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ORIGINAL ARTICLE

Cytokine inhibition and time-related influence of inflammatory stimuli on the hyperalgesia induced by the nucleus pulposus

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Abstract
Introduction The symptoms of lumbar disc herniation, such as low back pain and sciatica, have been associated with local release of cytokines following the inflammatory process induced by the contact of the nucleus pulposus (NP) with the spinal nerve.
Material and methods Using an animal experimental model of intervertebral disc herniation and behavioral tests to evaluate mechanical (electronic von Frey test) and thermal (Hargreaves Plantar test) hyperalgesia in the hind paw of rats submitted to the surgical model, this study aimed to detect in normal intervertebral disc the cytokines known to be involved in the mechanisms of inflammatory hyperalgesia, to observe if previous exposure of the intervertebral disc tissue to specific antibodies could affect the pain behavior (mechanical and thermal hyperalgesia) induced by the NP, and to observe the influence of the time of contact of the NP with the fifth lumbar dorsal root ganglion (L5-DRG) in the mechanical and thermal hyperalgesia.
Results The cytokines present at highest concentrations in the rat NP were TNF-α, IL-1β and CINC-1. Rats submitted to the disc herniation experimental model, in which a NP from the sacrococcygeal region is deposited over the right L5-DRG, showed increased mechanical and thermal hyperalgesia that lasted at least 7 weeks. When the autologous NP was treated with antibodies against the three cytokines found at highest concentrations in the NP (TNF-α, IL-1β and CINC-1), there was decrease in both mechanical and thermal hyperalgesia in different time points, suggesting that each cytokine may be important for the hyperalgesia in different steps of the inflammatory process. The surgical remotion of the NP from herniated rats 1 week after the implantation reduced the hyperalgesia to the level similar to the control group. This reduction in the hyperalgesia was also observed in the group that had the NP removed 3 weeks after the implantation, although the intensity of the hyperalgesia did not decreased totally. The removal of the NP after 5 weeks did not changed the hyperalgesia observed in the hind paw, which suggests that the longer the contact of the NP with the DRG, the greater is the possibility of development of chronic pain.
Conclusion Together our results indicate that specific cytokines released during the inflammatory process induced by the herniated intervertebral disc play fundamental role in the development of the two modalities of hyperalgesia (mechanical and thermal) and that the maintenance of this inflammation may be the most important point for the chronification of the pain.

Keywords Lumbar disc herniation · Dorsal root ganglion · Radicular pain · Hyperalgesia

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Introduction

The intervertebral disc is a biomechanical and biological active structure in the spine. The mechanism of radicular pain due to disc herniation is related to mechanical (nerve root compression) and chemical factors [inflammatory process induced by the contact of the nucleus pulposus

(NP) with the nerve fibers]. In animals and humans, pure compression of a non-inflamed nerve produces sensory and motor changes without pain, whereas pain is elicited with manipulation of an inflamed nerve [1, 2]. The effects induced by the contact of the NP with the epidural elements have been observed in experimental studies and compelling evidence of histological alterations, changes in the nervous electrical activity, and in the intraneural blood flux after such contact has been obtained [2–6]. The NP structure mainly comprises proteoglycans, collagen and support cells, which are able to produce inflammatory mediators after injury, such as cytokines [7]. In fact, these elements may be involved in the effects observed in clinical experimental models of back pain [7–9]. Besides locally produced cytokines, immunoglobulins (IgG, for instance), hydrogen, nitric oxide (NO) and enzymes such as phospholipase 2 (PLA2) might be responsible for the pathophysiological reactions [10–14]. In summary, the inflammatory effects induced by the contact of the NP with epidural elements are a complex event where numerous mediators and mechanisms play a role at various levels.

During the inflammatory process, nociceptive neurons (activated by nocive stimuli) become sensitized and begin to respond to stimulus that was not able to elicit responses previously. This phenomenon, known as hyperalgesia, is a common denominator of all inflammatory processes and is characterized by the decrease in the nociceptive threshold and increased activity in response to thermal and mechanical stimulation [15]. The role of local cellular mediators such as cytokines and chemokines in the inflammatory hyperalgesia was extensively studied and there is no doubt nowadays about their involvement in the etiology of the painful sensitization after tissue damage [16–20].

The objectives of the present work were: (1) to detect in normal intervertebral disc the cytokines known to be involved in the mechanisms of inflammatory hyperalgesia [17, 21, 22], (2) to observe if previous exposure of the intervertebral disc tissue to specific antibodies could affect the pain behavior (mechanical and thermal hyperalgesia) induced by the NP, and (3) to observe the influence of the time of contact of the NP with the fifth lumbar dorsal root ganglion (L5-DRG) in the mechanical and thermal hyperalgesia.

Materials and methods

All experiments were conducted in accordance with National Institute of Health Guidelines for the Welfare of Experimental Animals and with the methodology approved by the Ethics Committee of the author's institution. Each animal was used only in a single experimental group. Ninety-five male Wistar rats weighing 220–250 g were used in the study ($n = 95$). The animals were housed in temperature-controlled rooms (22–25°C) with an alternating 12 h light–dark cycle. Water and food were available ad libitum.

Table 1 Description of the protocols used in the study

Experiment 1: cytokine identification ($n = 10$ rats)
Group 1: in the NP
Group 2: in adipose tissue (control group)
Experiment 2: role of cytokines in thermal and mechanical hyperalgesia ($n = 30$ rats)
Group 1: treatment with anti-TNF-α antibody ($n = 5$ rats)
Group 2: treatment with anti-IL-1β antibody ($n = 5$ rats)
Group 3: treatment with anti-CINC-1 antibody ($n = 5$ rats)
Group 4: control group: treatment with non-immune serum ($n = 5$ rats)
Group 5: positive control group: no treatment ($n = 5$ rats)
Group 6: negative control group ($n = 5$ rats)
Experiment 3: effect of the time of contact of the NP with the L5-DRG ($n = 55$ rats)
Group 1: control group ($n = 15$ rats)
Group 2: hernia group ($n = 15$ rats)
Group 3: hernia removal group ($n = 15$ rats)

The study was carried out in three steps (Table 1). In the first step, the cytokines of the normal intervertebral disc of the rat sacrococcygeal region were detected. In the second step, we assessed the effect of inhibition with specific antibodies for the three cytokines found present at highest concentrations in the NP (TNF-α, IL-1β and CINC-1) on pain behavior (mechanical and thermal hyperalgesia). In the third step, we assessed the effect of time of contact of the NP fragment with the L5-dorsal root ganglion (L5-DRG) on pain behavior (mechanical and thermal hyperalgesia).

Surgical protocol

In all surgical procedures, the animals were anesthetized with an intraperitoneal (i.p.) injection of 10% ketamine (0.1 ml/100 g), 2% xylazine (0.07 ml/100 g) and 5% fentanyl 5 (0.001 ml/100 g).

The experimental model used in this study to reproduce the hyperalgesia seen in cases of intervertebral disc herniation—caused by the contact of the NP with the spinal nerves—consisted of a surgical procedure to harvest the NP from the coccygeal intervertebral disk followed by its implantation over the right L5-DRG. Briefly, a midline incision in the transition region between the fourth sacral vertebra and first coccygeal vertebra the intervertebral disc was bilaterally exposed and the NP removed through a transverse incision in the annulus fibrosus. The NP gel-like substance was collected, weighed on a precision scale (4–5 μg average weight) and placed over the L5-DRG. A hemilateral partial laminectomy exposed the DRG where the NP was placed. After this procedure, the surgical

wound was sutured on a single plane with muscle fascia and skin.

Behavioral tests

In the experiments 2 and 3, behavioral tests were performed to evaluate the decrease in the mechanical and thermal thresholds in the rats hind paws The objective of hyperalgesic evaluation was to determine the rat hind paw withdrawal threshold to a mechanical (electronic von Frey test) or thermal (Hargreaves plantar test) stimulus before and after the surgical procedures. All behavioral tests were performed by the same examiner without knowing the experimental group of the animal. Observations were performed on the day before surgery and weekly during the postoperative period according to the experimental group.

Electronic von Frey

Mechanical hyperalgesia was measured by the electronic von Frey method [23]. In a quiet room, rats were placed in acrylic cages (12 × 20 × 17 cm) with wire grid floors, 15–30 min before the beginning of the tests. During this adaptation period, the paws were tested (probed) two to three times. The test consisted of evoking a hind paw flexion reflex with a hand-held force transducer fitted with a 0.5-mm^2 polypropylene tip (electronic von Frey hair; IITC Life Science, Woodland Hills, CA). A tilted mirror placed under the grid provided a clear view of the rat hind paw. The investigator was trained to apply the tip in between the five distal footpads with a gradual increase in pressure. The stimulus was automatically discontinued and its intensity recorded when the paw was withdrawn. The maximum force applied was 80 g. The stimulus was repeated (up to six times, usually three) until the animal presented similar measurements (differences <10%). The end point was characterized by the removal of the paw in a clear flinch response after paw withdrawal. The animals were tested before and after the treatments. The results are reported as the Δ withdrawal threshold (in g) that was calculated by subtracting the average of the last three measurements after the treatments from the average of three measurements before the treatments.

Hargreaves plantar test

The thermal hyperalgesic threshold was also measured based on the latency to withdrawal evoked by exposing the right hind paw to a thermal stimulus [24]. The animals were placed in Plexiglas cages on top of a glass sheet. The thermal stimulus (IITC Life Science Inc., Woodland Hills, CA) was positioned under the glass sheet to focus the projection bulb exactly on the middle of the plantar surface of the animal. A mirror attached to the stimulus permitted visualization of the undersurface of the paw. After 15 min of adaptation, the paw withdrawal latencies were measured. A cutoff thermal latency of 12 s was set to prevent tissue damage.

Experiment 1: Cytokine identification and measurement

Ten animals were used for the detection of cytokines known to be involved in the mechanism of hyperalgesia (TNF-α, IL-6, CINC-1, IL-10, IL-1β) by the method of Safieh-Garabedian [25]. Adipose tissue was used as comparative reference, with cytokines being measured in a tissue fragment by the same method as used for the material from the intervertebral disk.

The fragments of the NP between the fourth sacral and first coccygeal vertebra and the adipose tissue were collected and homogenized in phosphate buffer (PBS) containing NaCl 0.4 M, 0.05% Tween 20, 0.5% bovine albumin serum (BSA), phenylmethylsulfonyl fluoride 0.1 mM, EDTA 10 mM and aprotinin 20 KI/mL (Sigma, USA). After homogenized, the samples were centrifuged (3,000 RPM/10 min) and the supernatant was collected for the quantification of the TNFα, IL-6, IL-1β, IL-10 and CINC-1 levels by ELISA (enzyme-linked immunosorbent assay). The plates with the samples were incubated for 12 h at 4°C with the antibodies against the four cytokines (10 μg/mL). In the next day, the plates were washed and incubated for 2 h in bovine albumin 1% in order to avoid non-specific bindings. After the incubation, the plates were washed again and different dilutions were performed. The diluted samples or the samples were incubated at 4°C for 24 h. The plates were washed three times in buffer solution and the polyclonal biotinilated antibodies against TNFα, IL-1β or rat IL-1ra, diluted 1/2,000, were then added (100 μL/sample). After incubation at room temperature for 1 h, the plates were washed again and 100 μL of avidin-HRP diluted 1:5,000 was added. After 15 min, 100 μL of the reagent *o*-phenylenediamine-2HCl (OPD, Sigma, USA) was added. The plates were kept in a darkened room for 15–20 min (at room temperature). The enzymatic reaction was interrupted with H_2SO_4 (1 M) and the absorbance curves were determined in 490 nm.

Experiment 2: Role of cytokines and cytokine inhibition in pain behavior

Thirty animals were used in the experiment 2 to study the role of the three cytokines present at highest concentrations in the NP (TNF-α, IL-1β and CINC-1) and the effect of their inhibition with specific antibodies in the mechanical and thermal hyperalgesia. The NP harvested from the sacrococcygeal region was bathed with the specific antibody for each experimental group before its implantation over the L5-DRG (Table 1).

Experimental groups were set up as following:

Group 1: anti-TNFα antibody (anti-TNF-α—R&D Systems Laboratory) ($n = 5$).
Group 2: anti-IL-1β antibody (anti-IL-1β—R&D Systems Laboratory) ($n = 5$),
Group 3: anti-CINC-1 antibody (anti-CINC-1—R&D Systems Laboratory) ($n = 5$),
Group 4: control—non-immune serum ($n = 5$),
Group 5: positive control—absorbable gel sponge + TNFα, IL-1β and CINC-1 (National Institute of Biological Standard and Control, NIBSC) ($n = 5$),
Group 6: negative control—absorbable gel sponge + saline ($n = 5$).

In the groups 5 and 6 (positive and negative controls, respectively), the surgery was performed the same way as described for the groups 1–4, except that the content of the NP was discarded and an absorbable gel sponge soaked in TNFα, IL-1β and CINC-1 (in group 5, positive control) or saline solution (group 6, negative control) was deposited on the L5-DRG.

Mechanical and thermal hyperalgesia were evaluated by the same examiner in a blind manner on the day before surgery and on the postoperative days 7, 14, 21, 28, 35, 42, and 49.

Experiment 3: Influence of time of NP contact with the L5-DRG

The objective of this experiment was to determine if the period of time of NP contact with the L5-DRG is important for the chronification of the mechanical and thermal hyperalgesia in the rat hind paw. After the first surgical procedure to remove the NP from the sacrococcygeal region and its deposition over the right L5-DRG a second procedure was performed 1, 3 and 5 weeks later, according to the experimental group, in order to remove the transplanted NP fragment. Fifty-five animals were divided into three experimental groups (Table 1): Group I—control group (sham-operated animals; simple resection of the NP from the sacrococcygeal junction and surgical exposure of the L5-DRG without NP transplantation. A second surgical procedure was performed to expose the L5-DRG); Group II—hernia group (surgical exposure of the L5-DRG with NP transplantation—also followed by a second surgical procedure to expose the L5-DRG. The transplanted NP was not removed); Group III—NP removal group (the transplanted NP was removed in a second surgical procedure after 1, 3 and 5 weeks of NP implantation). The groups were subdivided into three subgroups of five animals each according to the length of time before the second operation (1, 3 and 5 weeks after the initial procedure to implant the NP over the L5-DRG).

The behavioral tests (mechanical and thermal hyperalgesia) were performed 1 day before surgery and weekly after the first surgical procedure until 5 weeks after the second surgery. Testing was blind with respect to the different groups evaluated and was performed by the same examiner.

Statistical analysis

Results are reported as mean ± standard error mean (SEM) for groups of five animals. The statistical analysis used was one-way ANOVA followed by the Bonferroni test. Differences were considered statistically significant at $P < 0.05$.

Results

Experiment 1

The cytokines known to be involved in the mechanism of inflammatory hyperalgesia (TNF-α, CINC-1, IL-10 and IL-1β) were detected in the normal nucleus pulposus harvested from coccygeal intervertebral disc. However, IL-6 concentration was less and did not differ significantly from the adipose tissue (t test > 0.05). The concentration of TNF-α, CINC-1, IL-1β and IL-10 were significantly higher (t test < 0.05) than adipose tissue (Fig. 1). The three cytokines present at highest concentration in the normal NP were TNF-α, CINC-1 and IL-1β).

Experiment 2

Both the mechanical and thermal hyperalgesia observed in the animals that received the inert gel sponge soaked in TNF-α, IL-1β and CINC-1 was more intense than in the animals that received the sponge soaked in physiological saline containing no cytokines (Fig. 2) and lasted up to the fourth week, being more intense during the second week and then showing values similar to control at later time points. Note that inert gel sponge soaked with cytokines produced a higher hyperalgesia in the first 4 weeks ($P < 0.05$) compared to sponge soaked in physiological saline.

Treatment of the NP with a specific antibody for TNF-α, IL-1β and CINC-1 before its placement on the L5-DRG reduced the intensity of mechanical and thermal hyperalgesia compared to the group in which the NP was placed on the L5-DRG without treatment with a specific antibody (Fig. 3). The reduction of hyperalgesia had similar effects after treatment with specific antibodies for TNF-α and CINC-1. A more pronounced hyperalgesia effect was observed after treatment with antibody for IL-1β. Note that

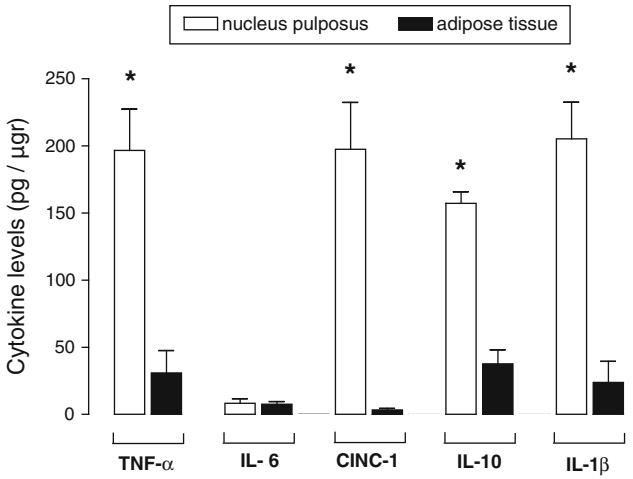

Fig. 1 Determination of inflammatory cytokines in the sacrococcygeal nucleus pulposus and adipose tissue of rats**.** Data are reported as the mean ± SEM of the material obtained from ten animals. The *asterisk* indicates a statistical difference ($P < 0.05$) between cytokine concentration in the nucleus pulposus and in adipose issue (Student's *t* test for unpaired data)

mechanical and thermal hyperalgesia was significantly lower in all groups treated with specific antibodies for TNF-α, IL-1β and CINC-1 compared to the group treated without specific antibody ($P < 0.05$).

Experiment 3: Influence of time of NP contact with the L5-DRG

The contact of NP with the L5-DRG induced both mechanical and thermal hyperalgesia in a time-dependent manner (Figs. 4, 5 and 6).

NP removal 1 week after the induction of hyperalgesia was followed by a decrease in the intensity of mechanical and thermal hyperalgesia similar to the one shown by the control group at the end of the assessment (Fig. 4). From the second week after NP removal, hyperalgesia was significantly lower in group III (removal group) than in group II (hernia group) (*t* test $P < 0.005$).

NP removal 3 weeks after the induction of hyperalgesia was also followed by a significant decrease in the intensity of mechanical and thermal hyperalgesia. However, the values did not return to basal levels when compared to control (Fig. 5).

NP removal 5 weeks after the induction of hyperalgesia did not change the intensity of either mechanical or thermal hyperalgesia (Fig. 6). In contrast to the other two experiments (NP removal after 1 and 3 weeks), there was no difference in the intensity of mechanical and thermal hyperalgesia between group II (hernia group) and group III (NP removal group) and the values were very similar. These results suggest that there was a chronification of the hyperalgesia induced by the inflammatory stimulus and, probably, the development of persistent pain.

Discussion

Our findings show levels of proinflammatory cytokines (TNF-α, CINC-1, IL-1β and IL-10) significantly higher in normal vertebral disc than adipose tissue. However, the concentration of IL-6 in the normal intervertebral disc was similar to adipose tissue. Although cytokines were found in large quantities in herniated nucleus pulposus [26, 27], it was unclear whether the cytokine secretion would be derived from inflammatory cells that infiltrated the herniated nucleus pulposus tissue or from disc-derived cells. The capacity of normal disc cells to secrete proinflammatory cytokines and interleukin-10 in the absence of inflammatory cells was observed in cultured murine disc [28]. TNF-α is spontaneously produced by intervertebral disc tissue in culture, and it has been found in the NP cells of rats and pigs [8, 18, 27]. In herniated disc, TNF-α is expressed at higher levels compared to asymptomatic or autopic controls [29].

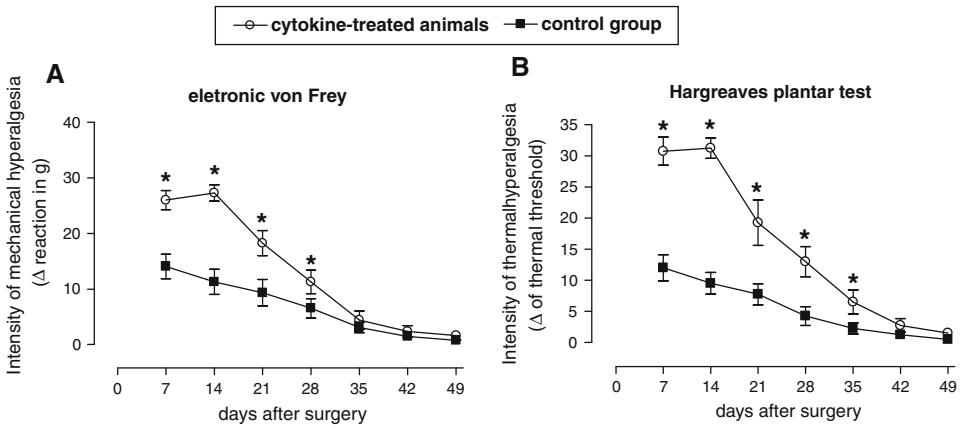

Fig. 2 Intensity of mechanical (electronic von Frey) and thermal (Hargreaves) hyperalgesia induced by placement of a sponge soaked in cytokines (TNF-α, IL-1β and CINC-1—positive control group) or saline solution (negative control group) over the L5-DRG. The *asterisk* indicates a statistically significant difference ($P < 0.005$) between the control and the treated groups

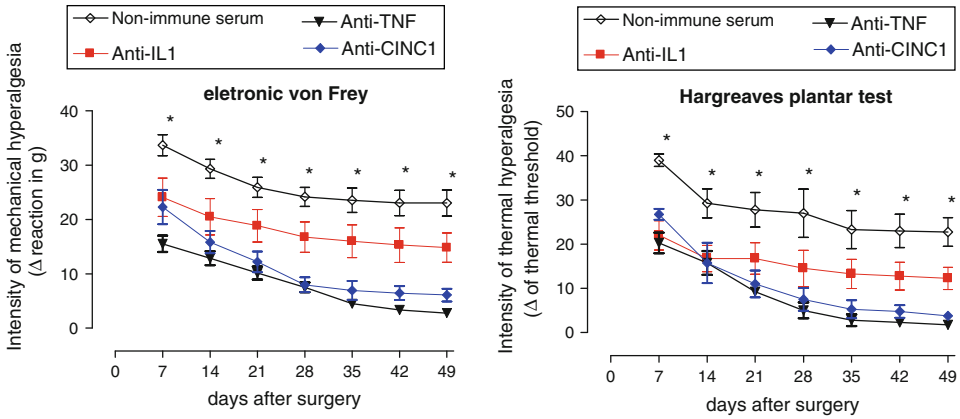

Fig. 3 Intensity of mechanical (electronic von Frey) and thermal (Hargreaves) hyperalgesia induced by placement of a sponge soaked in anti-IL-1β, anti-TNF-α, anti-CINC-1 antibody or in non-immune serum and placed over the L5-DRG. The *asterisk* indicates a statistically significant difference ($P < 0.005$) between the group treated with IL-1β, anti-TNF-α and anti-CINC-1 antibody and the group treated with non-immune serum

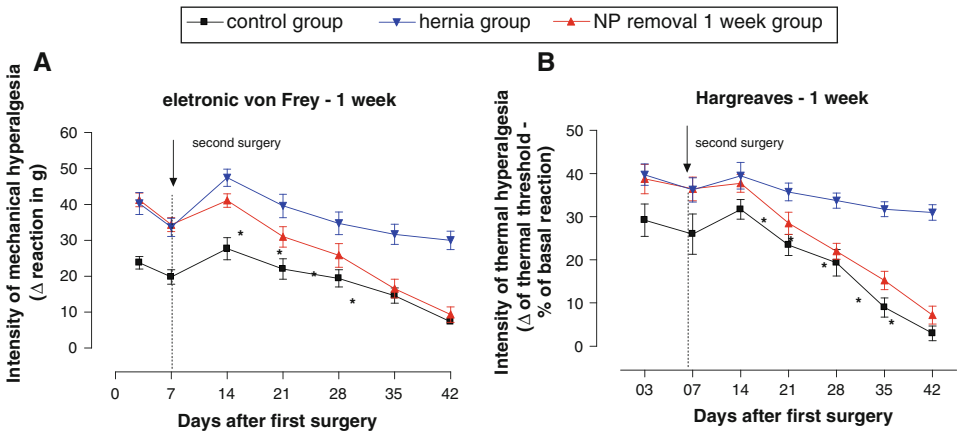

Fig. 4 Intensity of mechanical and thermal hyperalgesia after surgical removal of the nucleus pulposus (NP) 1 week after the induction of hyperalgesia. The *asterisk* indicates a significant difference ($P < 0.05$) (MANOVA, followed by ANOVA with Bonferroni test) between group II (hernia group) and group III (NP removal group). The results are reported as the mean ± SEM of five animals

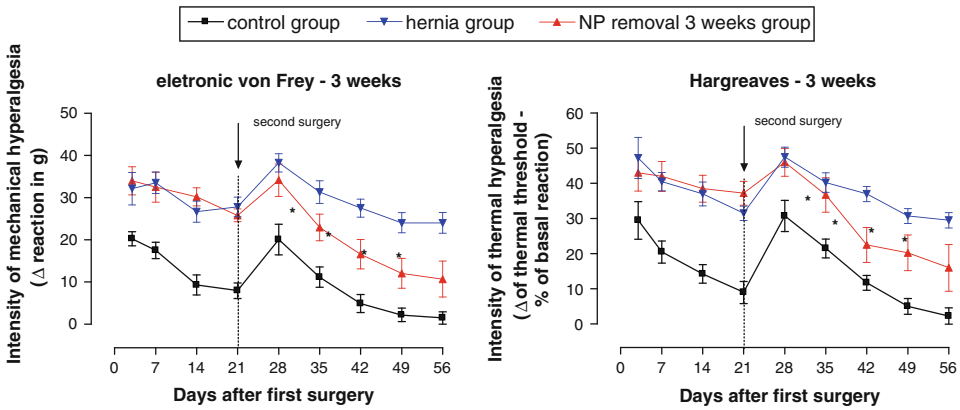

Fig. 5 Intensity of mechanical and thermal hyperalgesia after surgical removal of the nucleus pulposus (NP) 3 weeks after the induction of hyperalgesia. The *asterisk* indicates a significant difference ($P < 0.05$) (MANOVA, followed by ANOVA with Bonferroni test) between group II (hernia group) and group III (NP removal group). The results are reported as the mean ± SEM of five animals

Moreover, cytokines play a role in the generation of neuropathic pain both in the peripheral and central nervous systems [30–32] and participation of pro-inflammatory cytokines in the genesis of changes of nervous tissue and symptoms generated by lumbar disc herniation has been emphasized by several authors [32–34].

In the present study, the involvement of TNF-α, IL-1β and CINC-1 in the induction of hyperalgesia induced by contact of the NP of the intervertebral disc with the DRG was demonstrated by attenuation of the painful behavior by specific antibodies. Interestingly, the reduction in the intensity of the hyperalgesia by the antibody treatment was more intense during the acute phase (in the first weeks of observation), with a significant fall starting on the fourth week. However, the rate of release of the cytokines placed in the gel should be considered for interpretation of the results.

Of note, the different time points in which the treatment with each antibody reduced the intensity of hyperalgesia

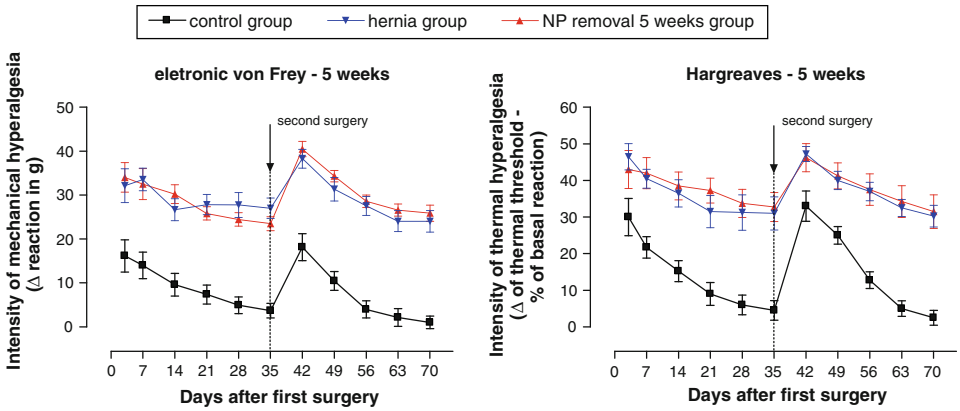

Fig. 6 Intensity of mechanical and thermal hyperalgesia after the surgical removal of the nucleus pulposus (NP) 5 weeks after the induction of hyperalgesia**.** There was no significant difference ($P < 0.05$) (MANOVA, followed by ANOVA with Bonferroni test) between group II (hernia group) and group III (NP removal group). The results are reported as the mean ± SEM of five animals

suggest that each cytokine may exert its maximum activity at a different time after the injury. On the other hand, it is also possible that the concentration of antibodies in the gel sponge at the beginning and at the end of the experiment could have helped to explain the differences in the kinetics observed in the groups treated with specific antibodies.

Several cytokines such as TNF-α, IL-1 and IL-6 are associated with increased neuropeptides in peripheral nerves [35], and the results observed with cytokine inhibition in our study suggest that blocking induction upstream of several cytokines is desirable for blocking the sensation of pain. This observation is in line with Studer et al. [35], whose in vitro study revealed that blocking cytokine activation blunts the production of factors associated with inflammation and disc matrix catabolism [36].

The present results show that mechanical and thermal hyperalgesia caused by contact of the NP with the DRG depended on the duration of this contact. The removal of the transplanted NP fragment 1 week after the first surgical procedure caused a reduction of the aversive response to thermal and mechanical nociceptive stimuli in such a way that the hypernociceptive response was similar to that presented by the control group (no contact between DRG and NP). When the second surgery was performed after 3 weeks, hyperalgesia was not reduced to the levels presented by the control group (no NP fragment on the DRG). At the second surgery 5 weeks later, however, there was no reduction of mechanical or thermal hyperalgesia. Thus, the intensity of hyperalgesia observed in the group which had the NP fragment removed after 5 weeks was similar to that of the hernia group, with the NP fragment in contact with the DRG throughout the study.

The inflammatory process may lead to sensitization of nociceptors, which are neurons specialized in detecting noxious stimuli or stimuli which may become noxious if not removed. This hyperalgesic state may be understood as an increased response to mechanical, thermal and chemical stimuli. This sensitization, described as increased efficiency of synaptic conduction, is evident in every patient with chronic pain due to changes in the central, as well as the peripheral nervous system [24]. Several chemical irritants, such as cytokines, matrix metalloprotease, fibroblast growth factor, leukotrienes, thromboxanes, prostaglandins, immunoglobulins, among others, known to cause inflammation, are considered to play a role in nociceptor sensitization [37].

The maintenance of the stimulus seemed to be fundamental for the establishment of the sensitized state and this may be related to a persistent hyperalgesic state induced by the prolonged presence of the NP fragment in contact with the DRG. Thus, the results obtained by us could be attributed to this phenomenon, since there is constant production of inflammatory mediators by the herniated tissue. This may lead to the persistent picture, as exemplified by the failure in decreasing the mechanical hyperalgesia when the NP fragment was removed after 5 weeks of hyperalgesia induction.

The clinical observation that surgical discectomy for carefully selected patients with sciatica due to lumbar disc prolapse provides faster relief from the acute attack [38] and that preoperative duration of leg pain exceeding 3 months predicted poorer outcome [39] could be supported by the results observed in our study. However, epidemiological and clinical studies show that most lumbar disc prolapses heal naturally with conservative treatment and with time, without surgery [38]. This fact remains as a gap in our knowledge about the cost-effectiveness of all forms of surgical treatment of lumbar disc prolapses. The results of our study indicate that, after a chronic pain mechanism was triggered, even the removal of the induced agent would not interrupt the pain-related phenomena. However, at present we do not know the length of time needed to trigger the chronic pain mechanism in humans.

In our study, cytokines (TNF-α, IL-1β and CINC-1) were identified in the NP of healthy Wistar rats; their inhibition by a specific antibody was able to reduce the hyperalgesia induced by NP and the reduction of hyperalgesia was related to the time of NP removal. These results suggest a temporal influence of the maintenance of the

inflammatory stimulus (represented by the contact of the NP with the L5-DRG) for the chronification of the hyperalgesic state in rats. The time of maintenance of the inflammatory stimulus may be important for the chronification of pain, which may be correlated with the presence of persistent hyperalgesia.

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Conflict of interest None.

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