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## ***Vasodilator effect of vascular endothelial growth factors (VEGF) in the conditions of bone tissue formation by the method of discrete traction in congenital segmental pathology***

**E.P. Vykhovanets<sup>1</sup>, T.I. Dolganova<sup>1</sup>, S.N. Luneva<sup>1</sup>, D.Yu. Borzunov<sup>1,2</sup>**

<sup>1</sup>Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation

<sup>2</sup>Tyumen State Medical University, Tyumen, Russian Federation

The development of the limbs in patients with congenital segmental defects is associated with defects in the vascular system of the same segment. In this case, vascular endothelial growth factors that participate in physiological angiogenesis may have effect on bone tissue formation in ontogenesis and participate in endochondral ossification. In this regard, the aim of the study was to compare the quantitative changes in growth factors (VEGF) and the contractile properties of arterioles under conditions of discrete distraction for congenital pathology of the lower leg bones. **Materials and methods** Subjects of the study were 12 patients with congenital pseudarthrosis of the lower leg (ICD-10 Q 74.0). Their serum was analyzed using an enzyme immunoassay. Microcirculation in the calf skin was examined by laser flowmetry (BLF21, Transonic Systems Inc., USA). We used the findings obtained from the study of blood serum in 103 somatically healthy people for control. **Results** Significant imbalance in serum concentrations of endothelial factors and their receptors was detected in the subjects with congenital pathology. As a result of surgical treatment, the imbalance of secretion of vascular growth factors and their receptors increased. Vasodilator effect in the precapillary vessels in the zone of the operated segment in patients with congenital pathology was not revealed by surgical intervention.

**Keywords:** endothelial growth factors, bone tissue, congenital segmental pathology, enzyme immunoassay, laser Doppler flowmetry

### INTRODUCTION

It is known that congenital segmental limb defects are associated with disorders of the vascular system of the same segment in all patients [1, 2]. It is believed that primary disorders of vascularization cause anomalies in the development of the osseous system [3]. Concomitant arterial hypoplasia is caused by dysplasia of the vascular wall in disorder of its basal (sympathetic) tone [4].

Vascular growth factors (VEGF) play a major part in embryonic vasculo- and angiogenesis [5], physiological angiogenesis [6, 7, 8, 9, 10], have an effect on bone tissue formation in ontogenesis [11], and participate in the transformation of the cartilage into bone (endochondral ossification) [12] in distraction regenerate [13].

It has been shown that an increase in the VEGF in the blood indicates its increased secretion [14, 15], and its activity is regulated by anti-angiogenic factor

in the blood, the soluble receptor 1 of the vasculo-endothelial growth factor (sVEGF-R1) [16, 17]. A number of studies show that VEGF can have an effect on the contractility of the vascular wall [18] by inducing NO-dependent relaxation in the arteries [19, 20, 21] or activating the production of prostacyclin by VEGF interacting with flk-1/KDR (VEGF-R2), one of the most powerful vasodilator agents [22]. Vasodilatation of resistive vessels with an increase in arteriovenous shunting is one of the mechanisms that trigger tissue hyperemia and, as a result, increased pressure in venules with stimulation of angiogenesis [23, 24].

The aim of the study was to conduct a comparative analysis of quantitative changes in vascular growth factors (VEGFs) and contractile properties of arterioles under the conditions of discrete distraction in lower limb bone congenital pathology.

### MATERIAL AND METHODS

The material of the study was 12 patients at the age from 7 to 18 years with congenital

pseudarthrosis of the tibia and associated anatomical shortening of  $2.5 \pm 1.1$  cm. Mean age of patients

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was  $13.1 \pm 0.59$  years. As far as the integrity of the bone was broken with osteotomy (corticotomy) during the intervention in the operating room, thereby causing skeletal trauma, the examinations of patients were conducted in accordance with the main stages of traumatic disease: before surgery, on days 3 to 5, 7 to 10, 12 to 14 and the 30th day after surgery [25, 26].

Etiologically, congenital defects were represented by Type I neurofibromatosis. Surgical intervention was performed according to the Masquelet method, based on the concept of a "biological" osteoinduction membrane arising after the application of the cement spacer during the first stage and acting as a "chamber" for the introduction of non-vascularized grafts at the second intervention. In addition, external fixators and principles of the Ilizarov non-free bone grafting were used [27, 28, 29].

The material for the enzyme immunoassay was patients' blood serum.

As a control norm, we used our data obtained from the study of blood serum in 103 practically healthy middle-aged subjects.

Permission was obtained from the Ethics Committee of the RISC RTO to conduct the research. The studies were carried out in accordance with the ethical standards of the Helsinki Declaration of the World Medical Association "Ethical Principles for Conducting Scientific Medical Research with Human Participation", as amended in 2000, and the "Rules of Clinical Practice in the Russian Federation" approved by Order No. 266 of the Ministry of Health of the Russian Federation of June 19, 2003.

*Immunochemical methods of studying blood serum* Growth factors were determined on the ThermoFisher equipment complex (USA): Multiscan FC detector, Shaker-401, WellWash automatic washer. To measure the concentration of the factors in the blood serum, kits for the enzyme immunoassay

(ELISA) were used. A brief description of the sets is given in Table 1.

The kits used refer to the non-competitive ELISA format, of which the "sandwich" method is a variation. A solution containing the antigen to be analyzed was added to the carrier with immobilized antibodies. During the incubation in the first stage, the antigen-antibody complex was formed on the solid phase. The carrier was then washed from unbound components and enzyme-labeled specific antibodies were added. The enzymatic reaction (color reaction) ran in the presence of a substrate (trimethylbenzidine), represented by an uncolored compound, which during the reaction with the intermediate "layer" of streptavidin is oxidized to a colored product at the final stage of the test. The intensity of staining depends on the number of specific antibodies detected. The result is evaluated spectrophotometrically at a wavelength of 450 nm.

Evaluation of the contractile properties of arterioles was carried out using laser Doppler flowmetry (LDF) on a BLF21 unit (Transonic Systems Inc., USA). To study the mechanisms involved in the regulation of tissue blood flow, detection of hidden microhemodynamic disorders and adaptive reserves of the microcirculation system, a local ischemic test was used with the sensor mounted on the leg, and the occlusion cuff on the thigh [30]. After the data were recorded at rest (KKrest, pf unit) and after 3 minutes of ischemia (KKpeak, pf units), the peak blood flow index was calculated (PI, % = KKpeak / KKrest  $\times 100$  %) – increase in capillary blood flow after cessation of 3-minute's occlusion;  $\Delta t$ , sec – the time interval from the occlusion cessation to the appearance of the maximum increase in the capillary blood flow; the period of half-recovery of the capillary blood flow ( $T_{1/2}$ , sec) and duration of reactive hyperemia (T, sec.) – the time interval from the appearance of the maximum increase in the capillary blood flow to its recovery to baseline values.

Table 1

Brief description of assay kits

Growth factor name	Company	Measuring range	Serum dilution	Total time of test
VEGF	Invitrogen Corporation (CIHIA)	n/a, ng/ml	1:2	4 h 00 min
VEGF-R2/KDR	eBioscience Platinum	7.104 – 30.861 ng/ml	1:50	3 h 30 min
VEGF-A	eBioscience Platinum	n/a – 0.0426 ng/ml	1:2	4 h 30 min
VEGF-C	eBioscience Platinum	n/a – 5.35 ng/ml	1:5	4 h 30 min
sVEGF-R1	eBioscience Platinum	n/a – 0.42 ng/ml	1:2	3 h 10 min
VEGF-R3/FLT-4	eBioscience Platinum	33 – 167 ng/ml	1:20	3 h 30 min

Note: n/a – unavailable or undetectable values

Statistical processing of the data was carried out using the Microsoft EXCEL-2000 and AtteStat data analysis package. Distribution of the asymmetry profiles of the calculated indicators was  $n = 12$  according to the modified Kolmogorov, Smirnov criteria as well as the asymmetry and kurtosis criteria showed that the normality hypothesis

deviates: the investigated indicators do not obey the "normal distribution law". The results of the studies were processed by the variation statistics method, used for small samples with acceptance of significance level of  $p \leq 0.05$ . Statistical significance of the differences was determined using the Wilcoxon test.

## RESULTS

There was a significant imbalance in serum vascular endothelial growth factors and their receptors (Table 2) in patients with congenital segmental tibial pathology. The concentration of VEGF, VEGF-A and VEGF-R2 receptor at the preoperative stage was significantly higher than the values of the reference group, while the VEGF-R3 receptor concentration was more than 2 times lower than the reference values.

VEGF-C concentration in the blood of both subjectively healthy children and children with congenital pathology was below the detection level (54 pg/ml). Serum concentrations of the soluble sVEGF-R1 receptor in somatically healthy children and children with congenital pathology were also below the detection interval equal to 0.42 ng/ml.

All parameters of capillary blood flow were reduced in congenital tibial pathology relative to the normal values before treatment: capillary blood flow

(KKrest) by 17 %, KKpeak and peak capillary blood flow index – 2 times lower; half-recovery time after ischemic test and duration of reactive hyperemia by 5-6 times (Table 3, **Figures 1 and 2**). This indicates a significant decrease in the reserve capacity of the capillary bed, dysfunction of the endothelium of the vessels of the precapillary link. [32]. Potential capacity of the capillary bed of the affected limb depends on the degree of preservation of its ability to maintain weight. In the absence of limb's weight-bearing, the blood flow growth index did not exceed 130 % while it was more than 230 % if the static load on the limb was possible. However, the calculated indices of the duration of the hyperemia and the half-recovery period of the KK ( $T_{1/2}$ ) were equally sharply reduced, i.e. the reactivity of the vessels of the precapillary link was determined by the etiology (congenital) of the affected tissues of the leg.

Table 2

Concentration of vascular endothelial growth factors at the stages of orthopedic treatment of patients with congenital defect of the tibia ( $M \pm m$ ,  $n$  – number of cases)

Factor name	Reference group (n = 103)	Before surgery (n = 12)	After surgery (n = 12)			
			3-5 days	7-10 days	12-14 days	30 days
VEGF, ng/ml	0.165 ± 0.03	0.178 ± 0.04*	0.212 ± 0.03*#	0.201 ± 0.03*#	0.183 ± 0.02*	0.199 ± 0.01*
VEGF-R2/KDR, ng/ml	11.3 ± 0.75	35.7 ± 1.30*	47.6 ± 0.26*#	46.8 ± 1.4*#	68.1 ± 1.4*#	54.5 ± 1.4*#
VEGF-A, ng/ml	0.12 ± 0.03	0.62 ± 0.05*	0.60 ± 0.04*	0.59 ± 0.02*	0.53 ± 1.0*	1.38 ± 0.01*#
VEGF-R3/FLT-4 ng/ml	102.01 ± 11	42.75 ± 4.7*	22.33 ± 0.04*#	26.83 ± 0.02*#	25.87 ± 0.02*#	15.34 ± 0.39*#

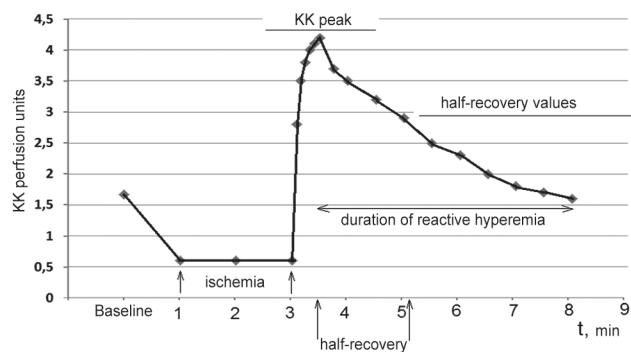
Note: \* – differences are significant when  $p \leq 0.05$  between the control values and the values obtained at the stages of orthopedic treatment; # – differences are significant at  $p \leq 0.05$  between preoperative values and values obtained at the stages of orthopedic treatment

Table 3

Parameters of laser Doppler flowmetry of the lower leg skin in patients with congenital defects of the tibia ( $M \pm m$ ,  $n$  – number of cases)

Parameters	Norm (n = 30)	Affected limb			
		Before treatment (n = 12)	After surgery (n = 12)		
			7-10 days	12-14 days	30 days
KK rest, pu	1.67 ± 0.14	1.4 ± 0.15	1.5 ± 0.15	1.55 ± 0.16	1.46 ± 0.17
KK peak, pu	4.11 ± 0.27	2.8 ± 0.19*	2.7 ± 0.59*	2.8 ± 0.38*	2.65 ± 0.96*
PI, %	298.0 ± 17.89	192.5 ± 34.2*	176.0 ± 46.5*	183 ± 52.1*	188.6 ± 40.0*
Δt sec	30.0 ± 1.27	25.0 ± 2.66*	10.0 ± 2.33*	10.0 ± 2.89*	10.0 ± 2.31*
T <sub>1/2</sub> sec	97.1 ± 5.52	35.1 ± 4.12*	15.4 ± 5.17*	15.0 ± 3.19*	15.0 ± 3.12*
T, sec.	330.0 ± 50.4	60.0 ± 8.11*	35.0 ± 7.11*	30.0 ± 5.16*	30.0 ± 4.11*

Note: KKrest, perf. units (pu) – capillary blood flow at rest; KK peak., perf. units (pu) – maximum perfusion of hyperemia (maximum blood flow after 3-minute's occlusion cessation), perf. units; PI – increase in capillary blood flow after cessation of 3-minute's occlusion; Δt – time interval from the removal of occlusion to the appearance of the maximum increase in capillary blood flow; T<sub>1/2</sub> – time of half-recovery of KKrest; T, sec – duration of reactive hyperemia. Difference in parameters relative to the values of the norm is shown,  $p \leq 0.05$

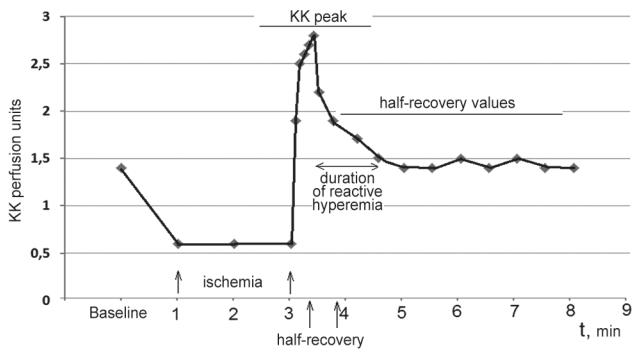


**Fig. 1** Dynamics of capillary blood flow of lower leg skin after a 3-minute's ischemic test in healthy subjects

High concentration of VEGF, VEGF-A and VEGF-R2 receptor in patients with a congenital etiology of the disease was not accompanied by a decrease in the contractile properties of the vascular wall, and there was no effect of vasodilation of the capillary bed. The results do not agree with the literature data that described that the factors VEGF, VEGF-A and VEGF-R2 receptor affect the contractility of the vascular wall, causing a vasodilator effect in patients with acquired cardiovascular pathology [18, 19].

In patients with congenital musculoskeletal pathology associated with the disorders of the adjacent vascular system, disturbances in vascular reactivity of the pre-capillary link are combined with a low concentration of the VEGF-R3 receptor.

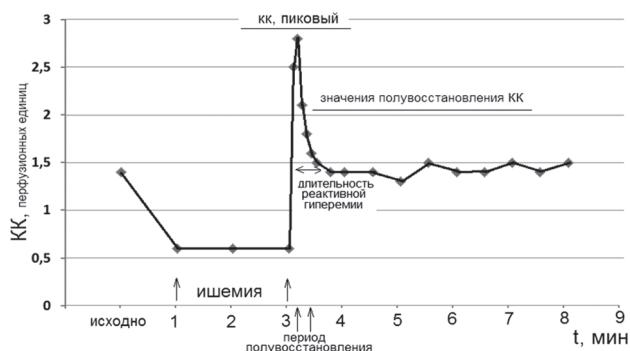
On days 3 to 5 after surgery involving the procedure of an inducing membrane and transosseous osteosynthesis, the concentration of VEGF growth factor and VEGF-R2 receptor continued to increase in patients' blood serum. After seven to 10 days post-surgery, VEGF-R2 values increased by 48%. The concentration of VEGF-A did not change during the first two weeks after treatment, but increased more than twice by the 30th day after the operation and showed a tenfold growth to the values of the reference



**Fig. 2** Dynamics of capillary blood flow in the affected lower leg skin after a 3-minute's ischemic test in a patient with a congenital defect of the tibia (before surgery)

group. But the surgical intervention also led to the condition that the initially lowered (more than twice) serum concentration of VEGF-R3 receptor continued to decrease, and on the 30th day after the operation, its level was only 15% of the values of the age norm.

As can be seen from Table 3, the capillary flow at rest and the skin peak blood flow in the zone of the operated segment remained stable during the course of the observations; there was no increase in the reserve capacities of the vascular bed. The ischemic test revealed a 2-fold decrease in the period of half-recovery and duration of reactive hyperemia (Table 3, Fig. 3).



**Fig. 3** Dynamics of capillary blood flow of the skin of the operated lower limb after a 3-minute's ischemic test in a patient with a congenital defect of the tibia (14 days after the operation)

## DISCUSSION

Vascular growth factors are of paramount importance in the formation of the collateral vascular network in the focus of chronic ischemia, which is a congenital pseudarthrosis of the tibia, and have an impact on all stages of neoangiogenesis. Therefore, the effectiveness of the growth factors of the VEGF family on post-ischemic angiogenesis directly depends on the factors that have an influence on their activity, and, first of all, on the number and availability of VEGF receptors. Among the growth factors of

the VEGF family and their receptors studied, the serum concentration of VEGF-R3 was of the highest significance as the surgical intervention in patients with congenital pathology was not accompanied by vasodilation of the vessels of the precapillary link in the zone of the operated segment.

Concentrations of the VEGF-R3 receptor and the impaired reactivity of the pre-capillary vessels had a unidirectional dynamics: a decrease in the VEGF-R3 receptor concentration was accompanied

by a decrease in the half-recovery period and duration of reactive hyperemia of the capillary bed after an ischemic 3-minute's test.

In congenital etiology of tibial defect, the increase in the blood filling of tissues along the main flow during Ilizarov treatment was poorly expressed and did not lead to an increase in reserve capacity of capillary blood flow, which, on the contrary, depends on the volume of the main arterial inflow in patients with acquired pathology [33].

A significant imbalance in serum vascular endothelial growth factors and their receptors was revealed in congenital pathology of the musculoskeletal system. Relative the age norm, on average, the concentration of VEGF is 10% higher, VEGF-A – 5 times higher, VEGF-R2 receptor – 3 times higher, while the VEGF-R3 receptor concentration is 2.4 times lower than the normal values.

As a result of surgical treatment, the imbalance in the secretion of vascular growth factors and their receptors has enhanced due to a further increase in VEGF concentration by 15%, VEGF-R2 – 1.5 times,

According to the literature, a pronounced effect of physiotherapeutic factors on angiogenesis (magneto-laser therapy) is noted in patients with acquired etiology of the disease and is manifested by vasodilation of arterioles due to increased parasympathetic activity. In patients with congenital orthopedic pathology, there is a disorder of the basal (sympathetic) tone of the arteries [4], and the effect of magneto-laser therapy on vasodilation of the vessels is not expressed [34].

## CONCLUSIONS

and a decrease in the VEGF-R3 receptor concentration by 2.5 times.

High concentration of VEGF, VEGF-A and VEGF-R2 receptor in patients with a congenital etiology does not cause vasodilation of the capillary bed after a 3-minute's ischemic test and a decrease in the contractile properties of the vascular wall.

A decrease in the concentration of the VEGF-R3 receptor is accompanied by a decrease in the half-recovery period and duration of reactive hyperemia of the capillary bed during surgical treatment in patients with congenital pathology.

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**Information about the authors:**

1. Evgenia P. Vakhovanets,  
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation,  
Email: vykhovanets.eva@mail.ru
2. Tamara I. Dolganova, M.D., Ph.D.,  
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation,  
Email: rjik532007@rambler.ru
3. Svetlana N. Luneva, Ph.D. of Biological Sciences, Professor,  
Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation,  
Email: luneva\_s@mail.ru
4. Dmitry Yu. Borzunov, M.D., Ph.D.,  
<sup>1</sup>Russian Ilizarov Scientific Center for Restorative Traumatology and Orthopaedics, Kurgan, Russian Federation,  
<sup>2</sup>Tumen State Medical University, Tumen, Russian Federation,  
Email: borzunov@bk.ru