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Research Article

Assessment of the severity of delayed changes in the state of the neuromuscular system during correction of local cold injury of III-IV degree with pCMV-VEGF165 plasmid preparation in an experiment

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Abstract

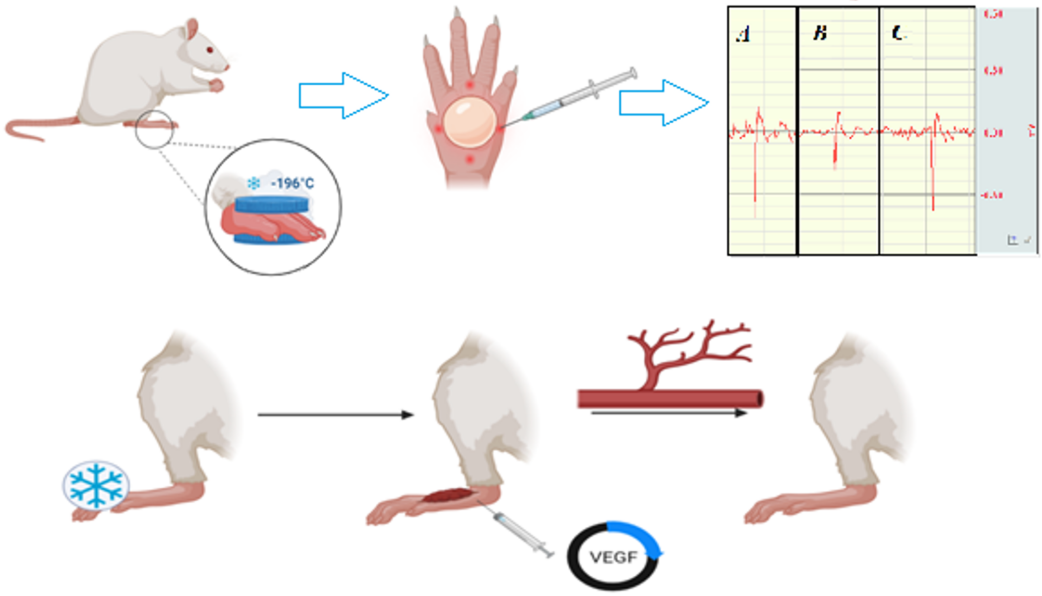
Introduction: The relevance of the topic is due to a wide range of clinical manifestations of frostbite, including long-term complications such as neuropathy, chronic pain, and functional impairments. Tissue recovery after frostbite depends on the degree of microvascular damage, and VEGF (vascular endothelial growth factor) is considered a promising agent for stimulating angiogenesis and improving tissue trophism. **The aim** of the study was to study the effectiveness of the use of a genetic construct – pCMV-VEGF165 plasmid in the correction of delayed complications of cold injury in rats.

Materials and Methods: Local frostbite of III-IV degree was modeled in 18 rats using a neodymium magnet cooled in liquid nitrogen. On days 2 and 7 after the injury, pCMV-VEGF165 (60 μg) was administered to the wound edges. The control group received a placebo. The state of the neuromuscular system was assessed using electromyography on days 28 and 60.

Results and Discussion: In the control group, a significant decrease in the amplitude of the M-response (1.75 times, p=0.001) and an increase in the latency period by 10% (p=0.004) were observed, indicating axonal damage and myelinopathy. The decrement index of the M-response increased (2.2 times), suggesting impaired neuromuscular conduction. In the pCMV-VEGF165 group, the amplitude of the M-response, latency period, and decrement index did not differ significantly from those in the intact animal group.

Conclusion: pCMV-VEGF165 promotes a reduction in the degree of tissue damage and accelerates their regeneration by restoring blood flow, confirming its effectiveness in preventing delayed complications of cold injury.

Graphical abstract



Keywords

EMG; frostbite; myelinopathy; VEGF

Introduction

In military medicine, cold injuries, including frostbite, have long been recognized as a significant cause of morbidity and mortality. An example is Hannibal's crossing of the Alps in 218 BC, when only 19,000 out of 38,000 people survived due to the cold, or Napoleon Bonaparte's invasion of Russia in 1812 (Handford et al. 2017). In the Great Patriotic War of 1941-1945, frostbite accounted for 2-4 % of the structure of combat surgical trauma (Pestov 2020). However, now technological advances have allowed people to travel and live in colder conditions, which has made frostbite more common among the civilian population nowadays. The frequency of frostbite in the Russian Federation on average is no more than 0.3 - 1% of all accidents, but in Siberia, the Far East and the Northern regions of Russia it reaches 20% of all cases of thermal injury (Medical professional non-profit organization All-Russian public organization Association of Combustiologists “World without burns” 2024).

The range of clinical manifestations of frostbite is quite wide: ranging from injuries that heal completely without any consequences, to injuries that lead to serious disability and amputation of limbs (Imray et al. 2009). However, even without significant tissue loss, patients may suffer from long-term effects after suffering frostbite. Due to the short periods of medical supervision and the fact that most case descriptions in the literature do not consider long-term consequences, it is difficult to specify the exact frequency of these conditions. Despite the limitations, a review of five different studies showed that 67% of patients have long-term complications from frostbite, the most common of which are neuropathy, chronic pain and functional disorders (Regli et al. 2021). Prevention and treatment of long-term complications of cold injury is an important and challenging task.

Pathological changes that occur in tissues when exposed to low temperatures can be divided into direct cell damage and indirect damage mediated by progressive ischemia. Intense exposure to cold leads to peripheral vasoconstriction. Further, intracellular ice

crystals form in the tissues, which leads to an imbalance of electrolytes and pH, cellular dehydration, and microvascular damage. Vasodilation after warming triggers a cascade of reperfusion injury through the generation of free radicals and the production of prostaglandins, thromboxane, bradykinin, and histamines. These vasoactive substances worsen endothelial damage, reduce blood flow and increase thrombosis. Together, these factors eventually lead to ischemia and tissue necrosis (McIntosh et al. 2019). The outcome of frostbite depends on the degree of microvascular damage in the tissue. After acute pathology, the long-term effects of frostbite are associated with vasomotor dysfunction. In particular, vasospasm leads to circulatory disorders, which leads to chronic pain and hypersensitivity to cold. In addition, nerve damage caused by cold is associated with neuropathic pain and ischemic neuritis (Gorjanc et al. 2018).

Improved understanding of the pathophysiology of frostbite has led to the development of various treatment regimens, including the widespread use of rapid warming in a vortex bath, thrombolytic therapy, surgical reconstruction under X-ray control and pain correction (Murphy et al. 2000; McIntosh et al. 2019). However, despite some promising therapeutic concepts, the paucity of data on the long-term effects of frostbite in the literature indicates the need for more in-depth studies of this pathology in all its aspects (Regli et al. 2021).

Neovasculgen® is a highly purified supercoiled form of pCMV-VEGF165 plasmid encoding Vascular endothelial growth factor (VEGF). The use of VEGF as a therapeutic agent for contact cold injury has prospects due to its ability to stimulate angiogenesis and improve tissue trophism, which may be critically important for the restoration of damaged areas after frostbite (Ishchenko et al. 2024). Experimental studies show that the use of VEGF can reduce the degree of necrosis in tissue ischemia and improve the restoration of neuromuscular function (Shvalb et al. 2011; Burleva and Babushkina 2016).

The purpose of this study was to study the effectiveness of the use of a genetic construct – pCMV-VEGF165 plasmid in the correction of delayed complications of cold injury in rats. In the course of this study, the hypothesis of therapeutic angiogenesis was tested through the use of Neovasculgen® to stimulate regenerative processes and reduce the frequency and severity of delayed complications.

Materials and Methods

The study was conducted on the basis of the Research Institute of Pharmacology of Living Systems (Belgorod). The experiment was performed according to The Rules of Laboratory Practice, approved by Order No.708n of the Ministry of Health of the Russian Federation of August 23, 2010, in strict compliance with The European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (Directive 2010/63/EU). The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (Minutes No 03/24 of March 18, 2024). Vivisection was performed in compliance with the ethical principles of treating laboratory animals outlined in The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (CETS No.123).

Experimental animals

The study was conducted on white female laboratory rats weighing 275±25 g. A total of 18 female rats were used in the experiment. The rats were obtained from the Stolbovaya Branch of the Federal State Budgetary Institution of Science “Scientific Center for Biomedical Technologies of the Federal Medical and Biological Agency” (Russia).

The rats were kept in polycarbonate cages on a litter of hardwood sawdust. Throughout the study, the animals were kept on a standard laboratory diet (granular feed for laboratory rodents) and tap filtered water ad libitum. The animals were kept in controlled environmental conditions (18-26°C and 30-70% relative humidity). A 12-hour lighting cycle was maintained in animal rooms.

Animals without signs of deviations in appearance were randomly selected in the experimental groups, so that the individual weight value deviated from the average value by no more than 20%. Each animal was assigned an individual number. The identification of the animals was carried out using individual tags on the body.

Drug under study

The tested drug (Neovasculgen®, NextGen JSC, Russia) is a lyophilizate for the

preparation of a solution for intramuscular administration containing deoxyribonucleic acid plasmid supercoiled pCMV VEGF165. The dosage form of the tested drug requires dilution with water for injection immediately before use and warming to room temperature. To prepare a solution for intramuscular administration, immediately before use, water for injection at room temperature in an amount of 2 mL was added to the contents of the vial, and the drug was completely dissolved. The prepared solution of the tested drug was examined immediately before administration for transparency and absence of undissolved particles, and its temperature was determined – before administration, the solution was to be at room temperature 22°C – 25°C. A single dose of the drug was 60 micrograms of pCMV-VEGF165 plasmids dissolved in 100 mL of water for injection: 0.025 mL each on the right and left, top and bottom from the bottom of the wound at a distance of 2 mm at an angle of 45° with a 30G needle (0.3-4 mm). The dose calculation for local administration was carried out based on the ratios included in the preclinical study of the drug Neovasculgen® based on the level of basal metabolism of humans and laboratory rodents.

Experimental model

In 12 mature female wild-type rats weighing 275±25 g, in accordance with international rules for the humane treatment of laboratory animals, a local cold injury of III-IV degree was simulated by contact of a neodymium magnet cooled in liquid nitrogen with the back surface of the distal posterior left limb (Aizawa et al. 2019). After anesthesia (Xyla+Zoletil in doses of 5 mg/kg + 10 mg/kg, respectively, intraperitoneally) the dorsal surface of the left hind limb was shaved with electric scissors, then depilation cream was applied for 3 minutes to remove the remaining hair. Neodymium magnets (diameter 15 mm; thickness 2 mm) were cooled in liquid nitrogen (-196°C) for 5 minutes, and another identical magnet was placed in polyurethane foam at room temperature. The left hind limb of the rat was fixed on a magnet embedded in foam with a film. Then the cooled magnet was placed on the back of the foot using tweezers, as a result of which, by means of magnetic force, the cooled magnet was pressed tightly against the skin. The exposure time of the cooled magnet on the skin was 1 minute, after which the magnet was removed and the paw was released. The frozen skin was warmed at room temperature (22°C). After recovering from anesthesia, the animals were returned to their cages without the wounds being bandaged.

Experimental groups

After modeling the cold injury, the animals were divided into experimental groups of 6 rats:

1) experimental group – on days 2 and 7 after modeling frostbite, rats in the experimental group were injected into the edges of the wound with a solution containing 60 micrograms of the drug under study (paravulnarly to the right and left, above and below the wound at a distance of 2 mm at an angle of 45 ° with a 30G needle (0.3-4 mm));

2) control group – the rats of the control group received a placebo injection (water for injection) in a similar way on days 2 and 7 after modeling the pathology;

3) intact group – animals that have not been exposed to cold.

EMG registration

The analysis of delayed complications of cold injury was carried out by assessing the state of the neuromuscular system by electromyography on the 28th and 60th days after modeling frostbite. EMG registration was performed using the Biopac MP150 software and hardware complex with an EMG100C amplifier (BIOPAC Systems, Inc., USA), and the data obtained was processed using the AcqKnowledge program, version 4.2.

The anesthetized animal was placed on the operating table in a position on its stomach, with its forelimbs, hind limbs, and tail fixed. Next, subcutaneous application of needle electrodes (EL450, EL452) was performed as follows:

1. Recording electrode (Vin+): into the projection of the distal portion of the vermiform muscle located between the 2nd and 3rd metatarsal bones through the interdigital space;

2. Recording electrode (Vin-): into the area of the middle part of the above muscle, at an angle of 30-60° to the axis of the first recording electrode;

3. Stimulating electrode (cathode): into the projection of the popliteal fossa;

4. Stimulating electrode (anode): at an angle of 90° to the axis of the cathode, 1.5-2 cm above it, bringing the end of the electrode to the spinal column;

5. The grounding electrode was applied arbitrarily to the withers area.

After applying the electrodes, electrical pulses were generated to obtain the action potentials of the motor unit: using the STM200-1 stimulator, 10 consecutive rectangular pulses were generated, with a voltage of 5V, with a duration of 100 ms.

In the subsequent analysis of the data obtained, the following indicators were analyzed:

– the amplitude of the negative phase of the M-response;
– duration of the negative phase of the M-response;
– the area of the negative phase of the M-response;
– amplitude P-P;
– the duration of the M-response;
– the area of the M-response;
– distal terminal latency;
– the amplitude of the negative phase of the 10th M-response;
– the decrement of the negative phase of the M-response (calculated using the following formula):

$$decrement = \frac{(AM1 - AM0)}{AM1} \times 100\,\%$$

where AM1 is the amplitude of the negative phase of the M-response after the first stimulation; AM10 is the amplitude of the negative phase of the M–response after the tenth stimulation.

Statistical data processing

All the data obtained were subjected to adequate statistical processing. Using descriptive statistics methods, data validation for the normality of the distribution using the Shapiro-Wilk criterion was verified. When analyzing the data, the intergroup differences were determined by parametric or nonparametric methods, depending on the type of distribution. In the case of a normal distribution, the Student's criterion was used to analyze the differences between the two samples. When the distribution was different from normal, the Mann and Whitney U-test was used. The differences were considered significant at $p<0.05$. The statistical analysis was performed using IBM SPSS Statistics 26 and Microsoft Excel 2010 software.

Results and Discussion

When analyzing the total baseline EMG, low-amplitude monophase potentials of short duration were recorded in all groups, indicating normal spontaneous activity, probably associated with normal end plate noise (Fig. 1). Single endplate adhesions were recorded in a number of animals: these changes were interpreted by us as a variant of the norm associated with the localization of the electrode needle near the neuromuscular junction in the endplate area.

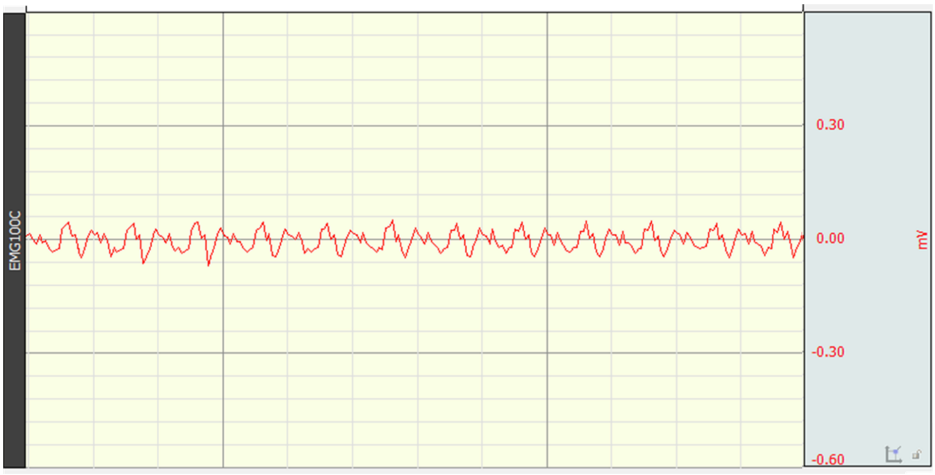


Figure 1. Example of total baseline EMG (intact animal).

The results of the analysis of needle EMG indicators are presented in Table 1. It should be noted that the most sensitive indicators were the following: the amplitude of the negative phase of the M-response (Fig. 2), the P-P span, distal terminal latency, and the decrement of the negative phase of the M-response.

Thus, in the control group on the 28th day of the experiment, a statistically significant decrease in the amplitude of the negative phase of the M-response was recorded by 1.86 times (p=0.004), which also statistically significantly differed from the indicators of the pCMV-VEGF165 group (p=0.013). In turn, this indicator in the group of intact animals and the pCMV-VEGF165 group was comparable, and reached values of 0.39 [0.35; 0.47] mV and 0.34±0.08 mV, respectively. By day 60, there was no statistically significant difference between the groups.

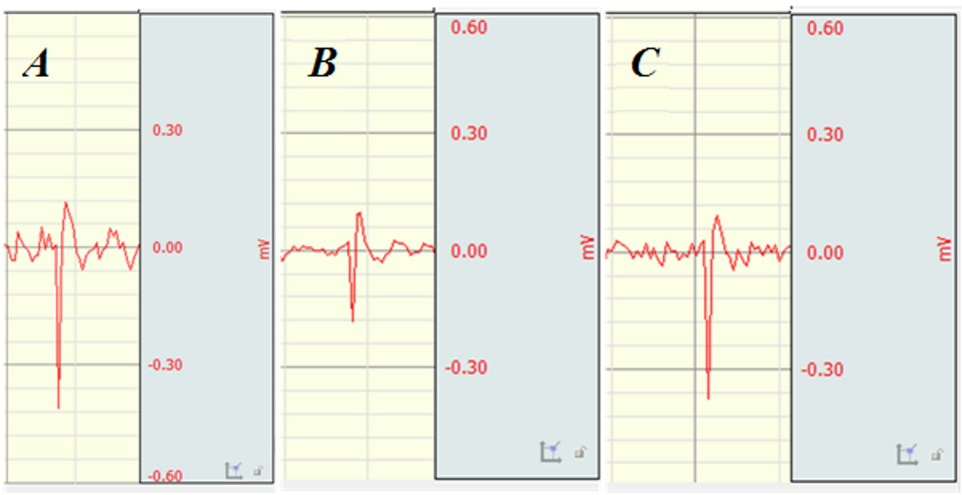


Figure 2. An example of registration of the M-response in experimental groups: A – the group of intact animals; **B** – the control group; **C** – the experimental group receiving pCMV-VEGF165.

The analysis of the amplitude of the M-response also revealed a statistically significant decrease on the 28th day of the experiment in both the control group and pCMV-VEGF165 by 1.75 (p=0.001) and 1.27 (p=0.047) times, respectively. However, it should be clarified that the noted difference with the pCMV-VEGF165 group is approaching the border of statistical significance, and with a larger number of observations it can be leveled.

It should be also noted that in the control group on the 28th day of the experiment, there was a statistically significant increase in the latency period by an average of 10% of the indicators of the group of intact animals (p=0.004). In some cases, polyphase responses with satellite potentials were also recorded in the control group.

In the group receiving pCMV-VEGF165 on day 28, the duration of the latency period also increased slightly; however, it did not differ statistically significantly from the indicators of intact animals. On day 60, on the contrary, the pCMV-VEGF165 group showed an increase in the latency period to 64.33±5.35 ms, which statistically significantly exceeded the indicators of the intact group (p=0.035).

Pronounced changes were noted when analyzing the index of the decrement of the negative phase of the M-response: this indicator increased statistically significantly by 2.2 times in the control group by both the 28th and 60th days on average. In the pCMV-VEGF165 group on days 28 and 60, this indicator did not differ statistically significantly from either those in the group of intact animals or the control group; however, it tended to increase.

Thus, analyzing the data from the results of electromyography in experimental groups, it can be noted that cold injury leads to delayed changes in the state of the neuromuscular system in the area of injury. Axonal damage is indicated by a decrease in the amplitude of both the negative phase of the M-response and the amplitude of the entire complex (Badalyan and Skvortsov 1986), which was observed on day 28 in the control group.

The disappearance of a statistically significant difference in this indicator by the 60th day indicates the completion of reparative processes and reinnervation in the area of damage. The absence of a statistically significant difference in the amplitude of the negative phase of the M-response in the pCMV-VEGF165 group indicates the ability of this drug to prevent and reduce the severity of damage to nerve fibers in the cold injury zone. This may probably be due to a faster restoration of microcirculation and a decrease in the severity of ischemic damage to nerve fibers.

The prolongation of the latency period in combination with the polyphase responses and satellite potentials recorded in a number of animals in the control group on the 28th day of the experiment may indirectly indicate the existing myelinopathy (Kapustina and Rusanova 2007). The absence of a statistically significant difference in latency on day 28 in the pCMV-VEGF165 group also indicates the ability of this drug to prevent and reduce the severity of damage to nerve fibers in the cold injury zone. The statistically significant difference in the pCMV-VEGF165 group on day 60 compared with the group of intact animals in the absence of other changes requires a more careful study on a larger number of subjects, and, in our opinion, cannot be interpreted as a sign of myelinopathy.

An increase in decrement in the control group on days 28 and 60 indicates the development of neuromuscular conduction disorders in the cold injury zone, which may be associated with structural or functional changes in the synapse and/or receptor apparatus due to their damage by ischemic, inflammatory or traumatic processes. In the pCMV-VEGF165 group, there was also a tendency to faster depletion of neuromuscular transmission on the 28th and 60th days of the experiment; however, there was no statistically significant difference with the group of intact animals. This may be due to a decrease in the degree of damage to the neuromuscular apparatus, as well as a faster restoration of blood flow in the area of cold injury due to the growth of new vessels.

Conclusion

Thus, pCMV-VEGF165 in the dose 60 micrograms has a beneficial effect on the development of delayed changes in the state of the neuromuscular system, which is probably due to a decrease in the degree of tissue damage and their faster regeneration

Table 1. Key EMG indicators in experimental groups

Group	Amplitude of the negative phase of the M-response, mV	Duration of the negative phase of the M-response, ms	The area of the negative phase of the M-response, mV*s	The amplitude of the M-response, mV	Duration of the M-response, ms	The area of the M-response, mV*s	Distal terminal latency, ms	Decrement of the negative phase of the M-response, %
intact group	0.39 [0.35; 0.47]	3.5±1.05	0.0004± 0.0001	0.56±0.11	25.67± 2.34	0.0012 [0.001; 0.0024]	59.83 [58.25; 62]	30.23± 7.0
control group, 28 day	**0.21±0.07***	5 [3.75; 5]	0.0004± 0.00004	**0.32± 0.07***	24±3.41	**0.0008± 0.0002***	**66±1.9***	**67.05± 15.6***
experimental group pCMV-VEGF165, 28 day	**0.34±0.08#**	4.33±0.82	0.0004± 0.0001	**0.44± 0.07*#**	**22.8± 1.94***	0.0009 [0.0009; 0.0016]	61.5±6.22	49.64± 28.59
control group, 60 day	0.4±0.18	2.5 [2; 3.5]	0.0004± 0.0002	0.53±0.21	26.17± 3.07	0.0014±0.0 004	58.5±10.8	**65.62± 30.46***
experimental group pCMV-VEGF165, 60 day	0.5±0.16	3±1.27	0.0005 [0.00027; 0.00096]	0.61±0.23	25.33± 3.14	0.0011 [0.0009; 0.0016]	**64.33± 5.35***	53.98± 26.19

***Note*: *** – $p<0.05$ when compared with the results of the group of intact animals, # – $p<0.05$ when compared with the results of the control group on the 28th day of the experiment.

due to the restoration of sufficient blood flow. To obtain more reliable data, the number of observations should be increased.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (Minutes No 03/24 of March 18, 2024).

Acknowledgements

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Data availability

All of the data that support the findings of this study are available in the main text.

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