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ORIGINAL ARTICLE

Pulsed Radiofrequency Effects on the Lumbar Dorsal Root Ganglion of the Domestic Porcine: Pilot Study

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Summary
Introduction. Pulsed radiofrequency (PRF) is a percutaneous minimal invasive procedure for chronic pain management that can be used when conservative treatment methods have been ineffective. The effectiveness of PRF was demonstrated in various good quality randomized control studies, but mechanisms of action are still unclear.
Aim of the Study. The aim of our study is to analyse the histological effects of PRF on the domestic porcine dorsal root ganglion (DRG), and evaluate the expression of biomarkers in gangliocytes of the subject(s).
Materials and Methods. A total 3 domestic porcines were investigated. Under general anaesthesia and X-ray control, DRG PRF was performed. Four lumbar DRGs (L_1, L_2, L_3, L_4) were randomly treated. The opposite side DRGs was used as control. One month after the procedure the animal was euthanized. The lumbar region of the spine was placed in 10% formaldehyde for a month. After this fixation DRG samples were prepared for slide analysis. They were embedded in paraffin in order to obtain 3mm thick sections, which were then cut by microtome and collected on slide glasses. Using standard immunohistochemical reactions, the materials were tinted to define biomarkers neurofilaments (NF), glial fibrillary acidic protein (GFAP), heat shock protein – 70 (Hsp-70) expression and apoptosis by transferase-mediated dUTP nick-end labeling (TUNEL) analysis.
Results. The number of cells with NF (26,0 ± 3,0 vs 16,1 ± 3,3; p<0,05), GFAP (12,0 ± 1,3 vs 3,2 ± 0,9; p<0,05) and Hsp-70 (10,0 ± 1,6 vs 4,2 ± 1,0; p<0,05) expression, were larger in the PRF side comparing with the control side. Additionally, glial cells in spinal ganglia of both sides demonstrated immunoreactivity. The instances of apoptosis were not significantly different, in statistical terms, between the control and experimental sides (18,0 ± 4,0 vs 20,0 ± 4,0; p=0,35).
Conclusions. PRF in spinal gangliocytes of lumbar region increases neural tissue cytoskeleton factors like NF and GFAP suggesting about active regeneration processes into the cells 1 month after the procedure.
Spinal gangliocytes one month after PRF treatment notably increases Hsp-70 expression suggesting about activation of cellular activity and inhibitory role reducing of oxidative stress.
Similar number of apoptotic cells in spinal ganglia of lumbar region after PRF and control side suggests about inhibitory role of PRF on programmed cell death and stimulation of cell survival.
Key words: pig, morphology, pulsed radiofrequency, dorsal ganglion root, growth factors-apoptosis, stress markers

INTRODUCTION

Radiofrequency (RF) is a percutaneous minimal invasive procedure, for chronic pain management, which can be used, when conservative treatment modalities, including medical and physical therapy are not effective. Nowadays, in clinical practice, pain physicians applied two modes of radiofrequency procedures: continuous or conventional RF (CRF) and pulsed RF (PRF). The application of PRF in management of chronic pain is a useful tool, because it's low invasive character, the target selective approach, the possibility of outpatient treatment and its safety.

The first use of RF in humans, treating trigeminal neuralgia, dates back from 1931, when direct current of 350 mAmp was delivered through a needle with 10 mm uninsulated tip[16]. This technique produced nerve tissue lesion with unpredictable size, which can be dangerous and lead to complications. In 1965 Rosomoff et al.[23] were described percutaneous lateral cordotomy for unilateral malignant pain. Shealy reported the first use of RF current for spinal pain[25]. He was performed RF lesioning of the medial branch of the dorsal ramus for facets joint pain, using 14G electrode introduced through a 12G needle. It may produce mechanical lesions besides the desired thermolesions, due to large needle diameter. In 1977 Uematsu described the RF lesion of the dorsal root ganglion (DRG), using the same electrode as Shealy and 75°C tip temperature, causing serious damage of the DRG[37]. A turning point came in 1980 when small-diameter electrodes for the treatment of spinal pain were introduced[28]. The system, known as Sluijter Mehta Kit (SMK) system, consists of 22G disposable cannula with a fine thermocouple probe inside. The small electrode size and temperature control, allows performed procedures with less risk for nerves mechanical injury. After that, the main idea was to create a method to apply RF at

sufficient intensity without letting the tip temperature rise to neurodestructive levels. This method has been named PRF. The history of pulsed radiofrequency usage in chronic pain management originated in 1998, when Sluijter et al. applied it to the dorsal root ganglion[27]. In this modified technique in 1 second 2 bursts of 20 ms each RF current are usually delivered. One cycle has active phase (20 ms) and silent period (480 ms) to allow for washout of the generated heat. During the procedure the temperature of tissues around not exceed 42°C, however output is usually set at 45V and as result not causing damage to nervous tissue.

Analgesic mode of action of CRF consist of destroying nerves and subsequently disrupt the transmission of pain signals through the spinothalamic tracts of the spinal cord. The effectiveness of PRF was demonstrated in various good quality randomized control studies, but mechanisms of action are still unclear.

In an animal study evaluating the histologic effects of pulsed and continuous RF at 42°C to rat DRG and sciatic nerve, showed no structural changes, but cause transient endoneurial edema and collagen deposition. Tissue returned to normal conditions by 7 days in nerve and 21 days in the DRG[21]. In another study PRF applied to dorsal root ganglia induces cellular stress as measured by expression of neuron activating transcription factor 3, sensory fibers appear to be selectively targeted by it[8]. PRF has been introduced as non-neurodestructive procedure, however should partly destroy the myelin envelope of nervous fibers[22]. It was demonstrated using transmission electron microscopy of rats DRG at acute stage after PRF. In the similar study on ultrastructural changes showed microscopic damage after PRF exposure including abnormal membranes and morphology of mitochondria and disruption, which says disorganization of microfilaments and microtubules[6]. Nowadays is popular theory that rapidly changing electric fields produced by PRF alter the transmission of pain signals via a pathway involving c-Fos, a so-called immediate early gene, in the superficial laminae I and II of the dorsal horn at the C5 and C6 levels[38]. Authors were observed a significant increase of c-Fos expression in the dorsal horn of rats that underwent active intervention compared with the sham-operated controls.

There are two types of cell death: necrosis and apoptosis. They are generally considered to be distinct forms of cell death. Both were initially identified based on characteristic changes in cell morphology[14]. Necrosis can result from oxidative stress, but apoptosis can be induced by activation of cystein proteases, cell surface receptor engagement, growth factor withdrawal, and DNA damage. Apoptosis or programmed cell death (PCD) is genetically determined process to destroy cells for the maintaining of cellular homeostasis in the tissue. Gangliocytes death is normal during nervous system development but is abnormal in disease or injury. It can lead to the nerve tissue degeneration. Apoptotic nerve cell death now appears as likely to underlie a number of neurological conditions including Alzheimer's disease, Parkinson's disease, stroke, hereditary retinal dystrophies and Amyotrophic Lateral Sclerosis. Despite subsequent development of numerous molecular markers, the morphological changes still remain the "gold standard" to define the mode of cell death[4]. In light microscopy dorsal root ganglion neurons can be classified into A- and B-cells based on their distinct morphological manifestations. A-cells are big and normally have one big central-located nucleolus, whereas B-cells are small and have more than one peripheral-located nucleoli[32]. A- and B-cells have different physiological functions. A-cells project myelinated fibers that mediate proprioceptive sensations, whereas B-cells give thin-myelinated or unmyelinated fibers, which transmit nociceptive sensations[17,33]. We are particularly interested in the role of apoptosis after DRG PRF. To evaluate the level of apoptosis transferase-mediated dUTP nick-end labeling (TUNEL) analysis was performed. The method of TUNEL was tested on a variety of tissues in which the migration of cells to their final destination is already delineated unequivocally or in tissues that are known for their active PCD. The results demonstrate that in many of these examples, where traditional criteria of apoptosis are missing, the topographical arrangement of nuclei labeled for DNA breaks by TUNEL is in perfect agreement with the expected location of PCD[40].

Glial fibrillary acidic protein (GFAP) is expressed in the central nervous system (CNS) and it has proved to be the most specific marker for cells of astrocytic origin. GFAP is the principal 8-9 nm intermediate filaments in mature astrocytes. As a member of the cytoskeletal protein family, GFAP is thought to be important in modulating astrocyte motility and shape by providing structural stability to extensions of astrocytic processes. Although the exact role of GFAP is unknown, it is involved in many important CNS processes and is partially responsible for neurological functions within the blood-brain barrier. GFAP has been shown to play a role in mitosis by adjusting the filament network present in the cell. During mitosis, there is an increase in the amount of phosphorylated GFAP, and a movement of this modified protein to the cleavage furrow[34]. Studies have also shown that GFAP knockout mice undergo multiple degenerative processes including abnormal myelination, white matter structure deterioration, and functional/structural impairment of the blood-brain barrier[19]. Biochemical studies of GFAP have shown MgCl2 calcium/calmodulin dependent phosphorylation at various serine or threonine residues by protein kinase C and protein kinase A[9], which are two kinases that are important for the cytoplasmic transduction of signals. These data highlight the importance of GFAP for cell-cell communication. Following injury to the human CNS caused by trauma, genetic disorders, or chemicals, astrocytes proliferate and show extensive hypertrophy of the cell body and processes, and GFAP is markedly upregulated. In contrast, with increasing astrocyte malignancy, there is a progressive loss of GFAP production[5].

There are multiple disorders associated with improper GFAP regulation, and injury can cause glial cells to react in detrimental ways. Glial scarring is a consequence of several neurodegenerative conditions, as well as injury that severs neural material. The scar is formed by astrocytes interacting with fibrous tissue to re-establish the glial margins around the central injury core[2] and is partially caused by up-regulation of GFAP[30].

Another condition directly related to GFAP is Alexander disease, a rare genetic disorder. The cellular mechanism of the disease is the presence of cytoplasmic accumulations containing GFAP and heat shock proteins, known as Rosenthal fibers[7]. Notably, the expressions of some GFAP isoforms have been reported to decrease in response to acute infection or neurodegeneration[12]. Reduction in GFAP expression has also been reported in Wernicke's encephalopathy[3]. Changes in GFAP expression have been reported in Down's syndrome, schizophrenia, bipolar disorder, depression[12], Hiv-1[18], varicella zoster[13].

Neurofilaments (NF) are the intermediate filaments of nerve cells and one of the major components of the neuronal cytoskeleton. The function of these cytoskeletal elements is the control of axonal caliber. Neurons with large diameter axons contain more NF, comparing with small diameter axons. Particularly motor neurons contain big amount of NF, where fast impulse conduction play important role. They can accumulate as a marker of a disease process. NF accumulations are seen in several neurological diseases, including Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and diabetic neuropathy[26,1,11,24].

Heat shock protein – 70 (Hsp-70) one of the heat shock proteins, which play vital role in protein folding and turnover. HSPs are traditionally classified into two groups: the high-molecular-weight HSPs and the small HSP family. To the first group belong the HSP90, HSP70, and HSP60 families. Under stress inducing conditions, production of many heat shock proteins increase dramatically in an attempt to protect cells from the effects of the stress inducing agent. They are preferentially induced in response to cell stresses including heat shock, oxidative stress, ultraviolet radiation, ischemia-reperfusion injury, viral infections, nutrient deprivation, and chemicals protecting cells from injury[20]. Hsp70 was demonstrated to have a disease suppressive role in experimental models of autoimmunity preventing disease[39] development and inflammatory effects, by the induction of IL-10 producing T cells.

AIM OF THE STUDY

According that dorsal root ganglia is the first structure of pain modulation, the aim of our study is to analyse the histological effects of PRF on the domestic porcine dorsal root ganglion (DRG), and evaluate the expression of biomarkers glial fibrillary acidic protein (GFAP), neurofilaments (NF), heat shock protein 70 (hsp-70) and apoptosis by TUNEL method in gangliocytes of the subject(s).

MATERIALS AND METHODS

General Conditions. A protocol used in this study was approved by the Animal Care Ethics committee, Riga, Latvia (N 41, 2012.26.01). A total 3 clinical healthy adult female domestic porcine weighting 59 to 65 kg were used for this pilot study. Animals were housed in standard farm in Jelgava district, with access to food and water *ad libitum*, and brought for the experiment to the Faculty of Veterinary Medicine, Latvian University of Agriculture, Jelgava, Latvia.

Pulsed Radiofrequency Procedure. After premedication with Azaperon (4 mg/kg i/m), Atropine (0.02 mg/kg i/m), and Ketamine (10 mg/kg i/m), induction with Pentobarbital (6 mg/kg i/v) the subject's porcines were placed in prone position and were intubated. Before intubation animals has received Pentobarbital (6mg/kg i/v). The animal's backs were shaved and prepared with antiseptic (96% ethanol). Following inhalation of Isoflurane (2Vol%) under X-ray control (C-arm, Philips) RF needle (Radiofrequency Cannula 22 guage, 5 mm active tip, S-1005, NeuroTherm) was introduced transforaminaly in order to maximally reach the DRG. Motor stimulation (2Hz) was positive between 0.5V-0.8V. The porcines were observed for muscles contraction. DRGs PRF was performed using a 10 cm long electrode and following RF generator (NeuroTherm 1100) settings: 42°C, 7 min, 5 p.p.s, 5ms, 45V. The temperature during all procedure was not exceeding 42°C and impedance was checked (180-220Ω). Four lumbar DRGs (L_1, L_2, L_3, L_4) were randomly treated. The opposite side DRGs was used as control.

Observation of the Animals and Tissue Preparation. Animals were checked daily for general condition and complications. The housing room was with 12h:12h light: dark cycle (lights on at 07:00 AM). 30 days after the procedure the animals was euthanized using pentobarbital (200 mg/kg, i/v). The lumbar region of the spine was separated and placed in a 10% buffer of formaldehyde for a month. After these fixations, using both sides' Th12-L5 hemilaminectomy methods, corresponding DRGs were prepared for slide analysis. They were embedded in paraffin in order to obtain 3mm thick sections, which were then cut by microtome and collected on slide glasses.

Immunohistochemistry. Multiple 3μm-thick sections of the paraffin-embedded DRGs were examined for immunohistochemistry. Prior to immunostaining, sections were deparaffinised and rehydrated. Sections were processed in microwave for 20 min in 4% citrate buffer (pH 10), quenched for 10 min with 3% H_2O_2 to block endogenous peroxidase activity, rinsed in phosphate-buffered saline (pH 7.4), pre-treated with nonimmune goat serum for 10 min to block a nonspecific antibody binding and than incubated for 2 h with the primary antibodies. The primary antibodies utilized in immunohistochemistry were monoclonal mouse anti-human neurofilament protein (NF, code-Nr. M 0762, dilution 1:100, DakoCytomation, DK), monoclonal mouse anti-human glial fibrillary acidic protein (GFAP,

code-Nr. M 0761, dilution 1:100, DakoCytomation, DK), mouse anti-HSP70 (HSP70, code-Nr. 33-3800, dilution 1:50, Invitrogen, UK).

TUNEL reaction was used for detection of apoptosis. In situ Cell Death Detection, POD (TUNEL, code-Nr. 11684817910, dilution 1:10, Roche, DE) was used. Deparaffinised sections (xylol 2 x 4 min, 99% ethanol 2 x 2 min, 95% ethanol 2 x 2 min and 70% ethanol 2 x 2 min) were rinsed with water (7-10 min) and transferred to PBS (pH 7.5) for 10 min. Subsequently slides placed into 50 ml PBS solution with 500 µl 30% hydrogen peroxide for 30 min on shaker to block the endogenous peroxidases. Afterwards tissue samples were washed with PBS (3 x 5 min), placed into microwave (700 W) for 10 min for fixation of antigen cooled to room temperature and rinsed with PBS. After that, slides were kept in refrigerator in 0.1% BSA (bovine serum albumin) solution with PBS for 10 min and than incubated in TUNEL mix (Tdt – mix of terminal deoxynucleotide transferase and DIG-labeled deoxynucleotide) for 1h at 37°C. Then the slides were rinsed with PBS 1:10, and incubated for 30 min at 37°C with POD (sheep anti-digoxygenin antibody coupled with horseradish peroxidase Fab fragment). Then the slides were washed with PBS, covered with DAB (diaminobenzidine chromogen) for 7 min, and then rinsed with running water for 5 min. Finally, haematoxylin and eosin staining was performed on each sample. Sections were covered with a polystyrene-based medium and coverslipped.

Analysis. For quantitative analysis we used a counting of cells with biomarkers expression, using light microscopy, in three fields of vision with X400 magnification. Counting was done by a blinded and experienced investigator. For all analysis significance level of <0,05 was applied. Obtained averages were compared for control and experimental group using t-test (either for equal variance for NF, or unequal one for GFAP and Hsp-70). For parameter TUNEL, the p-value was evaluated as probability to get total number of apoptotic cells in a whole series of probes.

RESULTS

DRG PRF increase expression of biomarkers NF, GFAP, Hsp-70. The number of apoptotic cells does not change with treatment.

The number of cells with NF, GFAP and Hsp-70 expression, were larger in the PRF side as compared with control side ($p< 0,05$) (Table 1).

Generally number of NF–containing gangliocytes was higher in the experimental side (26,0 ± 3,0) then in the control side (16,1 ± 3,3) (Figs. 1-2).

The same relation we observed also for GFAP positive gangliocytes in experimental side (12,0 ± 1,3) and in the control side (3,2 ± 0,9) (Figs. 3-4). Additionally, glial cells in spinal ganglia of both sides also demonstrated immunoreactivity. Cellular stress marker Hsp-70 showed obvious dominance of factor positive cells in the experimental side (10,0 ± 1,6) (Fig. 5) in comparison to the control side (4,2 ± 1,0) (Fig. 6).

Apoptosis appeared in approximately similar numbers in both control (18,0 ± 4,0) and experimental (20,0 ± 4,0) sides (Figs. 7-8) (Table 1) without any statistical difference between ganglias (p=0,35).

DISCUSSION

PRF is widely used for the treatment of chronic pain, although its mechanism of action is still not known. However, PRF was advocated as a non-destructive pain therapy, on the basis of the fact that patients treated with pulsed radiofrequency did not show clinical signs of nervous tissue destruction[29]. In our experiment we have not found changes in apoptosis after DRG PRF in the animals that were sacrificed 30 day after procedure. Thus, we suggest about PRF inhibitory effect on spinal ganglion that avoid cell death and stimulate cells survival. This is the first report about apoptosis research on PRF affected neuronal tissue and results seem to be very original and promising of view of tissue surviving. Recently, Podhajsky RJ et al[21] reported that exposure of the DRG in rats to PRF currents causes only transient endoneurial edema, without others structural changes, which resolved on day 21 after procedure. Higuchi et al[10] reported that exposure of the DRG in rats to PRF currents activates dorsal horn lamina I and II neurons, and this effect is not mediated with tissue heating. However, Erdine at al[6] demonstrated abnormal disruption of mitochondria, especially in smaller pain-carrying C-fibers.

Our study has shown multiple NF-containing gangliocytes in experimental side and small amount of NF-containing positive gangliocytes in control side. As NF is an important component of the neuronal cytoskeleton, our data suggest that NF accumulation after PRF might underlie disruption of axonal transport, with probably following worsening of pain signals transmission.

We found GFAP expression in gangliocytes, which was higher in treated side, comparing with untreated. It is the first biomarker, which react on nerve cells stress. Loss of GFAP impairs neuronal cells proliferation and delays nerve regeneration after damage[35]. According to this fact, we suggest about PRF regeneration affinity on gangliocytes.

We also found Hsp-70 expression in gangliocytes, which was significantly higher in PRF treated side, comparing with untreated. Hsp-70 was demonstrated to have a disease suppressive role by reducing of oxidative stress and inflammation. In experimental models in other authors was demonstrated reduction of inflammatory responses against Listeria monocytogenes via production of IL-10[15]. Via the same mechanism, another study confirmed that Hsp-70 protected rats from development of arthritis[34]. This finding indicates that the effect of PRF on the nerve tissues is anti-inflammatory and could explain PRF effectiveness in such pathologies like radicular pain and peripheral neuralgias, where inflammation of DRG or nerve root can be caused by injury or influence of inflammatory cytokines and others biologically active substances. In

addition, Hsp-70 has been reported to play important role activation in lymphocytes and macrophages, and provide the link between innate and adaptive immune system[36]. In another study suppression of metastatic tumor progression after immunization of mice with Hsp (gp96) has been introduced[31]. Due to this fact, it is not excluded that PRF activate also tissue immune response.

CONCLUSIONS

1. PRF in spinal gangliocytes of lumbar region increases neural tissue cytoskeleton factors like NF and GFAP suggesting about active regeneration processes into the cells one month after the procedure.
2. Spinal gangliocytes one month after PRF treatment notably increases Hsp-70 expression suggesting about activation of cellular activity and inhibitory role reducing of oxidative stress.
3. Similar number of apoptotic cells in spinal ganglia of lumbar region after PRF and control side suggests about inhibitory role of PRF on programmed cell death and stimulation of cell survival.

Conflict of interest: None

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Table 1. Mean number and standard deviation of different factors and apoptosis positive cells in the control and the experimental pigs spinal ganglia one month after PRF.

Factors	Control	Experimental	p-value
NF	16,1 ± 3,3	26,0 ± 3,0	<0,05
GFAP	3,2 ± 0,9	12,0 ± 1,3	<0,05
Hsp-70	4,2 ± 1,0	10,0 ± 1,6	<0,05
TUNEL	18,0 ± 4,0	20,0 ± 4,0	0,35

Abbreviations:
NF – neurofillament
GFAP – glial fibrillary acidic protein
Hsp-70 – heat shock protein 70
TUNEL – transferase-mediated dUTP nick-end labeling analysis

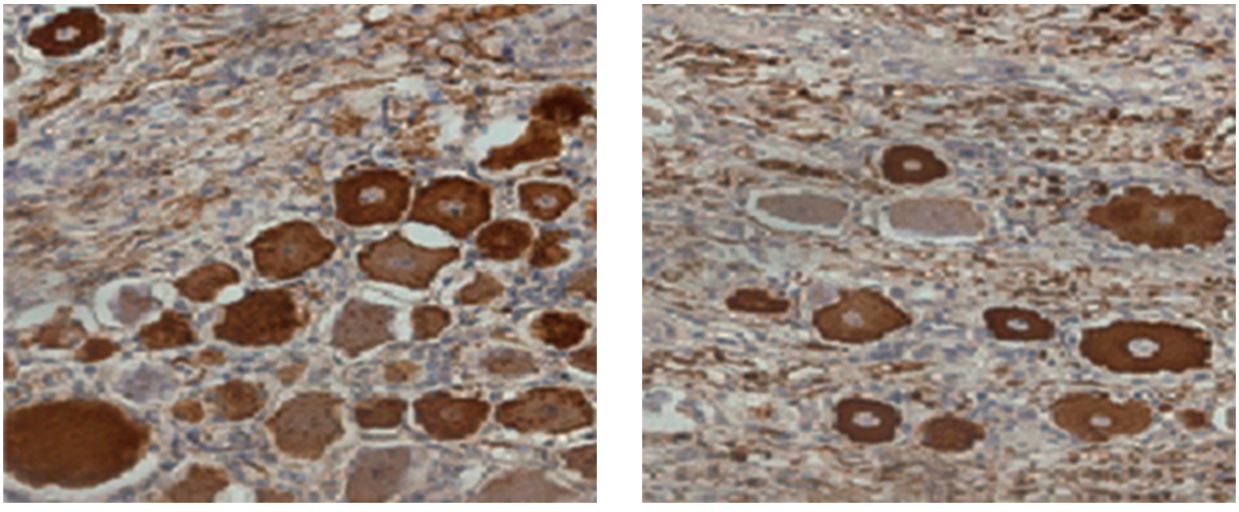


Fig. 1-2: multiple NF containing gangliocytes (1, L2dxt., ×400, NF IMH) in experimental side and small amount of NF positive gangliocytes - control side (2, L2sin., ×400, NF IMH) one month after PRF.

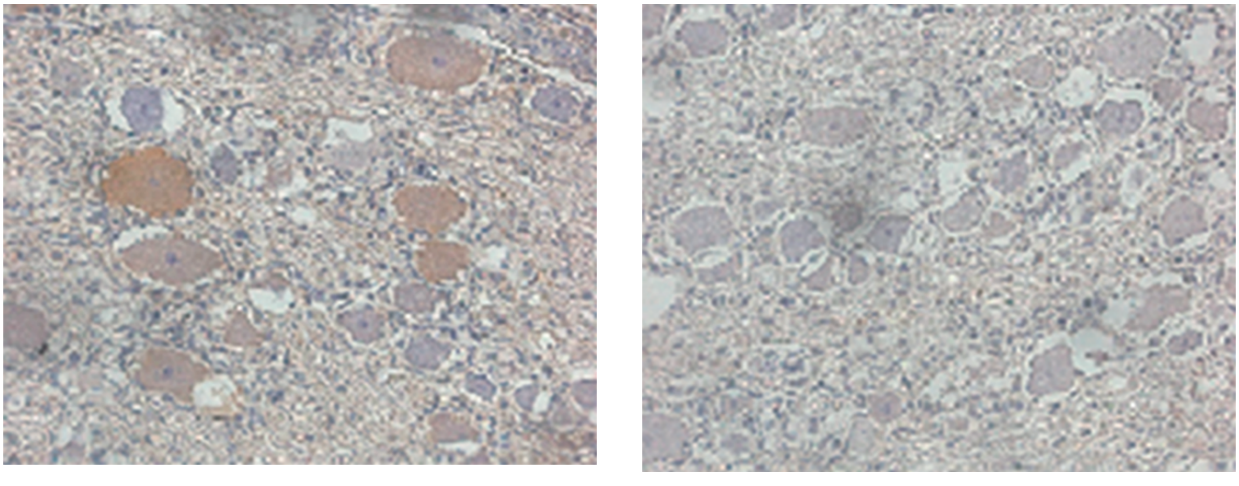
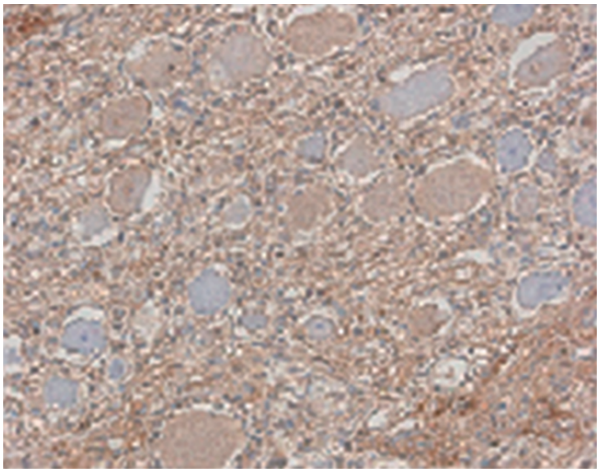


Fig. 3-4: multiple GFAP containing gangliocytes (3, L4dxt., ×400, GFAP IMH) in experimental side and small amount of GFAP positive gangliocytes - control side (4, L4sin., ×400, GFAP IMH) one month after PRF.



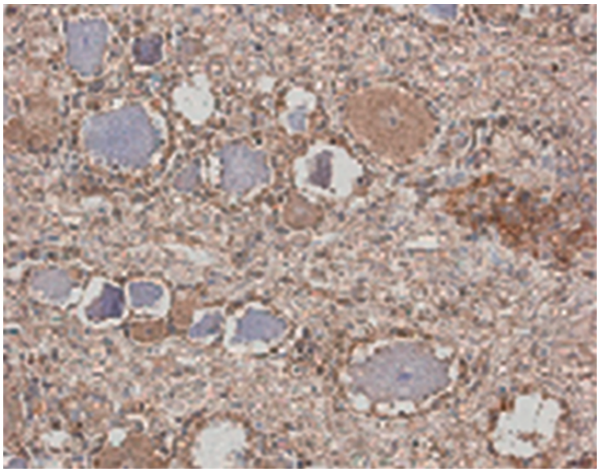
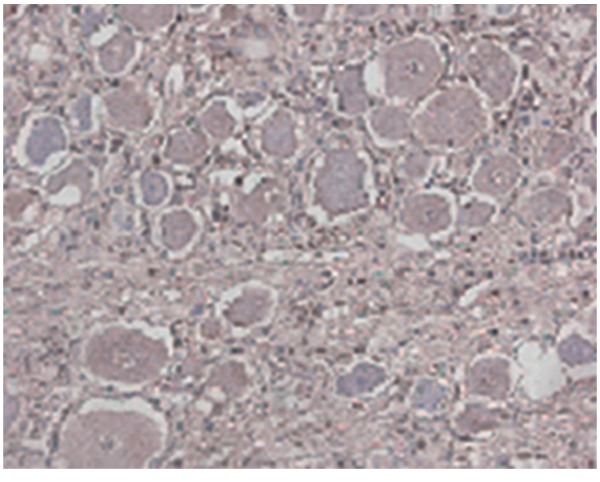


Fig. 5-6: multiple Hsp-70 containing gangliocytes (5, L4dxt., ×400, Hsp-70 IMH) in experimental side and small amount of Hsp-70 positive gangliocytes - control side (6, L4sin., ×400, Hsp-70 IMH) one month after PRF.



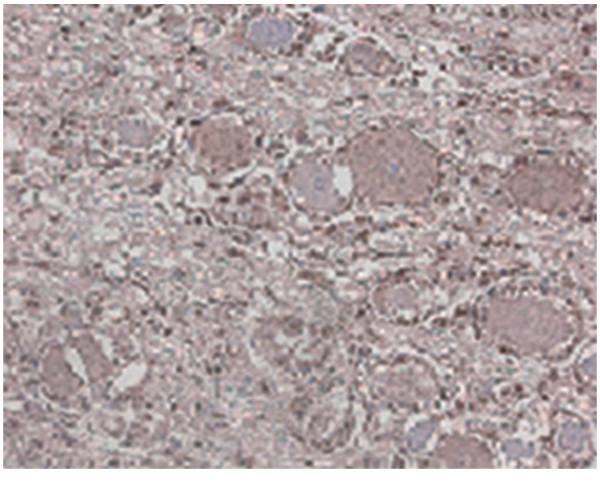


Fig. 7-8: note absence of difference in appearance of apoptotic cells both – experimental (7, L3dxt., ×400, TUNEL) and control (8, L3sin., ×400, TUNEL) sides in spinal ganglia one month after PRF.