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Cytokine expression in the epidural space: a model of non-compressive disc herniation-induced inflammation

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Abstract

Study Design—Animal study

Objective—Development of an animal model for the study of biochemical changes that occur in the epidural space after intervertebral disc herniation.

Summary of Background Data—Although strong evidence for an inflammatory component exists, the biochemical processes underlying pain following disc herniation remain unknown.

Methods—Epidural lavage was performed in 48 rats after L5 dorsal root ganglion (DRG) exposure at baseline and 3, 6, or 24 hours after placement of autologous nucleus pulposus (NP) (N = 15), saline (N = 15), or NP + an interferon-gamma antibody (anti-IFNγ; N = 18) directly onto the DRG. Multiplex assays quantifying interleukin (IL-)-1-α, IL-1β, IL-2, IL-4, IL-6, IL-10, TNFα, IFNγ and GM-CSF were performed. NP (N = 7) was also analyzed for these cytokines by placing NP into saline and measuring the relative concentration.

Results—Cytokines measured low at baseline (0–100pg/ml) in all groups. Compared to saline, NP application caused IL-6 elevation, peaking at T=3hr, that was prevented by anti-IFNγ. NP induced elevation of TNFα, peaking at T=24hr and was prevented by anti-IFNγ. IFNγ was elevated after NP at T=3hr and T=24hr. IL-1α was similar after saline versus NP. The concentrations of IL-1β and IL-10 were elevated at T=3hr, 6hr and 24hr in all groups without between-groups difference. The level of IL-4 peaked at T=3hr in the NP group and was different than saline and NP +anti-IFNγ groups but the time effect was insignificant. There was no change for GM-CSF. The concentration of cytokines measured in normal NP was < 2pg/ml for all cytokines except TNFα.

Conclusion—In this model of acute non-compressive disc herniation, NP caused the elevation of epidural IL-6, TNFα and IFNγ; all attenuated by IFNγ blockade. IL-1β and IL-10 were both significantly elevated by saline alone and their response was not prevented by IFNγ blockade. This model may prove useful for the study of the biochemical processes by which NP induces inflammation-induced nerve root irritation and radiculopathic pain.

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Keywords

Cytokine; Disc herniation; Inflammation; Epidural; Rat; IL-6; TNF-alpha; Interferon-gamma

Introduction

An association between low back and radiculopathic pain and herniation of nucleus pulposus (HNP) from the intervertebral disc has been recognized for decades,[1,2] but the specific mechanisms remain unknown. Traditional thought and utilization of compressive models focused on a mechanism by which mechanical pressure is placed on the dorsal root ganglion (DRG) or nerve roots. However, evidence increasingly supports a mechanism by which neural irritation may result from a combination of compressive and non-compressive etiologies or even in the absence of compression.[1,3–9] The observation in human studies that mechanical nerve root stimulation is painful only after prior exposure to nucleus pulposus (NP)[10,11] supports both this concept and the possibility that the inflammatory milieu resulting from disc herniation without compression may be sufficient to induce symptoms.[1,12] Furthermore, it has been shown that application of NP to the nerve roots or DRG can rapidly sensitize spinal dorsal horn neuronal responses[4,5,13,14] or primary afferent neurons.[15]

The mediators of neuronal sensitization are under investigation, and may include the recruitment of cytokines to the area of inflammation via immunomodulatory cells such as satellite glia or Schwann cells, and resident macrophages.[16,17] As NP has been sequestered from circulation since embryologic formation[18] it is immunologically naïve, thereby inducing an autoimmune-mediated inflammatory response once herniated.[1,12,19,20] It is likely that multiple modulators are involved in a single or in multiple inflammatory cascades. Evidence of this is provided by the clinical efficacy of broad immunosuppressive agents such as steroids and experimental findings that doxycycline, which can modulate expression of several different cytokines, is more effective at alleviating pain behavior than is blockade of a specific pro-inflammatory cytokine such as tumor necrosis factor-alpha (TNFα).[21]

Few studies have evaluated cytokines in the epidural space in humans with neural root irritation thought to result from disc herniation, partially because of difficulty in accessing this potential space in clinical practice.[22,23] Furthermore, clinical variability, including time to presentation, makes this problem difficult to address in clinical studies. An animal model may therefore prove beneficial for further investigation of the mediators involved in NP-induced inflammation and neural sensitization. Cytokine expression was therefore quantified in the epidural fluid around the fifth lumbar dorsal root ganglion (L5 DRG) using lavage at baseline and at various time-points following the application of autologous NP to the L5 DRG. In addition, because interferon-gamma (IFNγ) is a pro-inflammatory cytokine produced by multiple cell types[24–27] and can cause the upregulation of various cytokines via the activation of microglia and other cells,[26–28] the co-application of a soluble anti-IFNγ antibody with autologous NP was also investigated.

Methods and Methods

Animals

Forty-eight adult male Sprague-Dawley rats (300–400g, Charles River Laboratories) were used in this study. The animals were housed in a temperature-controlled room (22 ± 1°C) in a 12 hour light/dark cycle with food and water provided *ad libitum*. The experimental

procedures were approved by the Stanford University Institutional Animal Care and Use Committee.

L5 Dorsal Root Ganglion Exposure

Rats were anesthetized with isoflurane 3–4% in a chamber, then moved to mask anesthesia with 1.5–2.5% maintenance isoflurane during surgery. The isoflurane concentration was adjusted so that a strong paw pinch failed to evoke a withdrawal response prior to the incision. An incision over the L5-L6 spinous processes was made and the paraspinous muscles were dissected free from the left L5-L6 lamina. Using a dissection microscope and micro-rongeurs, the left L5 inferior articular facet was resected, but the superior articular process of L6 was left intact to minimize DRG trauma and bleeding. This procedure allowed visualization of most of the dorsal side of the left L5 DRG and has been previously described.[4,5] The ligamentum flavum was removed between L5 and L6 on the left side, but the dura was left intact.

Epidural lavage of dorsal root ganglion

Baseline lavage was performed following exposure of the left L5 DRG using a micro-pipettor to apply 50 μl of phosphate-buffered saline (pH = 7.4) to the DRG. The solution was allowed to sit for 5 seconds prior to aspiration back into the same pipette. The aspirate was placed into a microcentrifuge tube containing a mix of protease inhibitors (Complete, Roche Diagnostics, Indianapolis, IN; 1 tablet per 1.5ml PBS then aliquoted into 30μl per tube) and kept at −80°C prior to analysis. Multiple time points were analyzed; for 3, 6, and 24 hr post-surgery, animals were randomly assigned to one of three groups based on the solution applied to the DRG: saline only (n = 15), nucleus pulposus (NP) only (n = 15) or NP + co-application of a soluble anti-IFNγ (n = 18) (50 μl of 0.1 mg/ml; R&D Systems, MN, USA).

Coccygeal disc exposure

For animals in the NP or NP + anti-IFNγ groups, a coccygeal disc at the base of the tail was exposed through a skin incision approximately 10 mm in length and 7 mm in width on the dorsal surface of the tail.[5] Connective tissue overlying the disc was removed until the outermost surface of the annulus fibrosus was visible. The annulus was incised and the nucleus pulposus was excised with the tip of a #11 scalpel blade and placed onto the exposed L5 DRG immediately. The incision was not taken laterally past the flat surface of the tail, thus avoiding the vasculature, resulting in minimal bleeding. For the animals in the 24-hour experimental group, the spine skin incision was closed with wound clips and the tail skin incision was closed with a 6-0 prolene suture.

Measurement of cytokines in normal nucleus pulposus

In a separate experiment, autologous NP was extracted from a single coccygeal tail disc of multiple rats (n=7) using the same procedure as that described above and placed immediately into a microcentrifuge tube with protease inhibitor and frozen for later analysis. The entire NP was always extracted and placed into a constant volume of solution so that the relative concentration measured between samples was of consistent dilution.

Cytokine analysis

The concentrations of 9 cytokines and chemokines (IFNγ, IL-2, IL-4, IL-6, IL-10, GM-CSF, TNFα, IL-1β and IL-1α) were quantified in samples using a rat 9-plex inflammatory cytokine panel and the BioPlex 200 System (Bio-Rad, Hercules, CA), following the manufacturer's protocol in a 96-well plate format. This assay utilizes antibodies linked to

polystyrene beads containing different levels of fluorophores and has been validated against standard ELISAs.[29]

Statistical analysis

Analysis of variance (ANOVA) with *post-hoc* Bonferroni correction for multiple comparisons was used to compare mean cytokine concentrations across the 3 time points compared to baseline and to make comparisons between treatment groups. This analysis was performed using SPSS 9.0 software (Chicago, USA). A p-value < 0.05 was taken as statistically significant.

Results

Data are reported as mean ± standard error of the mean (SEM).

Normal nucleus pulposus

The concentration of TNFα and IL-1α were measured at 64 ± 13 pg/mL and 2 ± 1 pg/ml, respectively. All other cytokines measured were 0 ± 0 pg/ml in the autologous NP (data not shown).

Epidural dorsal root ganglion lavage

IL-6 expression after NP application to the L5 DRG demonstrated a rapid increase over baseline, peaking at 3hr ($p < 0.001$) and falling back to baseline by 24hr ($p = 1.00$), respectively, and was significantly greater compared to after saline application (Figure 1; $p < 0.005$). Soluble anti-IFNγ co-application prevented the enhancement of IL-6 when compared to saline; therefore, the saline and NP+anti-IFNγ mean results were not statistically different ($p = 1.0$).

IL-1β expression after saline, NP, and NP+anti-IFNγ application increased over baseline with a peak at 6hr (Figure 2). Although IL-1β levels were statistically significantly elevated at all time points compared to baseline ($p < 0.005$), there was no difference between NP ($p = 1.0$) or NP+anti-IFNγ ($p = 0.66$) treatment groups compared to saline.

IL-10 expression peaked at 6hr post saline application and the level of IL-10 was significantly elevated at all time points when compared to baseline in all groups (Figure 3; $p < 0.005$). However, there was no significant difference between the saline and either NP ($p = 0.93$) or NP+anti-IFNγ ($p = 0.68$) groups at any time point.

TNFα levels peaked at 24hr after NP application (Figure 4). Significant differences in expression over time were seen between baseline and 3hr ($p < 0.05$), 6hr ($p < 0.05$) and 24hr ($p < 0.001$). NP application caused a statistically significant increase in expression compared to the saline control group ($p < 0.05$). The co-application of anti-IFNγ prevented NP-induced elevation of TNFα levels, resulting in no statistically significant difference between the NP+anti-IFNγ and saline groups ($p = 0.32$).

The measured concentration of IFNγ was also significantly greater in the NP group compared to the saline group, peaking at 3hr ($p < 0.05$; Figure 5). Time differences of IFNγ concentration were seen between baseline, 3hr and 24hr ($p < 0.005$). There was also a significantly lower concentration of IFNγ after NP+anti-IFNγ compared to NP alone at 24hr; therefore, there was no difference between the saline and NP+anti-IFNγ groups at 24hr ($p = 0.63$).

The concentration of IL-1α was similar after both saline and NP treatment ($p = 1.00$) but was significantly elevated at 24hr ($p < 0.001$) in the NP+anti-IFNγ compared to the saline

group ($p < 0.05$; Figure 6). The measured concentrations were not significantly elevated compared to baseline at 3hr ($p = 1.0$) and 6hr ($p = 0.11$).

Although the concentrations of IL-2 (Figure 7) and IL-4 (Figure 8) increased over baseline (Figure 7) there was no statistically significant difference between groups over time. There was no significant change over time ($p = 1.0$) and no group difference ($p > 0.05$) for the concentration of GM-CSF (data not shown).

Discussion

In the present study the concentrations of several pro-inflammatory (TNFα, IL-1a, IL-1β, IL-6, GM-CSF), anti-inflammatory (IL-10), T-helper-1 (IFNγ, IL-2) and Th-2 (IL-4) cell-derived cytokines were measured over the course of 24 hours. This study provides an animal model to investigate the biochemical mediators by which exposure of NP results in neural root irritation. Prior animal behavioral data demonstrate pro-inflammatory cytokines can initiate or increase allodynia and hyperalgesia,[16,22,30–32] while anti-inflammatory cytokines can act as negative feedback regulators of these cytokines.[16,33] Thus regulation of the balance of pro- and anti-inflammatory mediators could potentially play a role in pain from neural root irritation following disc herniation.[5,14,34]

Nucleus pulposus-induced cytokine expression of IL-6, TNFα, IFNγ, IL-4

The concentrations of IL-6, TNFα, IFNγ and IL-4 were significantly elevated over time after application of NP compared to saline alone. Although there was a marked and significant elevation of IL-1β, IL-10 and IL-2 over time from baseline levels, there was not a significant difference between the saline and NP application groups, indicating that these cytokines are involved in the inflammatory response to surgery but their expression is not further enhanced by the presence of NP. This could represent either a physiologic ceiling effect of the expression of these cytokines or a divergent inflammatory cascade that varies from that which is mediated by NP.

Interleukin-6 is a pro-inflammatory cytokine produced by several cell types, including of the mast- and glial-cell lineages,[35–38] and has been shown to sensitize joint nociceptors[39] and induce hyperalgesia, possibly via the cyclooxygenase-prostaglandin-E2 pathway.[40,41] In the present study the concentration of IL-6 around the L5 DRG was significantly elevated by the surgery but to a significantly greater extent following NP application. This NP-induced enhancement of IL-6 was prevented by co-application of a neutralizing soluble IFNγ antibody, thus providing evidence of an inflammatory pathway that involves IL-6 in an IFNγ-dependent cascade induced by NP.

An NP-induced upregulation of TNFα was also observed in the present study. TNFα is a pro-inflammatory cytokine that plays a critical role in the inflammatory response initiation.[42,43] It is produced by various cell types including Schwann cells after nerve injury,[42,44,45] neutrophils,[46] and infiltrating and resident macrophages during inflammation.[8,47] TNFα may play a role in nociceptor sensitization, allodynia, and hyperalgesia[43,48,49] and a positive correlation between its tissue concentration and neuropathic pain has been observed.[50,51] In addition, there is prior evidence that NP-induced enhancement of spinal cord nociceptive responses may involve TNFα.[5] There was a small measurable concentration of TNFα in the autologous NP. Therefore, the TNFα measured in the epidural lavage may be the result of both an inherent supply from within the NP as well as from the production by inflammatory cells locally or recruited to the region in response to NP.

It has been shown that IL-6 and its receptor may be modulated by both TNFα and IFNγ[52] and there is evidence that IL-6 upregulation is a function of the concentrations of TNFα and IL-1β.[53,54] TNFα has been shown to induce the production of IL-1β, IL-6, and IL-8,[7] and there is evidence that IL-1β and IL-6 may act synergistically.[53] These findings may be in agreement with the results of the present study observation that IFNγ neutralization prevented the NP-induced enhancement of both IL-6 and TNFα. Although the present study did not observe a significant elevation of IL-1β after NP compared to saline, there was a very marked elevation in IL-1β levels over time following the surgery, an observation also consistent with these previous studies demonstrating complex interactions between TNFα, IFNγ, IL-6 and IL-1β.

The concentration of IFNγ after NP application was significantly greater than after saline. IFNγ was undetectable in the autologous NP lavage. Therefore, IFNγ production may be a mediator of an inflammatory response induced by NP exposure. It has been shown that IFNγ is produced by multiple cell types including natural killer cells, macrophages, activated type 1 helper T-cells, astrocytes and damaged neurons,[24–27] and is a key pro-inflammatory cytokine that can sensitize spinal cord dorsal horn neurons,[55–57] possibly via the activation of microglia.[26–28, 58]

Interleukin-4 has been shown to inhibit the expression of pro-inflammatory cytokines IL-1β, TNFα, and IL-6[59,60] as well as the activation of macrophages and microglia – accounting for its antinociceptive properties.[59,61] The concentration of IL-4, an anti-inflammatory cytokine, was significantly greater in the NP group compared to the saline group by ANOVA. However, because the *post-hoc* test did not find a significant time effect it cannot be concluded with confidence that NP induces the elevation of IL-4 over time compared to saline.

Study limitations

The epidural or intradiscal concentration of cytokines reported in the present study should not be taken as absolute values since there was some dilution upon lavage and placement of the sample into the microfuge tube containing protease inhibitor. However, since the volumes of lavage and protease inhibitor were known and were consistent across animals, this should not affect relative concentration comparisons. Theoretically, the dilution could have reduced the levels of the cytokines (except TNFα) to undetectable levels in the normal NP samples. This dilution was unavoidable, however, as any quantitative protein concentration assay requires some volume of fluid.

The amount of neutralizing soluble IFNγ antibody used in this study (50 μl of 0.1 mg/ml) was based upon results of *in vitro* neutralization studies provided by the manufacturer whereby the neutralization dose (ND_{50}) is typically 0.08–0.2 μg/ml of antibody in the presence of 2.5 ng/ml of recombinant rat IFNγ. Because no similar prior *in vivo* study had been performed, it was unknown how much neutralizing antibody would be required to prevent *in vivo* IFNγ activity after saline or NP application.

The study time points of 3, 6 and 24hrs following HNP may appear to be on a much shorter time span compared to the usual clinical presentation of patients with radiculopathy. However, with the use of standard allometric scaling of physiologic parameters one can estimate that 3, 6 and 24 hr time points in the 0.35kg rat are equivalent to as many as 7, 14 and 56 days, respectively, in the 75kg human.[62,63] The time points used in this study may therefore be clinically relevant to the time course of clinically significant HNP-induced radiculopathy.

Conclusions and future studies

The present study has provided an *in vivo* animal model by which to investigate the biochemical changes that occur in the epidural space contiguous with the dorsal root ganglia and nerve root following exposure of nucleus pulposus by disc herniation. Exposure of nucleus pulposus induced a significant elevation of IL-6, TNFα and IFNγ in a manner that is prevented by neutralization of soluble IFNγ. The surgery alone induced a significant elevation of expression of IL-1β, IL-10, IL-1α and IL-2 and NP did not cause further enhanced levels. The level of GM-CSF was unchanged over time following surgery. The animal model presented may be useful for further studies investigating not only the epidural but also the neuronal changes within the nerve root and dorsal root ganglion that occur following disc herniation. Finally, this model may enable the evaluation of various inflammatory modulators that may be developed for potential therapeutics. It will be important to determine the correlation of these findings with pain behavior in future studies.

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Key Points

1. The present study describes a new animal model of non-compressive disc herniation
2. Nucleus pulposus application to the L5 DRG resulted in rapid and marked elevation of epidural IL-6, TNFα, IFNγ and IL-4
3. IFNγ blockade prevented NP-induced elevation of IL-6, TNFα and IFNγ
4. There was a marked elevation of IL-1β and IL-10 in response to surgery that was not further enhanced by NP
5. IL-6, TNFα and IFNγ may interact in an important way in the development of neural root irritation caused by exposure of nucleus pulposus from a disc herniation

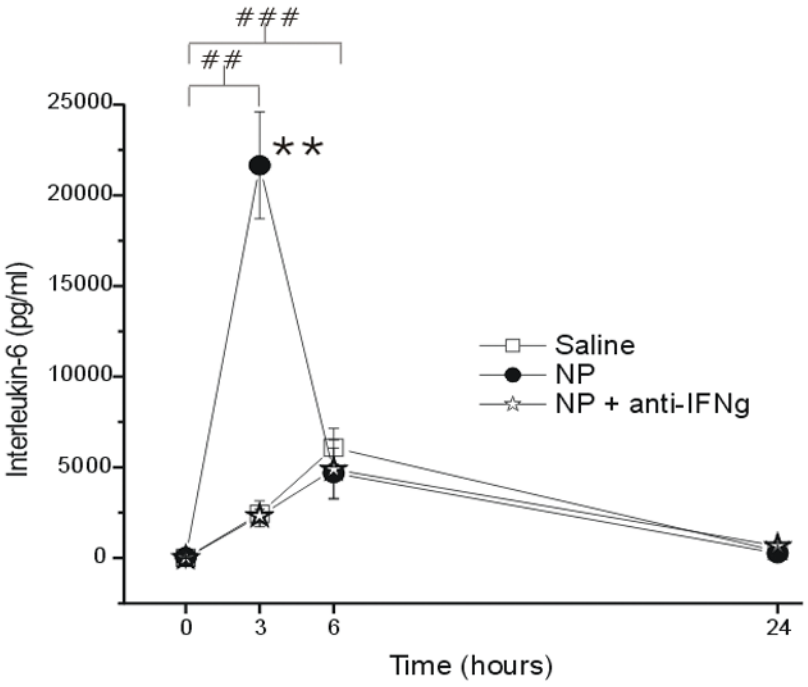

Figure 1.

The concentration of interleukin-6 (IL-6; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). ## $p<0.005$ at T=3hr and T=6hr compared to T=0hr; ** $p<0.001$ between-groups difference between NP and saline groups.

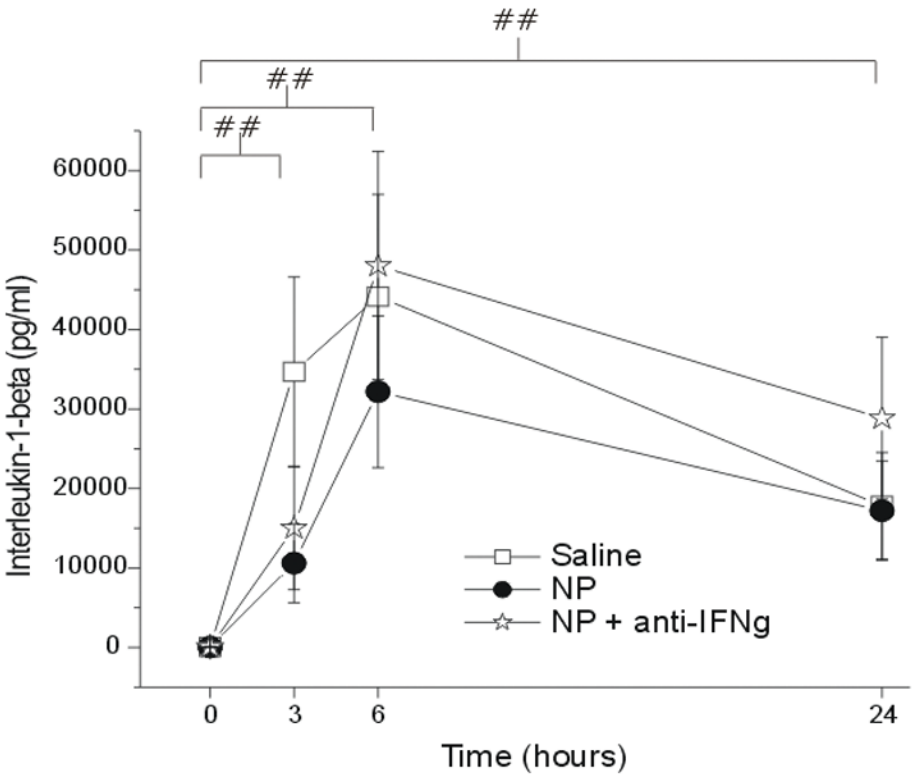

Figure 2.

The concentration of interleukin-1-beta (IL-1β; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). ## $p<0.005$ at T=3, 6, 24hr compared to T=0hr.

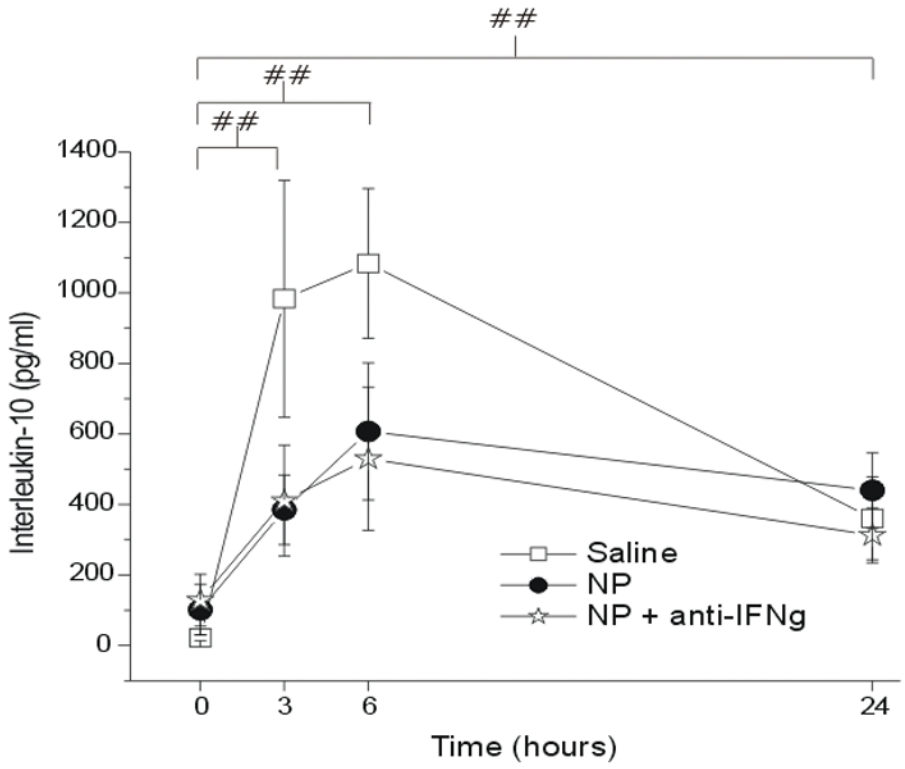

Figure 3.

The concentration of interleukin-10 (IL-10; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). ## $p<0.005$ at T=3, 6, 24hr compared to T=0hr.

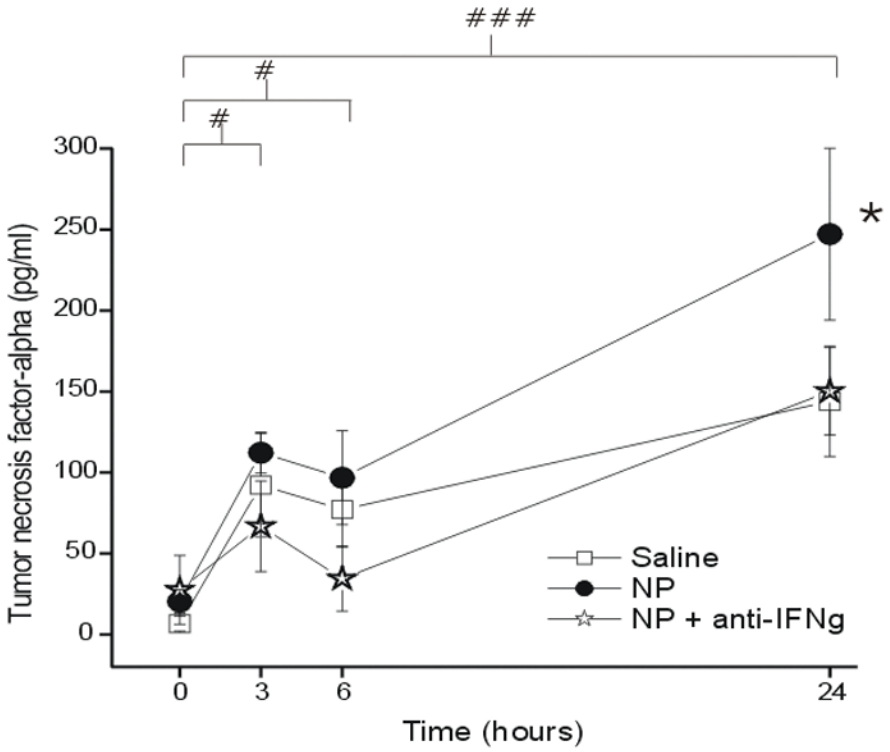

Figure 4.

The concentration of tumor necrosis factor-alpha (TNFα; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). # $p<0.05$ at T=3hr and T=6 compared to baseline; ### $p<0.001$ at T=24hr compared to baseline. * $p<0.05$ between-groups difference between NP and saline groups.

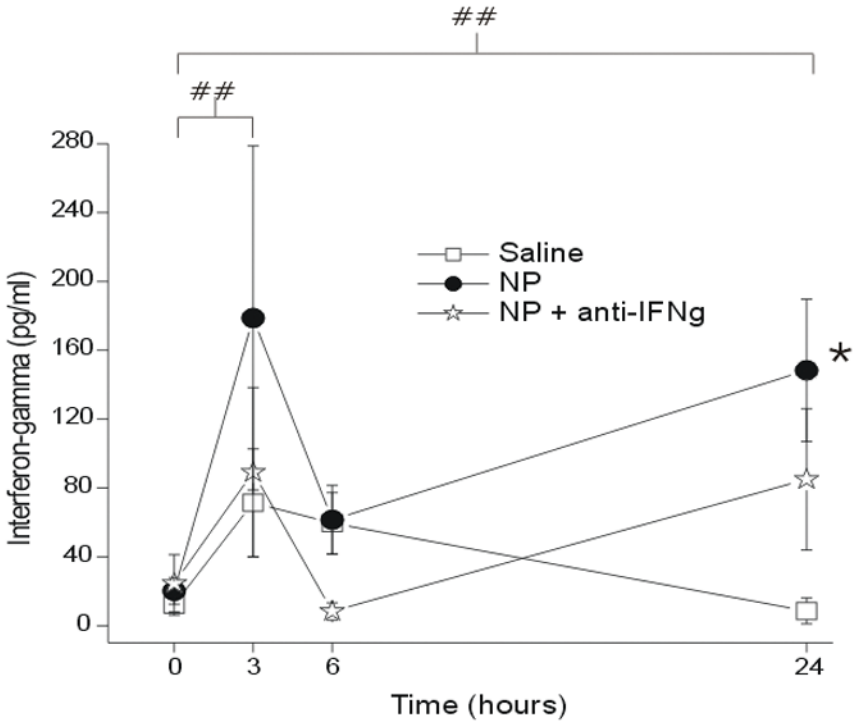

Figure 5.

The concentration of interferon gamma (IFNγ; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). ## $p<0.005$ at T=3, 24hr compared to T=0hr; * $p<0.05$ between-groups difference between NP and saline groups.

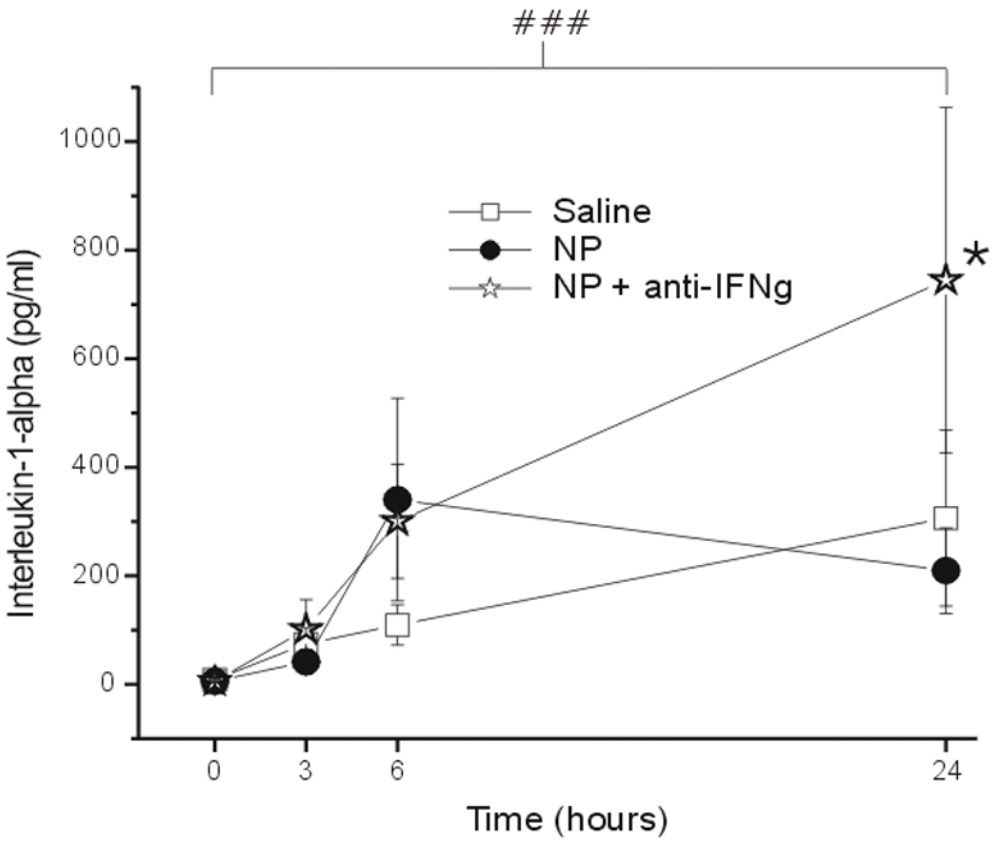

Figure 6.

The concentration of interleukin-1-alpha (IL-1α; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). ### $p<0.001$ at T=24hr compared to T=0hr; * $p<0.05$ between-groups difference between NP+anti-IFNγ and saline group.

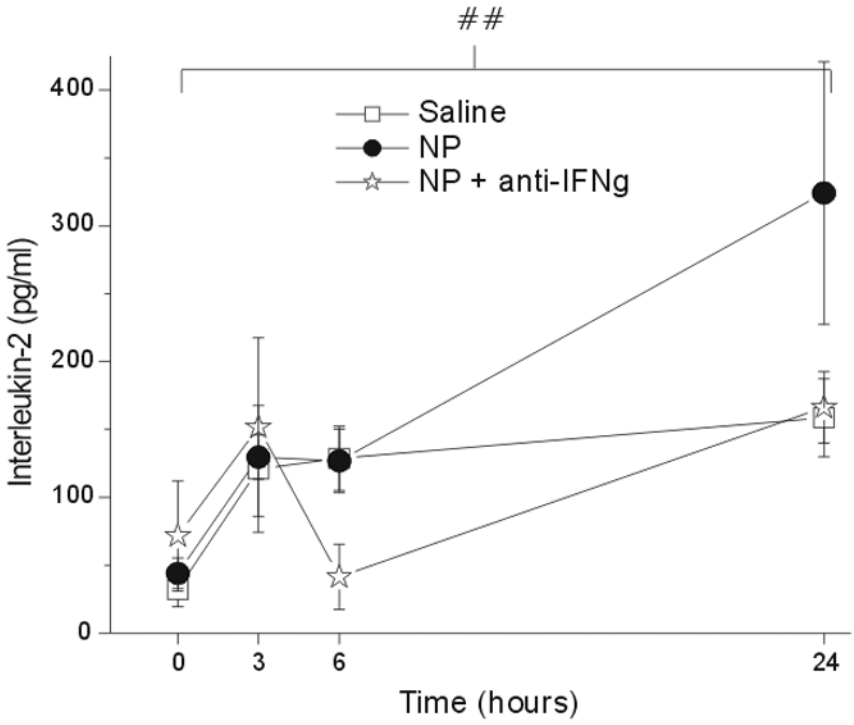

Figure 7.

The concentration of interleukin-2 (IL-2; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). ## $p<0.001$ at T=24hr compared to T=0hr.

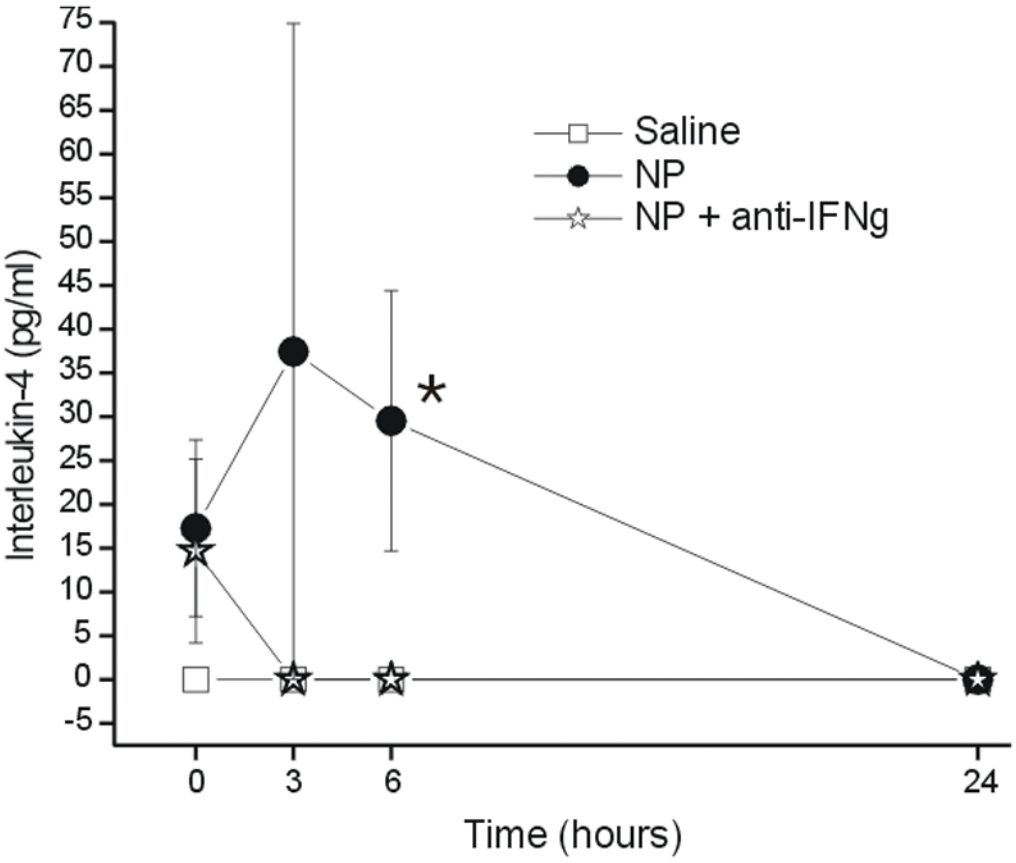

Figure 8.

The concentration of interleukin-4 (IL-4; y-axis, picograms/milliliter) measured in the epidural space overlying the L5 DRG at baseline (Time=0hr) and 3, 6 and 24 hr after the application of saline (N = 15), autologous nucleus pulposus (NP=15) or NP + a neutralizing antibody to interferon-gamma (anti-IFNγ; N=18). * $p<0.05$ between-groups difference between NP and saline groups.