

## The role of recovered thiols in venous endothel adaptation in case of autovenous reconstruction of lower limb arteries

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### ABSTRACT

**Aim.** To evaluate the role of thiol status and its correlative relationship with the level of nitric oxide metabolites and vascular endothelial growth factor A (VEGF-A) in the blood plasma of patients with critical lower limb ischemia (CLI) after autovenous reconstructions of femoropopliteal segment's arteries in the setting of venous endothelium of the arterial bed.

**Materials and methods.** 54 patients with critical lower limb ischemia had been examined and divided into groups: synthetic prosthesis, *in situ* autovenous bypass procedure and reversed vein autovenous bypass procedure. Peripheral venous blood was taken on the 1<sup>st</sup> and 10th days, and in 1, 3 and 6 months after the operation. Nitric oxide metabolites level was examined with photocolometric method by reaction with Griess reagent on a microplate analyzer (Awareness Technology, USA). VEGF-A number estimation was done by ELISA test (PersonalLab, Italy) with the use of Human VEGF-A Platinum ELISA. The level of thiol (SH-) groups was estimated with the use of Ellman's reagent (SERVA, Germany) on spectrophotometer (Saint-Petersburg, Russian Federation).

**Results.** The concentration of VEGF-A and the level of SH-groups increase on the 10th day and after 1 month in the group of patients operated on using a synthetic prosthesis. The level of NO metabolites, the concentration of VEGF-A, and the content of SH-groups increase statistically significantly and then decrease to the initial values in the group of patients operated on by reversed vein autovenous bypass procedure. In the group of patients operated on by the *in situ* method, the level of nitric oxide metabolites increases, the concentration of VEGF-A increases on the 10th day, the level of SH-groups increases, and a positive correlation was found between the content of SH-groups and the concentration of VEGF-A.

**Conclusion.** NO metabolites contribute to the build-up of SH groups and VEGF-A in patients operated by the "reversed vein" method, and in patients in the *in situ* group, the concentration of VEGF-A and the level of SH groups are not affected, which may be of clinical importance when prescribing NO donors.

The revealed patterns of change in the level of recovered thiols, nitrogen oxide metabolites, VEGF-A in combination with analysis of early and late postoperative complications make it possible to conclude the advantage of autovenous reconstruction of the femoropopliteal segment due to functional adaptation of the venous endothelium compared to the group of patients, operated on by the method of "synthetic prosthesis". Method *in situ* on biochemical and angiological indicators proved to be more favorable in terms of the clinical course, than the "reversed vein" method.

**Key words:** nitric oxide metabolites, vascular endothelial growth factor, protein and nonprotein thiol groups, autovenous reconstruction of lower limbs arteries, reperfusion endothelial injury.

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## Роль восстановленных тиолов в адаптации венозного эндотелия при аутовенозной реконструкции артерий нижних конечностей

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### РЕЗЮМЕ

**Цель.** Оценить изменения тиолового статуса, выявить корреляционные связи между содержанием SH-групп и уровнем метаболитов оксида азота (NO), васкулоэндотелиальным фактором роста А (VEGF-A) плазмы крови пациентов с критической ишемией нижних конечностей (КИНК) после аутовенозных реконструкций артерий бедренно-подколенного сегмента в условиях артериального русла.

**Материалы и методы.** Обследованы 54 пациента с КИНК, которые разделены на три группы: шунтирование с использованием синтетического протеза, аутовенозное шунтирование по методу реверсированной вены и аутовенозное шунтирование по методу *in situ*. Забор периферической венозной крови производили на 1-е, 10-е сут, через 1, 3 и 6 мес после операции. Уровень метаболитов оксида азота оценивали фотоколориметрическим методом по реакции с реактивом Грисса на микропланшетном анализаторе (Awareness Technology, США). Определение концентрации VEGF-A осуществлялось путем иммуноферментного анализа (Personal Lab., Италия) с использованием Human VEGF-A Platinum ELISA. Содержания тиоловых (SH-) групп определяли с помощью реактива Элмана (SERVA, Германия) на спектрофотометре СФ-2000 (г. Санкт-Петербург, Россия).

**Результаты.** Концентрация VEGF-A и уровень SH-групп возрастают на 10-е сут, через 1 мес в группе пациентов, оперированных с использованием синтетического протеза. Уровень метаболитов NO, концентрация VEGF-A, содержание SH-групп статистически значимо возрастают, а затем снижаются до исходных значений в группе пациентов, оперированных по методу реверсированной вены. В группе пациентов, оперированных по методу *in situ*, уровень метаболитов оксида азота повышается, концентрация VEGF-A увеличивается на 10-е сут, уровень SH-групп возрастает и выявлена положительная корреляционная связь между содержанием SH-групп и концентрацией VEGF-A.

**Заключение.** Метаболиты NO способствуют нарастанию SH-групп и VEGF-A у пациентов, оперированных по методу реверсированной вены, а у пациентов в группе *in situ* не влияют на концентрацию VEGF-A и уровень SH-групп, что может иметь клиническое значение при назначении доноров NO. Выявленные закономерности изменения уровня восстановленных тиолов, метаболитов оксида азота, VEGF-A в совокупности с анализом ранних и поздних послеоперационных осложнений позволяют сделать вывод о преимуществе аутовенозной реконструкции бедренно-подколенного сегмента за счет функциональной адаптации венозного эндотелия по сравнению с группой пациентов, оперированных с использованием синтетического протеза. Метод *in situ* по биохимическим и ангиологическим показателям оказался более благоприятным с точки зрения клинического течения, чем метод реверсированной вены.

**Ключевые слова:** метаболиты оксида азота, васкулоэндотелиальный фактор роста, белковые и небелковые тиоловые группы, аутовенозная реконструкция артерий нижних конечностей, реперфузионное повреждение эндотелия.

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## INTRODUCTION

The use of an autovenous conduit from the great saphenous vein (GSV) remains an acceptable method of open reconstructive vascular surgery. Currently, autovenous bypass is performed using the reverse vein method and the *in situ* method. The discussion about the advantages and disadvantages of both methods has not stopped for more than half a century: will the transformation of the autovein take the path of adaptation or maladjustment?

Published data describes a proposal on the possibility of considering the concentration of SH-groups as an indicator of the adaptive capabilities of the organism [1]. Since the 1960s, a large number of clinical studies have been performed in which a decrease in the concentration of SH-groups in the blood serum of patients with various diseases was obtained: cardiovascular disorders [2], diabetes mellitus [3], chronic renal failure [4], alcoholic cirrhosis [5] and other pathology [6]. The dynamics of changes in SH-groups against the background of autovenous reconstructions of the arteries of the lower extremities under conditions of reperfusion damage to the endothelium may turn out to be a marker for predicting the adaptive transformation of an arterialized vein.

It is known that the most important and early marker of intimal damage is endothelial dysfunction associated, first of all, with the inhibition of the production of nitric oxide (NO), the most important regulator of vascular tone [7, 8]. Nitric oxide, due to its functions, is a universal angioprotective substance on which structural changes in the vascular wall depend [9, 10]. It has been proven that NO helps to slow down the formation of neointima in the area of surgical anastomoses of an artery with a synthetic prosthesis in patients after surgery [11], therefore, it was suggested that treatment with drugs that stimulate the production of NO may have an inhibitory effect on the development of restenosis after surgery [12], acting as one of the mechanisms of long-term compensation of blood circulation. Since NO is a “short-lived” molecule, many of its protective properties are associated not only with direct action, but also with the endogenous protective systems activated by it, in

particular with the activation of vasculoendothelial growth factor (VEGF) [13].

In this regard, it is relevant to study the thiol status and identify correlations between the content of SH-groups and the level of metabolites of nitric oxide, VEGF-A in blood plasma in combination with the analysis of early and late complications in patients with critical lower limb ischemia (CLI) after autovenous reconstruction of the arteries of the femoral-popliteal segment to assess the degree of adaptation of the venous endothelium in the settings of the arterial bed.

## MATERIALS AND METHODS

The study included 54 patients with CLI (III–IV stage according to the Pokrovskiy – Fontaine classification), of whom there were 48 men (88.9%) and 6 women (11.1%). The average age was  $(64.6 \pm 6.6)$  years. All patients gave informed consent to participate in the study and to use their bioassay. The patients were divided into three groups depending on the type of graft used for revascularization of the arteries of the femoral-popliteal segment: group A – synthetic prosthesis, group B – reversed vein and group C – *in situ* autovenous bypass grafting. Conical prostheses made of 4/8 mm polytetrafluoroethylene were used as a synthetic graft in all cases. In patients with autovenous revascularization, the ipsilateral great saphenous vein was used in all cases. Clinical groups were matched by gender, age, stage of the disease, comorbidity, initial minimum diameter of GSV, Rutherford outflow score (Table 1).

Post-operative peripheral venous blood withdrawal for evaluation of the examined biochemical indicators had been done on days 1 and 10, and on months 1, 3 and 6.

The level of nitric oxide metabolites (the total concentration of nitrates and nitrites) was determined by the photocolometric method by the development of color in the diazotization reaction with sulfanilamide nitrate, which is part of the Griess reagent (NevaReaktiv, Russia). Nitrites diazotize sulfanilamide, and the resulting substance reacts with azo coupling with naphthylethylenediamine to form a pink compound, the color intensity of which is proportional to the total concentration of nitrites and nitrates in the sample [14].

Table 1

| Clinical characteristics of study groups                        |                      |               |             |
|---|----------------------|---------------|-------------|
| Parameter, unit of measure                                      | Group A              | Group B       | Group C     |
| Number of patients, <i>n</i>                                    | 18                   | 18            | 18          |
| Age, years  | 64.15 ± 6.5          | 64.9 ± 6.5    | 65.6 ± 6.9  |
| Male, <i>n</i> (%)  | 16 (88.9 %)          | 16 (88.9 %)   | 16 (88.9 %) |
| Female, <i>n</i> (%)  | 2 (11.1 %)           | 2 (11.1 %)    | 2 (11.1 %)  |
| <i>Disease state on Pokrovskiy – Fontaine classification</i>    |                      |               |             |
| Stage III, <i>n</i> (%)   | 11 (61.1 %)          | 10 (55.6 %)   | 11 (61.1 %) |
| Stage IV, <i>n</i> (%)  | 7 (38.9 %)           | 8 (44.4 %)    | 7 (38.9 %)  |
| <i>Comorbidities</i>  |                      |               |             |
| Ischemic Heart disease, <i>n</i> (%)                            | 12 (66.7%)           | 11 (61.1%)    | 14 (77.8%)  |
| Hypertensive disease, <i>n</i> (%)                              | 15 (83.3%)           | 16 (88.9%)    | 18 (100%)   |
| Cerebrovascular diseases, <i>n</i> (%)                          | 4 (22.2%)            | 3 (16.7%)     | 5 (27.8%)   |
| <i>Base Anatomic-Morphological Characteristics</i>              |                      |               |             |
| Great saphenous vein diameter on ultrasound duplex scanning, mm | -                    | 3.5 ± 0.6     | 3.7 ± 0.7   |
| Condition of outflow tract on Rutherford, points                | 6.1 ± 1.1            | 6.7 ± 1.4     | 6.5 ± 1.6   |
| <i>Type of reconstruction of infrainguinal segment arteries</i> |                      |               |             |
| Method  | Synthetic Prosthesis | Reversed vein | in situ     |
| Above knee joint cleft  | 13 (72.2 %)          | 10 (55.6 %)   | 13 (72.2 %) |
| Below knee joint cleft  | 5 (27.8 %)           | 8 (44.4 %)    | 5 (27.8 %)  |

The color intensity was assessed in the visible spectral region with registration on a StatFax 3200 microplate analyzer (Awareness Technology, USA) at a wavelength of 540 nm and expressed in nmol/mg protein.

Determination of the active form of human vascular endothelial growth factor A (VEGF-A) was carried out by enzyme immunoassay on Personal Lab units (Italy) using Human VEGF-A Platinum ELISA human VEGF-A (BMS277/2) (BioChimMac, Moscow, Russia). The intensity of the color measured at 450 nm is directly proportional to the concentration of VEGF-A present in the samples. The VEGF-A concentration in the samples was determined using a standard curve and expressed in pg/ml.

The level of intracellular and extracellular reducing agents was assessed by the change in the content of thiol (SH-) groups, which were determined using Ellman's reagent on an SF-2000 spectrophotometer (St. Petersburg, Russia) [15]. The reaction of sulfhydryl (thiol) groups with Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid, SERVA, Germany) breaks the disulfide bond in the reagent and forms 2-nitro-5-thiobenzoic acid, which at alkaline pH in water turns into a quinoid form and has a bright yellow color. The resulting thionitrophilic anion is quantified on a spectrophotometer at 412 nm. To calculate the level of reduced thiols in blood plasma (in μmol/ml), the molar extinction coefficient of the Ellman reagent was taken as 14150 M<sup>-1</sup> cm<sup>-1</sup>.

The statistical analysis of the results of the experimental study was carried out using the Statistica 10.0 pro-

gram. The normal distribution of data was checked using the Shapiro – Wilk test (W test). The results were presented as Me [Q 1 ; Q 3], where Me is the median, Q 1 is the first quartile (25%), Q 3 is the third quartile (75%), arithmetic mean and its error  $M \pm m$ . To analyze the statistical significance of differences between independent samples, the Mann – Whitney rank test (U test) was used. To check the equality of the medians of several samples, the Kruskal – Wallis test was used. Spearman's coefficient (*R*) was used to assess the rank correlation. The critical level of statistical significance of the differences in the null hypothesis (*p*) was taken equal to 0.05.

## RESULTS AND DISCUSSION

NO metabolites level does not change significantly in Group A and increases in post-operation period in groups B and C.

In groups B and C NO metabolites level significantly grows on days 1 and 10, in the period from month 1 to month 6 NO level is stable but statistically higher than before operations (Fig. 1).

Any operative intervention on arteries leads to endothelium damage which causes inflammation with adhesion and thrombocyte aggregation, proliferation and migration of smooth muscle cells into intima. Endothelium damage induces production of NO synthase-2 (NOS-2) in smooth muscle cells and endothelium [16, 17], which can explain the results reached in groups B and C. Venous conduit from great saphenous vein has functional endothelial lining and reacts on local and systemic molecular and hemodynamic

stimulants and regulates vascular tone and homeostasis which is not the case of synthetic prosthesis due to absence of endothelium in it and that is why NO metabolites level does not change in group A (Fig. 1).

Along with changes in the level of nitric oxide metabolites, vascular endothelial growth factor VEGF

is one of the well-studied indicators of angiogenesis, indicating the severity of the lesion [18]. It has been proven that VEGF ensures normal growth and development of the body, wound healing. However, high levels of VEGF were found in the development of cancerous tumors and rheumatoid arthritis [19].

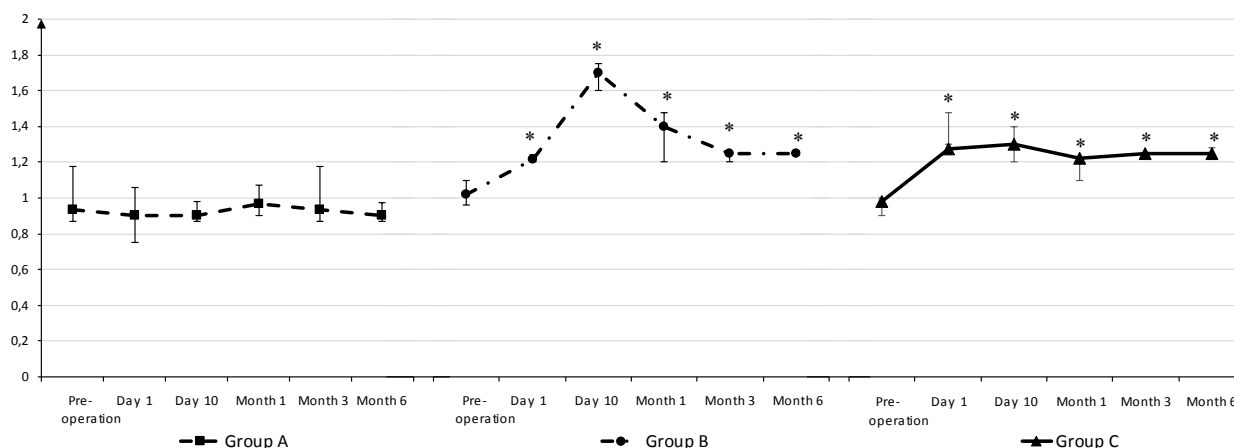


Fig. 1. NO metabolites level in blood plasma of patients with critical lower limb ischemia before and after femoropopliteal segment arteries reconstruction,  $Me [Q_1; Q_3]$ : \* significant differences in data between pre-operation and post-operation ( $p < 0.05$ )

The concentration of the active form of human VEGF-A in experimental group A increases statistically significantly from the 1st day to 6 months, reaching a maximum on the 10th day. In experimental group B, the amount of VEGF-A statistically significantly increased after 1 month relative to the values before surgery. In group C, it statistically significantly increases on the 10th day after surgery and decreases

in the period from 1 to 6 months relative to the values before surgery.

It is known that the survival rate of patients with a high level of VEGF is significantly lower than that of patients with a low expression of VEGF-A [18], therefore, the results obtained indicate a positive trend in autovenous bypass grafting using the *in situ* technique.

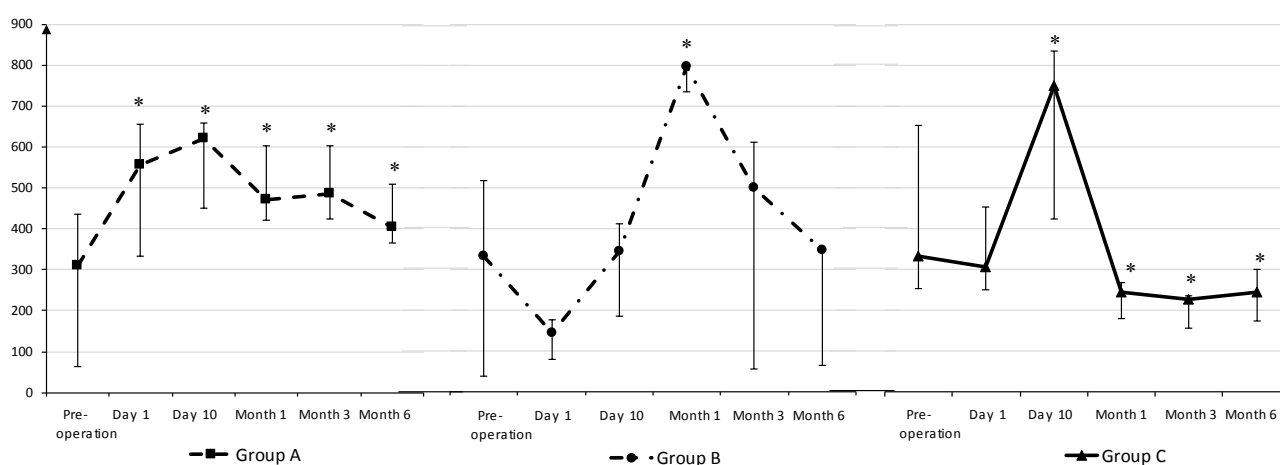


Fig. 2. Concentration of VEGF-A's active form in blood plasma of patients with critical lower limb ischemia before and after femoropopliteal segment arteries reconstruction,  $Me [Q_1; Q_3]$ , pg/ml: \* significant differences in data between pre-operation and post-operation ( $p < 0.05$ )

After surgery, the restoration of the body is accompanied by adaptation to new conditions. Currently, thiol-containing compounds (molecules containing-SH groups) are considered not only as a new marker of oxidative stress, but also as an indicator of homeostasis and adaptation under pathophysiological conditions [20].

The level of reduced thiols in the blood plasma of patients in groups A and B increases on the 10th day and

after 1 month, and in group C it statistically significantly increases from the 10th day to the 6th month (Fig. 3).

The degree of adaptation directly depends on the concentration of thiols: the higher the degree of adaptation, the higher the level of SH-groups in the blood serum. It follows from this that the functional adaptation of the venous endothelium is higher in patients operated on by the *in situ* method, compared with the reverse vein method.

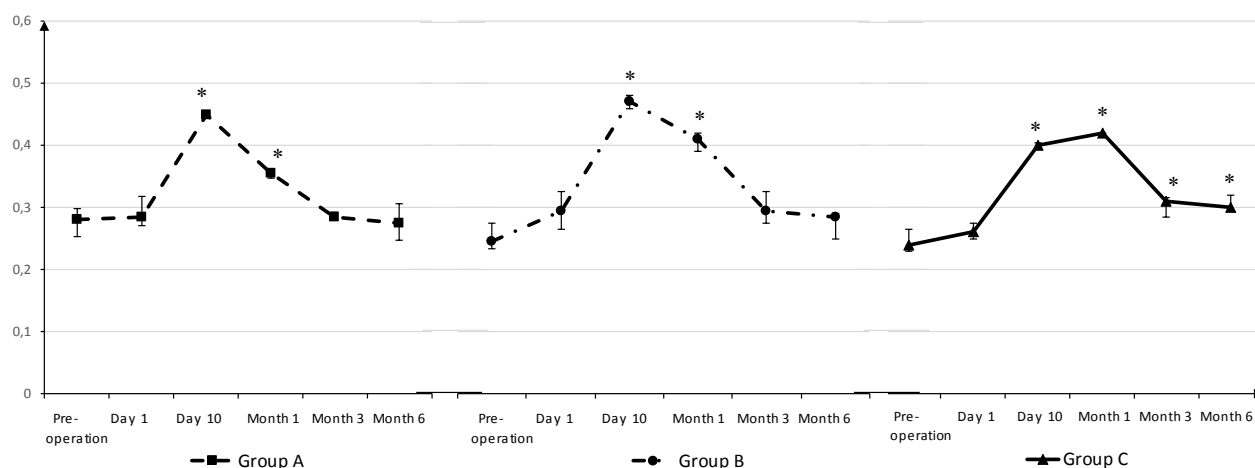


Fig. 3. Reduced thiols level in blood plasma of patients with critical lower limb ischemia before and after femoropopliteal segment arteries reconstruction,  $Me [Q_1; Q_3]$ ,  $\mu\text{mol/ml}$ : \* significant differences in data between pre-operation and post-operation ( $p < 0.05$ )

Thiols are able to form reversible mixed disulfide bonds between protein and low molecular weight SH groups. The results obtained in group C indicate that oxidative stress decreases, disulfide bonds are restored to thiol groups, and thiol-disulfide homeostasis is maintained, and the body's resistance to reconstructive surgery increases. Abnormalities in thiol-disulfide homeostasis can play an essential role as a biomarker

in the development of pathophysiological conditions. In patients in groups A and B, after mobilization of the body's defense reactions, which are expressed in an increase in the level of thiols, their decrease to the initial level is observed, which indicates adaptation or compensation without depletion.

The obtained biochemical results confirm the clinical observations given in Table. 2.

Table 2

| Clinical characteristics of early and late complications of patients in the study groups |         |         |          |
|--|---------|---------|----------|
|  | Group A | Group B | Group C  |
| <i>Short-term results (up to 30 days)</i>  |         |         |          |
| Thrombosis, $n$ (%)  | 2 (10%) | 2 (10%) | 1 (4.5%) |
| First-time passableness, %   | 90      | 90      | 95.5     |
| Second-time passableness, %  | 90      | 95      | 100      |
| Limb salvage, %  | 90      | 95      | 95.5     |
| Major amputations, $n$ (%)   | 2 (10%) | 1 (5%)  | 1 (4.5%) |
| Minor amputations, $n$ (%)   | 1 (5%)  | 5 (25%) | 2 (9%)   |
| <i>Long-term results (6 months)</i>  |         |         |          |
| Thrombosis, $n$ (%)  | 4 (20%) | 3 (15%) | 1 (4.5%) |
| First-time passableness, %   | 70      | 75      | 91       |
| Second-time passableness, %  | 75      | 80      | 95.5     |
| Limb salvage, %  | 80      | 85      | 95.5     |
| Major amputations, $n$ (%)   | 2 (10%) | 2 (10%) | 0        |
| Minor amputations, $n$ (%)   | 1 (5%)  | 1 (5%)  | 1 (4.5%) |



From the perspective of early and late postoperative complications, patients in group C have a more favorable clinical picture, as indicated by a high percentage of primary and secondary patency of bypasses, a decrease in mortality, and an increase in the degree of limb preservation. The most unfavorable postoperative period according to the list of declared angiological signs is in patients in group A (see Table 2).

Interestingly, in terms of biochemical parameters in group B, positive correlations were revealed: the

higher the level of nitric oxide metabolites, the higher the number of VEGF-A and SH-groups. Patients of group C showed a positive correlation between the amount of VEGF-A and free thiols, regardless of the level of nitric oxide metabolites (Table 3). Thus, in group B, NO metabolites promote the growth of SH-groups and VEGF-A, and in group C, they do not affect the concentration of VEGF-A and the level of SH-groups, which may be of clinical significance when assigning NO donors.

Table 3

| Correlation ratio (R) between NO metabolites level, VEGF-A, SH-groups of patients in pre-operation and post-operation periods |                      |                      |                       |
|---|----------------------|----------------------|-----------------------|
|   | Group A              | Group B              | Group C               |
| NO metabolites/VEGF-A   | $R = 0.1; p > 0.05$  | $R = 0.47; p < 0.01$ | $R = 0.15; p > 0.05$  |
| NO metabolites/SH-groups  | $R = 0.15; p > 0.05$ | $R = 0.48; p < 0.01$ | $R = 0.065; p > 0.05$ |
| VEGF-A/SH-groups  | $R = 0.40; p < 0.01$ | $R = 0.35; p < 0.01$ | $R = 0.41; p < 0.01$  |

## CONCLUSION

The obtained patterns of changes in the level of reduced thiols, nitric oxide metabolites, VEGF-A in patients with CLI indicate adaptation of the venous endothelium in the settings of the arterial bed during autovenous reconstruction of the femoropopliteal segment in comparison with the group of patients operated on using a synthetic prosthesis, which is confirmed by the analysis of early and late postoperative complications. The *in situ* method demonstrates the development of a more favorable clinical picture in terms of biochemical and angiological parameters than the reverse vein method.

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## Authors contribution

Kalinin R.E. – verification of the manuscript, critical revision of the manuscript for important intellectual content, final approval of the manuscript for publication. Abalenikhina Yu.V., Pshennikov A.S., Vinogradov S.A. – conception and design of the study, analysis and interpretation of data, drafting of the article. Abalenikhina Yu.V. – carrying out of biochemical tests. Pshennikov A.S., Vinogradov S.A. – supervision of patients in the Department of Vascular Surgery of the Regional Clinical Hospital of Ryazan, treatment and surgical correction of the magistral blood flow, sampling of biological material from patients.

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