

ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Microanatomical characteristics of arterial vascularization of the anterior cruciate ligament

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SUMMARY

Introduction/Objective The aim of this study was to examine the immunohistochemical features of the vascularization of the anterior cruciate ligament (ACL), as well as the quantification of capillaries within the three segments of the ACL; proximal, middle and distal. The quantification and metric characteristics of mast cells of the ACL are the second goal of this research.

Methods In total, 30 human ACL of 30 persons, obtained during routine autopsy, were examined under the microscope, following immunohistochemical reactions against CD34 of blood vessels and MastTrip of mast cells.

Results The middle genicular artery close to the ACL gave off branches for the supply of ligament itself. Each field of mm² contained an average number of 1113.84 (959–1240), microvessels in ACL proximal third, an average number of 1145.43 (924–1310) microvessels in ACL middle third, and an average number of 1134.55 (889–1451) microvessels in ACL distal third. An average number of mast cells of the ACL was 3.8 per mm². In the peripheral synovial zone of the ACL, we counted 12.6 mast cells per mm². An average area value of the mast cells was 124.7 μm², and an average value of shorter and longer axis of the mast cells was 11.2 × 15.0 μm.

Conclusion There was no statistically significant differences between the average numbers of intraligamentous microvessels of the ACL thirds ($p > 0.05$), confirming and supporting our hypothesis of uniform distribution of blood supply within the ACL.

Keywords: anterior cruciate ligament; intraligamentous microvessels; mast cells; immunohistochemistry

INTRODUCTION

The anterior cruciate ligament (ACL) is an intracapsular but extrasynovial ligament. The cruciate ligaments are covered by a fold of synovial membrane which incompletely divides the joint in the sagittal plane. The histological structure shows that the intercellular matrix of cruciate ligaments consists of parallel bundles of collagen fibers, separated by thin reticular fibers. Ligaments belong to the group of hypodense tissues, and the cells that can be found in the structure of ligaments have the characteristics of fibroblasts [1]. ACL injuries are associated with various risk elements related to the extrinsic and intrinsic factors. Intrinsic factors of ACL tear include morphology of intercondylar notch of femur and proximal plateau of tibia, and the distribution of blood supply within the ACL [2].

The popliteal artery (PA) is the main blood vessel that supplies the knee joint, and its injury is common in the knee luxation and fractures of the lower extremities' bones. After

originating from the PA, the middle genicular artery (MGA) pierces through the posterior part of articular capsule and enters the area of the intercondylar fossa, where it divides by providing numerous branches to both cruciate ligaments and menisci [3]. With its terminal branches, the MGA participates in the vascularization of cruciate ligaments, branching out into small blood vessels that form a periligamentous network within the synovial sheath surrounding the ligaments. From the synovial membrane the capillaries originate and penetrate the ligaments at right angles, where they are longitudinally oriented forming intraligamentous vascular network. The distal part of the ACL is also supplied by branches of the PA: inferior lateral genicular arteries (ILGA) and inferior medial genicular arteries (IMGA) [4].

The network of blood vessels surrounding the ACL is significantly more developed comparing with the intraligamentous area, whereas the distal part of the ligament is the least vascularized. The reason for this difference can be found in the specific histological structure of

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the distal part of an ACL ligament, i.e., there is a zone of fibrocartilaginous tissue, which results in a certain degree of compression of blood vessels, consequently leading to weaker vascularization of this part [4, 5]. The mast cells (MCs) are present in different tissues close to blood vessels, containing different granules with bioactive molecules (histamine, cytokines, heparin, and tryptase) [6].

Immunohistochemical methods of staining enable precise identification and analysis of the capillary network of three parts; proximal, middle and distal segment of the ACL, as well as the quantification and metric characteristics of MCs of the ACL.

METHODS

Immunohistochemical studies were performed on 30 isolated ACL specimens obtained from cadavers of persons of both sexes (18 males and 12 females) aged 36–72 years (mean age 58.6 years) during autopsies at the Institute of Pathology of the Medical Faculty of Belgrade, with the approval of the Institute of Pathology. The material for histochemical and immunohistochemical staining methods was prepared in a standard manner at the Institute of Histology and Embryology and the Institute of Pathology of the Faculty of Medicine in Belgrade. The ACL samples were fixed in 4% neutral buffered formaldehyde solution for 24 hours, in a volume 20 times greater than the volume of the immersed tissue. After the fixation was completed, the samples were divided into three thirds (proximal, middle and distal) knowing that the lower part of the ligament was marked with the blue thread. They are then further prepared by a routine procedure, which includes dehydration, impregnation, and molding in paraffin/paraplast (Bio-Plast plus, Bio-Optica, Milan, Italy). Each sample of 15 ligaments embedded in paraffin was cut transversely, and 15 ligaments were cut longitudinally, serially, on a microtome (RM 2255, Leica Microsystems GmbH, Frankfurt, Germany) until the ligament was completely cut and the order of sections was carefully marked. Tissue sections 4–5 μm thick were mounted on special high-adhesion glass slides (SuperFrost Plus, Dako, Glostrup, Denmark), dried for 60 minutes in a thermostat at 56°C and then stained. The sections underwent immunostaining by incubation with two primary antibodies, CD34 (antibody Dako Denmark A/S, M 7165, dilution 1:25), and anti-mast cell tryptase (Dako Denmark A/S, M 7052, dilution 1:100) according to the staining protocol.

During the staining process of ligament slices, negative controls were represented by tissue samples to which a non-immune serum was applied instead of primary antibodies. The intensity of the immune response was determined semiquantitatively by two independent researchers as strongly positive (+++), moderate (++) , weak (+) or negative (-). The number of microvessels identified by immunostaining was counted manually in the software system "Leica Interactive Measurements" (Leica Microsystems GmbH, Frankfurt, Germany), on 10 randomly selected fields of view of each section at $\times 400$ magnification (field

size 341.7 $\mu\text{m} \times 250 \mu\text{m}$), then the number for the area of 1 mm^2 was subsequently calculated. The number of immunostained MCs was counted with a similar procedure applied.

The analyses of descriptive statistics that we used were arithmetic mean values with standard deviations, and minimum and maximum values. We used the student's t-test for independent samples and one-way analysis of variance (ANOVA) followed by Bonferroni's corrective. The probability level of $p < 0.05$ was considered as a statistically significant difference. All statistical analyses were performed using the statistical program SPSS (SPSS for Windows, release 17.0, SPSS Inc., Chicago, IL, USA). The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade, Belgrade, Serbia (No. 1322/V-10, Date 20-05-2021).

RESULTS

Our research has shown that the MGA, branch of the PA, in the close vicinity of the ACL sent more small arteries for the vascularization of the ligament itself. The distal part of the ACL received branches from the ILGA and IMGGA, also originating from the PA (Figure 1). Precise measurements gave diameters of these branches, from 0.022 mm to 0.049 mm (in average 0.032 mm). Upon separation from the PA, ILGA, and IMGGA branched and formed a fine synovial arterial network on the ACL surface from which originated thin penetrating vessels for ligament tissue. Studying the ligament slices, we noticed small arteries, arterioles, precapillaries, capillaries, venules and small veins. Only the capillaries reached the deepest parts of the ligament. The MGA also sent penetrating nutrient branches to the femoral attachment of ACL, and surrounding bony tissue of the lateral femoral condyle. The ILGA and IMGGA branches

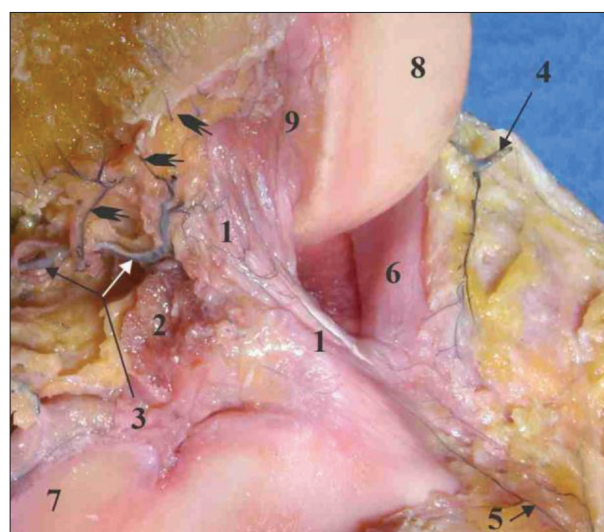


Figure 1. 1 – the anterior cruciate ligament; and 2 – the posterior cruciate ligament are supplied from the 3 – middle genicular artery, 4 – the inferior lateral genicular artery and 5 – the inferior medial genicular artery; note the femoral nutrient branches of the middle genicular artery (arrows), 6 – the lateral meniscus, 7 – the medial meniscus, 8 – the lateral condyle of femur, and 9 – the intercondylar fossa (medial view, dissection of knee specimen injected with India ink)

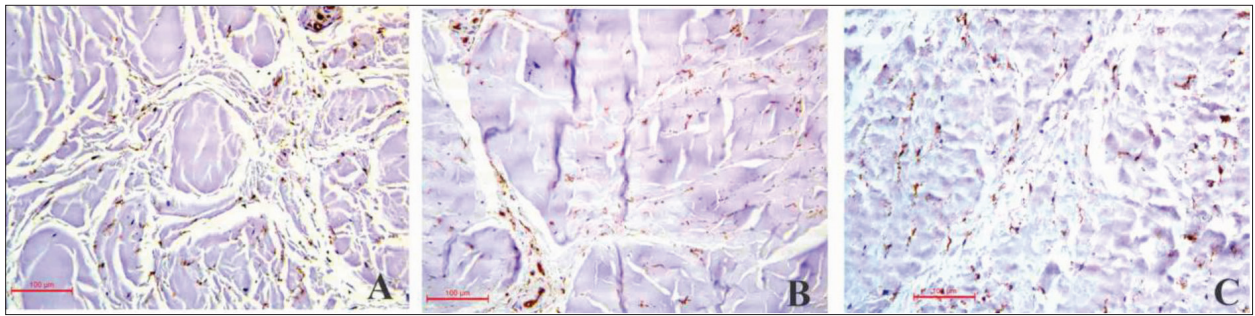


Figure 2. Intraligamentous microvessels in the transverse sections of A – proximal part; B – middle part; C – distal parts of the anterior cruciate ligament (CD34 immunostaining)

Table 1. Vascular density in three parts of anterior cruciate ligament: proximal, middle and distal third; mast cell density in synovial and intraligamentous tissues; and metric characteristics of tryptase positive mast cells

Anterior cruciate ligament	Proximal third	Middle third	Distal third
N° of microvessels/mm²: min-max (M)	959–1240 (1113.84)	924–1310 (1145.43)	889–1451 (1134.55)
Tissue	Synovial tissue		Intraligamentous tissue
N° of mast cells/mm²: min-max (M)	6–18 (12.6)		0–7 (3.8)
	Average shorter and longer, and mean diameter (µm): min-max (M ± SD)		Area value (µm²): min-max (M ± SD)
Metric characteristics of tryptase positive mast cells	11.2 × 15 (13.13 ± 0.9)		91.5–155.8 (124.7 ± 16.2)

supplied the tibial attachment of ACL, and the upper bony surfaces of the lateral and medial tibial condyles.

Dividing the longitudinal central axis of the ACL into thirds enabled us to analyze the partial vascularization of the ACL. The average microvessel intraligamentous density of the proximal third of the ACL counted in microscopic fields was 95.2 (82–106), and recalculated for the area of 1 mm² of ACL tissue was 1113.84 (959–1240) (Figure 2 and Table 1).

The intraligamentous vascular network of the middle third of the ACL, showed the average number of blood vessels per mm² of ACL tissue was 1145.43 (924–1310) (Figure 2 and Table 1).

The intraligamentous density of CD34 positive microvessels of the distal third of the ACL, the average number of blood vessels per mm² of ACL tissue was 1134.55 (889–1451) (Figure 2 and Table 1).

Testing the significance of the difference in the number of ACL microvessels, based on analysis of variance (ANOVA), by comparison on three thirds of the ACL, proximal, middle and distal, it was observed that all values are not statistically significant, $p > 0.05$, i.e., there was no statistically significant difference in the number of microvessels with regard to thirds of the ACL. Blood vessels are uniformly distributed regardless of the observed part of the ACL (Figure 3).

An average number of immunostained MCs of the ACL was 3.8 (0–7) per mm². In the peripheral synovial

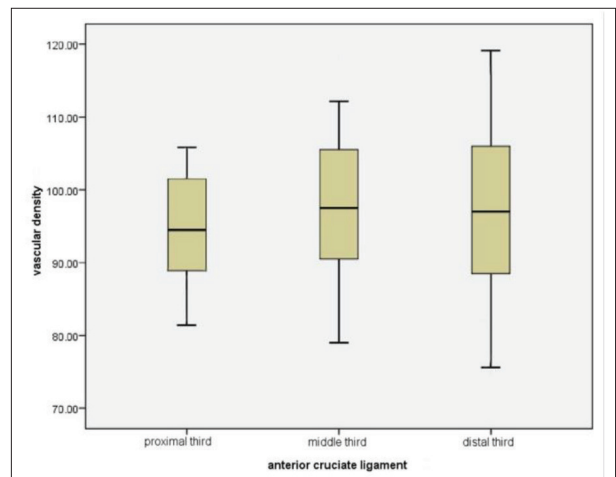


Figure 3. Distribution of number of microvessels in proximal, middle and distal third of anterior cruciate ligament

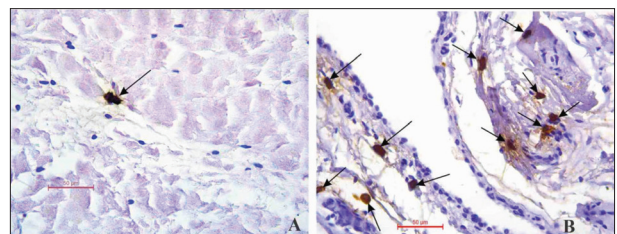


Figure 4. Tryptase-positive mast cells (arrows) in A – intraligamentous tissues; B – synovial tissues of the anterior cruciate ligament (anti-mast cell tryptase immunostaining)

zone of the ACL, reach in blood vessels, we counted 12.6 (6–18) MCs per mm² (Figure 4). An average area value of the MCs was $124.7 \pm 16.2 \mu\text{m}^2$ (91.5–155.8 μm^2), and an average value of shorter and longer axis of the MCs was $11.2 \times 15 \mu\text{m}$ (on average $13.13 \pm 0.91 \mu\text{m}$) (Table 1).

DISCUSSION

Our analysis of the ACL specimens related to their periligamentous vascularization are in agreement with already published results on this topic. The ACL receives arterial blood from MGA, ILGA and IMGA [1, 3]. The arteries originating from the MGA branch in the form of a network in the synovial tissue that surrounds the ligament. From the synovial tissue, small arteries deep into the ACL

and continue as longitudinally oriented intraligamentous vessels. Blood vessels are also found in the interfascicular connective tissue, between the longitudinal bundles of fibers, where they are protected from the forces of ligament stretching.

The ILGA and IMGA, branches of the PA, supply the bony parts and the skin above the lateral tibial condyle, and at the level of the upper border of the medial tibial condyle respectively [7, 8]. Developing and planning the perforating flaps in this region may be initiated including these arteries [9]. The ILGA and IMGA are also responsible for the blood supply to the distal part of the ACL, what was evident in our study. The surgeons should acquire a profound anatomical knowledge of the respective arteries and the extent of the perfused area in order to preserve the distal attachment of the ACL.

The blood supply to the ligament itself is much smaller than the vascular network of the synovial membrane. The network of periligamentous vessels within the synovial membrane extends the entire length of the ligament. The authors also describe these vessels as tortuous blood vessels, which allow them to withstand the demands of complex ligament movements [4, 5]. Thus, the synovial arterial network is very dense, and extends the entire length of the ligament, while in the ACL itself the capillary network is not so intense. In the ligament itself, capillaries follow the bundles of connective fibers. Our findings in the ACL arterial supply agree with this statement. These intraligamentous blood vessels of the ACL could rupture as a result of minor trauma creating an intraligamentous hematoma followed by knee pain and limitation of motion [10].

ACL injuries are among most frequent disabling injuries associated with athletic or other activities (e.g., different accidents). Most ACL injuries occur during sports like skiing, pivoting (e.g., soccer) or non-pivoting sports (e.g., running), that involve dynamic movements such as jumping, pivoting, and cutting. The ACL reconstruction procedure is currently the most common surgical technique for ACL tears. The procedure includes direct arthroscopy, preparation of graft material (e.g., bone-patellar tendon-bone graft), drilling a canal, and fixation of femoral and tibial sides of graft with titanium screws [11]. Both, ACL reconstruction, and non-surgical treatment (e.g., rehabilitation) are accepted procedures with the potential to restore acceptable knee joint function, offering good quality of life. The patients with a surgical procedure of ACL reconstruction report better knee function compared with the non-surgical group [12]. In the other study, most patients who had unsuccessful rehabilitation therapy after ACL injury reported instability, not optimal knee function, and pain during activity. They required and underwent a delayed ACL reconstruction [13]. Increased vascularity and remodeling of human ACL graft in the first year after surgery indicate its insufficient mechanical properties, and need for an extended rehabilitation program [14].

Complications of the ACL reconstruction have been described, including the vascular injuries. The surgeons should be aware about the possible injury to geniculate arteries [15, 16]. Vascular injury or avulsion of the MGA

can also occur during posterior cruciate ligament (PCL) reconstruction during open procedure with a posterior approach [17]. According to our investigation reported avascular necrosis of the medial femoral condyle, as a complication of ACL or PCL surgery, is the result of not preserved femoral nutrient branches of the MGA.

In case of a femoral avulsion tear, primary ACL repair in carefully selected patients possess apparent advantages over ACL reconstruction. Natural healing of the repaired proximal ACL tear is additionally achieved by using a high strength suture tape for the stabilization and protection of ligament from excessive elongation. Restoration of native ACL maintains its structural anatomical characteristics, proprioceptive and biomechanical functions. ACL primary repair is safe less invasive procedure, avoids graft-related complications, and faster rehabilitation is possible [18, 19]. Preserved periligamentous synovial sheath should be confirmed before the repair because of the mesenchymal stem cells which are evident in the tissue surrounding the blood vessels [20]. For revascularization, it is necessary that the site of graft attachment in case of its reconstruction is covered with a synovial membrane. The remnant tissue promotes formation of a fibrin-platelet scaffold, revascularization and ligamentization of ACL [21]. Primary repair promotes cell proliferation in the tendon-bone transitional zone and ligament portions, reduces osteoarthritis-like pathological changes, and maintains blood vessels within the ACL. Histological immunohistochemical research using CD34, the most sensitive endothelial marker of blood vessels, confirmed that the microvessels have a significant effect on tissue healing during the primary repair [18]. Our research performing CD34 immunostaining reported a fine synovial arterial network on the ACL surface from which originated thin vessels for the fibrous tissue of the ligament itself, and penetrating vessels for its femoral attachment and surrounding femoral bony tissue. The precursor cells from the surrounding tissue could invade the area and start fibroblast proliferation and collagen production within the repaired tissue as a basis for biological repair.

Studies using the injection technique have shown uneven vessel density in parts of the ACL. One group of authors claims that the vascularization of the proximal and distal parts of ligaments is rich, while between the vascular network of the central and distal part there is a completely avascular area. The coincidence of a poor vascularity and the occurrence of fibrocartilage in the ACL may be caused by the compression in area of the ligament related to the anterior border of the intercondylar fossa when the knee is in full extension. The proximal portion of the ACL has better vascularity. The MGA gives rise to ligamentous branches proximally, and courses distally along the dorsal aspect of the ACL [1, 3, 4, 5]. According to some authors, the poor healing potential of the ACL is a consequence of the weaker vascularization of the lower central parts of the ligament. According to the other study the distal portion of the ACL have many blood vessels and vascular-derived stem cells than in the middle third of the ligament [22]. One recent study quantified relative perfusion

of proximal, middle, and distal thirds of ACL injected with contrast using magnetic resonance imaging scanner. They demonstrated greatest mean relative perfusion within the proximal ACL region, followed by the middle third, and the least relative perfusion in the distal part [23].

To contrast previous studies, the present research has clearly shown the uniformity of vascularization of all parts, the three thirds of the ACL. The difference between the results of our research and the previous studies is probably in the method used in this study. Injection techniques can give unexpectedly unreliable results depending on the condition of the blood vessels and the quality of the injection material. Many of the earlier studies have been done on an animal model, which often cannot be extrapolated to humans, especially due to the different anatomy of the structures being compared. Because of the small number of analyzed cases in a number of papers the reported results are not relevant. In our opinion the immunohistochemical results are more relevant for analyzing an average ACL vascularization. Analyzing the position of extraligamentous synovial blood vessels approaching the ACL it was evident the existence of greater vascular network close to the proximal attachment of the ligament. We already stated that penetrating vessels from the synovium supply the femoral attachment of the ACL, and surrounding femoral bony tissue, but the intraligamentous vascular density was uniformly distributed through the ACL.

The MCs are present close to blood vessels in all connective tissues. Activation and degranulation of MCs comprise different granules with bioactive molecules (histamine, cytokines, heparin, and tryptase) into the local tissues. MCs can cause endothelial activation, vasodilatation and plasma extravasation, and contribute to neurogenic inflammation [24]. Activated MCs participate in tissue remodeling, by activating connective tissue cells and angiogenesis, by releasing histamine and the activation of other cells (macrophages and platelets) [6, 25]. Our research demonstrated very modest presence of MCs in the tissue of ACL, an average of 3.8 cells per mm². The peripheral synovial tissue of the ACL, reach in blood vessels, contained 12.6 MCs in average per mm². Our observations suggests that the periligamentous synovial sheath of the

ACL is reach in blood vessels, nerves and MCs, and has a great potential to promote ligament healing.

The limitations of our study could be related to the missing subgroup matching for the gender and age comparison. We formed only one group of the available ACL specimens in order to establish precise definitions on the variations of intraligamentous microvessels of these structures. Obviously higher values for length and cross-sectional area of the male comparing to the female ACL, and more prominent ability of the male relating to the female ACL to alter its morphology and mechanical properties during intense loading (e.g., a sport training), may suggest sex-based differences in ACL structure and microvascularization. The ACL is composed of fibrous tissue exposed to the various risk elements related to the extrinsic and intrinsic factors. Age related complex changes in microcirculation activate microvascular inflammatory processes and result in dysfunction. It is possible that ACL vascular density in older may differ from that of young persons. Future investigation should improve understanding of potential gender and age-related differences in ACL microvascular supply.

CONCLUSION

Comparing the three thirds of ACL, proximal, middle and distal, it was observed that all values were not statistically significant, $p > 0.05$; i.e., there is no statistically significant difference in the number of microvessels per third of the ACL. Blood vessels are uniformly distributed regardless of the observed part of the ACL. The highest presence of blood vessels and MCs was noticed in the peripheral synovial tissue of the ACL.

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Conflict of interest: None declared.

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Микроанатомске карактеристике артеријске васкуларизације предње укрштене везе

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САЖЕТАК

Увод/Циљ Циљ студије је био да се проуче имунохистохемијске карактеристике васкуларизације предње укрштене везе (ПУВ), као и квантификација капиларних судова у проксималном, средњем и дисталном сегменту ПУВ. Други циљ овог истраживања била је квантификација и одређивање мерних карактеристика мастоцита ПУВ.

Метод Тридесет хуманих ПУВ пореклом од 30 особа, добијених рутинском обдукцијом, проучавани су под микроскопом после имунохистохемијских реакција на ендотелни маркер CD34 крвних судова и мастоцитну триптазу присутних мастоцита.

Резултати Средња артерија колена (*a. media genu*) у непосредној близини ПУВ даје више малих артерија намењених васкуларизацији самог лигаментна. Просечан број капилара на квадратном милиметру површине поља препарата проксималне трећине ПУВ износио је 1113,84 (959–1240), средње

трећине 1145,43 (924–1310), док је код дисталне трећине ПУВ износио 1134,55 (889–1451). Просечан број мастоцита по квадратном милиметру препарата ПУВ износио је 3,8. У периферној синовијалној зони ПУВ постојало је просечно 12,6 мастоцита по квадратном милиметру. Просечна површина триптаза позитивних мастоцита била је 124,7 μm^2 . Просечна вредност краћег и дужег пречника ћелија била је 11,2 \times 15,0 μm .

Закључак Поређење три трећине ПУВ, проксималне, средње и дисталне, показало је да вредности капиларне густине у вези нису статистички значајне, $p > 0,05$; односно не постоји статистички значајна разлика у броју микросудова по трећинама ПУВ. Крвни судови се равномерно распоређују без обзира на посматрани део ПУВ.

Кључне речи: предња укрштена веза; интралигаментозни микросудови; мастоцити; имунохистохемија