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Nanoparticle fullerol alleviates radiculopathy via NLRP3 inflammasome and neuropeptides

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Abstract

The present study aimed to evaluate the analgesic effect of the antioxidant nanoparticle fullerol in a mouse radiculopathy and a dorsal root ganglion (DRG) culture models. Intervertebral disc degeneration causes significant hyperalgesia and nerve inflammation. Pain sensitization and inflammatory reaction were counteracted by fullerol when disc material was bathed in 10 or 100 μ M of fullerol prior to implantation. Immunohistochemistry showed similar massive IBA1 positive macrophage infiltration surrounding implanted disc material among groups, but IL-1 β and IL-6 expression was decreased in fullerol treated group. In the DRG explant culture, after treatment with TNF- α , the expression of IL-1 β , NLRP3, and caspase 1 was significantly increased but this was reversed by the addition of fullerol. In addition, fullerol also decreased the expression of substance P and CGRP in the cultured DRGs. Nanoparticle fullerol effectively counteracts pain sensitization and the inflammatory cascade caused by disc degeneration.

Graphical abstract

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Conflicts of interest: None

Intervertebral disc material caused a significant hyperalgesia and nerve inflammation, which were counteracted by fullerol administration. Similar massive macrophage infiltration was observed among groups, but IL-1 β and IL-6 expression was decreased in fullerol treated group. In the DRG explant culture, after treatment with TNF- α , the expression of IL-1 β , NLRP3, and caspase 1 was significantly increased but was reversed by the addition of fullerol. In addition, fullerol also decreased the expression of substance P and CGRP in the cultured DRGs.

Keywords

Fullerene; radiculopathy; DRG; inflammasome; hyperalgesia; low back pain

Background

Low back and leg pain is one of the leading causes of disability worldwide. The prevalence of low back pain increased 162% from 1992 to 2006 in the US and globally with estimated expenditures of \$100 billion in the US alone.^{1,2} Intervertebral disc (IVD) degeneration has a strong association with low back pain.³ Herniated disc leads to mechanical compression of nerves and a chemical inflammatory cascade that cause neurologic symptoms or radiculopathy. A wide range of pro-inflammatory mediators, including IL-1, TNF- α , IL-6, IL-8, and other chemokines, have been found within degenerated discs. The levels of these mediators are proportional to the grade of degeneration.^{4,5}

The mainstay of treatment for back pain remains symptomatic care with non-steroidal anti-inflammatory medications (NSAIDs), physical therapy, and pain management. Inflammatory mediators have become appealing therapeutic targets. Epidural steroid injection is a popular procedure performed to alleviate back pain and radiculopathy. Several clinical trials have shown that application of the TNF- α inhibitor etanercept onto the spinal nerve produces short term pain relief.^{6–10} Similarly, epidural injection of the receptor antagonist of LTB₄, a chemotaxis factor for neutrophils, attenuates painful discogenic radiculopathy.¹¹ However, no current therapy has delivered reliable complete symptom alleviation.

Fullerene (also named C₆₀) is composed of 60 carbon atoms that form a hollow sphere approximately 1 nm in diameter. It is characterized as a “radical sponge”, with an anti-oxidative efficacy several hundred-fold higher than other customary antioxidants. Fullerene’s biodistribution depends on surface functionalization. Water-soluble fullerenes were mainly distributed in liver, bone, muscle and skin of mice after 24 hours after

intravenous administration, and gastrointestinal tract following gavage or intraperitoneal injection.¹² Fullerenes have been implicated in treatment of tumor, oxidative stress, inflammation, and neurodegenerative diseases with little toxicity at low doses.^{13,14} In a previous study, we demonstrated that fullerol (the polyhydroxylated, water-soluble, biocompatible form of fullerene) effectively prevented the matrix degradation of nucleus pulposus (NP) cells under either H₂O₂ or IL-1 β stimulation, and that intradiscal injection of fullerol prevented IVD degeneration and increased water and proteoglycan content.¹⁵ In addition, fullerol suppressed the inflammatory responses of dorsal root ganglion (DRG) and prevented neuronal apoptosis by decreasing the level of reactive oxygen species (ROS) and upregulating anti-oxidative enzyme expression.¹⁶ Fullerol has also been shown to significantly suppress the TNF- α -induced production of pro-inflammatory cytokines in synovial fibroblasts, synovial infiltrating lymphocytes and macrophages.^{17,18}

In this study, we evaluated the *in vivo* efficacy of fullerol in alleviating mechanical hyperalgesia in a mouse tail disc implant radiculopathy model and elucidated the molecular mechanisms of its anti-inflammatory effect in an explant DRG culture model. Fullerol inhibits disc herniation induced neuronal inflammation via regulating the NLRP3 inflammasome and suppressing neurotropic peptide release.

Materials and Methods

Animal surgical procedure

The use of animals was approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines for use and care of laboratory animals. C57BL/6 mice (8–10 weeks old, male, 20–25g, Envigo, Indianapolis, IN) were housed in a 12-hour light/dark cycle with free access to food and water. In the first experiment, the animals were randomly divided into two groups: sham and disc implant groups (n=8/group). General anesthesia was induced with intraperitoneal injection of Ketamine/Xylazine (60–80/5–10mg/kg).

Briefly, using aseptic technique and a surgical microscope, the left L4/5 inter-laminar space was exposed. The NP with partial inner annulus fibrosus (AF) tissue was harvested from the coccygeal IVD of the same mouse, and then implanted over the exposed dura at left L4/5 inter-laminar space (Figure 1). In the second experiment, the animals were randomly divided into three groups: disc material bathed in 1) PBS, 2) 10 μ M and 3) 100 μ M fullerol solution (MER, Tucson, AZ) prior to implantation. The spine specimens were harvested at postoperative days 3, 7 and 21 for histological study.

Mechanical Hyperalgesia

The development of mechanical hyperalgesia in mice was assessed in each hind paw using an electronic Von Frey Anesthesiometer (IITC Life science, Woodland Hills, CA).¹⁹ Baseline data were collected on the day prior to the surgery. Each testing day followed an acclimatization to a noise and temperature controlled environment for at least one hour prior to testing. A rigid tip was slowly raised to stimulate the middle of the hind paw and pressure gradually increased until paw withdrawal was observed. Each hind paw was tested 5 times,

and an average computed of the 3 closest values deviating least from the median. A 10–15-minute break was provided between tests. All mice were tested an equal number of times. Testing was performed by a single experienced observer at the same time of day.

Isolation of DRGs

Twenty-one days after implantation, mice were euthanized with CO₂ asphyxiation followed by cervical dislocation. The left L5 DRG were immediately collected from the spinal column, and immersed in Trizol reagent for RNA isolation.

In vitro lumbar DRG explant culture was performed as described previously.²⁰ DRGs were treated with 1) serum free medium (SFM); 2) SFM + 25ng/ml TNF- α ; and 3) SFM + 25ng/ml TNF- α + 1 μ M fullerol. Specimen were harvested at 24 hours for RNA isolation and fixed with 4% paraformaldehyde for immunostaining.

Safranin-O staining

Lumbar discs were fixed with 10% neutral formalin followed by 0.25M EDTA decalcification for 2 weeks. Five μ m thick sections were stained with Safranin-O and fast green for the detection of proteoglycan as described previously.²¹

Immunohistochemistry

Immunostaining was performed as described previously.²² Tissue sections were prepared and stained with antibodies for IL1- β (Santa Cruz Biotechnology, Santa Cruz, CA; 1:100), IL-6 (Abcam, Cambridge, MA; 1:800), Ionized calcium binding adaptor molecule 1 (IBA1, 1:200) and GFAP (Novus Biological, Littleton, CO; 1:5000). Images were captured using a Nikon Eclipse E600 microscope (Nikon, Japan). Sections from 4 mice were evaluated for each group at each time point. Sections omitted primary antibody was used as a negative control.

For the CGRP and substance P staining, explant DRGs were fixed with 4% paraformaldehyde for 30 minutes followed by 30% sucrose overnight. specimens were embedded in OCT medium on dry ice and stored at -70°C . Five μ m sections were stained with antibodies against CGRP (calcitonin gene-related peptide, Sigma, St. Louis, MO, 1:4000) and Substance-P (R&D system, Minneapolis, MN 1:200).

The intensity of staining was quantified using an NIS element BR imaging software. Three serial sections from each of 4 mice were evaluated for each group at each time point. The same color hue settings were used throughout quantification. Several square regions of interest (ROI)s, with a linear length of 144.5 μ m that adequately represented the entire quantifiable cell area, were used and analyzed individually. A previously optimized object count threshold was loaded and maintained throughout the procedure for quantification of intensity. Each ROI per image was calculated and used to determine an average intensity.

Quantitative real-time reverse RT-PCR

RNA was isolated with Trizol reagent, and cDNA were synthesized using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacture's instruction. Gene

expression levels were determined by RT-PCR conducted on a iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA), and using SYBR Green Fluo FAST Mastermix (Qiagen, Valencia, CA). The mRNA expression of target genes was analyzed and normalized to 18S. Data analysis was performed on C_t values according to a modified method.²⁰ Fold changes in gene expression were presented as $2^{-(\text{average } C_t)}$. The C_t of each stimulated sample was related to the respective C_t of each control sample. Primer sequence for nlrp3, 5'-ATTACCCGCCCGAGAAAGG-3' and 5'-TCGCAGCAAAGATCCACACAG-3'; caspase 1: 5'-ACAAGGCACGGGACCTATG-3' and 5'-TCCCAGTCAGTCCTGGAAATG-3'. Other primer sequences were reported in previous study.²⁰

Statistical analysis

Hind paw withdrawal thresholds were analyzed with repeated measures ANOVA using SPSS software (IBM, North Castle, NY). Quantitative data are shown as mean $\pm$ standard error of the mean (SEM). All in vitro studies were performed in triplicate and repeated three times. Inter-group differences were analyzed using one-way ANOVA using Tukey's post hoc test. $p < 0.05$ was considered significant.

Results

Disc material induced mechanical hyperalgesia

The effects of disc material on hypersensitive withdrawal responses were first examined in 2 groups (n=8 per group). Implantation of autologous disc material harvested from the mouse-tail to the left L4/5 inter-lamina space produced pain hypersensitivity. As illustrated in Figure 2A and 2B, a sharp decrease in mechanical withdrawal threshold was observed in the disc material implantation group when compared to the sham operated group. In addition, the development of mechanical hypersensitivity in the experimental group was detected early within the first 3-days post-implantation. The withdrawal thresholds were significantly decreased compared to the pre-surgery baseline remaining persistent throughout the study period (15 days). The withdrawal thresholds decreased about 50% as compared with baseline (n=8, $p < 0.001$) and sham surgery.

Inflammatory response was observed in the disc implantation group

To evaluate whether the decreased withdrawal threshold after disc material implantation was due to neuronal inflammatory reaction, we measured pro-inflammatory cytokine mRNA level in the L5 DRGs with quantitative real-time RT-PCR at week 3 post-operatively. As shown in Figure 2C, both mRNA expression of il-1 β and tnf- α had a marked increase (3.81- and 2.06-fold; n=3; $p < 0.05$) in the implantation group as compared with the sham group, whereas the sod-2 mRNA expression was decreased about 4 fold (implant vs sham, $p < 0.05$) and the levels of il-6 and cox-2 mRNA increased about 1.6 fold as compared with the sham group ($p < 0.05$).

Fullerol alleviates mechanical hyperalgesia

Previous studies and the above results indicate that inflammation plays a role in the hyperalgesia induced by disc herniation. We tested the hypothesis that fullerol alleviates the

pain triggered by ectopic disc material. Prior to implantation, NP materials were bathed in either PBS, 10 μ M or 100 μ M fullerol (n=7). As shown in Figure 3, NP bathed in either concentration of fullerol significantly attenuated the mechanical hind paw withdrawal response bilaterally. A significant decrease in mechanical threshold was observed in the PBS group. There was a decrease in bilateral withdrawal threshold in the 10 μ M fullerol treated group ($p<0.05$, as compare with baseline) on POD2 with a significant improvement noted on POD6 in both contra- and ipsilateral hind paws. The improvement resulted in withdrawal responses similar to the baseline levels recorded on the ipsilateral paws. Interestingly, we noted that 10 μ M of fullerol provided a better analgesic effect on the contralateral paw than 100 μ M fullerol, but had a similar effect on the ipsilateral hind paws. This effect may be caused by irritation of high dose fullerol on the nerve. Actually, the mRNA level of il-1 β and cox-2 in DRGs was markedly increased in mice with 100 μ M fullerol treatment as compared with PBS group on POD21 ($p<0.05$, n=3). In contrast, the cytokine mRNA was decreased in 10 μ M fullerol group but this decrease only met statistical significance for TNF- α (Figure 3C). Thus, we chose the 10 μ M dose for all of our histological analyses.

Massive macrophages were detected around implanted disc material

Spine specimens were fixed and sectioned in the axial plane. Safranin-O staining was performed to detect disc material. As illustrated in Figure 4, disc material was observed over the left inter-laminar space, adjacent to the left dorsal horn of the spinal cord. Red staining indicated implanted disc tissue (green star). Hypertrophic chondrocytes were seen in the NP tissue by POD3. Some implanted tissue started to form bone-like tissue at the local site by POD6 in both groups, and more bone formation were observed on POD 21. Massive cell infiltrate was observed around the implanted tissue (Figure 4A & B) as early as POD3 and abundant cell infiltration by POD6. With immunohistochemistry, we confirmed massive macrophage infiltration (IBA1 positive brown staining, a microglia/macrophage specific protein marker) at the implant site (Figure 5A) surrounding the disc material. No significant difference was shown between groups indicating that disc tissue induced macrophage infiltration.

Fullerol elevated GFAP expression in the spinal cord

GFAP is an important astrocyte and glia cell marker. Although the function of this protein in neuropathic pain is not clear, its expression has been implicated in astrocyte activation.^{23,24} We tested the expression of GFAP in the spinal cord on POD3 and POD6. As shown in Figure 5B, an increase in GFAP staining was observed in the fullerol group, especially on the left side POD3 suggesting activation of astrocytes.

Fullerol attenuated the secretion of pro-inflammatory cytokines

Although there was no significant difference in the number of macrophages at the disc material implantation site, 10 μ M of fullerol was effective in suppressing pro-inflammatory cytokines IL-1 β and IL-6, and was safe with no apparent side effects. As shown in Figure 6 by immunohistochemistry, both IL-1 β and IL-6 expression (brown signal) were decreased at POD3 and POD6 specimens, in which the disc material was bathed in the fullerol solution prior to implantation. No significant positive signal was observed on POD21 (data not shown) for any groups. IgG was used as a negative control.

Fullerol reduced the expression of CGRP and substance P

CGRP and substance P are produced in both peripheral and central neurons. These neurotransmitters transmit pain signals. We hypothesized that fullerol attenuates pain via inhibiting the release of these neuropeptides. An increase of these proteins was observed in the DRGs treated with TNF- α , but this elevation was diminished by 1 μ M of fullerol, as shown in Figure 7A. Quantitative data was shown in Figure 7B.

Fullerol decreased inflammasome activation in explant DRG culture model

Previous and current data demonstrated that fullerol has anti-inflammatory effects on the inflamed DRG organ culture (Figure 5).²⁰ TNF- α treatment markedly increased the expression of IL-1 β secretion, which was effectively suppressed by administering fullerol for 24 hours. Since the inflammasome plays an important role in IL-1 β maturation, we tested the expression of NLRP3 and caspase 1 in this model. As shown in Figure 8, both NLRP3 and caspase 1 expression were increased by the TNF- α stimulation and this was inhibited by fullerol.

Discussion

Low back and leg pain is one of the most common conditions for clinical visits, with a high prevalence of 60–90% in one's lifetime. Current treatment is focused on alleviation of symptoms. Epidural steroid and TNF- α inhibitor injections are used to treat back and leg pain because of their anti-inflammatory properties. Fullerene and derivatives not only have anti-inflammatory effects, but also have potent anti-oxidative properties due to their “free radical sponge” capacities. As potential therapeutic agents for back pain, fullerenes are superior to growth factors, cytokines and enzymes, due to long-lasting activity and excellent cell membrane-penetration. In addition, fullerenes being biologically silent have minimum possible undesired side effects.^{13,17} We have previously shown that the water-soluble fullerene, fullerol has a protective effect by inhibiting neuroinflammation and cellular apoptosis in a neuron culture and a DRG explant models.²⁰ In this study, we extended *in vitro* findings to an *in vivo* radiculopathy model and evaluate analgesic effects and possible underlying molecular mechanisms of fullerol therapy.

A young and healthy disc is an aneural and avascular organ. NP tissue is located at the center of the IVD and is surrounded by concentric sheets of lamellar AF. The normal NP is immune privileged. In disc herniation, the herniated tissue mechanically impinges on the nerve root and chemically induces inflammation, causing neurologic symptoms. TNF- α , IL-1 β , IL-6, IL-17, and IL-8 have been found in degenerated discs.^{25–28} These pro-inflammatory cytokines induce a positive feedback loop and perpetuate an inflammatory environment by activating lymphocytes, macrophages and phagocytosis. The level of inflammatory mediator correlations with the severity of degeneration.^{29–31}

Animal studies from different laboratories have reported that epidural application of NP tissue causes inflammation and hypersensitivity of DRGs in rats.^{32–35} In our study, we created a mouse model of autologous tail disc NP implantation with some modifications. Since mouse NP is tiny and very soft, we included a small portion of inner AF with the NP.

In order to reduce surgical trauma to the DRG or nerve root, we did not perform hemilateral laminectomy to expose the DRG, which was common in the rat model. Instead, we exposed the left L4/5 interlaminar space and inserted tail disc tissue in the interlaminar space. As expected, reliable and reproducible mechanical hyperalgesia was observed, accompanied by abundant inflammatory cell infiltration and pro-inflammatory cytokine expression such as IL-1 β and IL-6 (Figure 6) on histological analysis. This validated the feasibility of autologous NP implantation in a mouse model, which is more economical and amenable to genetic manipulation for discogenic radiculopathy research.

Fullerene is a potent anti-oxidative and anti-inflammatory nanoparticle, which represents the third allotrope of carbon. It is a rising star in biomedicine. Numerous studies have reported their radical scavenging activities and anti-inflammatory properties via reduction of ROS levels and regulation of the NF- κ B signaling pathways.³⁵ It has been reported to ameliorate inflammatory responses in arthritis and disc herniation models.^{14,17,18} In the current radiculopathy model, fullerol diminished the release of inflammatory mediators (Figure 6) when disc NPs were bathed in fullerol solution prior to implantation. The fullerol treated group showed much less positive staining for IL-1 β and IL-6 expression as compared with PBS control (Figure 6). In isolated DRGs on POD21, mRNA levels of inflammatory cytokine showed a downward trend in the 10 μ M fullerol group, compared with PBS group. However, 100 μ M fullerol increased mRNA expression of il-1 β and cox-2, indicating a possible effect of high dose fullerol cytotoxicity (Figure 3). Interestingly, both PBS and 10 μ M of fullerol bathed tissue induced similar macrophage infiltration (Figure 4&5) but the mechanical hyperalgesia was significantly alleviated in the 10 μ M fullerol group as compared with the PBS control. Tang et al reported that fullerene participates in regulating the host immune system. They reported that fullerols as well as gadolinium-fullerols activated macrophages phagocytosis and cell viabilities.³⁶

Radiculopathy may be the result of a focal inflammatory reaction to exposed immune-privileged nucleus material irritating the nerve root. Increased release of pro-inflammatory mediators, including IL-1 β and inflammasomes in primary sensory neurons may cause this pain hypersensitivity.³⁷ The secretion of mature IL-1 β requires the processing of its precursor form by a cysteine protease, caspase-1, which is activated by caspase-1-containing multi-protein complex called inflammasomes.³⁸ The NLRP3 inflammasome is strongly associated with sterile inflammation. NLRP3 can activate caspase-1 to process pro-IL1- β into its mature biologically active form.^{39,40} We suspect that inflammasome may be one of the targets of fullerene's anti-inflammatory effect. As shown in Figure 8, the expression of NLRP3 and caspase 1 was increased in TNF- α treated explant DRGs, and fullerol reversed this increase along with a reduction in IL-1 β expression. Another possible explanation is that fullerol functions as a TNF- α inhibitor. A recent computational study indicated that fullerenes and metallofullerenes reside in the same pocket of the TNF- α dimer as a known TNF- α inhibitor SPD304a which would allow them to inhibit TNF- α biological activities. Fullerene derivatives have a larger affinity for this site than the known inhibitor.⁴¹ Due to its high safety and biocompatibility, fullerene or its derivatives have been used as carriers for tumor-specific drug delivery to treat cancer.^{42,43}

Peripheral inflammatory pain transduction is dependent on intra-neuronal processes within the spinal cord and DRG. Neurotrophic factors and substances involved in the pathophysiology of neuropathic pain include NGF, BDNF, substance P, and other chemokines.⁴⁴ Astrocytes and activated microglia play a role in nociceptive hypersensitivity by releasing modulators such as substance P, CGRP, and pro-inflammatory cytokines.^{28,45} In the current study, GFAP immunostaining positive signals were elevated in the fullerol treated group. This suggests fullerol mediation of astrocyte activation. To avoid confounding factors of in vivo context, lumbar DRGs was explanted and simulated with TNF- α to model the inflammatory condition. As shown in figure 8, fullerol markedly reversed the expression of CGRP and substance P in these DRGs. Both CGRP and substance P function as neurotransmitters in pain signal transduction. Elucidating the molecular mechanism of fullerol's downregulation of neuropeptide warrants further investigation.

Limitations of our study include a limited observation period of 3 weeks follow up in the mouse model. A longitudinal investigation should be conducted to monitor outcomes of long term pathology. Secondly, a large animal model should be implemented to better elucidate the molecular crosstalk between disc material and the inflammasome cascade using TNF- α inhibitors or agonists as positive or negative controls, respectively, which is the focus of our ongoing research. Future studies should strive to elucidate the inhibitory effect of fullerene on inflammasome and neurotransmitter modulation with various doses and routes of administration. Finally, further research is needed to elucidate the molecular mechanisms of inflammation-induced hyper nociceptive response secondary to disc herniation.

In summary, we present a modified mouse model of radiculopathy. We demonstrated that nanoparticle fullerol suppressed mechanical hyperalgesia by suppressing pro-inflammatory cytokines possibly by inhibiting inflammasome initiation and formation, or by inhibiting substance P and CGRP neurotransmission. This study lays the foundation for translational exploration of fullerene as a potential candidate for therapeutic development in the treatment of discogenic radiculopathy and neuropathic pain.

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Figure 1. Implantation of mouse tail disc material into left L4/5 inter-laminar space

The laminar space was exposed in the L4/5 spinal process. NP tissue with a small part of AF was excised from the coccygeal 9/10 disc, and then implanted in the inter-laminar space. The asterisk indicates exposed dura, and the small green arrows point to dorsal root ganglion (DRG). The implanted disc material is shown proximal to the dura and DRGs.

Figure 2. Implantation of disc material induced significant mechanical hypersensitivity and inflammation

Paw withdrawal thresholds were recorded prior to surgery followed by every other day up to 15 days post-operatively (A. Right paw, B. Left paw). A significant decline in threshold was noted on day 3, and remained a steady state thereafter ($n=8$, $p<0.01$). The thresholds in the sham surgery animals revealed no change compared to the baseline data. Symmetrical decline in withdrawal threshold in contralateral and ipsilateral paws in the implantation animals indicated the central nature of pain ($p>0.05$). C. Increased pro-inflammatory mRNA in the DRGs of disc implanted group. The ipsilateral DRGs were freshly isolated on POD21. The expression of target mRNA was measured with quantitative real-time PCR and normalized to 18s ($n=3$). The expression of pro-inflammatory cytokines IL-1 β , TNF- α , IL-6, and cox2 was significantly upregulated by disc material implantation (*, $p<0.05$).

while anti-oxidative enzyme superoxide dismutase 2 (SOD2) was significantly decreased compared to sham operative animals (* $p<0.05$).

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Figure 3. Fullerol alleviated mechanical hypersensitivity induced by implanted disc tissue
Paw withdrawal thresholds were recorded prior to surgery and every other day up to 21 days post-operatively (A. Right paws. B. left paws). Fullerol exhibited a profound alleviation of mechanical hypersensitivity. Ten μ M fullerol caused a significant alleviation of mechanical hyperalgesia back to baseline on POD6 (* p <0.01, n =7). Fullerol at 100 μ M showed alleviation on POD2 (# p <0.05). There is no significant difference between 10 and 100 μ M (p >0.05). C. Decreased pro-inflammatory cytokine mRNA in DRGs of 10 μ M fullerol treated group but not in 100 μ M fullerol group (*, p <0.05) as compared with PBS group on POD21.

Figure 4. Safranin-O staining showing implanted disc tissue and massive cell infiltration
 Safranin-O staining was performed on specimens harvested on POD3 and 6. Disc tissue was observed in the laminar space at both time points. Massive cell infiltration was observed on POD3 surrounding the disc tissue. Green stars indicate transplanted disc material. A, low magnification. B, high magnification of implanted disc area.

Figure 5.

A, Immunohistochemistry confirmed macrophage infiltration surrounding the disc material. Tissue sections at 5μm thick were subjected to immunostaining. Macrophages were detected by IBA1 antibody (macrophages/microglial) followed by biotinylated rabbit IgG. Brown color indicated IBA1 positive signal. No difference in IBA1 signal was observed between fullerol and PBS groups. **B, Immunohistochemistry showing astrocytes in the spinal cord.** Fullerol increased the expression of GFAP in the spinal cord as detected by GFAP specific antibody indicating activation of astrocytes. Bar graphs show quantification of positive signal intensity (*, $p < 0.05$).

Figure 6. Immunohistochemistry showing IL-1 β and IL-6 expression at disc implantation site Five μ m sections were immunostained with antibodies specific to IL-1 β (A) and IL-6 (B) followed by biotinylated IgG. The same process without primary antibody was used as a IgG control. The signal was visualized with DAB. Nucleus was counterstained with Hematoxylin. Images were captured with a Nikon Eclipse E600 microscope. Brown color indicated positive signal. Fullerol suppressed the inflammatory cytokine expression induced by disc material. Bar graphs show quantification of positive (brown) signal intensity (*, $p < 0.05$).

Figure 7. Fullerol inhibits the release of neuropeptide in a DRG organ culture

A, fullerol inhibited neurotransmitter substance P and CGRP release in DRGs. Freshly isolated DRGs were cultured with growth medium supplemented with NGF 10ng/ml for 24 hours, and then treated with TNF- α at 25 ng/ml with or without fullerol (1 μ M) for another 24 hours. The DRGs were fixed for frozen section. Tissue sections at 5 μ m were incubated with substance P and CGRP antibodies followed by anti-mouse biotinylated IgG. The signal was visualized with DAB. Brown color indicated a positive signal. TNF- α enhanced the

expression of CGRP and substance P, and this was reversed by fullerol. Scale bar=100 μ m. B, quantification of positive signals in A.

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Figure 8. Fullerol suppressed pro-inflammatory cytokine expression via inflammasome
 After treatment for 24 hours, DRGs were harvested for RNA isolation. The expression of IL-1 β , caspase 1, and NLRP3 was measured with real-time PCR and normalized to 18s. $TNF-\alpha$ stimulated the inflammasome complex, and this effect was blocked by fullerol (n=3, p<0.05). This implied that fullerol may suppress inflammation via inflammasome. * $TNF-\alpha$ vs control, # $TNF-\alpha$ vs $TNF-\alpha$ + fullerol.