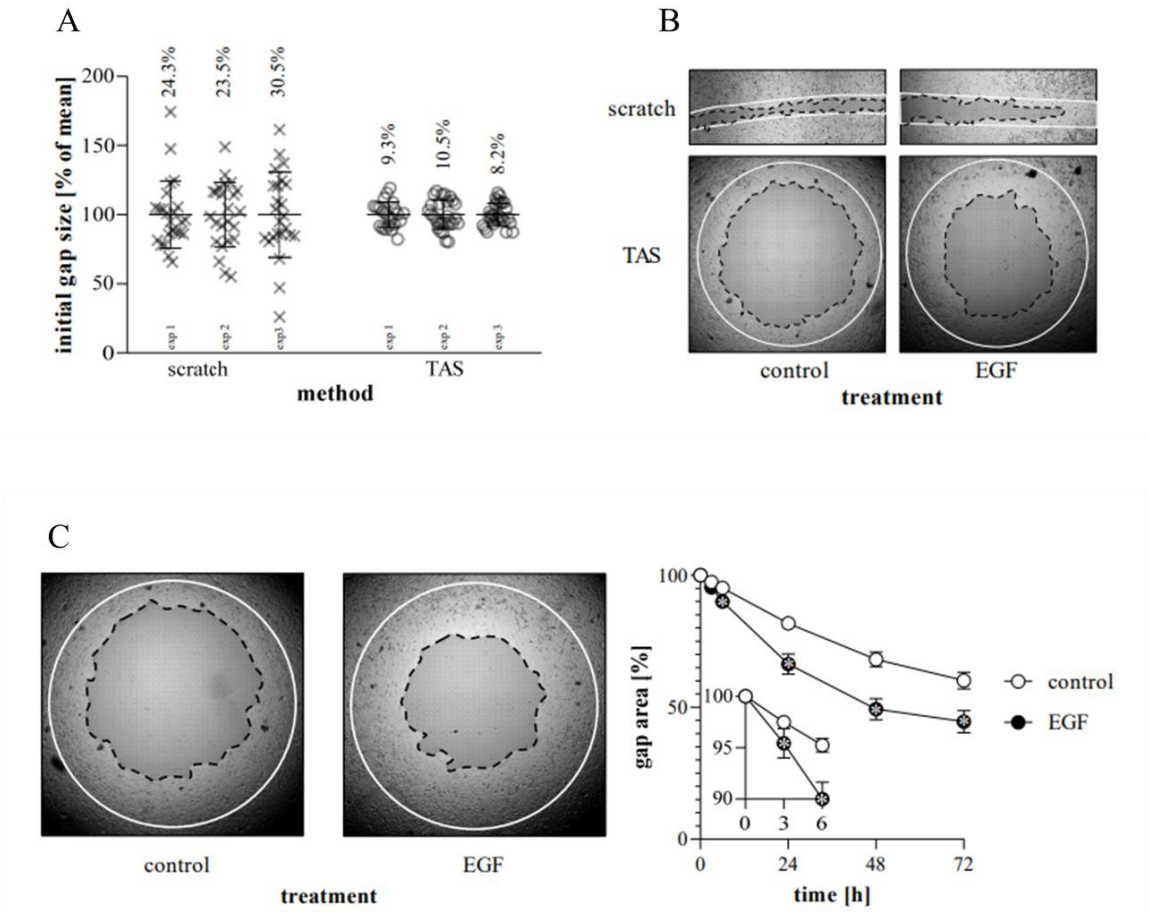

Figure 3. Comparison of the accuracy of TAS and conventional scratch assay. Differences in accuracy of scratch and TAS migration assays demonstrated on MRC-5 fibroblasts (A). The evenness of gap closure was investigated on control and EGF-treated MRC5 cells, as demonstrated in representative microscopic images (B). Effect of EGF treatment on gap closure using TAS assay (C).

Is too much salt harmful? Yes

Agócs et al. Pediatric Nephrology, 35(9), 1777-1785. (2020)

The contribution of high sodium intake to hypertension and cardiovascular morbidity and mortality is of great interest to the medical and scientific community. In the framework of the OTKA project, we published a comprehensive review aiming to collect the most recent data and answer the debating questions of the topic. The following topics were covered in the review:

- Sodium and blood pressure
- Sodium and cardiovascular morbidity/mortality
- Salt-sensitive hypertension and special risk groups in children and adults
- The effect of dietary salt overload on vascular function
- Extrarenal sodium handling and the impact of sodium on the immune system

The immunomodulatory role of sodium

Agócs et al. Orvosi Hetilap, 160(17), 646-653. (2019)

Increasing number of literature data uncovers the connection between excessive salt intake and immune-mediated diseases in various organs, including the skin, gut, and central nervous system. In this review, therefore, we discuss our current knowledge about the effect of high salt loading induced inflammatory mechanisms.

The effect of excessive salt intake on inflammation and tissue remodeling

Pajtók et al. XCVII. évfolyam, 4:457-508. (2022)

It has been shown that local Na^+ accumulation activates cells of the adaptive and innate immune systems. Independently of its etiology inflammation plays a pivotal role in the development of tissue fibrosis in almost all organs. Moreover, an increasing number of studies indicate that high salt intake influences the tissue remodeling of various organs, including the kidneys, peritoneum, lung, liver. Since the treatment of tissue fibrosis is unresolved, it is important to identify the molecular mechanisms as well as environmental factors that contribute to the development of excessive scarring. In light of new research findings, excessive salt intake may be one of these factors. In the present review, we summarize our current knowledge about the effects of increased salt intake on tissue fibrosis.

Effect of excessive salt intake on the development kidney fibrosis

Unpublished data

One of the main goals of the OTKA project was to examine the effect of high salt intake on the progression of chronic kidney disease. To this purpose, we used the unilateral urether obstruction (UUO, for 5 days) induced mouse model of kidney fibrosis in mice kept on normal (NSD) or high salt diet (HSD). After termination of the experiment, we compared the renal expression of the main markers of kidney fibrosis between the NSD and HSD groups. We found no difference between the kidney injury marker KIM-1 and NGAL. Similarly, there was no difference in the inflammatory marker COX-2 expression. Finally, our results showed that HSD did not alter the myofibroblast marker α-SMA and extracellular matrix marker fibronectin (FN).

Based on these results, the relationship between increased salt intake and renal fibrosis was not investigated further.

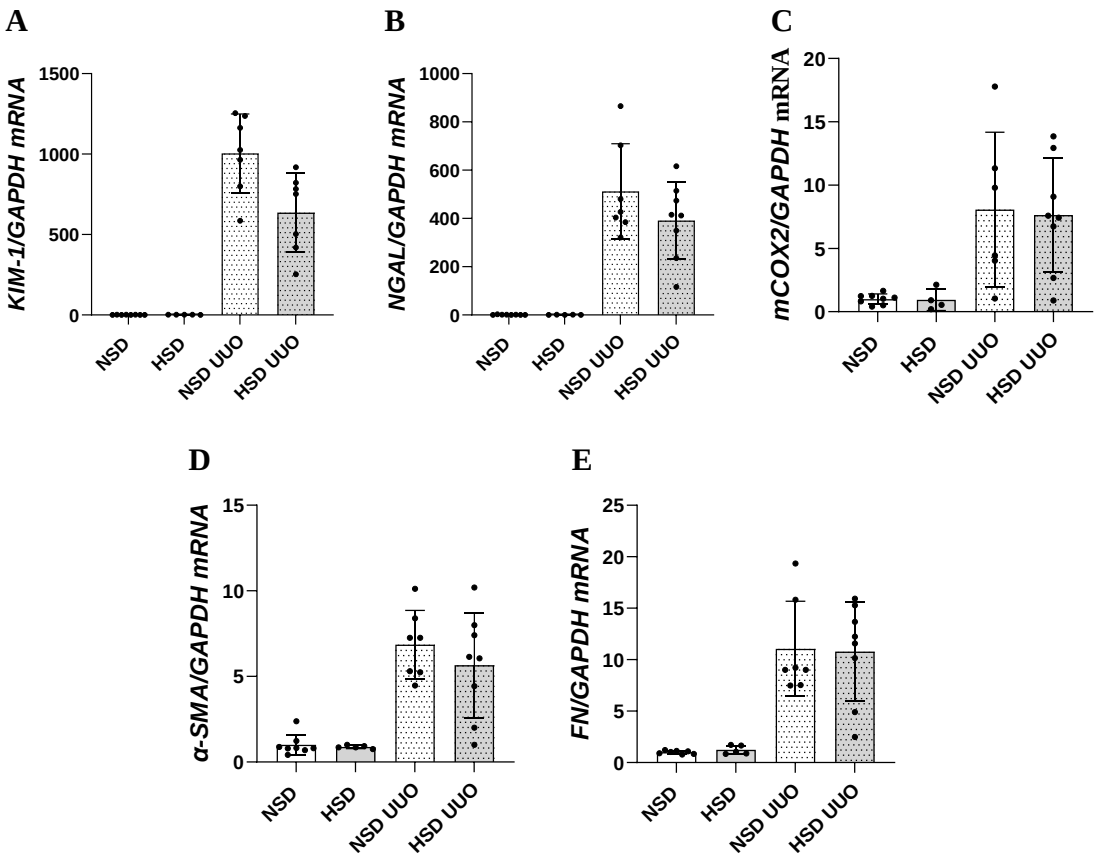

Figure 4. Effect of high salt diet on renal mRNA expression of KIM-1, NGAL, COX-2, α-SMA, and FN (A-E).

High salt environment disrupts the regulatory effect of podocin on the nephrin-nephrin distance

Unpublished data

The podocin encoding NPHS2 is the most frequently mutated gene in steroid-resistant nephrotic syndrome. Podocin homooligomerizes through C-terminal helical regions and binds nephrin in the glomerular slit diaphragm. It was formerly shown that podocin regulates the distance between two neighboring nephrin molecules in cis, i.e. the shortest dimension of the glomerular pore (3.5-4nm) High sodium diet has been shown to promote glomerular injury and proteinuria. We aimed to assess the effect of high salt environment on the nephrin-nephrin distance in the presence and absence of podocin. Our results showed that high salt environment did not influence the nephrin-nephrin distance in the absence of podocin. In accordance with our previous results, podocin decreased the nephrin-nephrin distance in the physiological salt environment, as reflected by an increased FRET efficiency between the extracellular YPet and mCherry tags. A high salt environment (+25 mM) completely disrupted this effect. Mannitol of similar osmotic concentration did not modify the effect of podocin High salt environment increases the distance between the nephrin molecules in cis by disrupting the organizing effect of podocin. The deleterious effect of high salt is not explained by increased osmotic pressure.

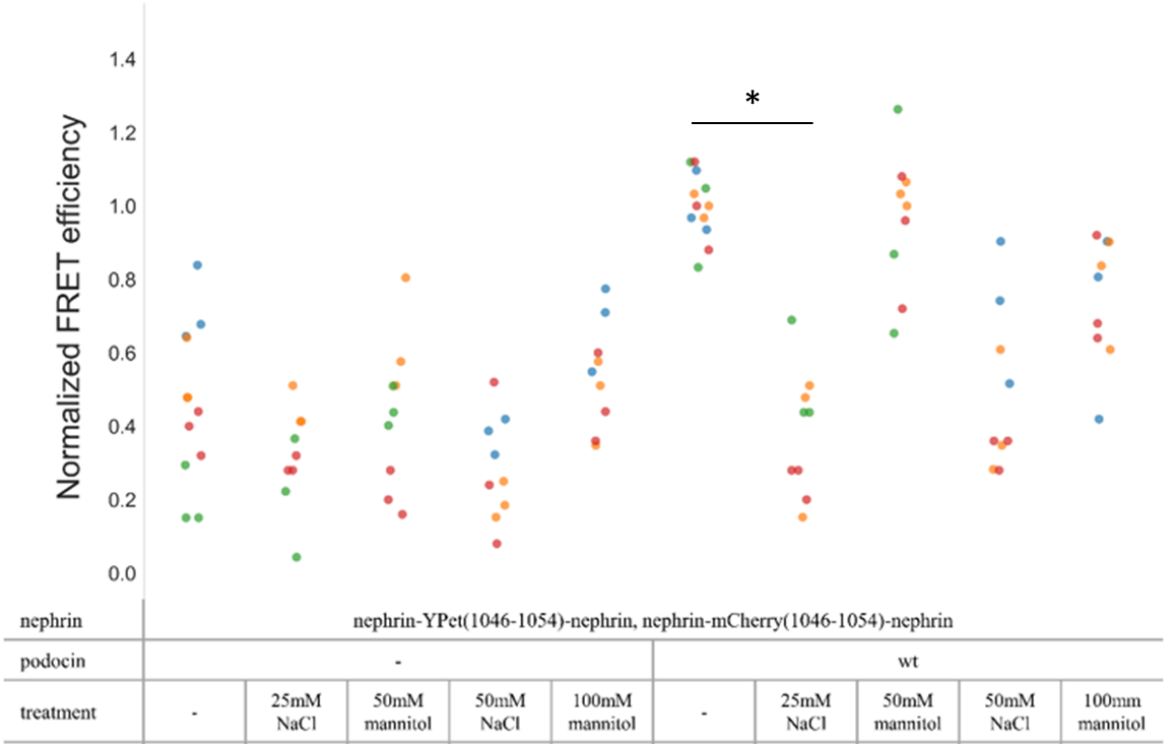

Figure 5. Effect of high salt on Normalized FRET efficiency values between the nephrin molecules with juxtamembranous labeling in function of the coexpressed podocin and added dietary salt or isotonic amount of mannitol. Data correspond to 9 to 12 measurements from three to four experiments for each coexpression. Values obtained from the same experiment are shown in the same color. In the absence of podocin, the treatment with either NaCl or mannitol could not affect the FRET efficiency. In the presence of podocin, both additional 25mM and 50mM NaCl decreased the FRET efficiency, while 50mM mannitol did not affect it. Treatment with 100mM mannitol decreased the FRET efficiency, but to a lesser extent than excess NaCl with equivalent osmotic pressure.

Summary

Our research provided insights into the molecular mechanism of sodium storage in the skin and also revealed how local sodium excess may influence the immune system and tissue remodeling. A deeper understanding of how a single dietary component affects our tissue homeostasis is the milestone for developing dietary based therapies to efficiently treat diseases.

PhD thesis in the framework of this OTKA project

- Dr. Csenge Pajtók: Effect of high salt diet on dermal tissue remodeling (2023)

PhD thesis that are in progress in the framework of this OTKA project

- Dr. Róbert Agócs: The role of the COX-2 pathway in skin glycosaminoglycan production during high salt load (2023)

Original articles and review articles in the framework of this project assigned with the OTKA identifier

- Agócs, R., Pap, D., Sugár, D., Tóth, G., Turiák, L., Veréb, Z., ... & Szabó, A. J. (2020). Cyclooxygenase-2 Modulates Glycosaminoglycan Production in the Skin During Salt Overload. Frontiers in physiology, 11, 561722.

  Q2 IF: 4.566

- Pajtók, C., Veres-Székely, A., Agócs, R., Szebeni, B., Dobosy, P., Németh, I., ... & Pap, D. (2021). High salt diet impairs dermal tissue remodeling in a mouse model of IMQ induced dermatitis. *PloS one*, *16*(11), e0258502.

  Q1/D1 IF: 3.58

- Veres-Székely, A., Pap, D., Szebeni, B., Őrfi, L., Szász, C., Pajtók, C., ... & Vannay, Á. (2022). Transient Agarose Spot (TAS) Assay: A New Method to Investigate Cell Migration. *International journal of molecular sciences*, *23*(4), 2119.

  Q1/D1 IF: 5.542

- Agócs, R., Sugár, D., & Szabó, A. J. (2020). Is too much salt harmful? Yes. *Pediatric Nephrology*, *35*(9), 1777-1785.

  Q1 IF: 3,651

- Agócs, R. I., Sugár, D., Pap, D., & Szabó, A. J. (2019). A nátrium immunmoduláns szerepe. The immunomodulatory role of sodium. *Orvosi Hetilap*, *160*(17), 646-653.

  Q3 IF: 0.428

- Dr. Pajtók Csenge, Dr. Pap Domonkos, Dr. Tulassay Tivadar. The effect of excessive salt intake on inflammation and tissue remodeling. 2022; XCVII. évfolyam, 4:457-508

Conference abstract connected to unpublished data

- Pajtók, Csenge ; Pap, Domonkos ; Veres-Székely, Apor ; Szebeni, Beáta ; Lévai, Eszter ; Szabó, Attila J. ; Vannay, Ádám ; Tulassay, Tivadar. A fokozott sóbevitel hatása a peritoneális fibrózisra. GYERMEKGYÓGYÁSZAT 72 : 3 pp. 189-189. , 1 p. (2021)

- Pajtók, Csenge ; Pap, Domonkos ; Veres-Székely, Apor ; Szebeni, Beáta ; Lévai, Eszter ; Szabó, Attila J. ; Vannay, Ádám ; Tulassay, Tivadar. Excess Sodium Chloride Induces Inflammation and Profibrotic Response in Peritoneal Cells. In: PhD Scientific Days 2021 (2021) Paper: 7842

- Pajtók, C ; Pap, D ; Veres-Székely, A ; Szebeni, B ; Lévai, E ; Szabó, AJ ; Vannay, A ; Tulassay, T. Excess sodium chloride induces inflammation and profibrotic response in

peritoneal cells. In: European Socitey of Paediatric Clinical Research 29th Meeting. (2021) 90 p. pp. 47-48.2 p.

- Csenge Pajtók; Apor Veres-Székely; Beáta Szebeni; Eszter Lévai; Csenge szász; Bokrossy Péter, Attila J. Szabó; Ádám Vannay; Tivadar Tulassay; Domonkos Pap. Effect of excess sodium chloride on the peritoneal fibrosis. Semmelweis Symposium 2022, Budapest, 2021.11.16-11.18. November 07-09, (2022)

Manuscript prepared for publication supported by the OTKA project:

CELLULAR AND MOLECULAR MECHANISM OF PERITONEAL FIBROSIS: THE ROLE OF HIGH SALT INTAKE

INTRODUCTION

End-stage kidney disease (ESRD) is a rapidly increasing global health burden. Due to ESRD, according to recent estimations in 2010 nearly 2.6 million people received renal replacement therapies worldwide [1]. Peritoneal dialysis (PD) and hemodialysis (HD) are renal replacement therapies for ESDR patients. During PD a dialysate fluid circulated in the abdominal cavity removes solutes and water from the body through the peritoneal membrane. PD has several advantages over HD because it offers better patient survival than in-center hemodialysis while, empowering patient autonomy because it can be done at home, and reducing the financial burden on healthcare systems [2]. However, on average 3 years after the onset PD up to 40% of patients develop peritoneal fibrosis (PF) leading to a decline of dialytic capacity and ultimately PD technique failure [3-5]. Despite the clear medical need, there is no specific therapy to treat or hinder PF. The underlying mechanisms of PF are not fully explored, therefore it has great importance to identify the factors and molecular mechanisms through which the development and progression of the disease can be influenced.

PF is a result of a complex pathological cross-talk between the effector cells of PF, including epithelial, endothelial, immune cells, and myofibroblast. In response to injury, they produce inflammatory cytokines and profibrotic growth factors, including interleukin (IL)-1ß, IL-6, monocyte chemoattractant protein (MCP-1) and transforming growth factor ß (TGF-ß), platelet-derived growth factor B (PDGF-B), and connective tissue growth factor (CTGF), as well. These factors activate peritoneal myofibroblasts leading to their increased proliferation and extracellular matrix (ECM) production, including collagens and fibronectin (FN). Activation of myofibroblasts are a part of the normal wound healing process, however in PD patients it becomes chronic and the excessive ECM production disrupts the integrity and function of the peritoneum.

Previously, it has been demonstrated dietary sodium (Na^+) can be stored in peritoneum and the local Na^+ excess can induce PF via osmotic stimuli [5,6]. However, little is known about the underlying cellular and molecular mechanisms.

In the present study, our goal was to reveal how the increasing sodium content induce inflammatory and profibrotic response in the main effector cells of PF. Our results may help to better understand the role of dietary salt intake in the development of PF.

METHODS

Peripheral blood mononuclear cells (PBMCs)

Isolation and culture on human PBMCs were approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics (31224-5/2017/EKU) after signing an informed consent. PBMCs from healthy adult donor were isolated by density gradient centrifugation using Histopaque-1077 (Merck). After isolation, the cells were placed into RPMI 1640 medium (ATCC) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin solution in humidified 95% air and 5% CO2 at 37°C. For LDH assay and real time PCRs PBMCs were seeded into 96 well plates (Sarstedt) at a density of 5×10^4 cells/well (n = 6 well/treatment group) and were cultured in medium with additional NaCl (10 mM, 20 mM and 40 mM) or as osmotic control additional mannitol (20 mM, 40 mM, 80 mM). Control cells were treated with cell culture medium alone.

Human Umbilical Vein Endothelial Cells (HUVECs)

LDH assay and real time PCRs HUVECs were seeded into 96 well plates (Sarstedt) at a density of 5×10^4 cells/well (n = 6 well/treatment group) and were cultured in medium with additional NaCl (10 mM, 20 mM and 40 mM) or as osmotic control additional mannitol (20 mM, 40 mM, 80 mM). Control cells were treated with cell culture medium alone.

Primary human peritoneal fibroblast cells (HPFCs)

Collection of biospecimens for the isolation and culture on HPFs complied with the directive of the Helsinki Declaration and were approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics (31224-5/2017/EKU). Primary HPFs were isolated from peritoneal biopsy specimens obtained when inserting a Tenckhoff catheter. After isolation, the cells were placed into RPMI 1640 medium (ATCC, Manassas, Virginia, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin solution in humidified 95% air and 5% CO2 at 37 °C. For LDH, MTT and SiriusRed assay and real-time PCRs HPFCs were seeded into 96 well plates (Sarstedt) at a density of 5×10^4 cells/well (n = 6 well/treatment group) and were cultured in medium with additional NaCl (10 mM, 20 mM, and 40 mM) or as osmotic control additional mannitol (20 mM, 40 mM, 80 mM). Control cells were treated with cell culture medium alone.

Primary human peritoneal mesothelial cells (HPMCs)

Collection of biospecimens for the isolation and culture on HPMCs complied with the directive of the Helsinki Declaration and were approved by Semmelweis University Regional and Institutional Committee of Science and Research Ethics (31224-5/2017/EKU). Primary HPMCs were isolated from peritoneal biopsy specimens obtained when inserting a Tenckhoff catheter. After isolation, the cells were placed into RPMI 1640 medium (ATCC, Manassas, Virginia, USA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific) and 1% penicillin/streptomycin solution in humidified 95% air and 5% CO2 at 37 °C. For LDH assay and real-time PCRs HPFCs were seeded into 96 well plates (Sarstedt) at a density of 5×10^4 cells/well (n = 6 well/treatment group) and were cultured in medium with additional NaCl (10 mM, 20 mM and 40 mM) or as osmotic control additional mannitol (20 mM, 40 mM, 80 mM). Control cells were treated with cell culture medium alone.

Ex vivo peritoneum samples

All experiments 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

(PEI/001/1731-9/2015). The mouse was housed in a temperature-controlled (22±1 °C) room with alternating light and dark cycles (12/12 hours) and had ad libitum access to food and water The peritoneal samples was collected from 7-8 week old male wild-type C57B1/6J mouse under general anesthesia subcutane injection of a mixture of 100 mg/kg ketamine (Richter Gedeon, Budapest, Hungary) and 10 mg/kg xylazine (Medicus Partner, Biatorbágy, Hungary). The peritoneum was dissected *ex vivo* and then the tissue samples were placed on 24 well plates (Sarstedt) (n = 8/treatment group) and were subjected to normal culture medium (Control), high NaCl medium (+40 mM) or high mannitol medium (+80 mM). After 24 hours the samples were collected for real time PCR measurements.

Immunofluorescence Staining

Characterization of isolated HPMC as well as HPFC cells was performed by immunofluorescence staining. Cells were seeded in cell culture chambers (Sarstedt) at a density of 10^4 cells/well and were incubated for 24 hours in 37 °C. After washing with WashPerm solution, slides were permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, California, USA) at room temperature for 15 minutes. Slides were incubated with primary antibody specific for α-SMA (mouse, 1:5000; MERCK, Darmstadt, Germany) or CK-18 (mouse, 1:1000; MERCK, Darmstadt, Germany) at room temperature for 1 hour. Thereafter the slides were washed and incubated with corresponding Alexa Fluor 488 conjugated secondary antibody (anti-mouse, 1:100; Invitrogen, Thermo Fisher Scientific) at room temperature in the dark for 30 minutes. Sections were analyzed with an Olympus IX81 fluorescent microscope system (Olympus, Japan).

Real-time polymerase chain reaction (PCR)

Real-time PCRs were performed in a final volume of 20 µl containing 0.5 µM of forward and reverse primers (Integrated DNA Technologies, Coralville, Iowa, USA), 10 µl of Light Cycler 480 SYBR Green I Master enzyme mix (Roche Diagnostics, Mannheim, Germany) and 1 µl cDNA on a LightCycler 480 system (Roche Diagnostics). Results were analyzed by Light-Cycler 480 software version 1.5.0.39 (Roche Diagnostics). The mRNA expressions were determined by comparison with the expression of *GAPDH* as a housekeeping gene from the same samples. The data were normalized and presented as the ratio of the mean values of their control groups.

SiriusRed collagen detection assay

For the SiriusRed assay, PMFs were seeded into 96-well plates (Sarstedt) at a density of 10^4 cells/well (n = 6 / group) and were subjected to culture medium with 10 mM, 20 mM, and 40 mM NaCl or as osmotic control 20 mM, 40 mM and 80 mM mannitol for 24 hours in 37°C. The assay was performed as previously described [23]. All reagents were purchased from Merck KGaA. Absorbance was determined at 544 nm by Hidex Chameleon Microplate Reader (Lablogic Systems, Sheffield, United Kingdom) using the MikroWin 2000 software. Data were normalized and presented as the ratio of their control values.

MTT Cell Proliferation Assay

MTT cell proliferation assay was performed using the Cell Proliferation Kit I (MTT) (Roche Diagnostics) according to the manufacturer's recommendations. Absorbance was recorded at 570 nm and 690 nm as background using a Hidex Chameleon Microplate Reader using the MikroWin 2000 program. Data were normalized and presented as the ratio of their control values.

LDH Cytotoxicity Assay

Reagents for *in vitro* LDH cytotoxicity assay [16] were purchased from Sigma-Aldrich. Absorbance was recorded at 570 nm and 690 nm as background in a Hidex Chameleon Microplate Reader using the MikroWin 2000 program. Data were normalized and presented as the ratio of their control values.

Statistical analysis

Statistical evaluation of data was performed by GraphPad Prism 7.0 Software (GraphPad Software, La Jolla, California, USA). After testing the normality with Kolmogorov–Smirnov test, ordinary one -way ANOVA- and unpaired t-test, Mann–Whitney or Kruskall–Wallis-test were used to determine the differences among groups. Data were normalized and presented as the ratio of the mean values of their control groups. $p \leq 0.05$ was considered as statistically significant. Values were expressed as mean+SD.

RESULTS

Characterization of primary peritoneal epithelial cells and fibroblast cells and the effect of high NaCl loading on cell viability

To investigate the profibrotic effect of increased Na^+ concentration, primary peritoneal epithelial (HPMCs) and fibroblast cells (HPFCs), and peripheral immune cells (PBMCs) were isolated. HUVEC cells were used to model the peritoneal endothelial cells. As a first step, we characterized the isolated HPMCs and HPFCs based on their specific biomarkers. According to our results, the peritoneal epithelial cells were positive for the epithelial cell marker CK-18 and negative for the fibroblast marker α-SMA (Figure 1/A). On the contrary, primary fibroblast cells were CK-18 negative and α-SMA positive (Figure 1/A). To investigate the effect of high Na+ environment on fibrosis-associated molecular markers we used increasing concentration of additional NaCl in cell culture media. As osmotic control, mannitol was used in equimolar concentrations. We also investigated whether the additional NaCl and mannitol treatments induce apoptosis of the cells by LDH assay (Figure 1/B). The result demonstrated that neither NaCl nor the mannitol increased the apoptotic marker LDH release of the cells (Figure 1/B).

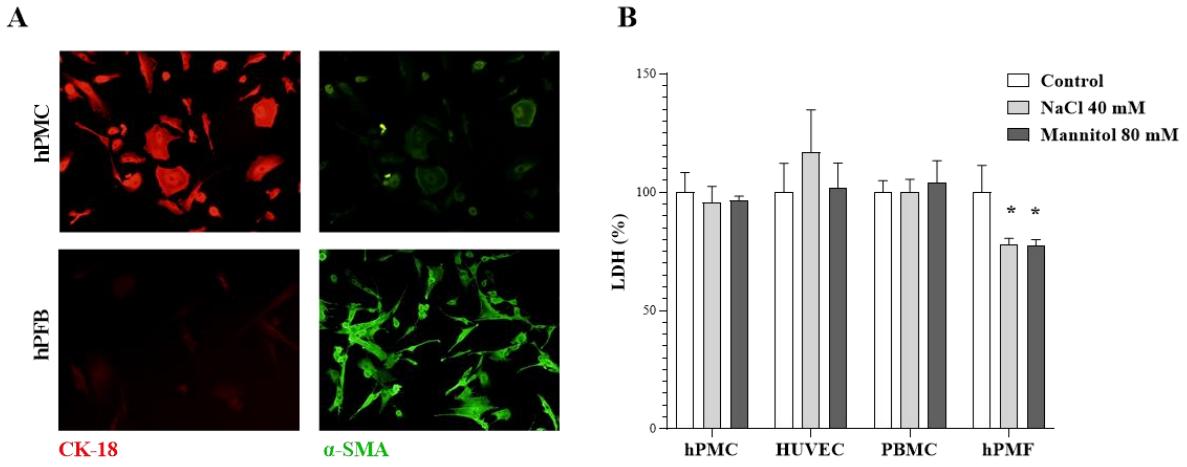

Figure 1. Characterization of peritoneal epithelial and fibroblast cells and the effect of

high salt and mannitol concentrations on cell viability. The presence of CK-18 (red) and α-SMA (green) on peritoneal epithelial and fibroblast cells was determined by immunofluorescence staining (A). The effect of supplementation of cell culture media with additional NaCl and mannitol on apoptosis was determined by LDH assays (A) (n=6). Results are presented as mean + SD. *$p < 0.05$ vs. control (One-way ANOVA or Kruskal-Wallis test)

The connection between inflammation and salt

Peritoneal cells are a potential source of inflammatory mediators, and high salt diet has been suggested to induce proinflammatory mechanisms in the peritoneal wall. Therefore, we investigated the cell-specific effect of high Na+ environment on their MCP-1, IL-1ß, and IL-6 expression. In HPMCs NaCl decreased the mRNA expression of *IL6* and mannitol increased the expression of *MCP1* (Figure 2/A, C). In HUVECs NaCl or mannitol increased the mRNA expression of *MCP1*, and *IL6* (Figure 2/D, F). In PBMCs NaCl increased the mRNA expression of *IL1B* and *MCP1* while mannitol decreased the expression of *IL6 MCP1* (Figure 2/A-C).

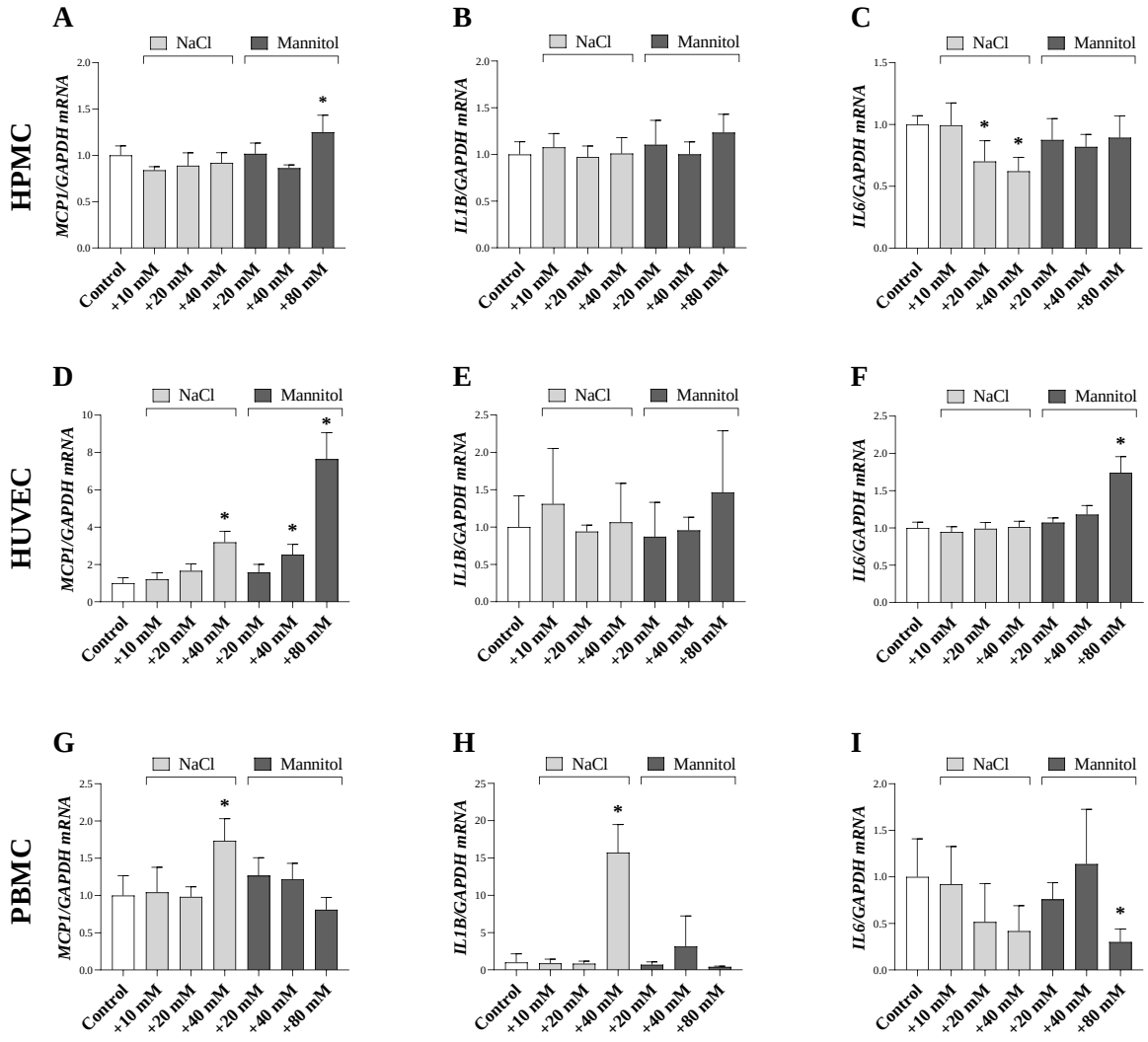

Figure 2. Effect of high Na^+ loading on inflammatory cytokine expression of peritoneal cells. After treatment with cell culture media supplemented with additional NaCl and mannitol, the mRNA expression of *MCP1* (A, D, G), *IL1B* (B, E, H), *IL6* (C, F, I) were determined by

real-time PCR by comparison with GAPDH as an internal control (n = 6). Results are presented as mean + SD. *p < 0.05 vs. control (One-way ANOVA or Kruskal-Wallis test).

Effect of high salt on the profibrotic growth factor production

In response to injury peritoneal cells produce growth factors, including CTGF, TGF-ß, and PDGF-B. Since previous data suggest the pathologic effect of sodium in the peritoneum, we investigated whether of high NaCl environment influences their expression. We found that NaCl and mannitol increased the expression of *CTGF* and *TGFB* in HPMCs (Figure 3/B, C). In HUVECs mRNA levels of *CTGF*, *TGFB* and *PDGFB* were increased by NaCl or mannitol (Figure 3/A-C). In PBMCs NaCl and mannitol increased the expression of *CTGF*, while decreasing the expression of *TGFB* and *PDGFB* (Figure 3/A-C).

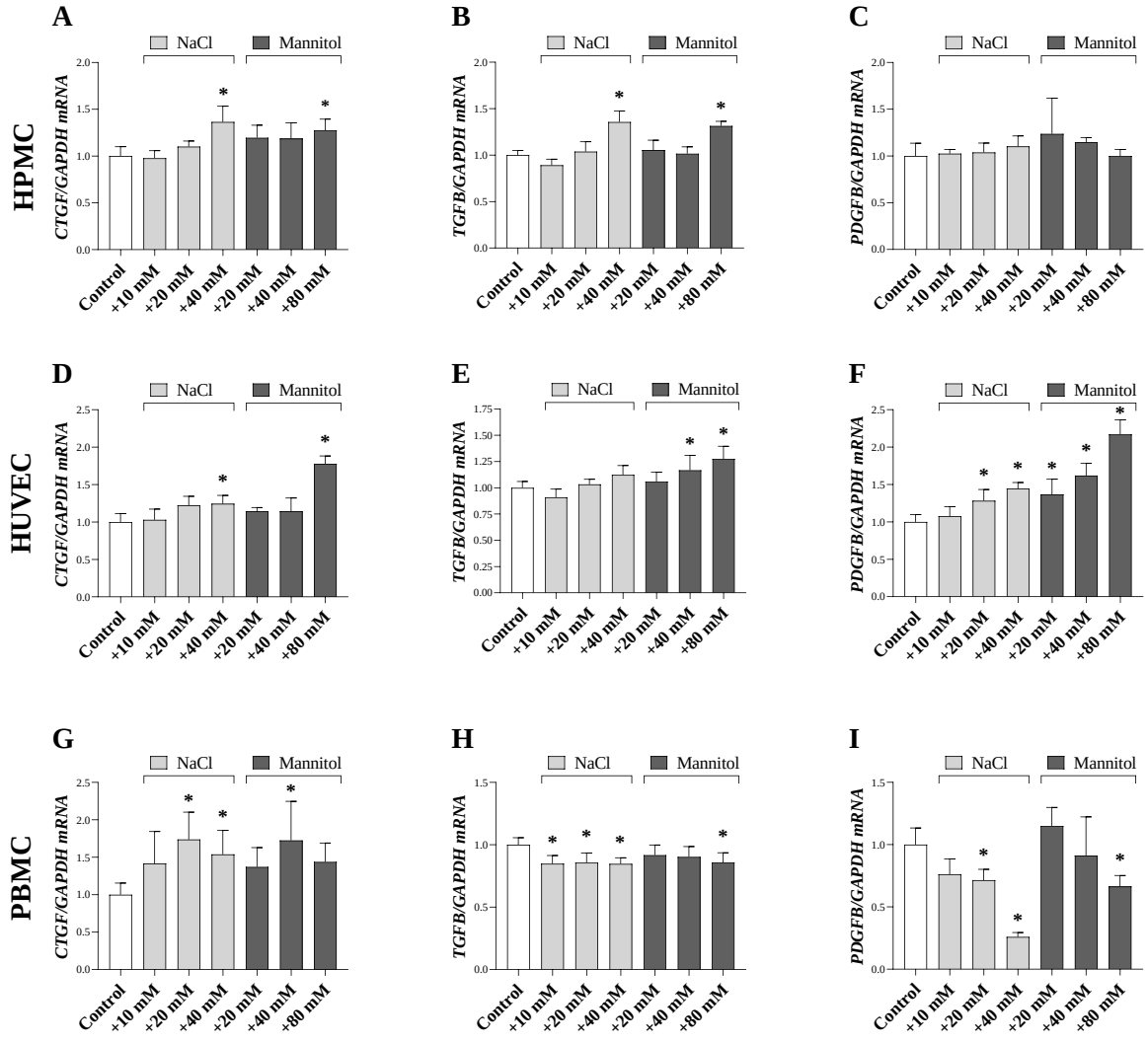

Figure 3. Effect of high Na^+ environment on profibrotic growth factor expression of peritoneal cells. After treatment with cell culture media supplemented with additional NaCl and mannitol the mRNA expression of *CTGF* (A, D, G), *TGFB* (B, E, H), and *PDGFB* (C, F, I) were determined by real-time PCR by comparison with GAPDH as internal control (n = 6). Results are presented as mean + SD. *p < 0.05 vs. control (One-way ANOVA or Kruskal-Wallis test).

Salt-induced growth factors and profibrotic response in the peritoneum

To demonstrate direct connection between high salt-induced growth factor production and peritoneal fibrosis we investigated the effect of CTGF, TGF-ß1, and PDGF-BB on the functional activity of HPFBs, including proliferation and collagen production. Both CTGF, TGF-ß1, and PDGF-BB treatment facilitated the proliferation of HPFBs (Figure 4/A). Furthermore, their collagen production was increased by TGF-ß1 and PDGF-BB (Figure 4/B).

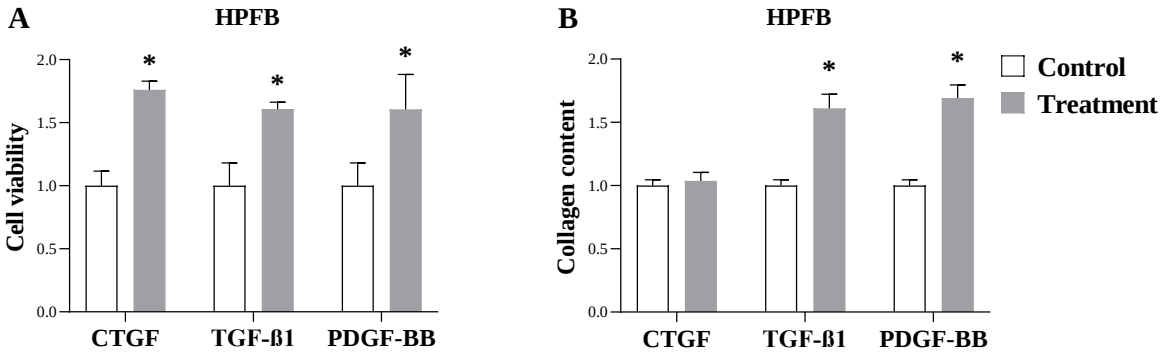

Figure 4. Effect of CTGF, TGF-ß, and PDGF-BB on proliferation and collagen production of HPFBs. (n = 6). The effect of CTGF, TGF-ß, and PDGF-BB on the proliferation was investigated by MTT (A). Growth factor-induced collagen production was measured by SiriusRed assay. Results are presented as mean + SD. *$p < 0.05$ vs. control (One-way ANOVA or Kruskal-Wallis test).

High salt loading induced epithelial mesenchymal transition of peritoneal epithelial cells

Mesothelial-to-Mesenchymal Transition (MMT) and Endothelial-to-Mesenchymal Transition (EndMT) contribute to the generation of fibroblasts, therefore, to the development of peritoneal fibrosis. We investigated the effect of high salt loading on the molecular markers of MMT and endoMT. In HPMCs both NaCl and mannitol decreased the mRNA expression of epithelial marker ECAD and increased the expression of mesenchymal (and fibroblast) marker SNAI1 and ACTA2 (Figure 5/A-C). Similarly, mRNA expression of SNAI1 and ACTA2 was increased by NaCl and mannitol in HUVECs (Figure 5/D, E).

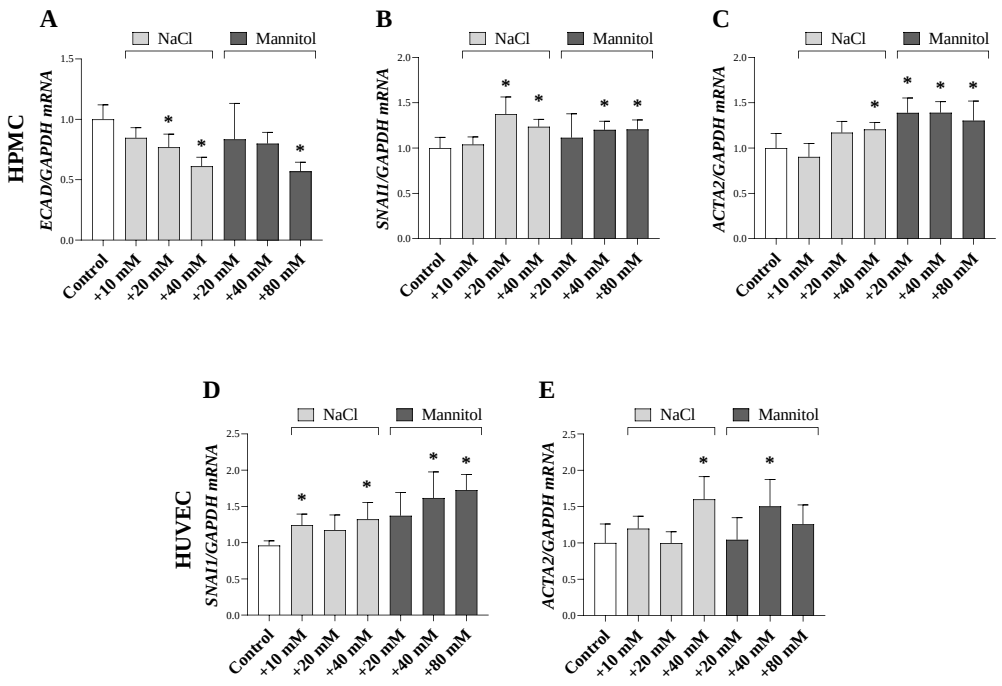

Figure 5. Effect of high Na^+ environment on mesenchymal transition of HPMCs and HUVECs. After treatment with cell culture media supplemented with additional NaCl and mannitol the mRNA expression of *ECAD* (A), *SNAI1* (B, D), and *ACTA2* (C, E) were determined by real-time PCR by comparison with GAPDH as internal control (n = 6). Results are presented as mean + SD. *p < 0.05 vs. control (One-way ANOVA or Kruskal-Wallis test).

Role of high salt in ECM production

Peritoneal fibroblasts are the main effector cells of peritoneal fibrosis via their ECM production. Our results showed that NaCl and mannitol increased the mRNA expression of ECM component fibronectin (FN) (Figure 6/A). Furthermore, NaCl increased the protein level of collagen-1 and 3 in HPFCs while mannitol did not affect it (Figure 6/B, C).

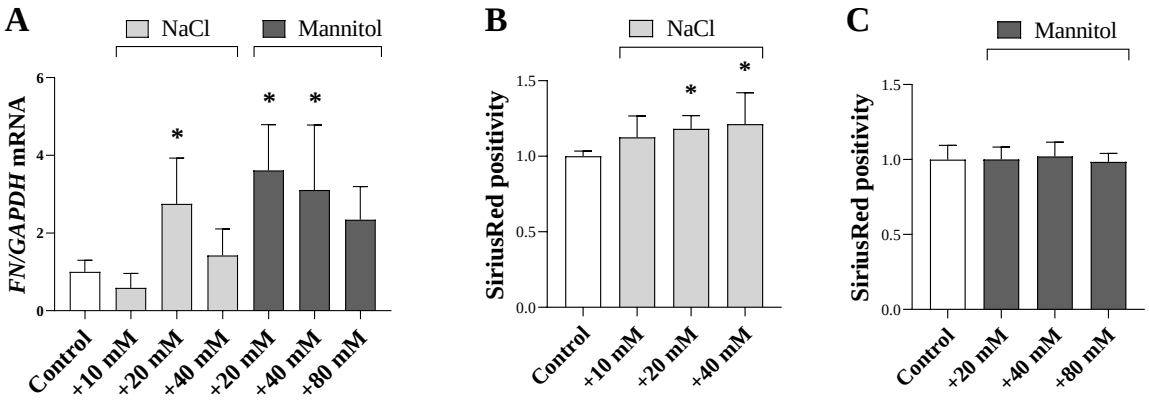

Figure 6. Effect of high Na^+ loading on the ECM production of peritoneal fibroblast cells. After treatment with additional NaCl and mannitol-supplemented media mRNA expressions of FN (A) was measured by real-time PCR, by comparison with *GAPDH* as internal control (n = 6). NaCl and mannitol supplementation induced collagen production (B) was measured by

SiriusRed assay (n = 6). Results are presented as mean + SD. *$p < 0.05$ vs. control (One-way ANOVA or Kruskal-Wallis test).

Ex vivo effect of high salt on the peritoneum

We also examined whether salt loading induced cell specific alterations in the peritoneal cells, including increased inflammatory cytokine, growth factor and mesenchymal marker expression, and elevated ECM production also can be detected in the intact peritoneum. Our results showed that NaCl increased the mRNA expression of inflammatory cytokine *Il1b* and *Il6*, profibrotic growth factor *Ctgf* and *Tgfb*, and EMT and endoMT marker *Acta2* and *Snai1*. mRNA expression of *Mcp1*, *Ctgf*, and *Acta2* was also increased by mannitol.

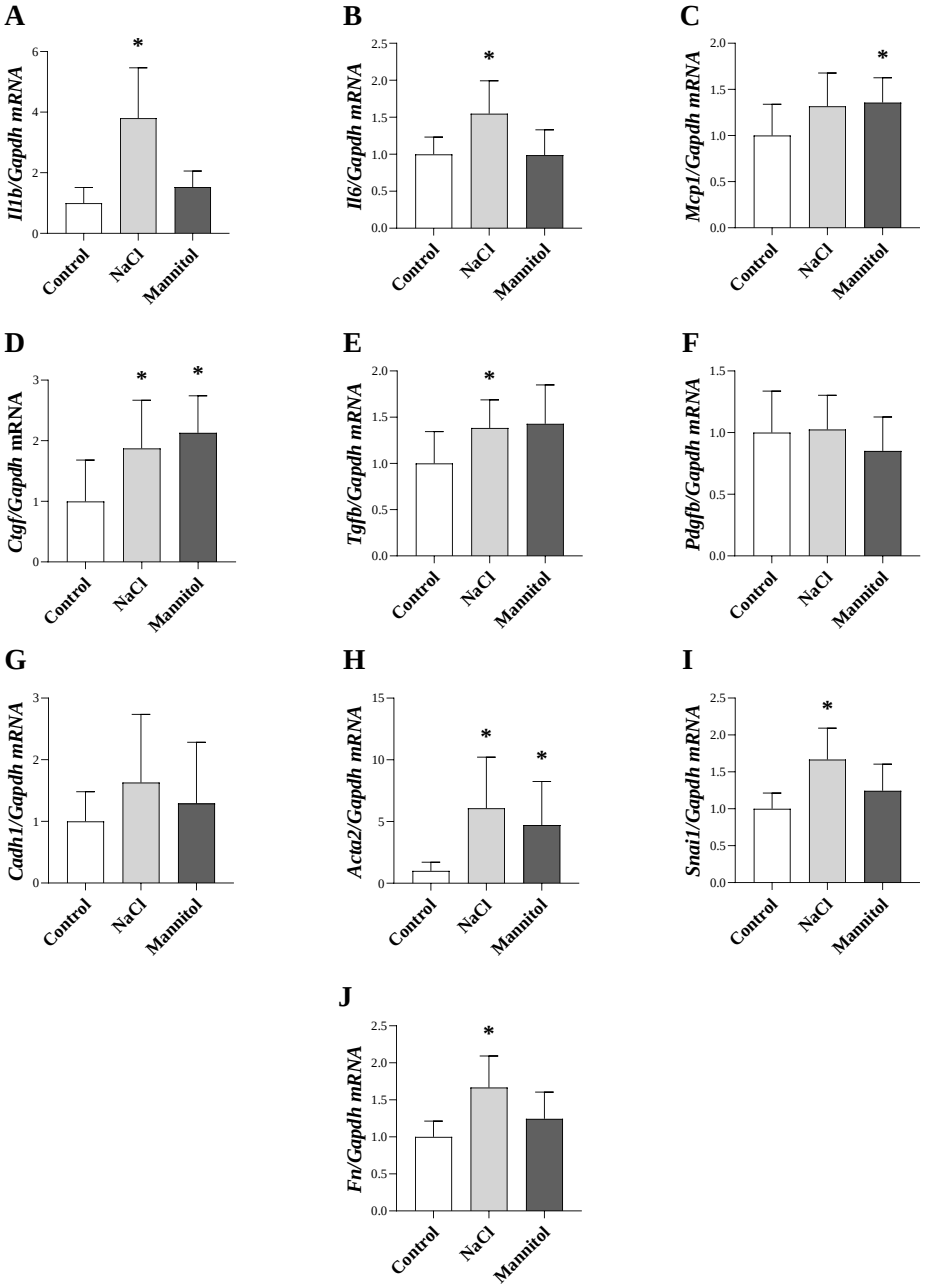

Figure 6. *Ex vivo* effect of high Na+ environment on the peritoneum. After treatment with culture media supplemented with additional NaCl and mannitol, the mRNA expression of *Il1b*, *Il6*, *Mcp1*, *Ctgf*, *TGFB*, *PDGFB*, *Ecad*, *Acta2*, *Snail1*, and *Fn* were determined by real-time PCR by comparison with GAPDH as internal control (n = 8). Results are presented as mean + SD. *p < 0.05 vs. control (unpaired t-test or Mann-Whitney U test).

Summary

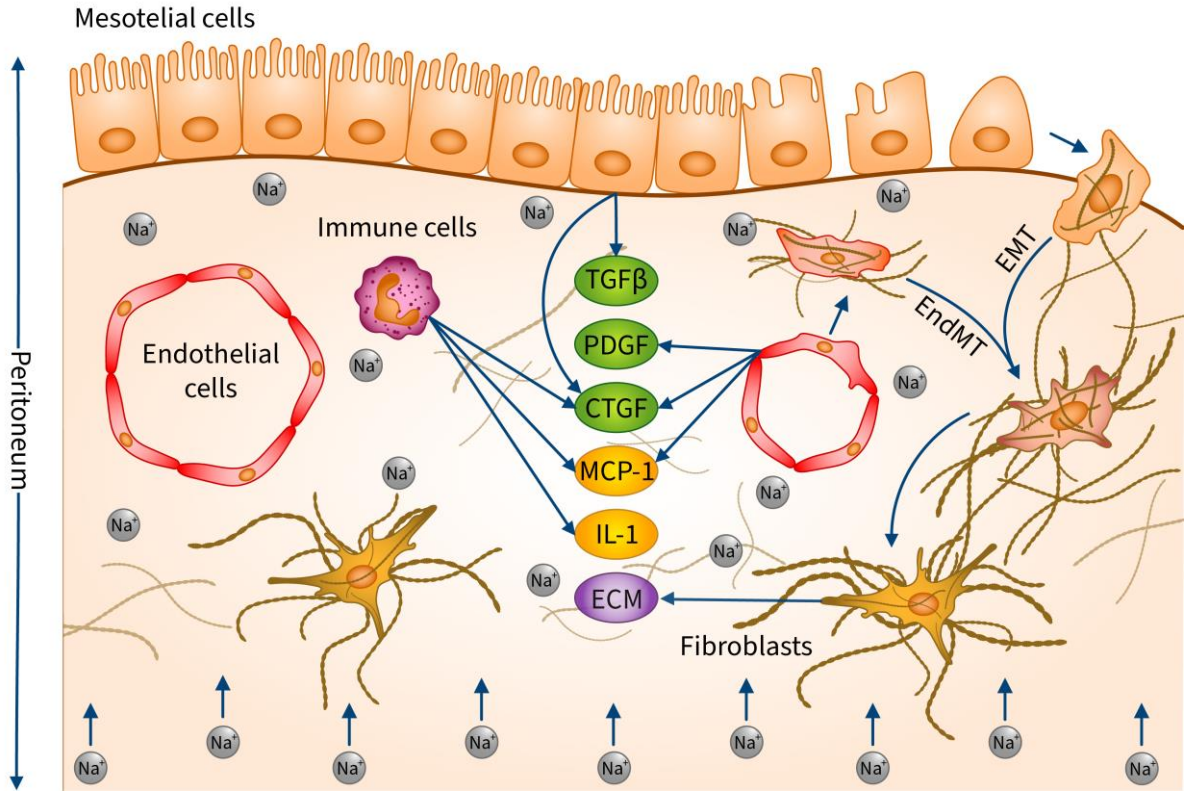

Figure 7. Effect of increased peritoneal sodium content on various peritoneal cell types.

DISCUSSION

One of the most important goals for ESDR patients on PD is the long-term preservation of the peritoneal membrane function. Therefore, it has great importance to identify factors that can damage the peritoneum. Since high salt intake has been suggested to promote PF, our goal was to understand the underlying cellular and molecular mechanisms.

Recently, the study of Sakata *et al.* found that high salt intake leads to increased sodium content and osmolarity in the peritoneal wall. Furthermore, their results suggested that peritoneal hyperosmolarity stimulates the expression of pro-inflammatory cytokines such as MCP-1, IL-1ß, and IL-6 and leads to excessive ECM depositions in the peritoneal membrane [5,6]. Although, these results suggested the pathologic effect of high salt intake in the peritoneum the underlying cellular and molecular mechanisms have not been revealed yet.

The peritoneal membrane forms the lining of the abdominal cavity composed of a continuous layer of mesothelial cells, together with endothelial cells of microvessels, leukocytes, and fibroblasts placed in the submesothelial region. Induction of PF is a complex

pathological event where immune and non-immune components of the peritoneal membrane sense the profibrotic stimuli and secrete inflammatory mediators leading to the recruitment of leukocytes and generation of myofibroblasts, cells of heterogenous origin with the ability to produce the extracellular matrix proteins (ECM). Defining the effect of a high sodium environment on the profibrotic response of peritoneal cells may lead to a better understanding of the relationship between salt intake and peritoneal pathologies.

Based on previous literature data, excessive dietary salt intake can increase sodium levels in the dermal interstitium and peritoneum by on average 30-50 mM. Therefore, to examine the effect of high sodium environment in the *in vitro* and *ex vivo* experiments we added 10, 20, or 40 mM extra NaCl supplementation to the cell culture media [7]. In order to distinguish between the effect of sodium itself and the sodium-induced osmotic changes, an equimolar amount of mannitol was used as an osmotic control.

MCP-1, IL-1ß, and IL-6 have been considered central mediators of pro-inflammatory processes via the recruitment of lymphocytes and monocytes to the site of injury that leads to a comprehensive immune response. Our results demonstrated that both high sodium or osmotic environment increased the expression of MCP-1 in HPMCs, HUVECs PBMCs (Figure 2/A, D and G), and in *ex vivo* peritoneal samples (Figure 6/A), as well. In accordance, with our data the role of hyperosmotic stress has already been connected to MCP-1 expression in Met5a immortalized peritoneal cell line upon exposure to hyperosmolar concentrations of NaCl, mannitol, or glucose [8]. Recently, Lee et al. investigated the effect of lentiviral overexpression of MCP-1 in the peritoneum, and their result demonstrated that, MP-1 facilitated the myofibroblast marker α-SMA and ECM marker fibronectin expression in the animal model of dialysis solution induced peritoneal scarring.

In the case of IL-1ß, we found a sodium-dependent increased expression level in PBMCs (Figure 2/H). The role of sodium in IL1ß expression was also supported by the *ex vivo* results (Figure 6/B). Our results are in line with previous data showing that NaCl and mannitol facilitated the IL-1ß expression in astrocytes and macrophages [9], however, its expression remained unchanged in NaCl-treated neutrophils evidencing also the cell-specific effect of the high salt environment [10]. The importance of IL-1ß in peritoneal pathologies is maybe due to its synergistic effect on inflammatory mechanisms. Indeed, IL-1ß can facilitate the initial inflammatory response by stimulating the production of MCP-1 and IL-6.

Interestingly, our *in vitro* data showed that high salt and osmotic environment rather decrease than increase IL-6 expression in the peritoneal cells (Figure 2/C, F and I). On the contrary t,he *ex vivo* results showed high salt medium, but not osmotic pressure increased the expression of *Il6* (Figure 6/C). The difference between *in vitro* and *ex vivo* results may be due to the intact cellular composition of ex vivo peritoneal samples. It is easy to accept that, IL-6 expression can be stimulated via other mmediators including IL-1ß that have superior ffect on the direct inhibitory effect of sodium. Recent studies supports this hypothesis demonstrating that, IL-1ß treatment increases the IL-6 secretion of HPMCs and HUVECs, as well [11,12]. IL-6 was demonstrated to induce PF by shifting acute inflammation into a chronic profibrotic state by changing the balance between anti-inflammatory Th2 cells and proinflammatory of Th1 cell into Th1 dominance [13]. Besides, IL-6 signaling drives STAT3 pathway activation which has crucial role in mesenchymal transformation of mesothelial cells, which is one of the central cellular mechanism behind PF [14-16].

Taken together our results demonstrate both inhibitory and enhancing effects of a high-salt environment and osmotic stress on the inflammatory response of various peritoneal cells, however, its overall effect is pro-inflammatory as supported by the ex vivo results (Figure 6. A-C)

Profibrotic growth factors, including CTGF, TGF-ß, and PDGF-BB play a key role in fibrogenesis via stimulation of fibroblast proliferation and ECM production [17]. Peritoneal

cells are a potential source of fibrogenic growth factors [18-21]. It was shown that, the glucose and its degradation products in dialysis fluid increase CTGF and TGF-ß production of HPMCs. As a positive feedback loop, TGF-ß treatment was also demonstrated to facilitate CTGF expression in HPMCs. The induction of PDGF-B expression in HUVECs by growth factors was also described.

According to our data, osmotic stress regardless of whether it was caused by NaCl or mannitol increased the expression of CTGF in peritoneal cells (Figure 3/A, D and G), and in ex vivo peritoneal samples (Figure 6/D), as well. Recent observations suggest the importance of CTGF in PF. Indeed, CTGF levels in dialysate samples have been reported to be associated with the extent of PF in PD patients [22]. Furthermore, recent observations demonstrated that neutralization of CTGF by FG-3019a monoclonal antibody ameliorates the chlorhexidine gluconate (CG) induced PF in mice. Investigating the underlying mechanisms they found that lack of CTGF blunted the effect of TGF-β1 on NIH/3T3 mouse embryonic fibroblast proliferation and ECM production [4], suggesting the synergistic effect of the two growth factors in the peritoneum. The fibrogenic effect of CTGF is strongly associated with its mitogenic properties. Indeed, CTGF-induced fibroblast proliferation was demonstrated on kidney, cardiac or lung fibroblasts, as well [23-26]. Nevertheless, its effect on peritoneal fibroblasts has not been elucidated yet. Therefore, as supportive data of our results we demonstrated that, CTGF induces the proliferation of HPFC. These further confirm that local Na excess through CTGF-mediated fibroblast activation contributes to PF (Figure 4/A).

Our finding showed that the both NaCl and its osmotic effect can affect the TGF-ß expression in peritoneal cells, however, in an opposite way. Indeed, NaCl or mannitol increased TGF-ß expression in HPMCs (Figure 3/B) while decreasing in PBMCs (Figure 3/H). However, overall NaCl increases its expression as it was demonstrated *ex vivo* in the intact peritoneal samples (Figure 6/E), making it reasonable to consider the high sodium environment as a factor that facilitates TGF-ß synthesis. The role of TGF-ß in PF has been previously suggested. Indeed, Duan *et al.* demonstrated that TGF-β induced Smad2/3 profibrotic signaling pathway is activated in biopsy samples of PD patients with PF [27]. In addition, overexpression of TGF-ß in the peritoneal tissue led to increased peritoneal collagen content in rodents. Due to a lack of literature evidence we experimentally demonstrated that TGF-ß can directly facilitate the proliferation and collagen production of peritoneal fibroblasts connecting more directly the ssodium-induced TGF-ß production to the development of peritoneal scarring (Figure 4/A, B).

According to our data, additional high salt loading had no clear effect on PDGFB expression. Both salt and mannitol to increased their expression in HUVECs and decreased in PBMC (Figure 3/F and I). In addition, its expression in HPMCs and *ex vivo* peritoneal samples remained unchanged (Figure 3/C and Figure 6/F). Despite the conflicting data role of PDGFB in peritoneal fibrogenesis can reasonably be assumed. Indeed, Study Seeger *et al.* described strong PDGFRß positivity in peritoneal biopsy samples from PD patients [28]. In accordance with these data, a recent study of Pranali *et al.* found that adenovirus-mediated overexpression of PDGF-B leads to PF. Furthermore, our results confirmed that similar to other organs, PDGF-BB increases the proliferation and ECM production of peritoneal fibroblasts (Figure 4/A, B).

Taken together, it can be reasonably assumed that, increased expression of CTGF, TGF-ß and PDGF due to high salt loading can contribute to the development of PF via activation of proliferation and ECM production of peritoneal fibroblasts.

Under pathological conditions, mesothelial cells tend to undergo a process termed mesothelial to mesenchymal transition (MMT) [29]. Also, endothelial-to-mesenchymal transition (EndMT) has been described [30,31]. Both MMT and endMT contribute to the formation and accumulation of myofibroblasts in the peritoneum [32]. The two processes are closely related and start with progressive loss of cells adhesion molecules, such as CK-18, and E-cadherin through induction of the transcriptional repressor Snail1 [29]. As a result of transition

cells acquire fibroblast-specific markers, such as α-smooth muscle actin (α-SMA), and also acquire the capacity to produce ECM components, including fibronectin [33]. Both MMT and endMT can lead to tissue fibrosis, therefore, in our next set of experiments, we investigated the effect of high salt on MMT and endMT. Our result showed that both salt and mannitol increased the expression of epithelial marker ECAD in HPMCs (Figure 5/A), and increase the expression of mesenchymal marker Snail1 and Acta2 in both HPMCs and HUVECs (Figure 5/B-E). Similarly, our *ex vivo* results also demonstrated the salt and osmotic stress-dependent increase of *Sanil1* and *Acta2* (Figure 6/H, I). Both MMT and endMT have been demonstrated to take a part in the development of PF [34-36]. Moreover, the connection between salt intake and mesenchymal transition in the peritoneum also has been suggested. Indeed, the observation of Pletinck et al. found that high salt intake induces MMT demonstrated by cytokeratin and α-SMA double-positive cells in the peritoneal membrane in rats [20]. These results suggest the role of high osmotic environment on both MMT and endMT and are in line with previous data showing that mannitol-induced hyperosmotic stress decreases E-cadherin and increases α-SMA expression in NRK-52E rat kidney epithelial cells [37].

Our observations demonstrated the potential role of peritoneal sodium accumulation in the mesenchymal transformation of both mesothelial and endothelial cells, thereby contributing to the generation of fibroblasts. It is important to note that high salt also can induce expression of CTGF, TGF, and PDGF (Figure 3.) which are considered to be the main inducers of mesenchymal transition in many organs, including the peritoneum suggesting an indirect effect of sodium in MMT and endMT.

Independently of their origin generation of myofibroblasts with the ability to produce and remodeling the extracellular matrix proteins (ECM) is central to fibrosis onset [31]. Our result showed that a high salt environment does not alter the proliferation of HPFC, however, it can stimulate their ECM production, including fibronectin and collagens.

Conclusion

Previous studies showed that high salt intake may lead to excess sodium accumulation in the peritoneum which induces PF and dysfunction [20]. Our study demonstrated that excess sodium may affect different peritoneal cell types in different ways. Overall, our results indicate that a high-salt environment can induce several pathological processes including, inflammatory cytokine and profibrotic growth factor production, mesenchymal transition and activation of peritoneal fibroblasts, that lead to PF and PD technical failure (Figure 7.). Our experimental data further strengthen that, salt restriction in PD patients might preserve peritoneal transport function.

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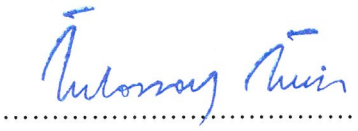

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