SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

STUDY OF THE ANGIOLOGICAL EFFECTS OF RHEOPHERESIS TREATMENT IN DIABETIC FOOT SYNDROME WITH HYPERVISCOSITY

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF KÁLMÁN LAKI

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Introduction

Rheopheresis is an extracorporeal selective double cascade filtration hematotherapy. During the procedure after preliminary plasma separation, the blood plasma flows through a rheofilter which is designed to block different proteins and macromolecules in its pores that are larger than 250-300 kDa. Thus rheopheresis can significantly reduce the blood plasma concentration of plasma constituents that increase blood viscosity and thus pathologically change the haemorheological status. The material of the MONET (Membranefiltration Optimised Novel Extracorporal Treatment) filter is polysulfone produced by Fresenius Medical Care AG & Co. KGAa, which can reduce the concentration of LDL circulating in the blood by 70%, the concentration of cholesterol and fibrinogen by 50%, the triglyceride level by 35-40%. In addition to all this, the treatment can remove a significant proportion of α-2-macroglobulin, von Willebrand factor, and circulating IgM from the circulation. An important and useful additional feature of the treatment is that the levels of HDL, which has a protective effect against atherosclerosis, albumin, which fulfills an important transport function and is responsible for maintaining normal colloid osmotic pressure, and total protein change to a negligible extent as a result of the treatment. The American Society for Apheresis currently recommends the treatment in the therapy of two diseases, which are age-related dry macular degeneration and sensorineural hearing loss. The rheopheresis treatment was used for the first time in Hungary in cooperation of the Intensive Care Unit and the Therapeutic Apheresis Department in Building “C” of the Internal Medicine Institute of the Clinical Center of University of Debrecen. The indication for the first treatments was age-related dry macular degeneration. The clinical results of the new therapeutic modality led to the conclusion that the treatment can also be used in other diseases affecting microcirculation if they are associated with hyperviscosity. Therefore, the treatment was introduced also in the therapy of hyperviscosity-associated, diabetes-induced lower extremity ulcers and diabetic

polyneuropathy resulting from microcirculation damage with ethical approval (IVU/10026-4/2020EKU, ETT-TUKEB).

Diabetic foot syndrome encompasses all lower limb symptoms that may appear as a complication of diabetes mellitus. As a result of macro- and microvascular damage, skin and nail changes may appear, the statics of the legs may change, lower limb ulcers and serious nerve damage may develop. These pathological deviations aggravate each other and aggravate the diabetic foot syndrome. Damage to the microcirculation and destruction of small blood vessels with a diameter of less than 500 μm such as precapillary arterioles and capillaries plays an essential role in the development of the disease and the appearance of all symptoms. The background of the damage is the disrupted mitochondrial processes due to intracellular hyperglycemia and the resulting significant release of reactive oxygen radicals. By inhibiting the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the released superoxide activates all 4 alternative metabolic pathways: the polyol pathway, the non-enzymatic glycation of proteins, the hexosamine pathway, and the protein kinase C pathway. During these mechanisms, extremely large amounts of free radicals are formed, which means a significant intracellular oxidative stress for the cells. As a result of abnormal prooxidant processes, the extracellular matrix is damaged, the hemorheological and antioxidant status changes, the production of harmful vascular mediators increases, and subclinical inflammation is induced.

One of the most sensitive cell types to damage is the endothelial cell layer that lines blood vessels. Diabetic polyneuropathy develops as a result of damage to the vascular network supplying the nerves, the vasa nervorum, and direct neuronal death. Most neuropathic patients initially experience severe, often unbearable pain, which gradually subsides with the gradual destruction of neurons, and the pain is replaced by sock-like numbness starting from the toes. As a result of the sensory damage, isomatrophy soon develops, the sensation of movement, touch and vibration, the transverse and longitudinal arch of the foot changes pathologically, and pathological

pressure points in the lower limbs favorable for diabetic lower limb ulcers are created. Due to neuropathy, patients are often unaware of injuries that appear on an overloaded limb for a long time, because of constant plantar pressure and stenotic circulation, the ulcer is unable to heal, and high blood sugar, immunological abnormalities, and disorders of lymphocyte function all provide an excellent breeding ground for ulcer infection. Ulcers on the lower limb often lead to inevitable lower limb amputation. Almost 85% of non-traumatic limb amputations are preceded by an ulcerative lesion of the lower limb. Reamputation is often required, and the five-year mortality of patients reaches 39-65%.

Due to the complexity of the diabetic foot syndrome, an interdisciplinary approach is necessary during its treatment and every opportunity should be seized in order to reduce the number of lower limb amputations resulting from diabetes. High mortality, expensive medical expenses, long hospital treatment, and a significant deterioration in quality of life represent a serious burden on both the patient and society, which justify extensive research into rheopheresis and similar therapeutic alternatives.

Aims

1. Our goal was to verify the function of rheopheresis treatment to restore hemorheological status and improve microcirculation even in cases of diabetic foot syndrome that did not require macrovascular restoration, or interventional radiological or vascular revascularization is no longer possible, or neuropathy is the dominant symptom. In the various symptoms of the disease, the common cause is hyperviscosity, which appears as a factor that increases damage.

2. We intended to set up an in vitro model, on which we can monitor the effects of diabetes mellitus causing endothelial damage, concerning inflammation and prooxidant effects, and thus determine biochemical markers that describe the effect of rheopheresis in vivo studies.

3. Examination of specific inflammatory cytokines as a function of rheopheresis treatment, to verify the anti-inflammatory effects of the treatment.

4. Complete analysis of cysteine pool which can be used to verify the positive change in glutathione balance as a result of rheopheresis treatment.

Material and methods

Clinical examinations

During the complete angiological examination of the patients, the ankle-brachial index on both sides was determined for each patient in order to investigate the criteria for inclusion in the study, and arterial and venous Color-Doppler examinations of the lower extremities were also performed. We also analyzed the flow curves and examined the plaques narrowing the vessel lumen. The aim of the venous ultrasound examinations was to rule out superficial venous insufficiency as an etiological factor behind the complaints. The superficial venous network was reviewed, and the presence of saphenofemoral reflux and insufficiently functioning perforator veins were ruled out. The examinations were performed using the 5-10 MHz linear vascular transducer of the Philips CX 50 ultrasound equipment.

Patients with neuropathic complaints were subjected to an ENG examination, where we obtained a complete picture of the neurological status of the nerves of the lower limb. In all cases, the ENG examination was performed by a clinical neurophysiologist using Keypoint Clinical System 9031A006401.

In addition to all this, general (blood count, kidney function) and immunological (Anti-beta-2-glycoprotein-I antibodies, anti-cardiolipin antibodies, Lupus anticoagulants, anti-cryoglobulin antibodies, anti-neutrophil cytoplasmic antibodies) laboratory tests were performed. We also examined the blood sugar (HgbA1C) and lipid profile (triglyceride, cholesterol, HDL-C, LDL-C).

Rheopheresis and hyperviscosity

Since the introduction of the therapy, we have used rheopoheresis in 23 patients. 10 patients had diabetic lower extremity ulcers, 10 patients suffered from diabetic polyneuropathy, 4 patients had both symptoms at the same time, and all patients had hyperviscosity. 3 patients had peripherial arterial disease. Hyperviscosity was diagnosed by determining the viscosity of whole blood and plasma, which was done at the Department of Operative Techniques and Surgical Research. The tests were performed using a Hevimet-40 viscometer (Hemorex International Kft., Budapest, Hungary) using the capillary viscometry method. A correction was also made for a hematocrit of 40% using the Mátrai formula.

The patients received rheopheresis treatment on two consecutive days, after infusion of physiological saline before each treatment. Two peripheral veins of the patients were cannulated, and then the blood plasma was separated from blood cells using the Art Universal system. The separated blood plasma flowed through a column containing a MONET (Membrane fitlration Optimized Novel Extracorporal Treatment) (Fresenius SE and Co. KGaA, Bad Homburg vor der Höhe, Germany) filter, and then, together with the shaped elements, was returned to the patients' circulation using the peripheral venous cannula. The rule of 40 ml/kilogram of body weight was used to quantify the plasma to be filtered. Citrate was used as an anticoagulant.

In vitro cell culture

The pathological effects of diabetes mellitus were modeled on HUVEC cell culture, with ethical approval (RKEB/IKEB 317-2012). The umbilical vein was cannulated and then filled with collagenase solution. It was incubated at 37 °C for 20 minutes, then after centrifugation for 8 minutes, the supernatant was aspirated, and the cells were suspended in M199 medium. We incubated the cell culture flasks with a 0.5% gelatin solution for 4-8 hours, on which the cells were spread for adhesion. For

cultivation, a continuous temperature of 37 °C and a 5% CO^2 atmosphere were provided for the cultivation of cells using a Galaxy 170 R incubator (Eppendorf, Hamburg, Germany). After confluency reached a minimum of 80%, cells were passaged at twice the basal area. Adhesion was terminated with trypsin-EDTA solution (Biosera, Nuaille, France) and the reaction was stopped with M11 medium. After centrifugation, another layering was finally performed. After the cell culture, a Flow cytometric analysis was performed once for phenotyping, during which the endothelial cells were marked with fluorophore-conjugated mouse monoclonal antibodies for identification. The analysis identified 97% of the cells as endothelial cells. The cells were then treated with a 30 mM/L glucose solution for 24 hours to simulate intravascular hyperglycemia.

Gene expression studies on the HUVEC model

After the hyperglycemic simulation, the gene expression levels of Il-6, Il-8, TNF-alpha, endothelin convertase enzyme, ET-1, and NO synthase were determined and compared with the control group. Cells were lysed and qRTPCR analysis was performed. We created cDNA with reverse transcriptase (LunaSrcipt RT SuperMix kit-PCR Biosystems, London, UK), and then after cleavage with TaqMan polymerase, the fluorescence of the reporter molecule was detected, thereby determining the cycle number for the detection threshold (Luna Universal Probe qPCR Master Mix -PCR Biosystems, London, UK). GAPDH was used as a housekeeping gene. The relative gene expression change was performed using the LIVAK method.

ROS release investigattion in the HUVEC model

The release of reactive oxygen species was examined by DCDFA staining (2',7'-dichlorofluorescin diacetate) (Sigma-Aldrich, St. Louis, MO, USA). For the investigation the cells were incubated for 6 minutes with a 100 microMol dye solution, and then the fluorescence intensity was measured (excitation = 485 nm, emission = 530

nm). After that we used a Clariostar microplate reader (BMG Labtech, Ortenberg, Germany). Here the results were also compared to a control group.

Examination of antioxidant status in the HUVEC model

The intracellular glutathione concentration of the endothelial cells was also determined, which was performed with the Glutathione Assay Kit (Cayman Chemical, Ann Arbor, Michigan, USA). Endothelial cells were treated with 30 mM glucose solution, and after 0, 6, 12, 24, 48, and 96 h, the cells were deproteinized to measure the concentration of reduced glutathione in the cells compared to the control group treated with a normoglycaemic solution. During the test, the spectrophotometric absorbance spectrum of the sample was determined at 414 nm.

Changes in concentrations of inflammatory markers as a result of rheopheresis treatment

Blood plasma Il-6, Il-8 and TNF-alpha concentrations were determined from the blood samples taken from the patients before and after treatments. Samples taken in sterile vacuum containers containing K-3 EDTA anticoagulant were centrifuged for 15 minutes at 3000 RPM, 4 °Celsius, and then the double sandwich ELISA technique was used. We used Abcam's ELISA Kits (Abcam PLC, Cambridge, UK). We set a standard, created a dilution series, and then created a plate map. The samples were reacted with the antibodies and HRP conjugate, then incubated for 60 minutes at a temperature of 37 °Celsius. After washing five times, the substrates were added, then incubated for another 10 minutes, and finally the reaction was stopped with the STOP solution and measured at 450 nm with a spectrophotometer.

Examination of vasoactive components as a function of rheopheresis treatment

The blood plasma concentrations of Endothelin-1 and Thromboxane B_2 were also determined during the study before and after the treatments. In both cases, the relevant ELISA Kits from Abcam (Abcam PLC, Cambridge, UK) were used, with code ab133030 for Endothelin-1 and ab133022 for Thromboxane-B_2. Following the manufacturer's recommendations, we created a dilution series and washed five times. In the case of endothelin-1 testing, we added 100 microliters of endothelin-1 antibody to the samples and incubated them for 30 minutes. After that, TMB substrate solution was added to them and after another 30-minute incubation time, the absorbance was measured at 450 nm. In the case of thromboxane B_2, 5 microliters of TXB_2 Alkaline Phosphatase Conjugatum and then 200 microliters of pNpp substrate were added to the samples, and after 45 minutes of incubation at room temperature, after the addition of the STOP solution, the absorbance was examined at 405 nm.

Changes in the SOD enzyme activity of red blood cells as a function of rheopheresis treatment

Photochem equipment and ACW Kit (Analytik Jena, Jena, Germany) were used for the determination. For 10 microliters of red blood cells, 1.5 ml of reagent 1, 1 ml of reagent 2 and 25 microliters of reagent 3 were added, according to the manufacturer's recommendation. SOD enzyme (Superoxide Dismutase, Sigma Aldrich, Germany) was used as a standard.

Changes in blood plasma glutathione pool as a function of rheopheresis treatment

A standard mixture (concentration: 10 µg/mL for each compound) was prepared from the standards cysteine, homocysteine, cysteineylglycine, γ-glutamylcysteine, cystine, and glutathione (168149-25 mg, 69453-10 mg, C01666-25 mg, G0903-25 mg, C8630-1 g, and PHR1359-500 mg) from Merck Life Science Ltd. (Budapest, Hungary). N-Acetylcysteine (A15409.14) was obtained from VWR

International Ltd. (Debrecen, Hungary). Briefly, 150 µL of standard mixture was combined with 300 µL of N-ethylmaleimide (NEM) solution (concentration: 100 µg/mL), 1020 µL of water and 30 µL of formic acid solution (0.01%, V/V). The reaction mixture was thermostated at 37 °C for 30 min. After cooling, standard solutions were prepared at the seven concentrations of 0.1, 1, 5, 10, 25, 50, and 100 ng/mL by dilution with water. The analyses were performed using the Dionex Ultimate 3000RS UHPLC system (Thermo Fisher, Waltham, MA, USA) coupled to a Thermo Q Exactive Orbitrap hybrid mass spectrometer equipped with an Acclaim Mixed-Mode HILIC-1 (2.1 × 150 mm, 3-µm particle size) analytical column. The flow rate was maintained at 0.3 mL/min. The column oven and postcolumn cooler temperatures were set to 25 °C ± 1 °C. Samples were thermostated at 25 °C ± 1 °C. The mobile phase consisted of water (A) and methanol (B) (both acidified with 0.1% formic acid). The gradient program was as follows: 0–1 min, 95% A; 1–6 min, →0% A; 6–10 min, 0% A; 10–10.5 min, →95% A; and 10.5–20 min, 95% A. The injection volume was 5 µL. The Thermo Q Exactive Orbitrap hybrid mass spectrometer (Thermo Fisher, Waltham, MA, USA) was equipped with a HESI source. The samples were measured in positive ion mode using the Selected Ion Monitoring (SIM) technique with the following inclusion list ([M + H]+): cysteine-NEM ($C_9H_{15}N_2O_4S$), 247.07525; N-acetylcysteine-NEM ($C_{11}H_{17}N_2O_5S$), 289.08582; homocysteine-NEM ($C_{11}H_{17}N_2O_5S$), 261.09090; cysteineylglycine-NEM ($C_{11}H_{18}N_3O_5S$), 304.09672; γ-glutamylcysteine-NEM ($C_{14}H_{22}N_3O_7S$), 376.11785; cystine ($C_6H_{13}N_2O_4S_2$), 241.03167; glutathione-NEM ($C_{16}H_{25}N_4O_8S$), 433.13931; and glutathione disulfide ($C_{20}H_{33}N_6O_{12}S_2$), 613.15979. The capillary temperature was set to 320 °C, and the spray voltage was 4.0 kV. The resolution was 35,000. The sheath gas flow rate and Aux gas flow rate were 32 AU and 7 AU, respectively. The differences between the measured and calculated monoisotopic molecular masses were less than 5 ppm in each case. The data were acquired and processed using Thermo Xcalibur 4.0 software (Thermo Fisher, Waltham, MA, USA).

Statistical analyses

As a limited statistic for the investigation of inflammatory markers, the pre- and post-treatment values were tested with a paired t-test/Wilcoxon test, a p-value of <0.05 was considered significant. During the statistical analysis of the in vitro results, endothelin-1, thromboxane B2 plasma concentrations, the data were expressed as the average of three experiments ± SEM. Statistical analysis was performed for multiple comparisons, the results were analyzed with ANOVA, and then for repeated measurements, they were analyzed according to the Bonferroni method. The threshold for significance was $p < 0.05$. The values of Il-6, Il-8, TNF-α, ET-1, TXB2, SOD enzyme activity before and after treatment were also compared with Wilcoxon rank sum non-parametric tests. The statistically significant threshold value was also $p<0.05$. Kruskal-Wallis and Wilcoxon tests were used during the statistical analysis of whole blood and plasma viscosity and thiol components. The Shapiro–Wilk test was used to determine whether the measured values were normally distributed. For normal distribution, Welch's t-test was used to test statistical significance. Otherwise, Kruskal–Wallis and Wilcoxon rank sum non-parametric tests were used. The p values were corrected using the Benjamini–Hochberg method. The significance threshold was set at $p<0.05$.

Results

Clinical results of Rheopheresis treatment in diabetic foot syndrome

As a result of the treatments, the patients' whole blood and plasma viscosities were in the normal or near-normal range. Considering the group of polyneuorpathic patients without lower extremity ulcers the subjective complaints of the patients significantly decreased as a result of the rheopheresis treatment, apart from one case. Rheopheresis treatment was given to patients whose complaints did not, or did not decrease sufficiently, despite the drug therapy used (gabapentin and pregabalin). Despite the aggressive pain relief, the patients complained of very severe stabbing, numbing, burning pain, which in several cases was reduced by a single rheopheresis treatment, thus reducing the patients' need for pain killer. In the case of two patients, the motor fiber amplitudes improved by an average of 0.8 mV, in three patients the improvement of the sensory fibers was demonstrated, in these cases the conduction amplitude increased by an average of 2.06 μV. In one case, the conduction speed of the sensory fibers increased by about 17.3 m/s, which represented an increase of 48.3%. In one case, the patient's complaints did not improve, and the ENG after treatment did not show any changes. The results of patients suffering from lower extremity ulcers show that after an average of 2 treatments, leg and leg ulcers began to heal. Partial or complete recovery was achieved in 10 cases. In three cases, we were unable to avoid partial amputation of the patients' lower limbs, but in both cases the problem was caused by the patients' poor compliance, and we initially achieved success with the treatment.

Results of in vitro investigations

Based on the results of in vitro investigations, incubation with a glucose solution with a concentration of 30 mmol/L for 24 hours significantly increases the expression of IL-6, IL-8, and TNF-alpha in endothelial cells. Furthermore, the intracellular mRNA levels of both endothelin-1 and endothelin-converting enzyme

were significantly increased, while the expression of the NO synthase enzyme was significantly inhibited by extreme hyperglycemia. As a result of the 24-hour treatment, the intracellular ROS radical production of the cells increased significantly. Examining the changes in the concentration of reduced glutathione in the endothelial cells, the intracellular glutathione concentration decreases significantly as a result of hyperglycaemia. Considering the function of time, in the first 24 hours comparing the samples with extremely high glucose concentration and the samples of the control group, the measurable difference in the concentrations of reduced glutathione gradually increases. After 24th hour, when the hyperglycemia was eliminated, the difference gradually began to decrease, although it remained significant.

Rheopheresis treatment effects on inflammatory cytokines and vasoactive substances

As a result of the rheopheresis treatment, measurable inflammatory cytokine concentrations in the blood plasma of the patients showed a significant decrease. The measurable value of IL-6 in the blood plasma decreased by an average of 44.35% after the treatments, compared to the values before Rheopheresis (Figure 19). The change was significant in 5 patients. Comparing the blood plasma concentrations before and after treatment with the Wilcoxon paired test, the difference is significant ($p=0.02$). The blood plasma level of IL-8 decreased significantly in 5 cases, by an average of 61.76% due to Rheopheresis (Fig. 20). Based on the Wilcoxon test, the difference is significant ($p=0.0022$) TNF-α decreased to an extreme extent, on average by 78.56%, as a result of the treatments. The decrease was significant in all cases (Figure 21). Based on the Wilcoxon rank sum non-parametric test, the difference is significant ($p=0.022$). When we measured the plasma concentrations of endothelin-1 in 6 out of 7 patients, we found a significant decrease after the treatments, while in the case of Thromboxan-B_2 a small but statistically significant decrease was observed in all 7 patients.

Effects of rheopheresis treatment on SOD enzyme activity and serum levels of total thiol and disulfide components

Although in 5 out of 7 patients, we experienced a large decrease (with an average value of 18.06%), measuring the SOD enzyme activity, in the case of our patient. In No. 2 we experienced a 16% increase while the enzyme activity of patient, while in No. 3 was almost the same (3, 13% increase), so we did not evaluate these test results as clear. The changes in the serum levels of the precursors involved in the glutathione cycle became complexly described by examining the thiol components. The investigations show that as a result of the treatment, the total ($p=0.04$) and protein-bound ($p=0.044$) forms of cysteine decreased significantly, while the free form of gamma-glutamyl-cysteine increased significantly ($p=0.044$). The free homocysteine content also showed a decrease. In the blood plasma concentrations of cystine and cysteinyl-glycine, except for one patient, an inverse proportionality can be discovered.

Discussion

The rapidly increasing prevalence of diabetes mellitus, the severity of complications, and their complexity all justify the introduction of new therapeutic alternatives. The clinical and laboratory results carried out by our research team confirm that rheopheresis, as extracorporeal hematotherapy, has many useful functions in the therapy of lower extremity symptoms arising from microcirculation damage in diabetic foot syndrome. In the first period of our research, we focused on clinical trials. Based on these, the hyperviscosity associated diabetes mellitus significantly worsens the healing tendency of existing lower extremity ulcers, while it increases the symptoms of polyneuropathy and their severity. Rheopheresis treatment can improve microcirculation by normalizing whole blood and plasma viscosity, which is proven by the healing of ulcers and the improvement of nerve functions. The subjective pain sensation of the neuropathic patients decreased, and the ENG tests performed objectively confirmed the beneficial effects of rheopheresis. In the doctoral dissertation,

little is said about the durability of the treatment. The COVID-19 pandemic that broke out in 2019, and the exclusive emergency care that was introduced at that time, significantly complicated the ambulatory follow-up care of our patients, so only generalities can be formulated in this work. According to our experience so far, the hyperviscosity-reducing effect of the performed rheopheresis lasts for 3-6 months after the treatments. In order to increase efficiency, close cooperation of patients, strict dietary restrictions, and adherence to drug therapy are necessary. In the case of patients with ulcers, proper regular wound care and orthotic relief are essential. To sum it all up based on our clinical results, the treatment went far beyond the normalization of the viscosity parameters, so we focused our studies on diabetes-induced endothelial inflammation and its treatment with rheopheresis. Using the in vitro HUVEC cell model, we complexly proved that hyperglycemia induces inflammation, increases vascular mediator production and pathologically changes antioxidant status. Although these findings have already been described in the literature, we considered it essential to examine the pathological processes on a single, complex model, where the processes can be examined in relation to each other. Given that the endothelial cells cannot be examined directly in the case of patients, the biochemical markers determined by simulation made it possible to monitor the treatment. It should be emphasized that instead of protein concentration, gene expression changes were performed during the study, the aim of which was to reveal the long-term damaging effects of diabetes. Based on these, hyperglycemia means significant oxidative stress for endothelial cells, has a significant inflammatory effect, and damages the antioxidant system. There have been many studies on the role of inflammatory cytokines. Based on blood plasma concentration determinations performed before and after rheopheresis, it can be said that the treatments can significantly reduce the amount of Il-6, Il-8 and TNF-alpha in the blood. The molecular weight of these cytokines is in a much smaller range than that which the used MONET filter can filter, so in principle direct filtration of cytokines is not possible. However, the intravascular transport of inflammatory cytokines is carried out by transport proteins, such as alpha-2-macroglobulin. Its molecular weight is high

enough to be unable to pass through the pores of the filter. Based on the investigation of vascular mediators, the concentration of endothelin-1, which has a vasoconstrictor effect, and thromboxane B2, which indicates prothrombotic activity, can also be significantly reduced by rheopheresis, which induces further beneficial effects. Summarizing the antioxidant tests, it can be said that measuring the activity of SOD did not yield significant results, so a more detailed study was necessary. Reduced glutathione, which as a cofactor of glutathione peroxidase is a tripeptide with one of the most significant antioxidant properties, can only perform its function intracellularly, creating a delicate balance. However, our blood tests did not allow direct measurement of reduced glutathione, so our task was to map the entire glutathione cycle and its most important intermediates and precursors. Based on the results of our studies, due to the filtration effects of rheopheresis, the glutathione cycle is clearly shifted towards the synthesis of reduced glutathione. This is determined by the significantly reduced amount of unstable and free-radical-forming cysteine in the blood and its protein-bound form. The treatment also reduces hyperhomocysteinemia, which is one of the best-known independent cofactors of atherosclerosis. On the other hand, free gamma-glutamyl-cysteine, which in itself has antioxidant properties and is also a direct precursor of reduced glutathione, showed a significant increase Based on the parameters determined during this study, the effects of the rheopheresis treatment can be objectively monitored in vivo as well. Based on the test results, it can be stated that rheopheresis can significantly reduce the concentration of pathological inflammatory cytokines, vasoconstrictors and vascular mediators with prothrombotic effects circulating in the blood. Its beneficial effect in the antioxidant status is proven by changes in the concentrations of the extracellular precursor of the glutathione pool. As a result of the treatment, there were significant changes in the concentrations of these precursors that induce an improvement in the antioxidant status.

New scientific results

I. Rheopheresis can effectively reduce whole blood and plasma viscosity in patients with diabetic foot syndrome.

II. The treatment has a beneficial effect in the therapy of diabetic sensorimotor polyneuropathy, it can improve nerve functions and reduce neuropathic pain.

III. The rheopheresis treatment promotes the healing of lower extremity ulcers arising from microvascular damage occurring as part of diabetic foot syndrome in cases where lower extremity vascular surgery or interventional radiological reconstruction is not possible.

IV. Rheopheresis can reduce endothelial inflammation in diabetic foot syndrome by filtering inflammatory cytokines.

V. The treatment reduces the blood plasma concentration of harmful vascular mediators, it also has an antithrombotic effect through the filtration of molecules with prothrombotic effects.

VI. Changes in the concentration of extracellular precursors and intermediates, which play an important role in the glutathione cycle, prove that the treatment has an antioxidant effect and can reduce the extreme oxidative stress that occurs during diabetes mellitus and its harmful effects.

Publication list

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Subject: PhD Publication List

Candidate: Kristóf Gál
Doctoral School: Kálmán Laki Doctoral School

List of publications related to the dissertation

1. **Gál, K.**, Pesti-Asbóth, G., Vass, M., Biró, A., Markovics, A., Homoki, J., Fidler, G., Paholcsek, M., Cziáky, Z., Németh, N., Gálné Remenyik, J., Soltész, P.: Monitoring and recovery of hyperglycaemia-induced endothelial dysfunction with rheopheresis in diabetic lower extremity ulceration with hyperviscosity.
   Diabetes and Vascular Disease Research. 19 (6), 1-14, 2022.
   DOI: http://dx.doi.org/10.1177/14791641221131788
   IF: 3.541 (2021)
2. **Gál, K.**, Veres, K., Halmi, S., Bozoki-Beke, K., Fekete, K., Homoki, J., Gálné Remenyik, J., Baráth, B., Varga, Á., Németh, N., Soltész, P.: The effect of rheopheresis treatment on the cytokine profile in diabetic foot syndrome with hyperviscosity in the aspect of clinical changes: a preliminary study.
   Clin. Hemorheol. Microcirc. 80 (2), 117-125, 2022.
   DOI: http://dx.doi.org/10.3233/CH-211188
   IF: 2.411 (2021)

List of other publications

3. Soltész, P., **Gál, K.**, Vass, M.: Rheopheresis.
   Focus Med. 24 (1), 17-19, 2022.
4. Soltész, P., Németh, N., **Gál, K.**, Vass, M., Diószegi, Á., Mechler, F., Fekete, K., Somogyi, V., Módis, L.: A rheopheresiskezeléssel szerzett első hazai tapasztalatok.
   Orv. hetil. 162 (10), 375-382, 2021.
   DOI: http://dx.doi.org/10.1556/650.2021.31889
   IF: 0.707
5. Laczik, R., **Gál, K.**, Soltész, P.: Cardiovascularis betegségek és a COVID-19 fertőzés kapcsolata.
   Érbetegségek. 28 (1), 5-8, 2021.

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6. Diószegi, Á., Vass, M., Flaskó, A., **Gál, K.**, Mechler, F., Káplár, M., Csiba, L., Soltész, P.: Analysis of the Correlation between Microvascular Involvement and Neuropathy in Association with Metabolic Disorders in Case of Diabetic Leg Syndrome.
Annals atherosc. res. 1 (2), 1-6, 2018.

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