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Study of the antioxidant status in patients with secondary lymphedema of the lower extremities under conservative treatment

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Abstract

Aim. To assess the antioxidant status in patients with secondary lymphedema of the lower extremities who undergo different types of conservative treatment.

Methods. The study included 90 patients with secondary lymphedema of the lower extremities and 30 healthy volunteers. Group 1 participants (n=30) received compression therapy and Vitamin E at a dose of 400 IU/day, group 2 participants (n=30) compression therapy and a micronized purified flavonoid fraction 1000 mg/day, group 3 (n=30) compression therapy alone. Group 4 (n=30) comprised healthy volunteers. The level of malondialdehyde, the activity of superoxide dismutase, glutathione peroxidase, catalase, and the level of non-protein thiols (SH-groups) were determined at inclusion in the study and then after 1 and 3 months.

Results. In patients with secondary lymphedema, the initial level of glutathione peroxidase was higher by 768.22%, catalase — by 420.5%, malondialdehyde — by 60%, and the level of SH-groups was lower by 65,71% compared with the group of volunteers. In the first group, there was a significant decrease of 36.1% in the level of superoxide dismutase and a significant increase of 89.9% in the level of glutathione peroxidase at the end of therapy when compared with the level after 1 month. In the second group, catalase level significantly increased — by 33.3%, superoxide dismutase by 17.6%, and glutathione peroxidase by 61.3% compared to baseline values. The biochemical indicators of the endothelium significantly increased when using a combination of micronized purified flavonoid fraction and elastic compression in comparison with elastic compression alone and a combination with Vitamin E. In the third group, there were no significant differences in the levels of biochemical indicators of endothelial function.

Conclusion. Increased formation of lipid peroxidation products along with a decrease in the activity of antioxidant systems was revealed in patients with lower extremity secondary lymphedema compared with healthy volunteers; the most effective therapy aimed at correcting endothelial cell dysfunction is the use of micronized purified flavonoid fraction and elastic compression.

Keywords: lymphedema, endothelial dysfunction, endothelium, oxidative stress, antioxidants, lipid oxidation, MPFF.

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Background. Lymphedema (LE) is a chronic pathology characterized by persistent edema due to the accumulation of high-protein fluid in the interstitial space. It occurs due to impaired outflow in the lymphatic system [1–4]. The development of LE is facilitated by acquired structural or functional defects of the lymphatic system [5].

Until a certain time, lymph outflow disorders were associated only with structural changes in the lymphatic system, such as with damage to lymphatic capillaries, vessels, and nodes. However, these changes do not reflect the functional state of the lymphatic system and prevent studying the methods of pathogenetic correction. That is why at present more and more attention of researchers is paid to the endothelium functional state (EFS) during the development of lymphatic system diseases. However, it is still unclear how the EFS of lymphatic vessels changes in pathology [3, 6, 7].

According to modern concepts, vascular system diseases, including lymphatic disorders, are caused by endothelial dysfunction [8, 9]. The pathophysiological mechanisms of the onset of endothelial dysfunction include the processes that develop under oxidative stress with increased generation of reactive oxygen species along with impaired func-

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tioning of the antioxidant system. According to the literature, regional oxygen deprivation develops in lymphedematous tissue with LE, followed by a reperfusion period, then the formation of reactive oxygen species increases, and the process of lipid peroxidation (LPO) in lymphatic vessels and interstitial tissue accelerates. The formation of LPO products in combination with a decrease in the antioxidant system activity leads to damage to endothelial cells and the development of a proinflammatory status [10].

Despite research on the lymphatic system's morphological and functional state, many issues of diagnostics and treatment of patients with lymphatic edema of the extremities remain unresolved [11].

The study of the pathophysiological mechanisms of the development and progression of diseases and conditions to create and implement methods for their correction is an urgent issue in contemporary lymphology. It is necessary to study sound approaches to correcting pathogenetic mechanisms based on evidential medicine, influencing the LE pathogenesis directly or indirectly [12].

To date, there are no studies that would demonstrate the dynamic changes in the LPO process at different times from the start of conservative treatment of LE. Major scientific communities assign the leading role in the treatment of LE to systematic conservative methods. The modern concept of these methods includes the complex application of physiotherapeutic, podiatric, rehabilitation, and pharmacotherapeutic methods [11]. Based on the pathogenetic foundations of the development and progression of secondary LE, the need for a targeted pharmacological antioxidant effect on the formation of free radicals is beyond doubt. According to the literature, bioflavonoids are pharmacological agents with antioxidant effects. In clinical practice, micronized purified flavonoid fraction (MPFF) preparations are used more often [13].

The study aimed to investigate the antioxidant status in patients with secondary LE of the lower extremities with various types of conservative treatment.

Materials and methods. The study was conducted within the research plan of the Ryazan State Medical University of the Ministry of Health of Russia, at the department of cardiovascular, X-ray endovascular, operative surgery, and topographic anatomy in 2019–2020. The study was registered on the ClinicalTrials.gov platform with an identifier NCT04360889 and approved by the local ethics committee of the Ryazan State Medical University (protocol No. 2 dated 10/08/2019).

The study included 120 participants aged 31 to 85 years, distributed later into four equal groups. All patients underwent examination and treatment at the Regional Consultative and Dispensary Polyclinic of the Regional Clinical Cardiological Dispensary.

Inclusion criteria for the study were secondary LE of the lower extremities of stages I–II according to M. Foeldi in patients of both genders.

Criteria for exclusion from the study were chronic venous diseases, venous thromboembolic complications, chronic diseases of the lower limb arteries, the history of an infectious disease within 3 months before inclusion in the study, diabetes mellitus, and its complications, decompensated heart, renal, and pulmonary failure.

A total of 90 patients with secondary LE of the lower extremities of stage II, meeting the inclusion criteria, underwent the envelope randomization procedure, after which they were distributed into three study groups. Group 4 consisted of 30 volunteers without pathology of the lymphatic and venous systems.

– Participants of group 1 (n = 30) received conservative therapy for 3 months (the 3rd class elastic compression and a drug with antioxidant activity, namely the reference antioxidant vitamin E, 400 IU/day).

– Participants of group 2 (n = 30) of the study received conservative therapy for 3 months of the follow-up (the 3rd class elastic compression and a drug with antioxidant endotheliotropic activity MPFF, 1000 mg/day).

– Participants of group 3 (n = 30) received only compression therapy (3rd class stockinette) for 3 months.

– Group 4 (n = 30) included apparently healthy volunteers without clinical data on the presence of pathology of the lymphatic and venous systems.

Patients of groups 1, 2, and 3 received compression therapy (compression class 3) at least 2 months before the start of follow-up and throughout the entire study period. To confirm the diagnosis, the case history was collected from all participants in the study, physical diagnostics were performed, and general clinical and special research methods were used (ultrasound examination of the veins of the lower extremities and soft tissues). The study was conducted in accordance with the principles of good clinical practice.

In patients meeting the inclusion criteria, 12 ml of blood was taken from the veins of the anterior ulnar region in the morning in the fasted state. Then, blood serum was obtained by centrifugation at a 4°C for 15 min at a speed of 3000 rpm. After that, the EFS biochemical markers were determined in the laboratory. They were determined at the time of enrollment in the study and then after 1 and 3 months. The EFS biochemical markers include the

Table 1. Comparison of biochemical markers of the endothelium functional state in apparently healthy people and patients with secondary lymphedema (LE) of the lower extremities before treatment

Indicator	Volunteers (n = 30), M ± m	Patients with secondary LE of the lower extremities (n = 90), M ± m	p
Superoxide dismutase, U/ml	0.03±0.005	0.03±0.009	0.89
Glutathione peroxidase, ng/ml	4.28±1.38	37.16±23	0.00077*
Catalase, ng/ml	0.073±0.037	0.38±0.11	0.0000001*
Malondialdehyde, ng/ml	229.5±6.8	368.32±10.57	0.0000001*
-SH- (non-protein), μmol/ml	0.35±0.12	0.12±0.02	0.0000001*

Note: * $p < 0.05$.

LPO end product malondialdehyde (MDA) and indicators of the enzymatic component of the antioxidant system, namely superoxide dismutase (SOD), glutathione peroxidase (GP), and catalase (CAT), as well as reduced non-protein thiols (-SH-groups) in blood serum.

The concentration of all indicators of the enzymatic component was determined using a competitive enzyme immunoassay. MDA and CAT were determined using USCN Life Science Inc. (catalog numbers CEA597GE and SEC418Hu, respectively). GP was determined using a kit from the supplier Abfrontier, Republic of Korea, catalog number LF-EK0110. The kit manufacturer for determining the total SOD was Cayman Chemical Company, and the catalog number was 706002. The content of SH-groups was determined on a Stat Fax 2100 spectrophotometer for plates (Awareness Technology, USA) by the reaction of the sulfhydryl group with Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic) acid]. The method involved preliminary mixing of the sample with chilled 5% trichloracetic acid. The levels of MDA, CAT, and GP were expressed in ng/ml, the concentration of SOD was presented in U/ml, and the level of SH-groups was presented in μmol/ml.

The results were analyzed using Statistica 13 software for statistical data processing. The Shapiro–Wilk test determined the type of data distribution. All groups were of normal distribution. Statistically significant intergroup differences were determined by Student's t-test and analysis of variance. The critical level of significance of the difference between the compared indicators was $p < 0.05$.

Results and discussion. The study participants were 31 to 85 years of age. The average age of the subjects was 62.18 ± 3.41 years in group 1, 58.14 ± 2.05 years in group 2, 60.10 ± 3.45 years in group 3, and 56.23 ± 4.08 years in group 4. The groups were comparable by age ($p \geq 0.05$). Among all study participants, women were present in 100% of cases.

Analysis revealed the etiological factors of secondary LE, namely trauma (open fractures of the bones of the lower extremities) in 30.2% of patients, surgical interventions (arthroplasty of the knee joints) in 24.3% of patients, and infection (recurrent erysipelas) in 45.5% of cases.

When comparing the concentration of EFS biochemical markers in volunteers and patients with secondary LE of the lower extremities, four indicators were significantly lower in volunteers, namely GP, CAT, MDA, and reduced non-protein thiols (-SH-). In patients with secondary LE before treatment, the GP level was higher by 768.22%, the CAT level was higher by 420.5%, the MDA level was higher by 60%, and the level of SH-groups was lower by 65.71% ($p < 0.05$) compared with the values of a group of volunteers. These data are presented in Table 1.

In group 1, during the study period, patients showed a significant decrease in the SOD level at the end of therapy by 36.1% ($p = 0.027$) compared with the level after 1 month from the start of treatment. The level of GP increased significantly after 3 months from the start of follow-up by 89.9% ($p = 0.032$) compared with the value after 1 month. Table 2 presents the comparison of biochemical markers of EFS in patients at different times from the start of treatment.

The changes in levels of EFS markers in group 2 of the study in patients who took the MPFF drug and received compression treatment are also presented in Table 2. The study revealed a statistically significant increase in the level of GP after 1 month from the start of the drug intake by 61.3% ($p = 0.00026$) compared with the initial level. In addition, when determining the CAT level, a statistically significant increase in its activity was detected compared with the initial value by 33.3% after 1 month ($p = 0.0075$) and by 27.37% after 3 months ($p = 0.0001$).

When determining the SOD level, a significant increase of 17.6% in the marker level after 1 month was registered ($p = 0.0359$) compared with the initial level.

The content of end products can determine LPO activity, that is, MDA. After 1 month from the start

Table 2. Comparison of biochemical markers of the functional state of the endothelium in patients of all groups at different times from the start of treatment

Indicator	Upon enrollment (V0), M ± m	After 1 month (V1), M ± m	After 3 months (V2), M ± m	$p_{V0–V1}$	$p_{V0–V2}$
Group 1					
Malondialdehyde, ng/ml	268.68±65.10	279.74±67.30	290.79±61.30	0.0824	0.1288
Glutathione peroxidase, ng/ml	14.28±13.35	11.99±7.58	22.77±17.07	0.4239	0.0319*
Catalase, ng/ml	0.21±0.21	0.19±0.16	0.19±0.12	0.6828	0.7850
Superoxide dismutase, U/ml	0.04±0.01	0.03±0.01	0.03±0.01	0.6497	0.0327*
Group 2					
Malondialdehyde, ng/ml	368.56±1.88	365.1±10.7	368.2±2.72	0.2826	0.6832
Glutathione peroxidase, ng/ml	45.34±16.86	73.14±8.36	69.85±7.21	0.00026*	0.0531
Catalase, ng/ml	0.37±0.05	0.492±0.13	0.470±0.05	0.0075*	0.0001*
Superoxide dismutase, U/ml	0.03±0.01	0.04±0.01	0.04±0.002	0.0359*	0.2649
Group 3					
Malondialdehyde, ng/ml	224.43±6.09	221.21±8.31	222.94±11.44	0.5020	0.5664
Glutathione peroxidase, ng/ml	4.61±2.37	4.84±1.83	6.76±2.61	0.5224	0.1211
Catalase, ng/ml	0.08±0.04	0.1±0.04	0.17±0.22	0.3328	0.3088
Superoxide dismutase, U/ml	0.03±0.01	0.03±0.005	0.03±0.01	0.5005	0.2190

Note: *$p < 0.05$.

of the study, the MDA level decreased by 1% in comparison with the indicator before treatment. During the study period, there were no significant differences in this group for this marker.

In group 3, there were no significant differences in the levels of EFS biochemical parameters. Table 2 also presents the changes with time of EFS biochemical markers in patients of this group at different times from the start of treatment.

When comparing the concentration of EFS indicators in patients of the test groups 3 months after the start of therapy, the advantage of complex pharmacotherapeutic (MPFF) and compression treatment (group 2) was noted compared with compression therapy only (group 3) and the use of vitamin E (group 1). All four EFS indicators were significantly higher in the group 2 after 3 months from the start of the study, compared with the group 1 (SOD ($p = 0.005485$), GP ($p = 0.000001$), MDA ($p = 0.000163$), and CAT ($p = 0.000001$)). The indicators of SOD ($p = 0.020222$), GP ($p = 0.000001$), MDA ($p = 0.000001$), and CAT ($p = 0.000247$) were significantly higher compared with the group 3.

According to the study results, the indicators of three biochemical markers of EFS (GP ($p = 0.00077$), CAT ($p = 0.0000001$), and MDA ($p = 0.0000001$)) were significantly higher in patients with secondary LE compared with apparently healthy volunteers. These results are associated with a compensatory increase in the level of enzymes of the antioxidant system (GP, MDA, CAT) and the secondary product of LPO (MDA) in patients with secondary LE of the lower extremities.

The results indicate an increased synthesis of reactive oxygen species and acceleration of LPO processes in patients with secondary LE. Evidently, the increased production of reactive oxygen species and LPO products in the edematous tissues of patients is so great that LPO products can be detected in the circulating blood. In addition, the literature describes cases of an increase in the MDA level in the blood of patients with end-stage renal failure [14].

The results obtained are in agreement with the literature data. So, according to W.G. Siems et al., who studied oxidative stress in LE patients, the level of the LPO specific aldehyde marker MDA was approximately 3 times higher in the blood serum of LE patients in comparison with the control group [10]. Furthermore, according to M. Ohkuma, the level of lipid peroxides, the primary product of LPO, was increased in the dermis of patients with LE of the lower extremities [15].

Cysteine is especially sensitive to oxidation; it serves as a constituent of proteins (protein thiols), as well as non-protein sulphydryls (non-protein thiols), most of which are accounted for by glutathione [16]. Thus, a decrease in the level of reduced thiols indicates the formation of -S-S-bonds between glutathione molecules under the action of GP and activation of the antioxidant defense link.

In group 1, the SOD level decreased significantly by 36% ($p = 0.027$) after 3 months from the start of therapy, compared with the SOD level after 1 month. Also, the GP level after 3 months from the start of follow-up increased by 89.9% ($p = 0.032$) compared with this indicator after 1 month. Thus, the results obtained in this study group were associated with the intake by the patients of one of the key components of the non-enzymatic component of the antioxidant system, namely the vitamin E, which can regulate the LPO process independently.

Vitamin E is one of the non-enzymatic antioxidants that serve as scavengers ("eliminators") of reactive oxygen species. Its antioxidant effect is based on the inhibition of lipid oxidation. The LPO process consists of three stages: initiation, elongation, and termination (chain breaking). Prooxidants, such as a hydroxyl radical, scavenge allylic hydrogen to form a carbon-centered lipid radical at the initiation stage. The latter interacts with oxygen in the elongation phase, resulting in the formation of a lipid peroxide radical that binds hydrogen from another lipid molecule, resulting in the formation of a completely different new molecule and lipid hydroperoxide [17]. Finally, at the chain-breaking stage, antioxidants, such as vitamin E, create non-radical products due to which LPO by-products do not accumulate.

In cells, tocopherol is incorporated into membranes where it is concentrated. Consequently, when using vitamin E, cell membranes are more resistant to the action of prooxidants. Therefore, reactive oxygen species may not penetrate the cell but inactivate on the membrane, and as a result, intracellular enzymatic antioxidant systems are not activated (GP and SOD).

It is noteworthy that with a prolonged course of oxidative stress, the antioxidant protection from vitamin E turns out to be insufficient, which leads to the activation of GP by month 3 from the start of treatment. GP is known to catalyze the reaction of H_2O_2 inactivation $2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$. Consequently, the activation of GP decreases the level of hydrogen peroxide, and as a result, there is no activation of CAT, and the reaction $2H_2O_2 = 2H_2O + O_2$ forms no O_2. O_2 is a source of superoxide anion, which functions as a substrate for SOD; therefore, GP activation leads to a decrease in the amount of H_2O_2 and superoxide anion, and therefore the SOD level in patients' decreases by the month 3 of treatment.

During the course of treatment of patients with secondary LE of the lower extremities in group 2 with the use of MPFF and compression therapy, the main biochemical markers of EFS change. The levels of all three markers of the enzymatic component of the antioxidant system (GP, CAT, and SOD) increased significantly after 1 month from the start of follow-up in comparison with the baseline values, and a significant increase in CAT was registered after 3 months compared with the baseline indicators. The comparison of the concentration of EFS indicators in patients of the groups 1, 2, and 3, 1, and 3 months after the start of therapy, the advantage of complex pharmacotherapeutic treatment (MPFF), and compression treatment (group 2) was noted. MPFF has endotheliotropic and antioxidant effects and activates a non-enzymatic component in the antioxidant system

Thus, further study of changes in EFS during various types of treatment of secondary LE of the lower extremities has its prospects in the pathogenetic correction of this disease and remains an urgent issue for modern angiology and lymphology.

According to the research results, a patent was obtained for invention No. 2720815, "Method for correction of endothelial dysfunction in patients with secondary LE of the lower extremities."

CONCLUSIONS

1. The study of the antioxidant status in patients with secondary lymphedema of the lower extremities with various types of treatment expands the fundamental understanding of the pathogenesis of the disease under study. It contributes to the development of lymphotropic antioxidant trend in the treatment of lymphedema, which can be added to systematic conservative methods in the treatment of this disease.

2. Based on the study results, the conclusion can be made on the increased formation of reactive oxygen species and the acceleration of LPO processes in patients with secondary lymphedema compared with healthy volunteers.

3. The study reveals a significant increase in the levels of four indicators of the EFS (SOD, GP, CAT, and MDA) with the use of complex conservative treatment as a combination of MPFF and compression therapy compared with compression treatment without pharmacotherapy and the use of vitamin E with the compression therapy.

4. MPFF has endotheliotropic and antioxidant effects in relation to patients with acquired lymphatic pathology of the lower extremities.

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