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The therapeutic effects of transferring remote ischemic preconditioning serum in rats with neuropathic pain symptoms

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ABSTRACT

Background and objectives: Neuropathic pain is defined as pain caused by damage to the nerve as a result of a lesion or disease. It has been shown that ischemic preconditioning exerts a protective role in various tissue injuries; however, the effect of transplantation of remote ischemic preconditioning serum (RIPCs) on neuropathic pain symptoms has not been studied. The aim of this project is to investigate the effect of RIPCs transfusion by different routes of administration on neuropathic pain symptoms. Our secondary aim was to demonstrate the role of Schwann cells in the regeneration of sciatic nerve injury and to evaluate the change in the number of glial cells in the spinal cord dorsal horn.

Methods: The sciatic nerve partial ligation method was used to induce neuropathic pain. Changes in neuropathic pain symptoms were assessed by measuring thermal hyperalgesia and mechanical allodynia. To determine the possible therapeutic site, alterations in the number of spinal cord lumbar posterior horn microglia and astrocytes were evaluated by ionized calcium-binding adapter molecule 1 (iba1) and glial fibrillary acidic protein (GFAP) immunostaining. Myelin basic protein immunohistochemistry was also used to assess Schwann cell immunoreactivity in the sciatic nerve.

Results: In rats that underwent partial sciatic nerve ligation, neuropathic pain symptoms developed on average on day 12 and persisted up to day 21 ($p < 0.0001$). RIPCs administered intravenously for five days reduced thermal hyperalgesia more than intraperitoneal and subcutaneous administration ($p < 0.05$). Both central glial cells appear to play a role in the effect of RIPCs. RIPCs treatment increases Schwann cell remyelination.

Conclusions: Our results showed that intravenously administered RIPCs remarkably improved the neuropathic pain symptoms, thermal hyperalgesia and mechanical allodynia. Further studies are needed to evaluate the role of RIPCs transfusion on glial cells.

1. Introduction

The pathophysiology of neuropathic pain has not been fully elucidated [1]. It has been shown that abnormal ectopic impulses

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originating from damaged nerve fibres as a result of nerve damage cause a change in the number of different ion channels (TRPV1, voltage-gated sodium channels, TRPM8, etc.) in the free nerve endings of both damaged and undamaged afferent fibres (A-delta and C fibres). It is also known to cause a continuous release of neurotransmitters in the central nervous system, leading to changes in the number of receptors and new synaptic connections in the dorsal horn of the spinal cord [2,3]. Histopathological examinations showed an increase in microglia infiltration and astrocyte reactivity in the spinal cord dorsal horn [4,5].

In the treatment of neuropathic pain, agents that inhibit the excitatory impulses formed in the ascending pathways or increase the activity of the descending inhibitory pathways are used. However, current treatments are not effective enough [6,7]. Therefore, there is a need for new drugs in the treatment of neuropathic pain.

Murry et al. showed that short-term ischemia is protective against subsequent injury and named this phenomenon as ischemic preconditioning (IPC) [8]. It has also been shown that ischemic conditioning in a remote organ can protect another organ [9,10]. However, remote ischemic conditioning is transmitted not yet well understood. The most likely mechanism is that the substances responsible for the protective effect are transported through the blood [11,12]. In our previous study, we were able to convey the protective effect of ischemic preconditioning by serum [13]. It has been shown that the protective effect of ischemic conditioning does not occur in the diabetes model [14]. The way to use the protective effect of ischemic preconditioning in diabetic patients may be to transport protective agents via serum. It has been shown that ischemic conditioning may also be protective against optic nerve damage where the ischemia-reperfusion injury is not prominent [15].

In a recent study, the effect of ischemic postconditioning on sciatic nerve transection has been determined [16]. However, there are no studies showing the effect of remote ischemic preconditioning serum (RIPCs) transfusion from healthy animals in the treatment of neuropathic pain. The primary aim of our research is to investigate the possible effect of RIPCs on thermal hyperalgesia and mechanical allodynia in neuropathic pain model induced by partial sciatic nerve ligation (PSNL) and to determine the variation of the effect with different routes of administration. Our secondary aim is to demonstrate the role of Schwann cells in the regeneration of sciatic nerve injury and to evaluate the change in the number of spinal cord dorsal horn glial cells (astrocytes, microglia).

2. Materials and methods

2.1. Animals and ethics

In our study, 120 male Sprague-Dawley rats, 8–12 weeks old, weighing 250–300 g, with the same biological and physiological characteristics were used. The animals were bred in Trakya University Experimental Animals Research Unit and housed in standard laboratory conditions (12–12 h light-dark cycles and temperature (21 $\pm$ 2 °C)) in an individually ventilated cage system (Tecniplast, Buguggiate, Italy). Local "Animal Care Ethics Committee" approval (2020.06.01) was obtained for the study. Experimental procedures in this study were in accordance with the National Academies Press (NAP) Guide for the Care and Use of Laboratory Animals [17].

2.2. Partial sciatic nerve ligation model of neuropathic pain

2.2.1. Surgical procedure

Under anaesthesia (ketamine 100 mg/kg/i.p. - xylazine 10 mg/kg/i.p.), the sciatic nerve was dissected from the surrounding tissue in the proximal thigh area of the right leg. As described by Seltzer et al., 8-0 silk with a reverse cut mini-needle was inserted through the nerve to cover 1/3–1/2 of the nerve thickness and tied tightly [18]. In the sham group, the sciatic nerve was left intact, then the muscle tissue was closed with 4–0 chromic catgut and the skin was closed with 4–0 silk suture.

2.3. Behavioural experiments

2.3.1. Assessment of mechanical allodynia

The dynamic plantar aesthesiometer (Ugo Basile, Varese, Italy) device was used to measure mechanical allodynia measurements. A 0.5 mm diameter rigid filament of the device was applied vertically to the plantar surface of the hind paw of the animal with increasing force (0–50 g in 20 s). The force they lifted the hind paw was automatically recorded. The cut-off force was determined to be 50 g. The changes in the paw withdrawal force after transfer of the RIPCs were converted to the percentage of maximum possible effect (MPE%) using the formula below.

MPE% = [(post-drug force – pre-drug force)/cut-off force – pre-drug force] $\times$ 100.

2.3.2. Assessment of thermal hyperalgesia

A cold/hot plate analgesia meter (Ugo Basile, Comerio, Italy) device was used to evaluate thermal hyperalgesia. The rats were placed on an electrically heated surface maintained at a temperature of 55 $\pm$ 0.1 °C, and response latencies for jumping or paw licking were recorded. The cut-off time was set as 25 s to prevent tissue damage. Then, test latencies were converted to the MPE% similarly with the formula in 2.3.1.

2.3.3. Assessment of motor coordination

Motor coordination was assessed using with the rotarod apparatus (Commat, Ankara, Turkey). The cylinder of the device was adjusted to rotate at 16 rpm. The time the animals fell off the cylinder was recorded. The cut-off time was set at 180 s.

2.4. Obtaining remote ischemic preconditioning serum

Under anaesthesia, a latex tourniquet was wrapped to both hind legs of the animals from a region close to the body to induce complete ischemia. The resulting ischemia was visually controlled by cyanosis of the hind paw. The ischemic preconditioning procedure was performed as four cycles of 10 min of ischemia and 10 min of reperfusion [16]. After 24 h, blood was collected from the heart under anaesthesia (ketamine 100 mg/kg/i.p. - xylazine 10 mg/kg/i.p.) and transferred to a dry tube. After 30 min, RIPCs were obtained by centrifugation at 2000×*g* for 10 min. Sufficient RIPCs were obtained from two healthy animals for each treated group (n = 8 rats). Pooling was performed using RIPCs from two animals. The pool of RIPCs to be transferred was prepared daily. RIPCs were applied immediately at 100 μL/50 g body weight.

2.5. Immunohistochemical analysis

2.5.1. Spinal cord lumbar dorsal horn

The L4-5 lumbar spinal cord tissues were fixed in 10 % neutral-buffered formalin for 24 h immediately upon removal. Tissue samples were processed according to the routine protocol of dehydration in ethanol, clearing in toluene and embedding in paraffin. Paraffin-embedded tissue samples were sectioned at 5 μm thickness using a rotary microtome.

Immunohistochemistry was performed as previously described [19]. Paraffin sections were deparaffinized then subjected to antigen retrieval using boiled citrate buffer (10 mM; pH 6.0, Thermo Fisher, Waltham, MA, USA). Sections were immersed in 3 % (v/v) methanolic hydrogen peroxide for 10 min to block endogenous peroxidase activity. To evaluate lumbar spinal cord astroglial and microglial activation, the slides were incubated for 1 h at room temperature with the following primary antibodies: rabbit polyclonal glial fibrillary acidic protein (GFAP) antibody (1:2000, ab7260, Abcam, Cambridge, MA, USA), ionized calcium-binding adapter molecule 1 (iba1) antibody (1:300, ab153696 Abcam, Cambridge, MA, USA). Primary antibodies were washed in phosphate buffer solution (Thermo Fisher, Waltham, MA, USA), followed by application of horseradish peroxidase (HRP)-conjugated secondary antibodies. Immunoreactivity was visualized with diaminobenzidine (DAB; Thermo Fisher, Waltham, MA, USA) and counterstained with hematoxylin. For quantitative analysis, dorsal horns were first identified by hematoxylin and eosin (H&E) and immunohistochemical staining at a low magnification of ×40. We investigated GFAP positive astrocytes and Iba 1 positive microglial cells located mainly in laminae I, II, and III according to the Rexed laminae system [20,21]. The shape, area, cell diameter, cell process length, and thickness were evaluated on dorsal horn astrocyte and microglia [22]. For each rat, 3–5 representative areas (at 400x and 200x magnification, respectively) were taken in each dorsal horn. A threshold for positive staining was determined for each image, including all cell bodies and processes. Results were reported as the average number of cells in the dorsal horns. The stained sections were examined with an Olympus BX51 (Olympus Corp., Tokyo, Japan) light microscope, and images were captured with a CCD spot camera (Olympus Corp., Tokyo, Japan). Slides were scored using a double-blinded protocol.

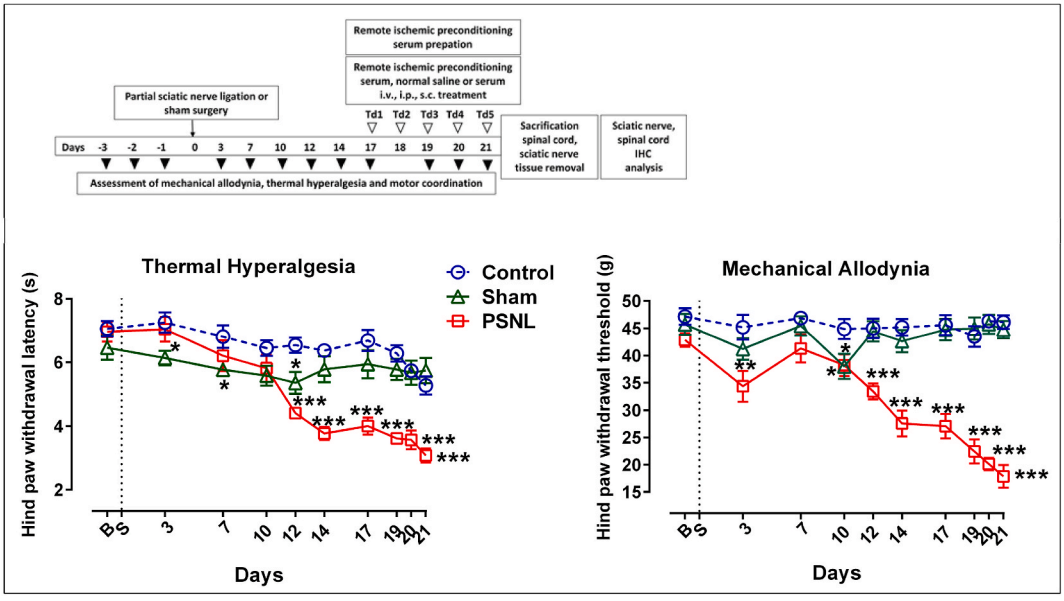

Fig. 1. Experiment design and time course of the development of mechanical allodynia and thermal hyperalgesia after partial sciatic nerve ligation. PSNL: Partial sciatic nerve ligation; Td: Treatment day. B; Basal values before surgery, S; Partial sciatic nerve ligation or sham surgery; IHC; Immunohistochemical analysis. *p < 0.05, **p < 0.001, ***p < 0.0001 versus control group. Two-way repeated measures ANOVA, followed by Bonferroni's correction was used. Data are presented as mean ± SEM (n = 7–8).

2.5.2. Sciatic nerve

Tissue preparation and immunohistochemical analysis were performed as described above. To investigate the effects of RIPCs on nerve fibre myelination, slides were stained with rabbit polyclonal anti-myelin basic protein carboxyterminal end antibody (MBP) (1:200, ab65988; Abcam, Cambridge, MA, USA). Hematoxylin counterstaining was applied to all sections. MBP immunoreactivity was examined by using light microscopy using 400 × objectives. The degree of axonal demyelination or remyelination was scored according to the formula (MBP-immunopositive area \ total section area x 100) [16].

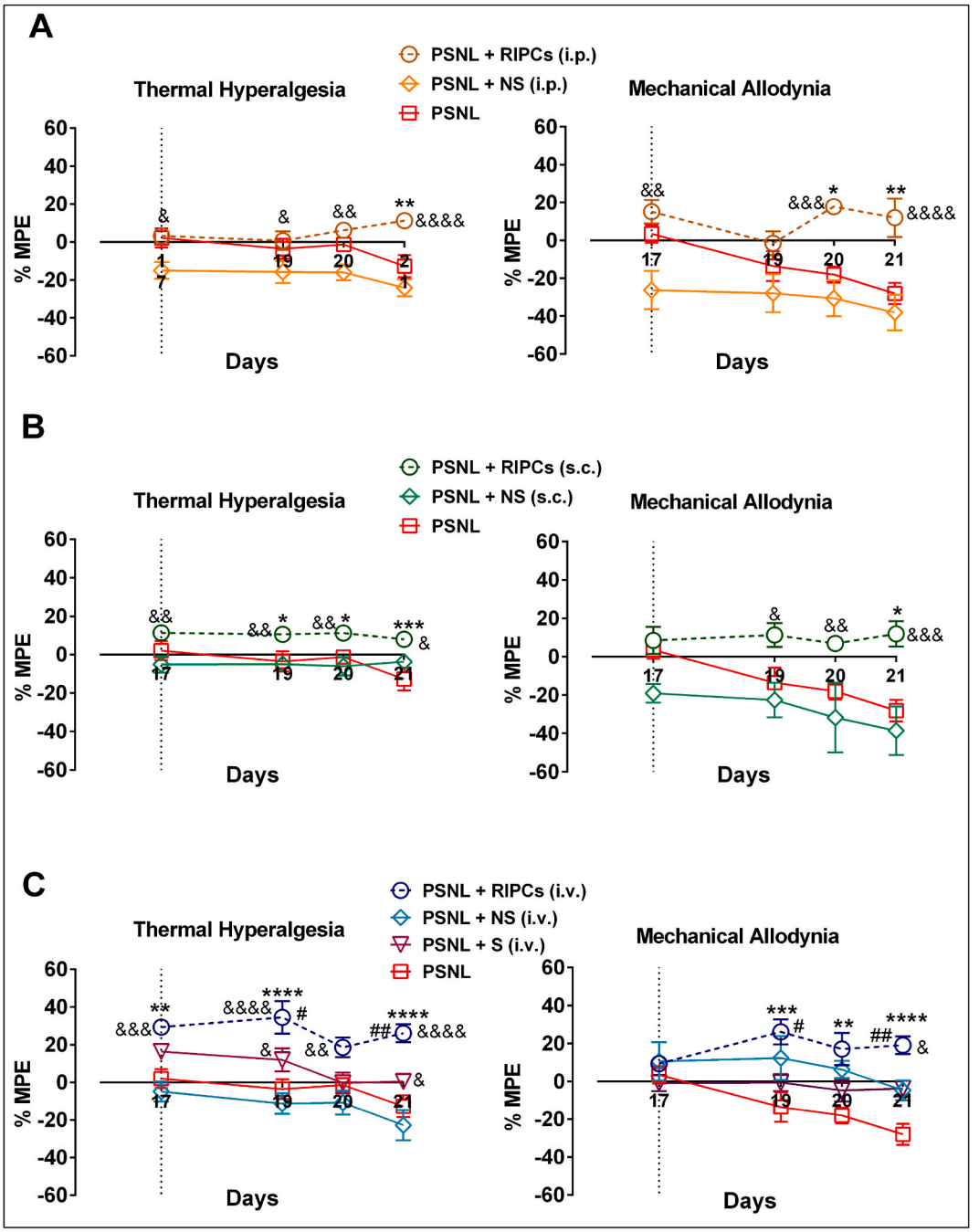

Fig. 2. Effects of intraperitoneal (A), subcutaneous (B), and intravenous (C) administration of remote ischemic preconditioning serum on thermal hyperalgesia and mechanical allodynia. PSNL; Partial sciatic nerve ligation, RIPCs; Remote ischemic preconditioning serum, NS; Normal saline, S; Serum, %MPE: maximal possible effect. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus PSNL group; &$p < 0.05$, &&$p < 0.01$, &&&$p < 0.01$, &&&&$p < 0.0001$ versus PSNL + NS group; #$p < 0.05$, ##$p < 0.01$ versus PSNL + S group. Two-way repeated measures ANOVA followed by Bonferroni correction was used. Data are presented as mean ± SEM (n = 5–8).

2.6. Study design

Thirty rats were used to obtain RIPCs and ten rats were used to obtain serum (S) without RIPCs. The remaining 80 animals were divided into groups as described: control, sham, PSNL, PSNL + RIPCs (i.p.), PSNL + RIPCs (s.c.), PSNL + RIPCs (i.v.), PSNL + normal saline (NS) (i.p.), PSNL + NS (s.c.), PSNL + NS (i.v.), PSNL + S (i.v.).

After three days of baseline measurements, PSNL surgery was performed on day 0 except in the control and sham groups. Behavioural tests (thermal hyperalgesia, mechanical allodynia, and motor coordination) were performed on days 3, 7, 10, 12, 14, 17, 19, 20, and 21. Treatment groups were given RIPCs obtained from healthy rats for five days between days 17–21. So, the 17th day after surgery was accepted as the first day of treatment (Td1). In the behavioural experiments, the measurements taken on the 14th day after surgery were used as the "predrug time" when calculating the MPE.

In the PSNL + S (i.v.) group, where the effect of anaesthesia (ketamine 100 mg/kg/i.p. - xylazine 10 mg/kg/i.p.) was evaluated, serum obtained from healthy rats without RIPC was administered for five days between 17 and 21 days. RIPCs, serum and NS were administered at 100 μL/50 g body weight 1 h prior to behavioural testing. In the i.v. treatment groups, rats were placed in the restrainer and RIPCs were administered via the tail vein. At the end of the behavioural experiments on day 21, euthanasia was performed by decapitation under anaesthesia (ketamine 100 mg/kg/i.p. - xylazine 10 mg/kg/i.p.). The sciatic nerve was rapidly isolated and a piece of nerve approximately 5 mm long containing 8-0 silk thread was removed. The spinal cord was removed, and the lumbar region was isolated [23]. The experimental design is shown in Fig. 1.

2.7. Statistical analysis

Normality was tested using the Shapiro-Wilk test. Two-way repeated measures analysis of variance (ANOVA) on time was used to assess antinociceptive effects between groups, followed by *Bonferroni* correction. Immunohistochemical data were analyzed by one-way analysis of variance, followed by Tukey test. Values of $p < 0.05$ were considered to be statistically significant. All data are expressed as mean ± standard error of the mean (SEM). GraphPad Prism version 6.0c (GraphPad Software Inc., La Jolla, CA, USA) was used to perform the statistics.

3. Results

3.1. Effects of partial ligation of the sciatic nerve

Compared to the control group, decreases in hind paw withdrawal latency and thresholds were observed in the PSNL surgery group on day 12 (Fig. 1, $p < 0.0001$). Neuropathic pain symptoms persisted until day 21 (Fig. 1).

3.2. Effects of transferring remote ischemic preconditioning serum administration by different routes on thermal hyperalgesia and mechanical allodynia

A Intraperitoneal administration

Compared to the PSNL group, intraperitoneal administration RIPCs attenuated thermal hyperalgesia on day Td5 (day 21 of the experiment) and mechanical allodynia on days Td4 and Td5 (Fig. 2A, $p < 0.01$; $p < 0.05$ and $p < 0.01$, respectively). RIPCs treatment decreased thermal hyperalgesia on days Td1, Td3, Td4, Td5 ($p < 0.05$, $p < 0.05$, $p < 0.01$, $p < 0.0001$, respectively) and mechanical allodynia on days Td1, Td4, Td5 ($p < 0.01$, $p < 0.001$, $p < 0.0001$, respectively) compared to the NS-treated group (Fig. 2A).

B Subcutaneous administration

Subcutaneously administered RIPCs attenuated thermal hyperalgesia on days Td3, Td4, and Td5 ($p < 0.05$, $p < 0.05$, $p < 0.001$, respectively) and mechanical allodynia ($p < 0.05$) on day Td5 of the treatment compared to the PSNL group (Fig. 2B). RIPCs treatment decreased thermal hyperalgesia on days Td1, Td3, Td4, Td5 ($p < 0.01$, $p < 0.01$, $p < 0.01$, $p < 0.05$, respectively) and mechanical allodynia on days Td3, Td4, Td5 ($p < 0.05$, $p < 0.01$, $p < 0.001$, respectively) compared to the NS-treated group (Fig. 2B).

C Intravenous administration

Intravenously administered RIPCs attenuated thermal hyperalgesia on days Td1, Td3, Td5, ($p < 0.01$, $p < 0.0001$, $p < 0.0001$, respectively) and mechanical allodynia on days Td3, Td4 and Td5 ($p < 0.001$, $p < 0.01$ and $p < 0.0001$respectively), compared to the PSNL group (Fig. 2C). RIPCs treatment decreased thermal hyperalgesia on days Td1, Td3, Td4, Td5 ($p < 0.001$, $p < 0.0001$, $p < 0.01$, $p < 0.0001$, respectively) and mechanical allodynia on day Td5 ($p < 0.05$) compared to the NS-treated group (Fig. 2C). RIPCs group attenuated both thermal hyperalgesia and mechanical allodynia on days Td3 and Td5 ($p < 0.05$, $p < 0.01$, respectively) compared with the group given serum without ischemic preconditioning (Fig. 2C).

3.3. Comparison of the effects of intraperitoneal, subcutaneous, and intravenous administration of remote ischemic preconditioning serum on thermal hyperalgesia and mechanical allodynia

Intravenously administered RIPCs increased %MPE in the thermal hyperalgesia test on days Td1, Td3, and Td5 (Fig. 3, $p < 0.001$, $p < 0.0001$, $p < 0.05$, respectively) and in the mechanical allodynia test on day Td3 ($p < 0.01$) compared to i.p. administration. When intravenously administered RIPCs were compared to subcutaneously administered RIPCs group, increased %MPE in the thermal hyperalgesia test increased on the 1st, 3rd, and 5th day of treatment (Fig. 3, $p < 0.01$, $p < 0.0001$, $p < 0.01$, respectively). Partial sciatic nerve ligation or sham surgery and remote ischemic preconditioning serum administered intraperitoneally, subcutaneously, and intravenously did not affect motor coordination (Figure supplement 1 (Fig. S1))

3.4. Effects of transferring remote ischemic preconditioning serum on spinal cord lumbar dorsal horn glial cells

3.4.1. Astrocytes

GFAP immunopositive astrocytes were found in the more superficial regions of the dorsal horn (Fig. 4). Weak staining and scattered astrocytes were seen in the spinal cords of the control and sham groups (Fig. 4a–b). In the PSNL group, the number of astrocytes was slightly increased (Fig. 4c). However, GFAP staining density was significantly increased, mainly due to astrocyte hypertrophy (Fig. 4d–j). In all PSNL groups, increased GFAP immunostaining was observed in the grey matter of the spinal cord ipsilateral to the lesion and specific to the spinal segments in which the sciatic nerve is distributed. Astrocyte secondarily branches (and cellular bodies to a much lesser extent) were mainly detected. Fig. 4k shows counts of GFAP immunopositive astrocyte, demonstrating no significant difference between PSNL performing groups and control groups, except PSNL + RIPCs (i.v.) group on day 21 ($p < 0{,}001$). Although the number of cells increased in the PSNL + RIPCs (i.v.) group, the staining density and the number of extensions were observed to be decreased (Fig. 4g and i).

3.4.2. Microglia

Iba-1 immunopositive rat microglial cells were found in laminae I, II, and III of the spinal cord dorsal horn. They represented an amoeboid spherical shape, morphologically. Fig. 5 shows iba-1 immunohistostaining of the experimental groups. Scattered and weakly stained microglial cells were observed in the spinal cord of the control and sham groups (Fig. 5a–b). PSNL increased the immunoreactivity and also the number of active microglia with long extensions. In addition, PSNL induced hypertrophy and elevated immunoreactivity microglia cells in the spinal cord (Fig. 5c–j). Fig. 5k shows the number of iba-1 immunopositive microglial cells. Although the number of cells increased in all PSNL performed groups, the staining density and the number of extensions were decreased in the PSNL + RIPCs (s.c.), PSNL + RIPCs (i.v.) treatment groups, and PSNL + S (i.v.) group.

3.5. Effects of transferring remote ischemic preconditioning serum on the injured sciatic nerve

Fibres were regularly arranged in control and sham animals (Fig. 6a–b). MBP immunoreactivity was observed in all axons in the sciatic nerve. However, markedly decreased MBP immunoreactivity and derangement of nerve fibres with significant axonal swelling was observed in all of the PSNL groups. The number of inflammatory cells also increased in injured sciatic nerves, an indication of sciatic nerve damage (Fig. 6c–j). PSNL + RIPCs (s.c.) and PSNL + RIPCs (i.v.) treatment groups partially reversed the histological damage ($p < 0.0001$ and $p < 0.05$, respectively) (Fig. 6g and i). On the other hand, MBP expression was still lower in all PSNL groups than in the control group (Fig. 6k).

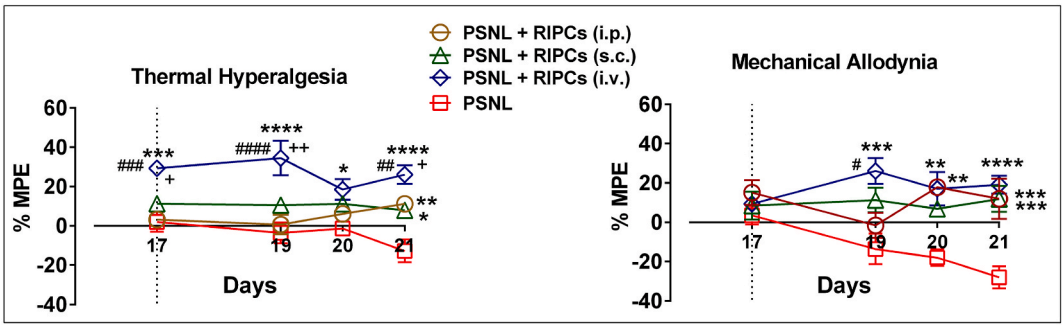

Fig. 3. Comparison of intraperitoneal, subcutaneous, and intravenous administration in thermal hyperalgesia and mechanical allodynia. PSNL; Partial sciatic nerve ligation, RIPCs; Remote ischemic preconditioning serum, NS; Normal saline, S; Serum, %MPE: maximal possible effect. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ versus PSNL + RIPCs (i.p.) group; &$p < 0.01$, &&$p < 0.0001$ versus PSNL + RIPCs (s.c.) group. Two-way repeated measures ANOVA followed by Bonferroni correction was used. Data are presented as mean ± SEM (n = 6–8).

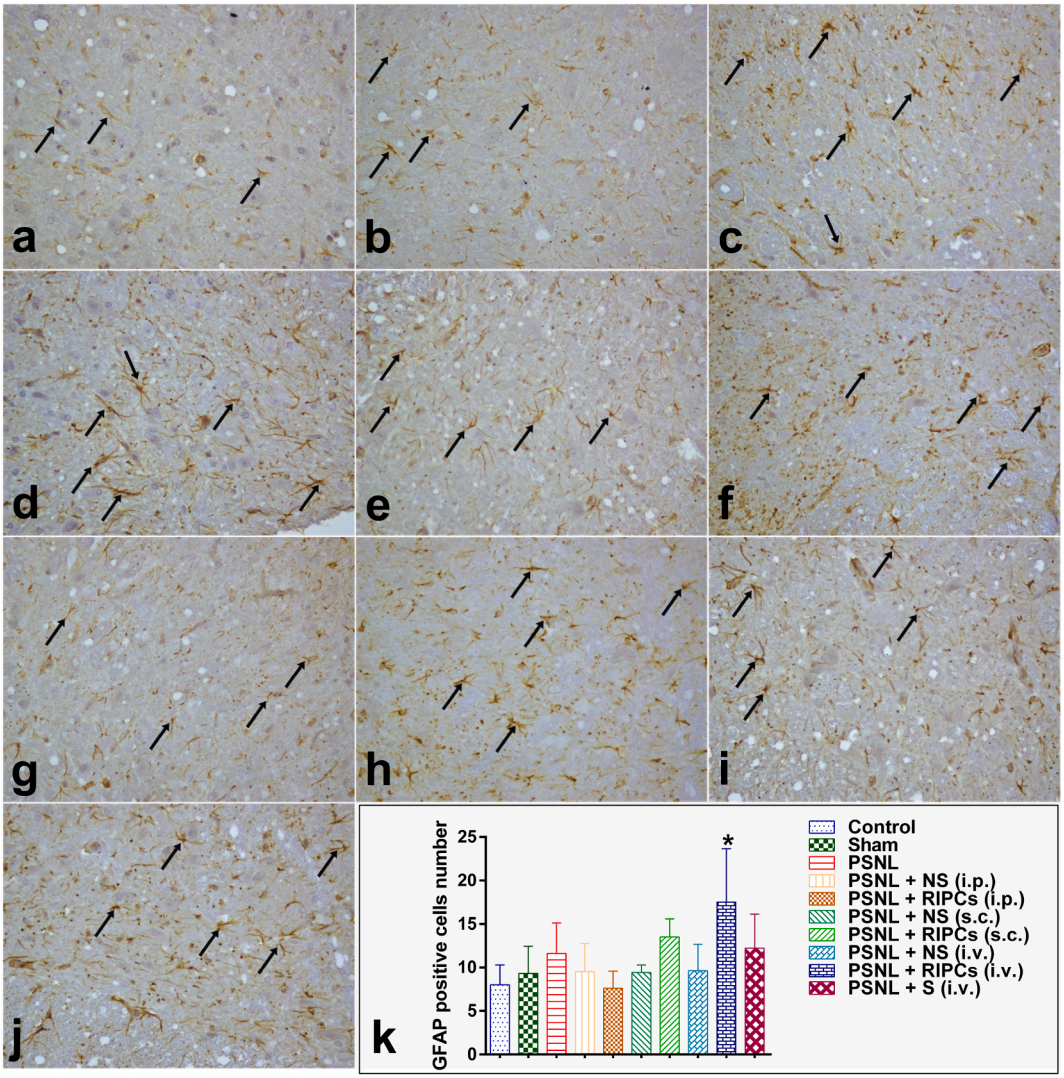

Fig. 4. GFAP immunoreactivity and the number of GFAP positive cells in the section. a- Control, b- Sham, c- PSNL, d- PSNL + NS (i.p.), e– PSNL + RIPCs (i.p.), f- PSNL + NS (s.c), g- PSNL + RIPCs (s.c.), h- PSNL + NS (i.v.), i- PSNL + RIPCs (i.v.), j- PSNL + S (i.v.). x400, Hematoxylin counterstaining. PSNL; Partial sciatic nerve ligation, RIPCs; Remote ischemic preconditioning serum, NS: Normal saline, S; Serum; Arrow; Astrocyte. *p $<$ 0.001 versus control group. One-way ANOVA test, post hoc Tukey test. Data are expressed as mean ± SEM (n = 5–6).

4. Discussion

In the treatment of sciatic nerve incision, in addition to surgical repair, ischemic postconditioning applied within 6 h has been shown to increase nerve healing [16]. The effect of RIPCs transfusion in the treatment of neuropathic pain due to partial sciatic nerve injury is unknown. In this study, we demonstrated the antinociceptive effect of RIPCs administered by different routes on thermal hyperalgesia and mechanical allodynia, which are symptoms of neuropathic pain, and the role of glial cells (astrocytes, microglia, and Schwann) in this effect. On day 12, the animals in which we performed PSNL surgery developed thermal hyperalgesia and mechanical allodynia. The time of onset of neuropathic signs after PSNL surgery was consistent with previous studies [24,25]. Transfusion of RIPCs administered by different routes for five days ameliorated thermal hyperalgesia and mechanical allodynia.

When the effectiveness of different administration routes was compared, we determined that intravenous RIPCs transfusion exerted more powerful effect on thermal hyperalgesia. It is not known exactly which substances are produced by RIPC. It is believed that the protective substances have a low molecular weight (less than 15 kDa) and a peptide structure [26]. The superiority of the intravenous route suggests that some preservatives may not reach the site of action sufficiently when administered intraperitoneally or subcutaneously. This may be because substances administered intraperitoneally enter the portal circulation and undergo first-pass elimination. In addition, slower absorption and lower peak plasma concentrations may occur with intraperitoneal and subcutaneous administration [27]. The mechanisms contributing to thermal hyperalgesia and mechanical allodynia differ are different [28–30]. In

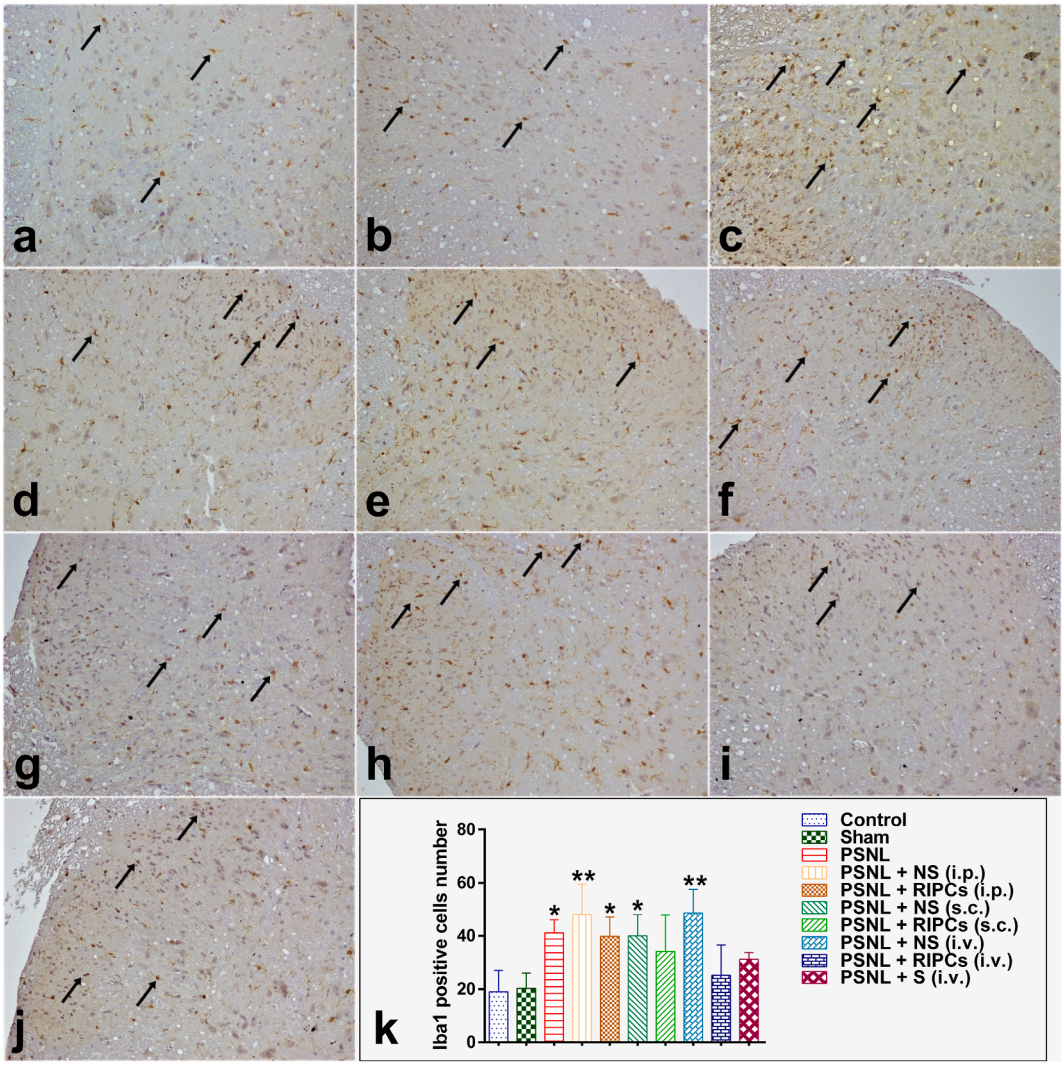

Fig. 5. iba-1 immunoreactivity and the number of iba 1 positive cells in the section. a- Control, b- Sham, c- PSNL, d- PSNL + NS (i.p.), e– PSNL + RIPCs (i.p.), f- PSNL + NS (s.c), g- PSNL + RIPCs (s.c.), h- PSNL + NS (i.v.), i- PSNL + RIPCs (i.v.), j- PSNL + S (i.v.). x400, Hematoxylin counterstaining. PSNL; Partial sciatic nerve ligation, RIPCs; Remote ischemic preconditioning serum, NS: Normal saline, S; Serum; Arrow; Microglia. *p $<$ 0.01, **p $<$ 0.001 versus control group. One-way ANOVA test, post hoc Tukey test. Data are expressed as mean ± SEM (n = 5–6).

the PSNL model, blockade of TRPV1 channels in the dorsal horn of the spinal cord for five days was shown to play a role in the development of thermal hyperalgesia but not mechanical allodynia [29]. TRPV1 channels are thought to contribute to astrogliosis in the late phase after PSNL application [29]. It can be hypothesised that some of the substances produced by RIPC prevent the development of thermal hyperalgesia by acting on TRPV1 channels.

The development mechanism of neuropathic pain is not fully understood. In addition to neurons, glial cells are also known to contribute to the development of neuropathic pain [31,32]. Following PSNL surgery, stimuli from the damaged nerve trigger the activation of glial cells at the level of the spinal dorsal horn [33,34]. Astrocytes and microglia cells, whose activity is increased in the spinal dorsal horn are thought to, contribute to the development of neuropathic pain by secreting various cytokines [32,35].

Clark et al. found an increase in microglial cells in the ipsilateral dorsal horn of the lumbar spine on day 14 after PSNL surgery in rats. They stated that the rise spread to both dorsal spinal areas on the 50th day [33]. Shibata et al. found that iba-1 immunoreactivity increased in lamina 1–2 of the dorsal horn of the lumbar spinal cord, microglial cells assumed an amoeboid structure, and the branches became short and thick in mice that underwent PSNL surgery on day 21 [34]. Similarly, we determined that the number of microglia cells in the ipsilateral lumbar spinal cord increased on the 21st day after PSNL surgery, and there was an increase in iba-1 immunoreactivity. In addition, our morphological findings were similar except that the microglia branching was longer. S.c. and i.v. administered RIPCs caused a decrease in the number and branching of microglia for five days. No significant reduction was observed with the i.p. application. Interestingly, i.v. serum also reduced the number of microglia.

Garrison et al. showed that GFAP synthesis increased in astrocytes located in the ipsilateral lumbar region of the spinal cord in

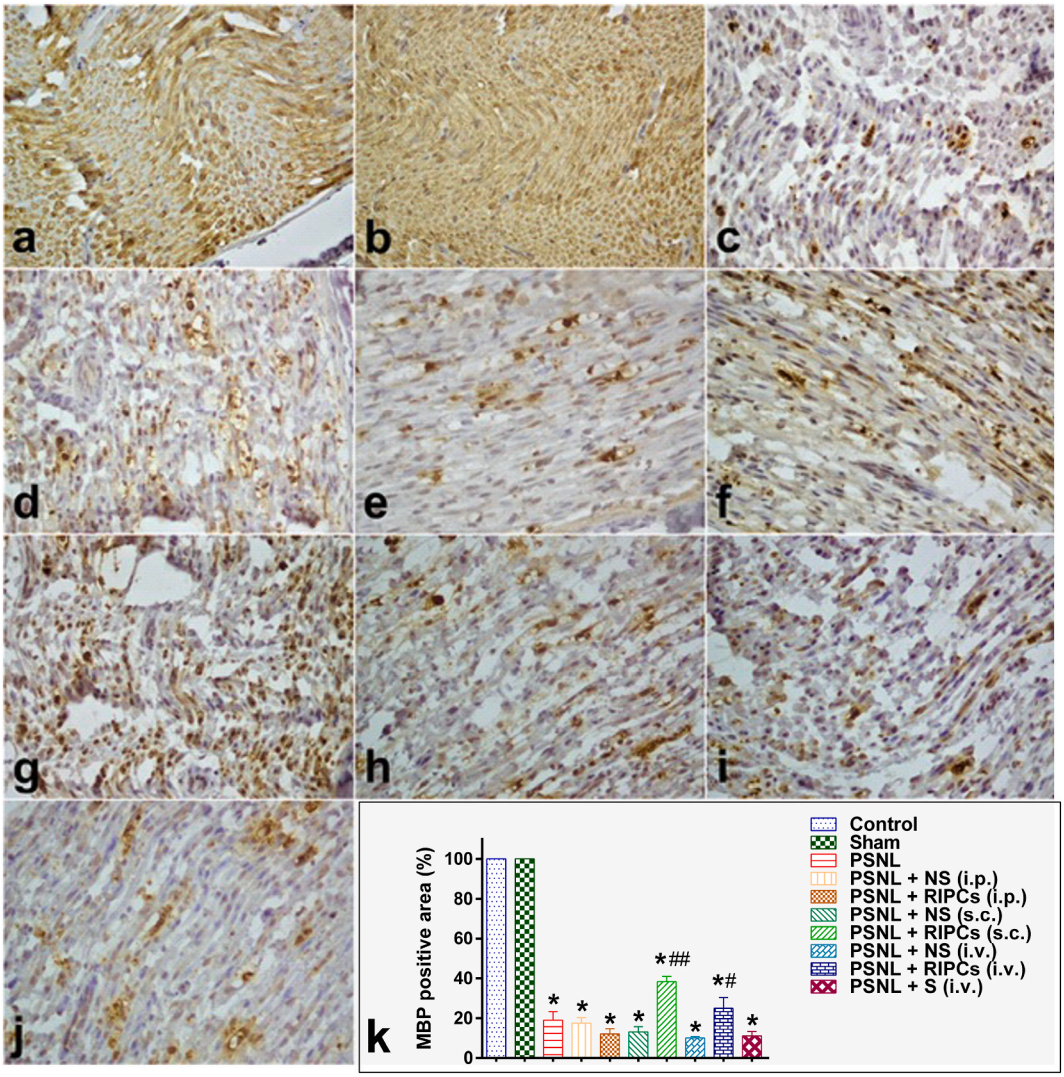

Fig. 6. MBP immunoreactivity and the ratio of MBP positive area on the sciatic nerve section. a- Control, b- Sham, c- PSNL, d- PSNL + NS (i.p.), e– PSNL + RIPCs (i.p.), f- PSNL + NS (s.c), g- PSNL + RIPCs (s.c.), h- PSNL + NS (i.v.), i- PSNL + RIPCs (i.v.), j- PSNL + S (i.v.). x400, Hematoxylin counterstaining. PSNL; Partial sciatic nerve ligation, RIPCs; Remote ischemic preconditioning serum, NS: Normal saline, S; Serum. *$p < 0.0001$ versus control group; $^{\#}p < 0.001$, $^{\#\#}p < 0.0001$ versus PSNL group. One-way ANOVA, post hoc Tukey test. Data are expressed as mean ± SEM ($n = 5–6$).

neuropathic rats at day 10 (Bennett's method), and found that astrocyte extensions were hypertrophic, but there was no numerical increase in astrocytes. In addition, they indicated for the first time that there is a correlation between the intensity of GFAP staining and the development of hyperalgesia [35]. Microglial cells have been shown to play a pivotal role in the first seven days of increased pain following neuropathic pain surgery (Seltzer method), and astrocytes are responsible for the findings in the late period when pain behaviour is established [34]. A recent study showed that the intensity of GFAP staining in the ipsilateral lumbar region after neuropathy surgery (Kim and Chung method) increased from day 3, peaked on day 7 and continued until day 21, and this increase was shown to be prevented by aquaporin-4 receptor blockade [36].

Our study found that GFAP staining intensity in the ipsilateral dorsal horn of the lumbar spinal cord increased on day 21 in the PSNL group. Similar to previous studies, we attributed the increase in GFAP staining intensity to astrocyte hypertrophy rather than an increase in astrocyte number. We found that the staining intensity decreased in the treatment groups. We also found that the number of astrocytes was increased in the group treated with RIPCs (i.v.). Therefore, we speculate that a decrease in active astrocytes and an increase in inactive astrocytes can prevent neuropathic pain.

In addition to central sensitisation, peripheral sensitisation also plays a role in the development of neuropathic pain. Schwann cells are responsible for providing support and myelinization to neurons [37]. It is known that damage to the myelin sheath formed by Schwann cells leads to the development of neuropathic pain [37]. Reactive oxygen species secreted from macrophages as a result of

partial sciatic nerve injury stimulate Schwann cells through transient receptor potential ankyrin 1 (TRPA1). It has been shown that stimulated Schwann cells cause neuroinflammation and mechanical allodynia through H_2O_2 [38]. Zhou et al. investigated the effect of ischemic postconditioning in the sciatic nerve slice, and observed Schwann cell remyelination at 4, 8, and 12 weeks based on the number of MBP-expressing cells. They reported that ischaemic postconditioning caused an increase in the number of cells expressing MBP but still did not reach sham levels at 12 weeks [16]. Similarly, we observed that MBP staining intensity was decreased to 20 % of its initial value in the PSNL surgery group. MBP staining intensity increased in the groups treated with intravenous and subcutaneous RIPCs (30 %, 40 %, respectively) but was still lower than the sham group. In the s.c. route of administration, absorption is slower. Therefore, a lower plasma peak concentration can be expected. However, due to the prolonged absorption, it can be assumed that the active substances will remain in the blood for a longer period compared to i.v. administration. It can be speculated that the prolonged presence of the unknown protective substance in the blood may further increase MBP expression [39].

Taking all the data together, we can say that RIPCs administered by different routes for five days improved thermal hyperalgesia and mechanical allodynia, and this effect was strong in the intravenous treatment group. We can speculate that with other routes of administration, the active substances do not adequately reach the treatment area or do not produce a sufficient peak serum concentration. Considering that the intravenously administered serum is also effective against thermal hyperalgesia, we conclude that substances acting against thermal hyperalgesia are relatively present in the serum without ischemic conditioning and increase with RIPC. Since agents that suppress astrocyte or microglia activities were not used in our study and temporal analysis was not performed, it could not be clearly determined on which cell RIPCs acted. However, the activity of both glial cells decreased, especially in the intravenous treatment group; thus, both cells appear to play role in the effect of RIPCs. In addition, RIPCs treatment may also act by increasing Schwann cell remyelination.

A limitation of our study is that spinal cord and sciatic nerve samples were taken only at the end of the experiments. The fact that agents suppressing the activity of glial cells were not used is another limitation. I.p. and s.c. serum groups were not included in our study not to increase the number of animals used. Behavioural experiments were performed 1 h after injections, but no repeated measurements were made afterwards. In addition, RIPCs were administered in a constant volume; the effect of RIPCs applied in different volumes should be investigated in further studies.

5. Conclusions

In summary, demonstrated that transfusion of RIPCs improves the thermal hyperalgesia and mechanical allodynia that neuropathic pain symptoms in rats. We also determined that the intravenous route can better transfer some active substances. Further studies are needed to evaluate the role of RIPCs on glial cells.

Author contribution statement

Ozgur Gunduz: Ruhan Deniz Topuz: Melike Sapmaz-Metin: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Zekiye Gulfem Yurtgezen: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Oktay Kaya: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper. Abdullah Erkan Orhan: Conceived and designed the experiments; Performed the experiments; Wrote the paper. Ahmet Ulugol: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e20954.

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