EVALUATION OF HYPERALGESIA AND HISTOLOGICAL CHANGES OF DORSAL ROOT GANGLION INDUCED BY NUCLEUS PULPOSUS

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ABSTRACT

Objective: To evaluate the hyperalgesia and histological abnormalities induced by contact between the dorsal root ganglion and the nucleus pulposus. Methods: Twenty Wistar rats were used, divided into two experimental groups. In one of the groups, a fragment of autologous nucleus pulposus was removed from the sacrococcygeal region and deposited on the L5 dorsal root ganglia. In the other group (control), a fragment of adipose tissue was deposited on the L5 dorsal root ganglia. Mechanical and thermal hyperalgesia was evaluated on the third day and the first, third, fifth and seventh weeks after the operation. A L5 dorsal root ganglion was removed in the first, third, fifth and seventh weeks after the operation for histological study using HE staining and histochemical study using specific labeling for iNOS. Results: Higher intensity of mechanical and thermal hyperalgesia was observed in the group of animals in which the nucleus pulposus was placed in contact with the dorsal root ganglion. In this group, the histological study showed abnormalities of the dorsal root ganglion tissue, characterized by an inflammatory process and axonal degeneration. The histopathological abnormalities of the dorsal root ganglion tissue presented increasing intensity with increasing length of observation, and there was a correlation with maintenance of the hyperalgesia observed in the behavioral assessment. Immunohistochemistry using specific labeling for iNOS in the group of animals in which the nucleus pulposus was placed in contact with the dorsal root ganglion showed higher expression of this enzyme in the nuclei of the inflammatory cells (glial cells) surrounding the neurons. Conclusion: Contact between the nucleus pulposus and the dorsal root ganglion induced mechanical and thermal hyperalgesia and caused histological abnormalities in the dorsal root ganglion components. These abnormalities were characterized by an inflammatory and degenerative process in the structures of the dorsal root ganglion, and they presented increasing intensity with longer periods of observation.

Keywords – Spine; Intervertebral Disk; Low Back Pain; Hyperalgesia; Intervertebral Disk Displacement; Wistar Rats

INTRODUCTION

The symptoms caused by disk herniation are related to mechanical compression of the lumbar nerve roots[1-3] and the inflammatory process provoked by the components of the nucleus pulposus. Nerve root compression occurs through extravasation of the content of the damaged intervertebral disk into the vertebral canal, which is a rigid and limited space. The inflammatory process

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Declaramos inexistência de conflito de interesses neste artigo

relating to lumbar disk herniation is caused by contact between the biochemical components of the nucleus pulposus and the nerve tissue[4-7]. The nucleus pulposus is a gelatinous structure present inside the intervertebral disk and surrounded by the fibrous ring. It is practically acellular and non-vascularized; it does not have lymphatic vessels and is composed of proteoglycans and water. Since it does not have any contact with the systemic circulation after embryogenesis, it is recognized as a foreign body when exposed to the immune system, thus triggering an inflammatory response characterized by an infiltrate of neutrophils, macrophages and T and B cells, around the herniated intervertebral disk[2,8]. The presence of pro-inflammatory cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor-α (TNFα) has also been demonstrated in patients with lumbar disk hernias, thus corroborating the studies that have shown that there is an inflammatory component in the physiopathology of disk herniation symptoms[9,10].

It has been reported that the nucleus pulposus has the capacity to cause abnormalities in the structure of dorsal root ganglia that are characterized by an inflammatory process and cell apoptosis[11,12]. The aim of this study was to experimentally evaluate mechanical and thermal hyperalgesia and histological abnormalities in the dorsal root ganglia induced by contact with the nucleus pulposus.

MATERIAL AND METHODS

Twenty male Wistar rats weighing between 220 and 250 g were used, which were supplied by the Central Vivarium of the Ribeirão Preto School of Medicine, USP. Before carrying out the experiment, the animals were kept for two days in the local vivarium for acclimatization in cages measuring 40 x 60 x 20 cm, with a maximum of six animals per cage, under controlled temperature conditions (22 to 25°C) and dark/light cycling (12 x 12 h), with free access to water and food. The research project was approved by the local ethics committee for animal experimentation, and the study was conducted in accordance with the international ethical standards for the use of laboratory animals.

To carry out the surgical procedure, the animals were anesthetized by means of intraperitoneal injection (i.p.), with a 10% solution of ketamine (Ketamina Agener®), at a dose of 0.1 ml per 100 g of rat body weight, 2% xylazine (Anesedan®), at a dose of 0.07 ml per 100 g

of body weight, and 5% fentanyl (Fentanest®) at a dose of 0.001 ml per 100 g of body weight. Before the operation, all the animals were administered a single dose of Veterinary Pentabiotic (benzathine benzylpenicillin) 600,000 UI, procaine benzylpenicillin 300,000 UI, potassium benzylpenicillin 300,000 UI, dihydrostreptomycin sulfate 250 mg and streptomycin sulfate 250 mg (Small Size Veterinary Pentabiotic®) intramuscularly, at a dose of 0.1 ml per 100 g of rat body weight. For the procedure of dorsal root ganglion removal for the histological study, intraperitoneal anesthesia was used, consisting of a solution of 4% chloral hydrate, at a dose of 1 ml per 100 g of body weight.

The experimental model for disk hernia was used in accordance with the method described by Grava et al[13], which consisted of removing a fragment of the nucleus pulposus from the sacrococcygeal region and then placing it in contact with the dorsal root ganglion of the fifth lumbar root (Figure 1). Removal of the nucleus pulposus material from the intervertebral disk was done in the sacrococcygeal region (base of the rat's tail) by means of an incision in the midline of the transition region between the fourth sacral vertebra and the first coccygeal vertebra. The intervertebral disc was exposed bilaterally and the nucleus pulposus material was removed by means of a transverse incision above the fibrous ring. This gelatinous material was collected, weighed on a precision balance (average weight of 4 to 5 mg) and then deposited at the location determined for this study (dura mater, dorsal root ganglion or nerve root). After concluding the procedure, the surgical wound was sutured as a single layer including the muscle fascia and the skin. The surgery to place nucleus pulposus material on nerve tissues was carried out with the aid of an optical lens with ten times magnification of the observation field. The structures (dura mater, dorsal root ganglion or nerve root) were exposed for the nucleus pulposus material to be deposited, by means of right-side partial hemilateral laminectomy and removal of the transverse vertebral process at the level established.

In the control-group animals, the same procedure was performed to remove the nucleus pulposus from the sacrococcygeal region and expose the L5 dorsal root ganglion, but a fragment of adipose tissue was placed on the ganglion.

Two experimental groups with 10 animals in each group were formed. Both groups were subjected to surgery to remove the fragment nucleus pulposus from the
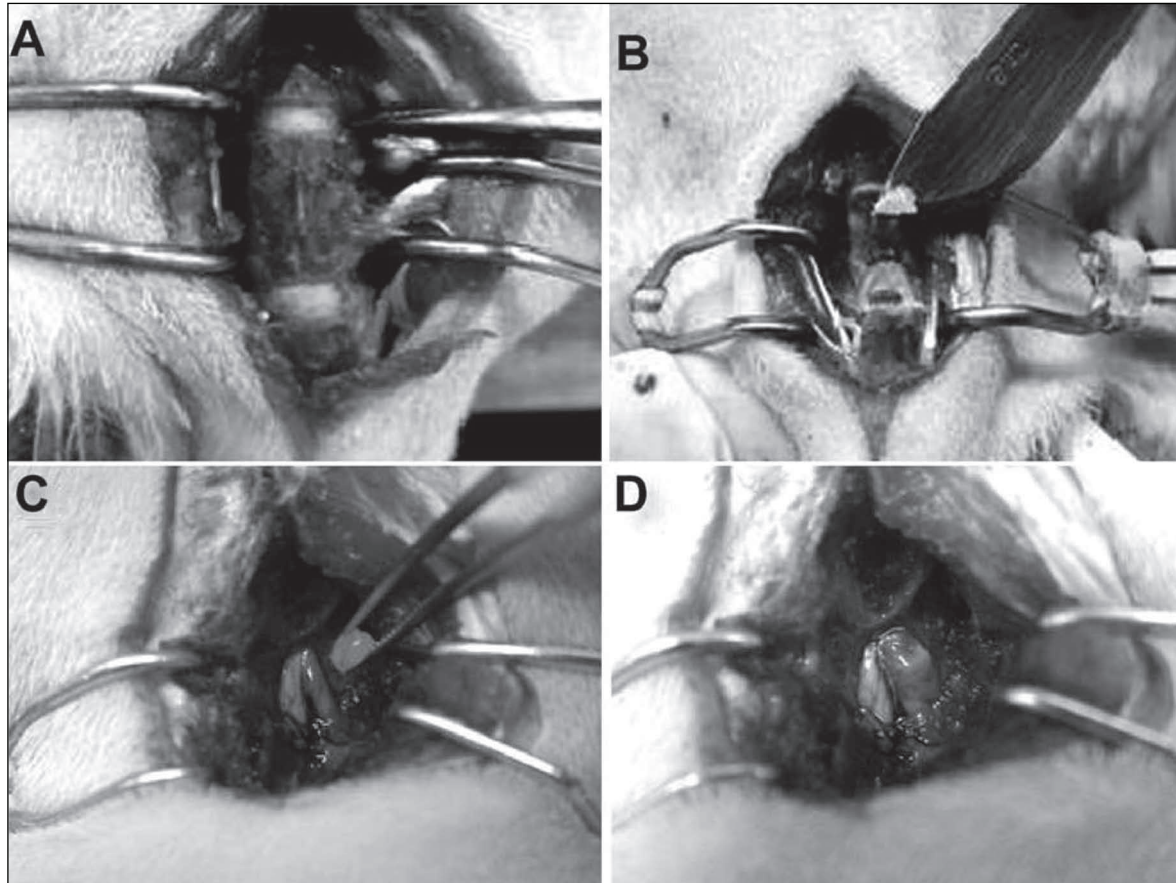
Figure 1 – Photographs illustrating the surgical procedure to place a fragment of the nucleus pulposus in contact with the L5 dorsal root ganglion. A) Surgical approach in the sacrococcygeal region to remove the fragment of nucleus pulposus. B, C and D) Surgical sequence of exposure of the L5 dorsal root ganglion and subsequent placement of the nucleus pulposus onto the nerve tissue.

intervertebral disk in the sacrococcygeal region. The nucleus pulposus material was placed on the L5 dorsal root ganglion in group I, while group II (control) received a fragment of adipose tissue of similar size to the fragment of nucleus pulposus that was used in group I.

After concluding the surgical procedure, the animals were evaluated by means of behavioral tests in order to assess the mechanical hyperalgesia (electronic von Frey test)[14] and thermal hyperalgesia (Hargreaves test)[15] on the third day and one, three, five and seven weeks after carrying out the surgical procedure to induce the inflammatory symptoms of the disk hernia. The hyperalgesia was assessed by an independent evaluator who did not participate in the surgery on the animals and did not have any information regarding the experimental group or the procedure that was carried out.

Increasing pressure test on the rats' paws (electronic von Frey test)

The mechanical hyperalgesia was evaluated by me-
ans of the increasing pressure test on the rats' paws, known as the electronic von Frey test (nociception)[14]. The method consisted of using an electronic anesthesiometer (model 1601C, Life Science Instruments®), which had a force transducer connected to a digital force counter expressed in grams(g). The precision of the apparatus was 0.1 g and it was calibrated to register a maximum force of 150 g and to maintain the precision of 0.1g up to a force of 80g. The contact between the force transducer and the paw was achieved by means of a disposable polypropylene pointer of 0.5 mm in diameter, adapted to the transducer. The animals were placed in acrylic boxes measuring 12 x 20 x 17 cm, in which the floor consisted of a net of mesh size 5 mm2, made of non-malleable wire of 1 mm in thickness. This was done 15 minutes before the experiment, to allow adaptation to the environment. Mirrors positioned 25 cm below the experimentation boxes made it easier to view the soles of the animals' paws. The investigator applied a linearly increasing force to the soles of the rats' paws,

through the net mesh, until the animals produced a response of withdrawing and shaking the paw that had been stimulated. The stimuli were repeated up to six times, generally until the animal had presented three similar measurements with a clear shaking response after withdrawing the paw.

Hargreaves thermal hyperalgesia test (plantar test)

The thermal hyperalgesia was evaluated by means of the plantar test described by Hargreaves et al[15]. This test consisted of heating up the plantar region of the rear paws of the rats, by means of a directed infrared light source, until the animal presented the behavioral pattern of paw withdrawal, or until the heating of the paw reached the predetermined time limit (cutoff time of 12 seconds).

The animals were placed in individual acrylic compartments and positioned over a surface of special glass that allowed homogenous passage of light and heat for a five-minute period, for adaptation to the environment. After this time, an infrared light source was placed under each of the animal's paws and activated together with an electronic chronometer, until the animal withdrew the paw. When this event occurred, the light source and clock stopped automatically.

Three measurements were made at time intervals of 5 to 10 minutes. The latency measurements of paw withdrawal were then expressed as percentage assessments relative to the control paws (contralateral paws) at each observation time. For situations in which the animals did not present a reaction, a maximum cutoff time for the experiment of 12 seconds was stipulated, so that their physical integrity would be preserved.

Two animals in each experimental group were sacrificed in the first, third, fifth and seventh weeks after carrying out the surgical procedure, with the aim of removing the L5 dorsal root ganglion in order to perform histological evaluations.

The L5 dorsal root ganglia were removed from both sides. The ganglion from the side contralateral to the operation was used as a control in the histological evaluations. The material was processed for histological evaluations, and HE staining was applied for examination under an optical microscope. Hoechst staining was used for the immunohistochemical evaluation, which was performed under a fluorescence microscope.

The behavioral test results were compared by means of statistical evaluation using multivariate analysis of variance (MANOVA), followed by Student's t test with Bonferroni inequality. The significance level laws set at $p < 0.05$.

RESULTS

The evaluation on the intensity of the mechanical and thermal hyperalgesia at the different evaluation times is represented in Figure 2, which illustrates the statistical difference that was seen throughout the observation period between the two experimental groups. This demonstrates the influence of contact between the nucleus pulposus and the nerve root ganglion of the fifth lumbar root on induction of hyperalgesia. In the group of animals in which the nucleus pulposus was placed in contact with the L5 dorsal root ganglion, a small reduction in intensity of the mechanical and thermal hyperalgesia was noted over the course of the observation period, while in the animals that received adipose tissue deposited on the dorsal root ganglion, the reduction in the intensity of hyperalgesia was greater.

The histological assessment on the L5 dorsal root ganglion that was in contact with the fragment of nucleus pulposus showed that an inflammatory process was present. This was characterized by proliferation of Schwann cells around the neurons and slight degenerative abnormalities of the neuronal bodies of some neurons of the ganglion, shown by eccentric nuclei and enlargement of the cytoplasm, and this differed from the ganglia that were used as controls. The control ganglia presented a nucleus in the central region, homogenous cytoplasm and few satellite cells. The observed abnormalities indicated that a progressive degenerative process was occurring in some neurons, which evolved towards a low-intensity inflammatory process in the first week (Figure 3), and moved into an intermediate stage in the third and fifth weeks (Figure 4), in which an intense inflammatory process was observed, with major proliferation of glial cells, cytoplasm of lacy appearance and the presence of some apoptotic neurons.

Starting in the fifth week, a degenerative process of greater intensity was observed. This was characterized by cells with eccentric or pyknotic nuclei, intense cell proliferation and cell adhesion to neurons (Figure 4).

Seven weeks after the operation, the observed appearance consisted of irreversible degeneration induced by the inflammatory process, with apoptotic neuron nuclei and greater numbers of glial cells (Figure 5). Intense
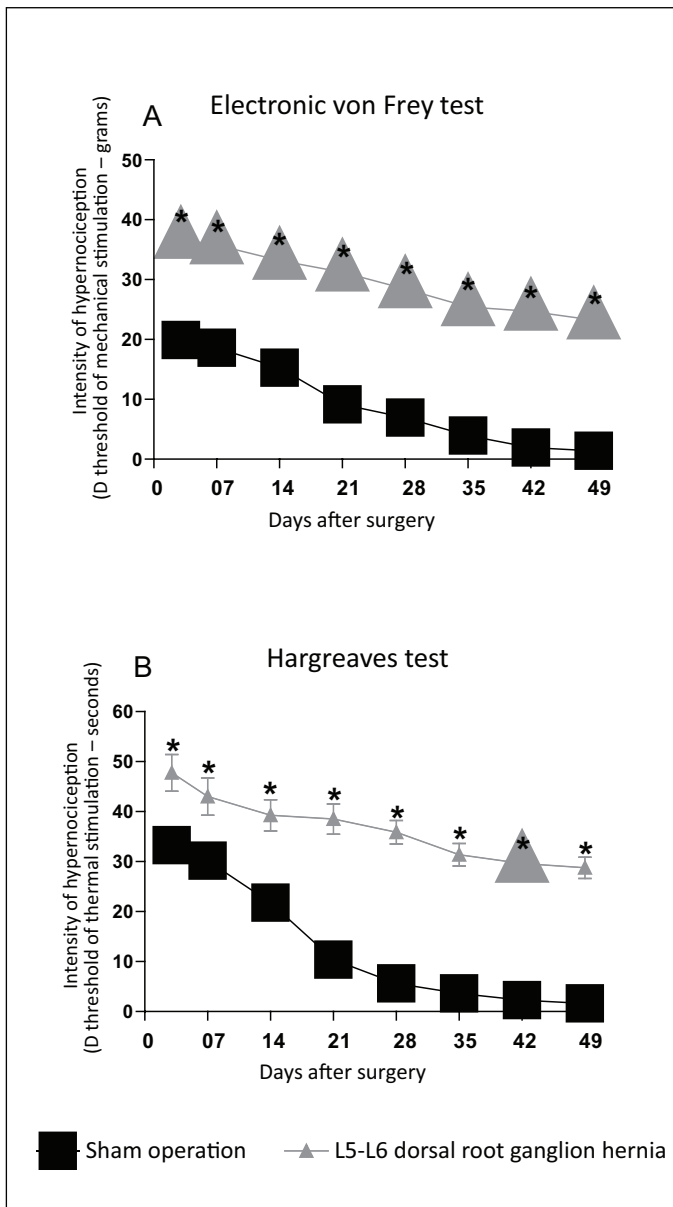
Figure 2 – Intensity of mechanical hyperalgesia (electronic von Frey test) and thermal hyperalgesia (Hargreaves test) in the experimental groups. The asterisk (*) indicates a statistical difference (Student's t test for unpaired data; $p < 0.05$).

staining was observed on the slides from the group in which the nucleus pulposus was in contact with the dorsal root ganglion, thus highlighting the presence of many inflammatory cell nuclei (glial cells) surrounding the neurons. This differed greatly from what was observed in the control group (Figure 5).

Immunohistochemical evaluation with specific labeling for iNOS (Figure 5) demonstrated that the expression of this enzyme was greater in the nuclei of the inflammatory cells (glial cells) surrounding the neurons, thus contrasting with the observations in the control group.

Histopathological abnormalities of the dorsal root ganglion tissue, characterized mainly by an inflammatory process and axon degeneration, were observed. This process had been induced by contact between the nucleus pulposus and the dorsal root ganglion, and it presented increasing intensity with time and correlated with maintenance of the hyperalgesia that was observed in the behavioral assessment.

DISCUSSION

A new field of study and research emerged after it was observed that placing the nucleus pulposus in contact with nerve elements, without mechanical compression of the nerve structures, would induce structural and functional abnormalities in these nerve elements[6]. Although the exact mechanism involved in the genesis of the hyperalgesia produced by contact between the nucleus pulposus and the nerve structures is not fully clear, the results from different studies have demonstrated that the nucleus pulposus has the property of inducing inflammatory abnormalities in tissues[8,16]. In a study carried out to develop an experimental model, we observed that contact between the nucleus pulposus and the dura mater, nerve root or dorsal root ganglion would provoke the appearance of mechanical and thermal hyperalgesia[13]. We also observed that using the L5 dorsal root ganglion for contact with the fragment of nucleus pulposus produced the greatest intensity of hyperalgesia, as measured by the methods used. Hence, this was the reason for choosing this structure for carrying out the present study.

Changes to the structure of the dorsal root ganglion after it has come into contact with the nucleus pulposus have been reported. Such changes have been observed after only three hours of contact, and are characterized by deformation of the cell nuclei, apoptosis and an inflammatory process[11,12].

The abnormalities observed in our study corroborate the results described in the literature[17-19]. They were characterized by the presence of an inflammatory process with proliferation of Schwann cells around the neurons and degenerative abnormalities of the neuron bodies, and these changes were progressive over the course of time. At the last evaluation, the abnormalities presented characteristics of irreversibility and degeneration induced by the inflammatory process.

The increase in the expression of the enzyme nitric oxide synthase (NOS) in the nuclei of the inflamma-
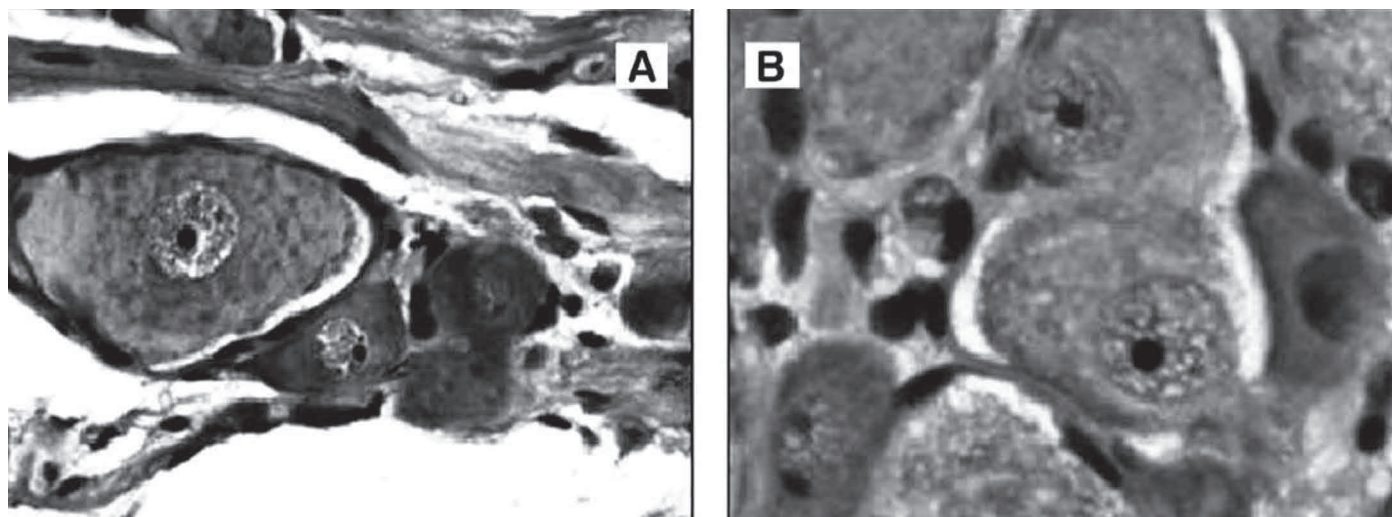
Figure 3 – Photomicrograph of histological section through the L5 dorsal root ganglion (40x magnification; HE staining). A) Ganglion in contact with the nucleus pulposus, viewed one week after the surgical procedure. Note the proliferation of Schwann cells surrounding the neurons, which present eccentric nuclei and cytoplasm enlargement. B) Ganglion from control animal. Note the nucleus in the central region, homogenous cytoplasm and presence of few satellite cells.

tory cells surrounding the neurons suggests that local production of nitric oxide had increased at the disk hernia site. Nitric oxide (NO) is formed from oxidation of the terminal nitrogen atom of the amino acid L-arginine, through the action of NOS. There are at least three well-defined isoforms of this enzyme: neural NOS (first described in neurons); induced NOS (iNOS; present in activated leukocytes); and endothelial NOS (first observed in endothelial cells)[20]. The activity of iNOS is capable of producing NO in micromolecular concentrations, in contrast with the constitutive enzyme, which only produces this mediator in nanomolar concentrations. Synthesis of iNOS may be activated by cytokines, such as TNF-α, IL-1β and interferon-α, β and γ, which are released during the inflammatory or infectious process[21,22]. The origin of the NO in the inflammatory process is unclear, but it may result from endothelial cells, neutrophils and macrophages(23). NO
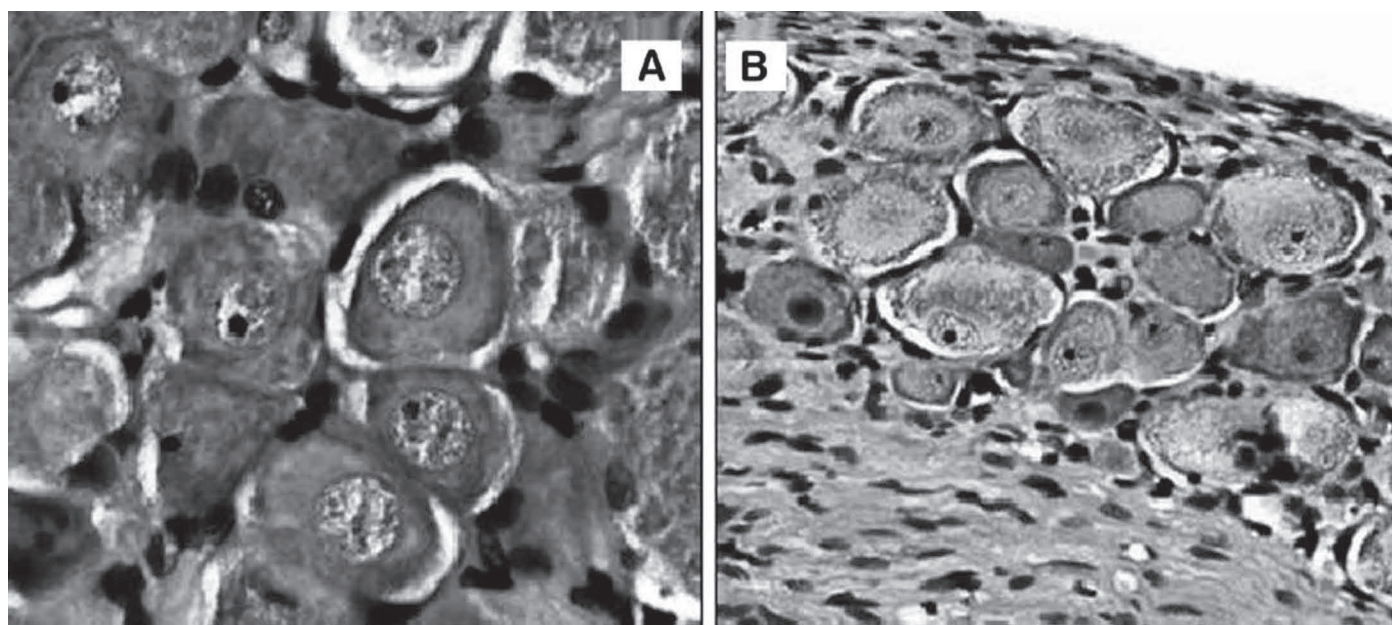

Figure 4 – Photomicrographs of L5 dorsal root ganglia made three weeks (A) and five weeks (B) after the surgery. A) Note the intense inflammatory process with major proliferation of glial cells, cytoplasm with lacy appearance and presence of some apoptotic neurons (40x magnification; HE staining). B) More accentuated degenerative process, characterized by cells with eccentric or pyknotic nuclei. Intense proliferation and adhesion of glial cells around the neurons (20x magnification; HE staining).
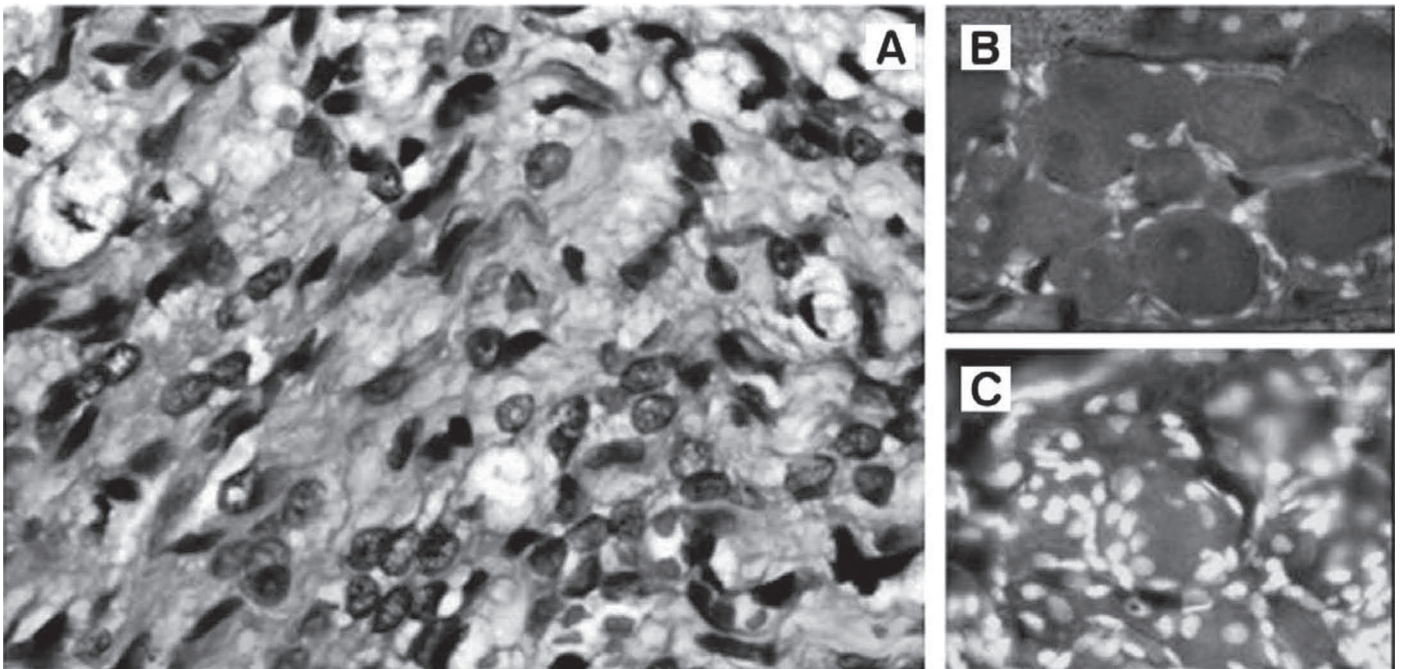
Figure 5 – Photomicrograph of L5 dorsal root ganglion made seven weeks after the surgery. A) Note the irreversible appearance of the degeneration induced by the inflammatory process in the ganglion (40x magnification; HE staining). B and C) Histological sections with Hoechst staining, examined under fluorescence microscope, showing labeling for iNOS in the neuron nuclei and glial cells. Control group animal (B) and animal with dorsal root ganglion in contact with the nucleus pulposus (B) presenting increased numbers of glial cells.

may modulate the inflammatory response, thus acting as a pro-inflammatory substance[24]. The pro-inflammatory role of NO produced by the activity of iNOS has also been demonstrated[25].

CONCLUSIONS

Contact between the nucleus pulposus and the L5 dorsal root ganglion resulted in histological abnormalities of the ganglion tissue, characterized by an inflammatory process and axonal degeneration. These histopathological changes to the dorsal root ganglion tissue presented increasing intensity with longer observation periods, and presented a correlation with maintenance of the hyperalgesia that was observed in the behavioral assessment.

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