Assessment of efficacy of platelet-rich plasma application in regeneration of the facial nerve in rabbits

Procena efikasnosti primene plazme obogaćene trombocitima u procesu regeneracije facijalnog nerva kunića

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Abstract

Background/Aim. The injuries of the facial nerve lead to paralysis of the mimic musculature, which is conditioned by functional disorders accompanied by deformity of varying degrees depending on the intensity and location of the injury. Surgical treatment is a method of choice to treat an injured nerve. Injuries in the parotid lodge area are repaired by direct neurosuture in combination with platelet-rich plasma (PRP). **Methods.** The experimental study was carried out on 48 chinchilla male rabbits (*Oryctolagus cuniculus*), of about the same weight (2,500–3,000 gr), aged between 3 and 4 months in two surgical stages, in two different periods – six and ten weeks after the first surgical procedure. The animals were divided into four groups: Group I (suture); Group II [suture and fibrin glue (FG)]; Group III (suture and PRP); Group IV (sutures, FG, and PRP). Each group had two subgroups based on the duration of the experiment (six and ten weeks). A part of the dissected nerve in the length of 5 mm was subjected to histologic verification, where the number of axons and Schwann cells was de-termined and expressed numerically based on the histological sample of the tissue of the observed nerve. The extent of the presence of connective tissue and the degree of neovascularisation is shown by the description of histological samples by grades (connective tissue 1-4, neovascularisation 1-3). **Results.** Our results showed that all parameters of regeneration of damaged nerve showed a significantly higher regeneration efficiency after six and ten weeks of intervention in groups treated with PRP therapy with or without using FG. **Conclusion**. The use of PRP and the stimulating effect of activated growth factors results in the regeneration of the facial nerve in the sense of replication of the Schwann cells and the number of axons, with a high degree of neovascularization and minimal proliferation of connective tissue, which histologically corresponds to a healthy nerve.

Key words:
animals, laboratory; facial nerve injuries; histological techniques; nerve regeneration; platelet rich plasma; rabbit.

Apstrakt

Uvod/Cilj. Povrede facijalnog nerva dovode do paralize mimičke muskulature lica, što je uslovljeno funkcionalnim poremećajima praćenim deformitetom različitog stepena, u zavisnosti od intenziteta i lokacije povrede. Hirurško lečenje je metoda izbora za lečenje povređenog nerva. Povrede u predelu parotidne lože se saniraju primenom direktnog neurošava u kombinaciji sa plazmom bogatom trombocitima (PBT). **Metode.** Eksperimentalna studija sprovedena je na 48 činčila zečeva (*Orictolagus cuniculus*) muškog pola, približno iste težine (2 500–3 000 gr), starosti između 3 i 4 meseca, u dva hirurška zahvata, u dva različita perioda: šest i deset nedelja nakon prvog hirurškog zahvata. Životinje su podeljene u četiri grupe: Grupa I (šav); Grupa II [šav i fibrinski lepak (FL)]; Grupa III (šav i PBT); Grupa IV (šav, FL i PBT). Svaka grupa imala je po dve podgrupe na osnovu trajanja eksperimenta (šest i deset nedelja). Deo diseciranog nerva u dužini od 5 mm podvrgnut je histološkoj analizi, kojom je određen i numerički izražen broj aksona i Švanovih ćelija. Stepen prisustva vezivnog tkiva i stepen neovaskularizacije prikazani su gradacijom histoloških uzoraka (vezivno tkivo 1-4, neovaskularizacija 1-3). **Rezultati.** Svi parametri regeneracije oštećenog nerva pokazali su da je

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značajno veća efikasnost regeneracije posle šest i deset nedelja intervencije postignuta u grupama lečenim PBT terapijom, sa ili bez upotrebe FL. **Zaključak**. Upotreba PBT i stimulatorno delovanje aktiviranih faktora rasta dovodi do regeneracije facijalnog nerva, u smislu replikacije Švanovih ćelija i broja aksona, sa visokim stepenom neovaskularizacije i minimalnom proliferacijom vezivnog tkiva, što histološki odgovara zdravom nervu.

Ključne reči:
životinje, laboratorijske; n. facialis, povrede;
histološke tehnike; nervi, regeneracija; plazma bogata
trombocitima; zečevi.

Introduction

The facial nerve is exclusively a motor nerve with intrapetrous sensory and neurovegetative functions.

In the human body, there are 10 to 14,000 fibers (7,000 of which are motor). Injuries of the facial nerve can be intracranial, intratemporal, and extracranial. Sunderland [1] described five degrees of damage to the peripheral nerve fiber. This classification system explains the course of physiological events associated with all types of lesions that involve the facial nerve.

The result of injuries is the paralysis of the mimic muscles, defined as the complete absence of all voluntary movements in parts of the nerve field area, that causes severe esthetic and functional problems to the patient. As a special type of nerve injury, there is an iatrogenic injury most commonly caused by surgical interventions. Lacerations and contusions arise as a result of stinging wounds and blunt injuries, leading to nerve contusions with preserved continuity much less often than lacerations. Compression and stretching occur in the growth of the pathological substrate around the nerve. Burning injuries are caused by the effects of missiles and are most often localized in the temporal bone pyramid [2].

Iatrogenic damage to the facial nerve is most often caused by direct surgery. Intraoperatively, the nerve can be injured directly or indirectly [2].

Iatrogenic injuries occur as a result of numerous neurosurgical, maxillofacial (parotidectomy) [3], and otorhinolaryngeal (ORL) (mastoidectomy) [4] operations. Injuries to the facial nerve can occur when bone fractures and jaw fractures occur, when bone fragments damage or completely interrupt nerves [5]. Injuries of soft tissues in the parotid region can lead to nerve injury of varying degrees [6]. Depending on the pathological changes, the time of injuries, and the severity of damage to the facial nerve, there are several surgical procedures to be performed: decompression, neurosuture in various segments of the nerve, neuroanastomosis with other nerves (hypoglossus, glossopharyngeus) [7], as well as neuroplastic, plastic, and reconstructive surgical procedures.

Choosing the method of surgical treatment depends on the following: "the height" of the nerve lesion, the time elapsed from the moment of injury, the integrity of the distal and proximal part of the nerve, the size of the defect, the age of the patient, presence of degenerative and vascular diseases, as well as the patient's desire [2].

A direct neurosuture of the nerve can be done if the defect is less than 2 cm or 2.5 cm large. For larger facial nerve defects of 2.5 cm, in which no primary reconstruction with a nerve graft of up to 72 hrs has occurred, the nerve endpoints should be marked by the end of treatment and reconstructed after three to six weeks.

Gavron and Clemis [8] state that up to a year after the injury is a good time for hyoglossus-facial anastomosis with the "cross-face" method. Conley and Baker [9] present this problem of time imbalance from the injury and believe this method can be successfully applied no later than two years after the injury. A spontaneous or reflex function of the nerve can rarely be recovered.

Recovery of the facial nerve function is slow, while postoperative sequelae are inevitable [2].

Surgical interventions that have been used so far (epineural and fascicular neurosuture) [10] in order to recover the motor activity of the facial nerve have not fully yielded results, and further research is needed to discover new techniques for the regeneration of the injured facial nerve [11].

The possibility of using PRP growth factors in the process of nerve regeneration, which have so far been used in the regeneration of soft tissues and bones, is being considered [12].

Platelet-rich plasma (PRP) is autogenic concentrated human platelets in a small plasma volume. Platelets are natural deposits of many growth factors released from alpha granules in the form of fundamental proteins: three isomers of platelet-derived growth factor (PDGF) – PDGFαα, PDGF ββ, and PDGF αβ, two transforming growth factors (TGF) β (TGF β1 and TGF β2), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). Growth factors are essential for the onset of tissue repair and regeneration and have a hemostatic and anti-inflammatory effect [13, 14].

The aim of the study was to present the feasibility of using PRP in the regeneration of injured facial nerves at the experimental level, which could later be used in clinical practice.

Methods

This experiment was approved by the Ethics Committee of the Faculty of Medicine in Belgrade under number 5603/2 in March 2013. This study was conducted on 48 chinchilla male rabbits (*Oryctolagus cuniculus*) of about the same weight (2,500–3,000 gr) aged between three and four months, from the farm of the Institute of Medical Research, Military Medical Academy, Belgrade, Serbia. The pathohistological verification of definitive preparations af-

ter the completion of the experiment was done at the Institute of Pathological Anatomy of the Faculty of Veterinary Medicine, Belgrade, Serbia.

Surgical procedure

This experimental study was carried out in two surgical stages, in two different periods, six and ten weeks after the first surgical procedure, in order to monitor the optimal period of nerve regeneration. Four groups (I–IV) of six rabbits were formed, with each group divided into two subgroups depending on the time interval: six weeks (subgroup A) and ten weeks (subgroup B). The results of all four groups were compared while each animal was controlling itself.

Surgical procedures were carried out in aseptic conditions. Rabbits were anesthetized with a combination of ketamine (Ketamidor® 10% injection; 35 mg/kg i.m.), Acepromazine (Promace® injection; 0.1 mg/kg i.m.), and atropine (Atropine Sopharma®, Genotropin; 0.04 mg/kg i.m.). After that, the left half of the face and skin was disinfected with Kodan® (kodan tincture), thus creating the conditions for the first phase of the experiment.

The incision was made with a preauricular-submandibular cut and by blunt preparation through the tissue of the parotid gland identifying the facial nerve trunk directly in front of the stylomastoid foramen. The nerve resection was made before bifurcation, after which the first phase of the surgical experiment began. That implies that one of the following methods of nerve reconstruction was performed: suture – perineural microsuture (nylon 9–0) (Group I); suture and fibrin glue (FG) (Group II); suture and PRP (Group III); suture, FG, and PRP (Group IV). After completing this surgical procedure, the skin was sutured with nylon 5–0.

In the second phase, after six and ten weeks, the same surgical procedure was performed on the opposite side, and that represented the control group. On both sides, the same surgical procedures (nerve identification and nerve resection of 5 mm) were made. On the left side of the resection, there was 2 to 3 mm on both sides from the suture site. On the right side, part of the nerve was resected in the area in front of the trunk at the same length. Further procedure implied special packing of the resected parts of the nerve and carrying them to the Institute where histological analysis and preparation of PRP was done.

PRP was prepared from 5 mL of blood taken from the ear vein from each rabbit, with 0.4 mL citrate, by double centrifugation in laboratory conditions by a special technological process [14].

From 5 mL of taken blood, 0.3 mL of PRP was obtained, which was supplemented with antifibrinolytic (transemic acid 1–5 mg per 0.5 mL cryoprecipitate) and calcium chloride ($CaCl_2$) (0.05 mL 10% $CaCl_2$ per 1 mL PRP) as an activator, applied to the place where the nerve section was made. FG in the form of "Beriplast®" was used in groups II and IV. The number of platelet counts from the peripheral blood of one rabbit was $683,680 \pm 186,229 \times 10^3/\mu L$, while in PRP, this number was $2,677,583 \pm 1,201,418 \times 10^3/\mu L$.

The indicated components were applied directly, successively through two syringes, to the site where the nerve section was made, followed by the instantaneous conversion of the liquid into the gel. In the second phase of the experiment, six and ten weeks after the first phase, a certain portion of the nerve was dissected, which was further treated under standard conditions.

Histological analysis

After the section, parts of the nerve in the length of 5 mm were immediately fixed 24 hrs in a 4% buffered formalin solution. Then they were washed with water and dehydrated in alcohol of increasing concentration (70% to absolute), then lyophilized in xylol and molded into paraffin. Paraffin blocks were cut with a microtome on a sample thickness of 3 to 5 μm. The cross-sections were visible by hematoxylin-eosin staining. Coloring takes place according to the procedures specified in the manufacturer's instructions in several stages. Positive immunoreactivity for the S100 was recorded as nuclear staining. Our products were photographed in the form of a digital microphotograph from a digital microscope and then analyzed by the software (Imagel) program.

In terms of an objective assessment of the effectiveness of regeneration of the damaged nerve depending on the applied method, the following histological characteristics were analyzed: the number of newly created axons, degree of the presence of connective tissue, degree of neovascularization, and the number of Schwann cells.

The number of axons and Schwann cells was determined and expressed numerically based on the histological sample of the tissue of the observed nerve.

The extent of the presence of connective tissue and the degree of neovascularization was shown by the description of histological samples by grades. Connective tissue: 1 – in trace; 2 – in groups; 3 – around individual axons; 4 – around a number of axons. The degree of neovascularization: 1 – no blood vessels; 2 – individual blood vessels; 3 – blood vessels.

Statistical analysis

In the analysis of the results of the pathohistological tests, we used non-parametric tests adapted to small samples and data available in the form of ranks based on a small number of assumptions most commonly performed in practical research.

The nonparametric Kruskal-Wallis test was used to estimate the significance of group differences.

For simultaneous comparison of sample pairs within groups, the Wilcoxon test was used to determine with certainty which samples or methods of reconstructing the damaged facial nerve had a statistically significant difference in the effects.

Results

In all parameters of regeneration of the damaged nerve, our results show that after six and ten weeks of intervention, significantly higher regeneration performance was achieved in groups treated with PRP with or without using FG (Tables 1 and 2).

Comparing the results of the experimental and control groups, we obtained the following results for the indicated test parameters. On the radar diagram of effects in the me-

dian space of the results of nerve regeneration, depending on the applied method and the results measured in the control group, we note that the values closest to those in the control group record the methods of applying PRP with or without using FG, which speaks in favor of their statistically significantly higher efficacy in relation to the number of newly-formed axons (Figure 1), the degree of the presence of connective tissue (Figure 2), neovascularization (Figure 3), and the number of Schwann cells (Figure 4) relative to other methods.

Table 1

Comparative overview of patohistological characteristics of the damaged nerve regeneration six weeks after intervention

Pathohistological characteristics	Groups			
	I	II	III	IV
Number of newly created axons	22[a]	26.5[b]	86.5	87
Degree of presence of connective tissue	4[b]	3.5[b]	2	2
Degree of neovascularization	1[b]	1[b]	3	3
Number of Schwann cells	10[b]	12[b]	83.5	82

Description of the groups and histological methods are given in the paragraph Methods.
Statistical significance: [a]$p < 0.05$ vs. Groups II, III, and IV (Kruskal-Wallis and Mann-Whitney-Wilcoxon test);
[b]$p < 0.05$ vs. Groups III and IV (Kruskal-Wallis and Mann-Whitney-Wilcoxon test).

Table 2

Comparative overview of pathophysiological characteristics of the damaged nerve regeneration 10 weeks after intervention

Pathohistological characteristics	Groups			
	I	II	III	IV
Number of newly created axons	22[a]	26.5[b]	88	89
Degree of presence of connective tissue	3.5[b]	3.5[b]	2	1.5
Degree of neovascularization	1[b]	1[b]	3	3
Number of Schwann cells	9.5[b]	12[b]	84	84

Description of the groups and histological methods are given in the paragraph Methods.
Statistical significance: [a]$p < 0.05$ vs. Groups II, III, and IV (Kruskal-Wallis and Mann-Whitney-Wilcoxon test);
[b]$p < 0.05$ vs. Groups III and IV (Kruskal-Wallis and Mann-Whitney-Wilcoxon test).
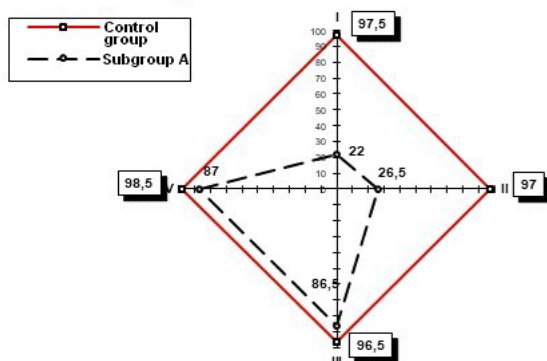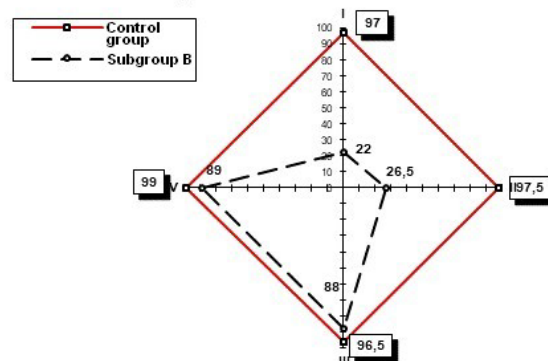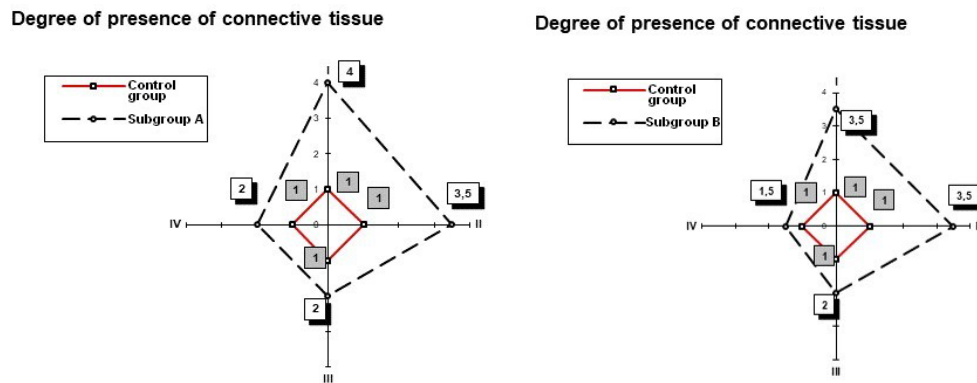

Fig. 1 − Comparison of the effects of the applied methods and results of the control group six weeks after intervention (subgroup A) and ten weeks after intervention (subgroup B) in terms of the number of newly created axons.
Description of the groups and histological methods are given in the paragraph Methods.

Degree of presence of connective tissue

Degree of presence of connective tissue

**Fig. 2 − Comparison of the effects of the applied methods and results of the control group
six weeks after intervention (subgroup A) and ten weeks after intervention (subgroup B)
in terms of the degree of binding of connective tissue.
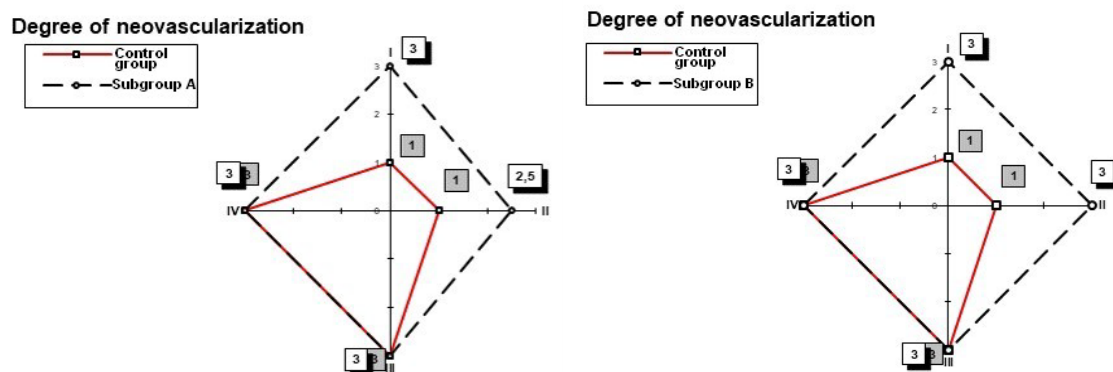Description of the groups and histological methods are given in the paragraph Methods.**

Degree of neovascularization

Degree of neovascularization

**Fig. 3 − Comparison of the effects of the applied methods and results of the control group six
weeks after intervention (subgroup A) and ten weeks after intervention (subgroup B)
in terms of the degree of neovascularization.
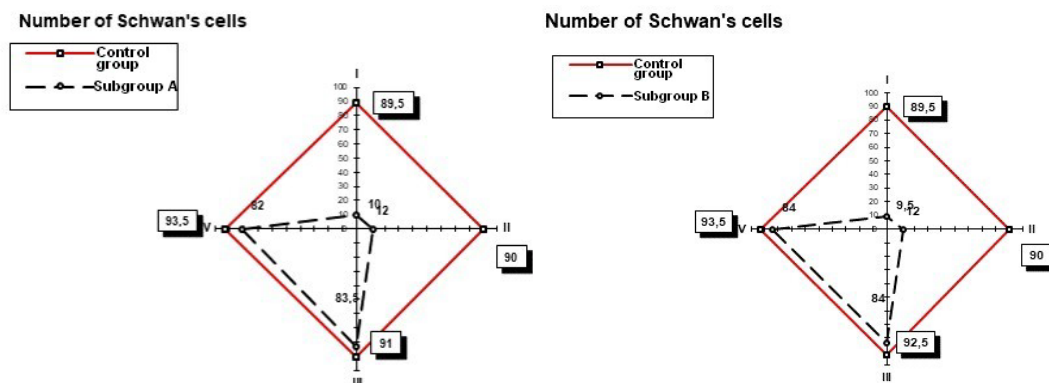Description of the groups and histological methods are given in the paragraph Methods.**

Number of Schwan's cells

Number of Schwan's cells

**Fig. 4 − Comparison of the effects of the applied methods and results of the control group six
weeks after intervention (subgroup A) and ten weeks after intervention (subgroup B) in terms
of the number of Schwann cells.
Description of the groups and histological methods are given in the paragraph Methods.**

Discussion

Injuries of peripheral nerves always lead to incomplete recovery due to the formation of connective tissue that creates a mechanical barrier in the process of axonal regenera-tion. Parts of the injured nerve due to scar healing lead to adhesion to the surrounding soft tissue. Despite the surgical methods of treatment of peripheral nerve injury, they lead to reduced functionality and prolonged recovery. Reduction of epineural degeneration and perineural scarring by using bio-

active factors that regulate connective proliferation and stimulating axonal regeneration on the distal portions of the injured nerve could lead to a rapid and complete recovery of the peripheral nerve [15].

Growth factors in the PRP are a class of natural biological mediators with local and systemic effects that regulate cell migration, coupling, and proliferation and also provide the accumulation of an extra cell matrix by binding to specific cell surface receptors [15].

The effect of PRP in the process of nerve regeneration in wounded nerves is preserved for now only within experimental studies, which showed different results in recent years and opened a new chapter in the polemics of the effectiveness of the PRP application [15].

Based on the published works from 2010 forward, primarily on experimental models for the regeneration of peripheral nerves with a special emphasis on the motor sciatic nerve and the results obtained on that occasion, it can be concluded that the application of PRP in the regeneration of peripheral nerves could be applied in clinical practice [16, 17].

The model of the study was the dominant sciatic nerve of rats and rabbits, after a section of the nerve, according to standardized protocols. Reconstruction was done through a suture or allograft in combination with PRP [16,18,19]. After a certain period, electromyographic examinations were performed, followed by histological analysis of the prepared part of the nerve. All results preferred the use of PRP in relation to control groups where it was not used [20, 21].

When it comes to degenerative changes, there are clinical studies based on the direct injection of PRP into the part of the damaged nerve [22, 23].

Favorable results were obtained after the application of PRP in patients with carpal syndrome, where electromyography tests were almost identical to animal model tests [24].

In the case of injuries of joint cartilage and muscle tendon, the PRP found a wide application in sports medicine with beneficial effects in the process of regeneration of cartilage hyaline with an improvement of function and slowing degenerative processes [23–25].

Platelet-rich plasma combined with bone substitutes or bone autografts can be used in jaw bone reconstruction in experimental and clinical studies [26, 27].

After a series of experimental studies of the effect of PRP on bone regeneration, clinical studies have confirmed the justification of the application of this method, especially when it comes to bone defects after fracture, in alveolar ridge enlargement, sinus elevation for the placement of dental implants after surgery of mandibular and maxillar cysts or tumors, where the resection of the jaws is necessary [26–30].

A large meta-analysis covering the period ending with 2014, with published papers in the field of clinical application of PRP in the bone regeneration of jawbone defects after tooth extraction (199 teeth in 156 patients), shows a positive effect of the PRP application. However, as this sample is still small for definitive conclusions, the results must be carefully interpreted with a proposal to work on a better standardiza-

tion of experimental design in order to better understand the effects of the effectiveness of the PRP application [31].

The primary aim of our study was to give a contribution in terms of a more effective understanding of the use of PRP in the process of nerve regeneration.

Based on the statistical parameters, we concluded that regardless of the time interval (six and ten weeks), there is no difference in relation to the parameters tested.

The number of axons, Schwann cells, and newly created blood vessels are significantly dominant in Groups III and IV, which explains that the FG we used in Group IV together with PRP has no function in the process of nerve regeneration but is rather used as a tissue adhesive whose properties are proven much earlier.

Groups III and IV, in which PRP is used, have almost identical results, which supports the effectiveness of using this biological agent.

When it comes to the degree of connective tissue formation, the results indicate the smallest percentage of representation in Groups III and IV (a lower value indicates better results based on the graded gradation).

Based on all the results, we concluded that there is no statistical significance in relation to the period of the regeneration process. We also concluded that a six-week period could represent the optimum time in the process of nerve regeneration, as the examined values did not significantly change ten weeks after the reconstruction of the nerve was performed.

Conclusion

Our results suggest that using PRP promotes facial nerve regeneration in an animal model of facial nerve axotomy. Laboratory parameters indicated that the platelet count in the PRP was up to four times higher than in peripheral blood, which means that the growth factor concentration was significantly higher in PRP, which can explain its efficiency in the healing process. Based on the obtained histological analyses, we noticed that six weeks after the application of PRP in the process of reconstruction of the facial nerve in experimental animals, the number of Schwann cells and newly formed axons was approximately the same as in the healthy nerve (over 95% success rate). The degree of neovascularisation is identical to that of a healthy nerve, suggesting the efficacy of VEGF from the applied PRP. The connective tissue is present in traces, and knowing that the healing process greatly inhibits the scarring connective tissue, this parameter undoubtedly confirms the effectiveness of this method due to the increased concentration of growth factors that prevent the process of creating the scar tissue. Bearing in mind all these parameters, we can suggest that the PRP enters the standard surgical procedure in the process of regeneration of damaged facial nerves regardless of the type of injuries and under what circumstances it was created, with the recommendation that further experimental studies support the clinical application of PRP.

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