ORIGINAL ARTICLE

Bone healing during electrostimulation

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Abstract

Aim To investigate the effect of direct epineural electrical stimulation of the nerve on the nature of reparative processes in the bone stump.

Methods Three series of experiments were carried out with amputation of the thigh in the middle third and muscle plasty. In the 1st and 2nd experimental series a perineural catheter was brought to the stump of the sciatic nerve, through which mechanical irritation of the nerve was performed for 20 days daily for 20 minutes. In the 2nd series, an electrode was added to the nerve and epineural electrical stimulation was performed daily for 20 days. Animals of the 3rd series served as control. The observation periods were 1, 3, 6 months. Histological research method with filling vessels with ink-gelatin mixture was applied.

Results In the 1st series, there was a sharp distortion of the reparative process, which consisted of a violation of microcirculation, changes in shape, resorption of the cortical diaphyseal plate, fractures, deformations. In most experiments of the 2nd series, organotypic stumps were formed with normalization of microcirculation. In the 3rd series, results of the formation of the stump were better than in the 1st, but worse than in the 2nd series.

Conclusions Painful nerve irritation after amputation leads to a significant disturbance of microcirculation and reparative regeneration at the end of the bone stump with the development of pathological restructuring of bone tissue. Electrostimulation of the nerve improves microcirculation and reparative regeneration of the bone tissue.

Key words: amputation, bone regeneration, epineural stimulation, microcirculation

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INTRODUCTION

Amputation of a limb is a severe physical and psychological damage to the patient. It is accompanied by a trauma of peripheral nerves with the ascending reaction of the central nervous system, it worsens the functioning of vessels with irritation of perivascular sympathetic plexus, changes the nature of proprioceptive imposition and significantly impairs the static-dynamic stereotype, which carries a potential threat of various complications (1). The leading one among them is the pain syndrome caused by nerve crossing (2). Studies (3-5) demonstrated that 72% of patients developed post-amputation pain syndrome within the first 8 days after amputation. After 6 months, it was observed in 65% of patients, and after 2 years – in 60% of patients (6).

The formation of a residual limb, ensuring the possibility of adequate prosthetics, is no less challenging. It is caused by difficulties in creating favourable conditions for healing of all residual limb tissues (7). The very idea of the conditions necessary for the full course of reparative processes and specific parameters of the residual limb suitability after their completion are beyond the surgeons' competence. Especially it concerns the backbone of a residual limb – its bone base (8). Despite the large number of studies on amputations and post-amputation pain syndrome, diseases and defects of stumps (3-6, 8-10), there are only few reports (11, 12), which highlight the processes of reparative regeneration at the end of the bone remnant of the future working organ. The issues of shaping the end of the residual limb in concomitant post-amputation pain syndrome remain unexplored. Pain syndrome takes place from the first days after amputation (5, 6), and in many cases even before it, due to the involvement of nerves in its occurrence (13).

Thus, the issue of bone residual limb formation, as well as the treatment of pain syndrome caused by peripheral nerve injury, remains to date a complex, unresolved problem (13). In this regard, it seems reasonable to develop and implement new methods of impact on the tissue structures to optimize the process of healing (9) and functioning of the residual limb.

In recent years, a strategy has been developed to treat peripheral nervous system injury through intratissue and direct electrical stimulation of nerve trunks, providing an opportunity to improve the blood supply to tissues and eliminate the disorders inherent in neuropathies (14-21). In this regard, it seemed appropriate to study the effect of direct electrical stimulation of the nerve trunk on the reparative process in the bone residual limb in experiment, since amputation sharply disturbs the blood supply to the bone and is often complicated by neuropathies (13).

The aim of this study was to investigate the impact of direct epineural stimulation on the reparative processes in the bone residual limb. It is the first study about this topic.

MATERIAL AND METHODS

Material and study design

Experiments were performed in vivarium of National Pirogov Memorial Medical University (Vinnytsia, Ukraine) from 2018 to 2021 in accordance with the principles of humane treatment of animals as set out in the European Community directives (86(609) EEC) and the Declaration of Helsinki on Humane Treatment of Animals.

Three series of experiments with mid-third femoral amputation and muscle plasty were performed on 54 rabbits. In the 1st and 2nd experimental series, a perineural catheter was placed to the sciatic nerve stump during amputation, and mechanical irritation of the nerve was performed for 20 days daily for 20 minutes. In the 2nd series, an additional electrode was attached percutaneously to the nerve and sutured to the skin. Epineural electrostimulation with a frequency of 8 Hz/sec and an amplitude of 20-40 mA was performed daily for 20 days along the installed electrode, 2 times a day. After the course was finished, the perineural catheter and the epineural electrode were removed. The animals of series 3 served as controls. The follow-up periods were 1, 3, and 6 months, 5 animals for each period. To study the peculiarities of the reparative process at the early stages of the experiment, an additional study was carried out in the 2nd series at 7, 14, 21 days, 3 animals for each period.

An approval of the Ethics Committee of the Scientific and Research Institute of Rehabilitation of National Pirogov Memorial Medical University was obtained for this study (approval No 3/2023).

Methods

A method of the investigation was histological with infusion of vessels with ink-gelatin mixture. After appropriate wiring, 15-20 microns thick sections were made and stained with haematoxylin and eosin and picrofuchsin. Enlightened sections of 100-150 μm thickness were also made. Histological sections of nerve fibres were stained with haematoxylin and eosin and impregnated with silver.

A morphometric study was performed using digitized images of the preparations on a panoramic scanner (3DHISTECH, Hungary) using the image analysis software Orbit Image Analysis and Panoramic Viewer. To estimate the amount and structure of bone tissue, the area (S) of osteogenic, chondroid and fibrous tissues in the specimen and their percentage of the specimen area were evaluated.

Statistical analysis

The statistical method based on analysis of variance (ANOVA) was used to analyse biomedical information by identifying differences between sample means for three groups of animals.

RESULTS

1st experimental series – 15 observations

In the term of 1 month, in 5 observations, a cylindrical form of the bone stump without the formation of a bone closure plate was found in all observations. In three observations, the medullary canal was closed by dense fibrous tissue, and in two observations, partially by dense fibrous tissue, partially by a network of endosteal formed immature bone beams. Between the beams there was cellular-fibrous tissue. The bone sawbones had fine serrations. Significant avascularity of the ends of the cortical diaphyseal plate (Figure 1A) and bone marrow was evident. Only some of the haversian canals were dilated. They contain vessels enlarged in diameter. In the medullary canal there was cell-poor oedematous loose fibrous tissue with a large number of tissue cysts, sinusoidal vessels and sharply dilated branches of the feeding artery throughout. The cortical diaphyseal plate was focally thinned and spongy.

In the term of 3 months, in 5 observations, the bone stump was cone-shaped. Compared with the previous period, significant changes in the cortical diaphyseal plate were observed.

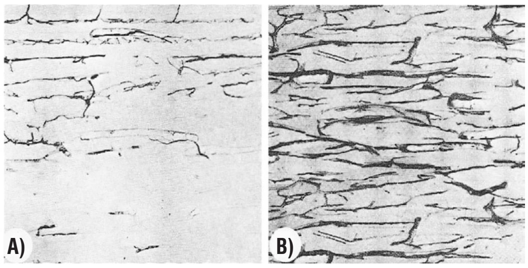

Figure 1. Microphotographs. Cortical diaphyseal plate, 1 month old: A) with avascularity, B) with significantly dilated vascular channels. Enlightened slices. x18 (Shevchuk V., 2021)

If avascularity of the plate and bone marrow was preserved, the process of bone atrophy with spongification and sharp thinning on the endosteal surface, especially the terminal one, was progressively increased. In the areas of thinning, cellular-fibrous osteoblastic tissue with the presence of osteoclasts was revealed. In other areas there were pronounced dystrophic changes. Diffusely located macrophages, lymphoid and plasma cells were seen in the resorption zone. Significant enlargement of the vessels of the cortical diaphyseal layer and branches of the *arteria (a.) nutricia*. There progresses deficit of the microvascular network, impaired vascular permeability, disappearance of the cellular composition of vascular channels, bone tissue resorption along the vascular channels and endosteal surface against the background of the absence of proliferative processes. Fatty bone marrow was replaced by oedematous fibrous connective tissue with tissue cysts and sinusoids. The narrowed end of the stump was overgrown with fibrous and fibrous-bone tissue. The process of remodelling continued. On the periosteal surface there were erosions, osteoclasts.

In the term of 6 months, in 5 observations, a cylindrical stump was formed in three observations, with extensive resorption of the cortical diaphyseal plate and fractures in the zone of greatest resorption. The endosteal regenerate at the end of the residual limb was represented by bone beams of varying maturity. The interstitial spaces contained wide varicose altered sinusoids. Between the beams of the endosteal regenerated, large branches of the feeding artery passed from the medullary canal into the soft tissue fringe (Figure 2). In the distal and proximal parts of the medullary canal, the di-

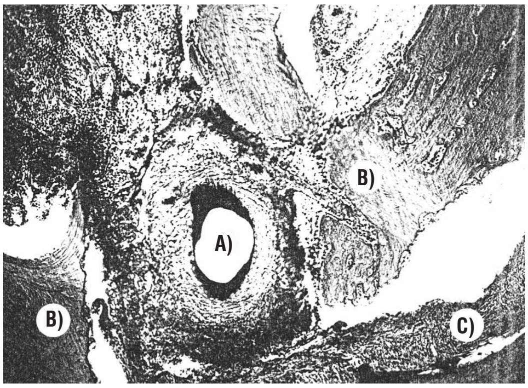

Figure 2. Microphotograph. A) branch of the feeding artery B) passing through the endosteal regenerate C) into the fibrous tissue surrounding the stump. Haematoxylin and eosin staining. x90 (Shevchuk V., 2021)

lated branches of *a. nutricia*, veins, sinusoidal vessels, and tissue cysts were found. There was a wall oedema of medullary contents represented by loose fibrous tissue. Completeness of reparative regeneration was not observed in any of these cases. In two observations of this term, the diaphyseal form was disturbed due to the formation of a bone regenerate along the periosteal surface and destruction of its end (Figure 3A). In one of them, resorption and spongification of the cortical diaphyseal plate over a large extent and its replacement by bone regenerate was noted. In another observation, the periosteal bone-cartilage overgrowth consisted of hyaline cartilage and a network of immature beams. In the medullary canal of the end stump endosteal bone formation was represented by a network of bone

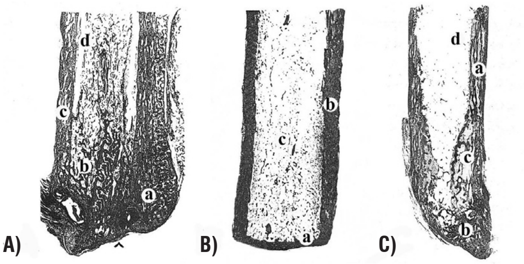

Figure 3. Histotopograms. Stumps of experimental (A, B) and control (C) groups.
A) Cylindrical residual limb: resorption of the cortical diaphyseal plate over a large extent and its replacement by bone regenerate (a), endosteal bone beams (b), spongy cortical diaphyseal plate (c), oedema in the proximal part of the medullary canal (d), dense connective tissue surrounding the residual limb – arrow; B) cylindrical stump: bone closure plate (a), cortical diaphyseal plate (b), content of the medullary canal (c); C) the residual limb with a deformed cone-shaped end due to asymmetry: thickened spongy cortical diaphyseal plate (a), cone-shaped end (b), endosteal formed bone beams (c), oedema in the proximal part of the medullary canal (d). Haematoxylin and eosin staining. x2.5 (Shevchuk V., 2021)

beams. The medullary canal was closed by a not fully formed fibrous plate, through the thickness of which branches of the feeding artery passed; the cancellous bone tissue here had different degrees of maturity. The shape of the fibrous-bony structures closing the medullary canal is irregular. In the medullary canal, bone beams were seen at a considerable distance, in the interbody spaces there was loose fibrous tissue, sinusoidal vessels, and tissue cysts. The formed bone closure plate was not observed in any of the observations. The examination of the nerve and surrounding tissues revealed a picture of sciatic nerve neuritis in all observations.

There were pronounced oedema, degeneration of nerve fibres, infiltration of the nerve trunk with lymphocytes, arteritis and obliteration of arteries. A consequent exudation caused thinning and fragmentation of nerve fibres, pronounced oedema of epineurium, perineurium, endoneurium. Hypertrophy of haemocytes with vacuolization of their cytoplasm was noted.

2nd experimental series – 24 observations

In order to examine in detail, the dynamics of the reparative process at early terms, additional observations were carried out in this series at 7, 14 and 21 days.

In the term of 7 days, in 3 observations, at one week in all three observations at the end of the medullary canal fibroreticular tissue with single primitive beams, tissue cysts and sinusoidal vessels was revealed. Bone marrow, interlayers of oedematous loose fibrous tissue, tissue cysts, branches of feeding artery were preserved in higher parts of the canal. The cortical diaphyseal plate was avascular throughout 2 cm in the lucid preparations. The smooth end surface of the file was covered by fibrous tissue, the collagen fibres of which were transverse to the longitudinal axis.

In the term of 14 days, in 3 observations, after 14 days, at the level of the file, starting from the inner surface of the cortical diaphyseal layer, more mature bone beams located in 2-3 rows were revealed. They were transverse to the axis of the limb and overlapped the entrance to the medullary canal. Above the beams there was a zone of fibroreticular tissue. The lower surface of the beams, together with the fillet, was bordered by fibrous tissue. The ends of the cortical diaphyseal plate of the stump remained avascular. However, positive dynamics consisting in staining and enlargement of a part of the haversian canals and carcass exiting into the perivascular spaces was noted. In the proximal part of the canal there were bone marrow, tissue cysts, small branches of the feeding artery.

In the term of 21 days, in 3 observations, after 21 days, revascularization of the cortical diaphyseal plate occurred on the lumen preparations. Most of the vascular canals were sharply dilated. They contained a network of micro vessels enlarged in diameter, some of them sinusoidal. At the entrance to the canal, a bone barrier plate with a horizontal arrangement of bone beams was formed.

In the term of 1 month, in 5 observations, after 1 month, the stump shape was cylindrical, the contours of the cortical diaphyseal fillet were slightly jagged. The cortical layer with significantly enlarged canals containing both single, increased in diameter vessels and capillary network (Figure 1B). At the entrance to the canal, a compact bone barrier plate overlapping it was formed. The zone of fibroreticular tissue above the lamina disappeared. Bone tissue of the last osteon-beam structure ($p_{1,2}$<0.05; $p_{2,3}$>0.05). In the space between the bone beams, fibrous tissue with carcass-infused vessels and tissue cysts was loose. In proximal sections fatty bone marrow with microcirculatory network characteristic for diaphysis appeared: The distal parts of the bone marrow were filled with fatty and partially hematopoietic bone marrow with sinusoids and tissue cysts.

In the term of 3 months, in 5 observations, at 3 months of age, the stump shape was cylindrical. In four observations the medullary canal was closed by the bone cortical closure plate. Beams of endosteal bone formation were not detected. The reparative process was completed ($p_{1,2}$<0.05; $p_{2,3}$<0.05). In these observations the intraosseous microcirculation and the state of the medullary tissues were completely normalized (Figure 4A). In the fifth observation, the osseo-bar bone layer had an osteon-beam structure and consisted of not quite mature bone tissue. There was marginal resorption of the cortical diaphyseal plate. At the level of this area, fibroreticular tissue with single bone beams, tissue cysts and sinusoids can be seen in the medullary canal among the fatty bone marrow. Such a picture indicates a tendency to complete reparative processes.

In the term of 6 months, in 5 observations, four observations included cylindrical stumps with a bone closure plate of mature bone tissue, reduction

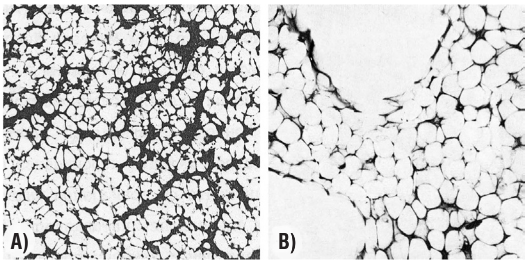

Figure 4. Microphotographs. Fatty bone marrow, 3 months: A) with normalized microcirculation, B) with avascularity. Haematoxylin and eosin staining, x90 (Shevchuk V., 2021)

of large vessels, normalization of microcirculation, and full completion of the reparative process was observed ($p_{1,2}$<0.01; $p_{2,3}$<0.05) (Figure 3B). In one case there was a stump with an enlarged base due to a small periosteal regenerate. The lamina here was represented by insufficiently mature bone tissue. In the distal part of the medullary canal there was fatty bone marrow, tissue cysts, sinusoids. At the level of periosteal regenerate partial resorption of cortical diaphyseal plate and its uneven thinning in this zone was determined. The reparative process was not fully completed.

The truncated sciatic nerve had a thickening at the end. Dystrophic changes, chaotic arrangement of fibres forming a tangle with growth bulbs and spirals were observed in all terms. No inflammatory phenomena were observed.

3rd (control) series – 15 observations

In this series of experiments, the results of residual limb formation in all terms were worse than those of the 2nd series, but slightly better than the 1st series. The cylindrical shape of the stump was preserved in 13 out of 15 cases. In two cases it was cone-shaped. The bone closure plate was immature at one month of age ($p_{1,3}$>0.05; $p_{2,3}$>0.05), and at 3-6 months of age, only two observations consisted of mature and the remaining eight consisted of immature bone tissue ($p_{1,3}$<0.05; $p_{2,3}$<0.05). Avascularity of the cortical diaphyseal plate and bone marrow (Figure 4B). in eight observations was preserved even at 3 months of age. In most observations, there was a sharp spongification of the cortical diaphyseal plate, its focal resorption, and fractures with the presence of immature bone beams. Intraosseous circulation was represented by dilated microvessels in the form of sinuses and small tissue cysts. In two cases at 6 months of age, bone resorption phenomena progressed along the course of the vascular canals on the side of the medullary cavity and periosteal surface, leading to focal saponification, thinning of the cortical plate, and cone-shaped changes in the stump shape (Figure 3C). The end of the residual limb was closed with fibrous bone tissue. In these observations, as in the others in this series, there was no completion of microcirculation and reparative regeneration (Table 1).

Table 1. Quantitative assessment of tissue structures of the amputated stump at different times of the experiment

Group animals	Tissue structure	Quantitative assessment of tissue structures of the amputated stump (%) follow up		
		1 month	3 months	6 months
1st series	osteogenic	38.2±2.48	42.8±6.38	51.4±4.45
	chondrogenic	28.4±3.34	25.4±2.88	22.8±2.39
	fibrogenic	33.5±2.07	31.8±7.79	25.8±4.44
2nd series	osteogenic	49.6±3.36	64.2±2.28	78.8±2.59
	chondrogenic	31.2±4.97	26.4±2.96	16.8±4.82
	fibrogenic	19.2±5.02	9.4±4.62	4.4±4.51
$p_{1,2}$		< 0.05	< 0.05	< 0.01
3rd series	osteogenic	43.4±4.28	52.8±4.38	65.8±4.38
	chondrogenic	34.2±4.82	27.6±2.7	19.8±3.63
	fibrogenic	22.4±5.32	19.6±2.09	14.4±3.85
$p_{1,3}$		> 0.05	> 0.05	< 0.05
$p_{2,3}$		> 0.05	< 0.05	< 0.05

p, statistical difference

Nerve trunk with thickening at the end. Heterogeneous maturation of connective tissue was traced. There were nonvascular zones formed by clusters of thick bundles of collagenous fibers. In two observations with cone-shaped and three with cylindrical stumps there was infiltration of nerve trunk with lymphocytes, oedema of epineurium, perineurium and endoneurium. In the remaining observations there was no inflammatory process of the nerve.

DISCUSSION

The conducted study revealed a significant disturbance of the processes of reparative regeneration and remodelling of the bone stump caused by pain syndrome and the development of sciatic neuritis. In all experiments of series 1 and part 2 there was a violation of the bone microvascular network, increased extravascular circulation, inhibition of proliferative processes, activation of resorption, which led to degenerative-dystrophic and atrophic changes in bone and bone marrow tissue. The formation of the bone closure plate at the end of the bone stump was disturbed. Healing of the fillet was performed by immature bone or fibrous-bone tissue. The anatomical shape of the residual limb was disturbed in 9% of 30 cases. Completeness of the reparative process was observed in only 2 (6.7%) of the 30 observations. These data coincide with the results of Shevchuk et al. study (24) suggesting that the processes of bone residual limb reparative regeneration were without any effect on the nerve and bone residual limb. Similar disorders of bone tissue reparative regeneration in fractures accompanied by nerve damage were noted by (25). In this regard, the author emphasized the need to find various ways to influence the nerve in order to optimize bone reparative regeneration.

In the experimental series, under the influence of nerve electrostimulation, a significant dramatic expansion of the vascular network of the cortical diaphyseal layer, medullary cavity, large branches of the feeding artery, soft tissue vessels and periosteum, and rapid development of anastomoses took place at an early stage (2 weeks) after amputation. Such simultaneous and powerful inclusion of all possible ways of bone blood supply compensation into the revascularization process affected not only the reparative regeneration of the end of the bone stump but also the remodelling of the cortical diaphyseal layer and the medullary contents. In all cases of this series, an anatomical cylindrical shape of the stump with a compact bone lamina and close to normal microcirculation were formed. Completeness of the reparative process in the experimental series was noted in all observations at 3-6 months.

The obtained results confirm the clinical data (26, 27) on the improvement of arterial and venous circulation under the influence of electrical nerve stimulation. No phenomena of inflammation of the truncated nerve were found in any observation of this series. Thus, the anti-inflammatory effect of direct electrostimulation of the nerve is confirmed, which is in agreement with the data (28), which studied its effect on the neuroskeleton.

The presented morphological studies of features of the course of the reparative process at the end of the stump under the influence of electrical nerve stimulation can significantly deepen and complement experimental (29) and clinical (30) data on the course of osteogenesis.

In our opinion, the listed effects of direct electrostimulation of the nerve can be mainly explained by its influence on the occurrence and course of pain syndrome after amputation. This opinion is indirectly confirmed by the data (2) proving the normalization of reparative regeneration processes at the end of the bone stump during long-term relief of pain syndrome after amputation.

The data of morphometric studies confirm the positive effect of electrical stimulation of the nerve on the reparative process (15, 16). Although it should be noted that the presence of cartilage and fibrous tissue during bone residual limb healing cannot be considered a manifestation of regenerative tissue pathology, it only indicates less favourable conditions in which the reparative processes proceed.

In conclusion, it should be noted that painful irritation of the nerve caused after amputation leads to significant disturbance of microcirculation and reparative regeneration at the end of the bone stump with the development of pathological reorganization of bone tissue. Electrostimulation of the truncated nerve improves microcirculation and reparative regeneration of bone tissue with rapid formation of the bone closure plate, preservation of the organotypic shape of the stump and completion of the reparative process.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

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