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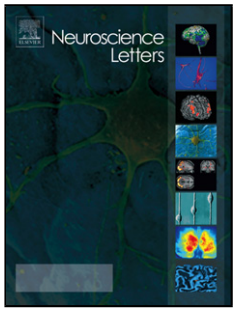

Anti-nociceptive and anti-inflammatory effects of sulforaphane on sciatic endometriosis in a rat model

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Anti-nociceptive and anti-inflammatory effects of sulforaphane on sciatic endometriosis in a rat model

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Running title: Anti-nociceptive and anti-inflammatory effects of sulforaphane

Highlight

- SFN alleviated pain by sciatic endometriosis evidenced by the increase in paw withdrawal threshold and paw withdrawal latency.
- IL6, IL-1β and TNF-α levels were decreased by SFN.
- SFN induced DOX2 and INOS suppression and Keap1 and Nrf2 upregulation.

Abstract

Endometriosis of sciatic nerve is a common gynecological disease. Here we aimed to study the anti-inflammatory and anti-nociceptive role of sulforaphane on sciatic nerve endometriosis. The sciatic nerve endometriosis rat model was constructed by autologous implantation of uterine tissue. Sulforaphane was administered intraperitoneally at the dose of 5, 15, 30 and 60 mg/kg/day for 28 days. Behavioral testing was performed at day 7, 14, 21 and 28. At day 28, rats were sacrificed, followed by collecting superficial dorsal horn tissues and lesions. Quantitative real-time PCR and Western blot were performed to assess COX2, Keap1, Nrf2 expression in collected tissues. Enzyme-linked immunosorbent assay was conducted to assess the expression of pro-inflammatory cytokines. Sulforaphane alleviated pain of sciatic endometriosis as evidenced by the increase in paw withdrawal threshold and paw withdrawal latency. Sulforaphane also inhibited ectopic endometrial tissue growth in sciatic endometriosis rat, shown as the shrinkage of lesion size and decreased VEGF levels. IL6, IL-1β and TNF-α levels were decreased by sulforaphane. Sulforaphane induced DOX2 and INOS suppression and Keap1 and Nrf2 upregulation. Sulforaphane alleviates pain induced by sciatic endometriosis, which is mediated by inhibiting inflammation.

Keywords: sciatic nerve endometriosis; sulforaphane; inflammation; Keap1; Nrf2

Introduction

Endometriosis, which is a common gynecological disease affecting 5%-10% of women of fertile age, causes chronic pain. The frequent sites of ectopic glandular or stromal cells accumulation include peritoneum, the pelvic, the rectovaginal septum, ovaries, the pleura, pericardium, and even the brain. Although rare, endometriosis of the sciatic nerve, e.g. around the sciatic nerve, near the sciatic notch, and under the nerve sheath, is difficult to treat. Inflammation is the major cause of sciatic nerve endometriosis [4, 14]. Therefore, except for surgically removing the ectopic lesions, strategies to suppress inflammation are mainly used for treatment of sciatic nerve endometriosis. However, these therapies are limited by pain relapse and side effects shortly after the cessation of the therapy. There is an unmet need to develop robust preclinical models, which recapitulate the human pain perception to test new therapies on these models to achieve a better therapeutic outcome.

Sulforaphane is an isothiocyanate naturally occurring in the cruciferous vegetables. The anti-diabetic, anti-cancer and anti-microbial efficacies of sulforaphane have been demonstrated. Recently, sulforaphane was also found to exert potent anti-inflammatory effects. For example, sulforaphane was shown to suppress bacterial lipopolysaccharide (LPS)-induced inflammation, characterized by elevated expression of TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), and COX-2, therefore exerting neuroprotective effects [3]. Sulforaphane also protects against renal fibrosis and ischemia-reperfusion injury through inflammation modulation [17, 20]. Additionally, another significant application of sulforaphane is to alleviate pain [5, 13, 16, 19]. However, despite the potential of sulforaphane in reducing inflammation and pain, the use of sulforaphane in clinical management of sciatic nerve endometriosis has not been demonstrated.

An important mechanism of the action of sulforaphane is the activation of Keap1-Nrf2 pathway [10, 11, 17, 20], and tremendous evidences have suggested that sulforaphane exerts anti-cancer activities through the Keap1-Nrf2 pathway. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a common target for anti-inflammatory strategies and it plays a critical role in cellular defense against electrophilic insults and oxidative stress [1]. Kelch-like erythroid cell-derived protein with CNC homology (ECH)-associated protein 1 (Keap1), which is a partner of Nrf2, participates in the attenuation of inflammation-associated pathogenesis [8].

Herein, we aimed to evaluate the efficacy of sulforaphane in alleviating pain of sciatic nerve endometriosis. A rat sciatic nerve endometriosis model was used based on implanting autologous uterine tissue at the sciatic nerve. Behavioral tests, as well as assessments of lesion growth, inflammatory factors expression were performed. Keap1-Nrf2 expression was also evaluated to verify the mechanism of sulforaphane in treating sciatic nerve endometriosis. Our data could provide evidences on the potential of sulforaphane in the therapy against sciatic nerve endometriosis.

Materials and methods

Animals

The animal study was approved by the Ethics Committee of Shandong University, Qilu Hospital. Adult female Sprague–Dawley rats, with the weight of 180-220 g and the age of 14–18 weeks were acquired from Beijing VitalRiver Biotech Company. The rats were kept in individual cages in a room with 12 h light: 12 h dark cycle, 21–23ºC, and 65%–70% humidity. Rats were allowed free access to food and water.

Rat sciatic nerve endometriosis model

The method for rat sciatic nerve endometriosis was conducted as previous reported [4]. Rats were anesthetized using isoflurane and a 2-cm incision was made in the abdomen, after which the right uterine horn was exposed. A piece of uterus with the length of 5 mm was dissected from the uterine horn and immersed in phosphate saline. After positioning the rats on the left side, a 1-cm long incision along the thigh bone was made at the right sciatic nerve. The collected uterine tissue was longitudinally opened and wrapped around the nerve. Sham operated rats received exposure of the right sciatic nerve but received no implantation. The incisions were closed using 6.0 silk and gentamicin was injected into to prevent infection. Sulforaphane dissolved in phosphate-buffered saline (PBS) at the dose of 5, 15, 30 and 60 mg/kg/day were intraperitoneal injected for 28 days. For the vehicle control, PBS was injected.

Behavioral testing

The Von Frey filament test [12] was used to measure the force sufficient to make a rat retract its paw away from a stimulus, defined as paw withdrawal threshold (PWT), as a response to mechanical stimulus. Before testing, rats were positioned on a wide-gauge, wire-mesh surface with over 15 min of habituation. The thermal hyperalgesia test [18] was also performed. A radiant heat using a high-intensity lamp bulb (Osram 58-8007, 8 V, 50 W) was applied to rat's hind paw to determine thermal hyperalgesia. paw withdrawal latency (PWL) was defined as the latency in the paw withdrawal response to the heat source. To avoid burning the skin, the maximal time of exposure was 20 s. Each test was repeated three times. At 4 days prior to inducing sciatic endometriosis, baseline threshold measurements were acquired. Behavioral tests were performed at day 7, 14, 21 and 28. At day 28, rats were sacrificed, and superficial dorsal horn tissues and lesions were acquired.

Western blot

Western blot was performed to examine protein expressions of VEGF, COX2, iNOS, Keap1 and Nrf2. The collected tissues were homogenized and centrifuged. The supernatant was loaded in SDS-PAGE gel followed by transferring to a PVDF membrane. After blocking with 4% fat milk, primary antibodies against VEGF, COX2, iNOS, Keap1 and Nrf2 were added and incubated the membrane for 1 h. All primary antibodies were acquired from the Abcam (Cambridge, UK). Then, the membranes were washed with TBST (Tris-buffered saline with 0.1% Tween 20) and the secondary goat-anti-rabbit was used to incubate the membrane for 1 h at room temperature. ECL Plus was then added and the intensity of protein bands were analyzed using Image J software. The value of beta-actin was used to normalize protein expression.

Quantitative real-time PCR

Total RNA was extracted from the collected tissues using Trizol agent (Invitrogen, Carlsbad, CA, USA). qRT-PCR was performed in a PRISM 7500 Real-Time PCR System (ABI, Carlsbad, California, USA) using a SYBR Green kit (Takara, Otsu, Shiga, Japan). Beta-actin was used as the house keeping gene. Quantification of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method. The primers used in the present study are listed in Table S1.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to examine the levels of VEGF, IL6, IL-1β and TNF-α in the superficial dorsal horn using the manufacturer's recommendations (Abcam).

Statistical analysis

Data are presented as mean ± standard deviation (SD). One- or two-way analysis of variance (ANOVA) followed by Tukey's post hoc test were used for analyzing the

differences. Results with $p < 0.05$ were considered significant. All statistical analyses were performed in GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA).

Results

Anti-nociceptive effects of sulforaphane on sciatic endometriosis induces mechanical hypersensitivity and thermal hyperalgesia

We constructed the rat sciatic nerve endometriosis model by implantation of uterus tissue (named ENDO rats) and the Sham group received surgery but no implantation. We first evaluated whether sulforaphane exerted anti-nociceptive effects on sciatic endometriosis induced mechanical hypersensitivity and thermal hyperalgesia. The toxicity profile of sulforaphane was tested by behavioral tests in Sham operated rats (Figure S1A&B) and dose-dependent therapeutic effects of sulforaphane were examined, which showed that 30 and 60 mg/kg sulforaphane exerted comparable effects (Figure S2A&B). We therefore used 30 mg/kg sulforaphane for the following therapy. Rats were injected with 30 mg/kg sulforaphane daily from 3 days prior to surgery and until 4 weeks after surgery. Two behavioral tests, Von Frey filament test (Figure 1A) and thermal hyperalgesia test (Figure 1B) were used. Our result indicated that ENDO rats demonstrated dramatically decreased PWT and PWL ($p<0.001$, $n=6$), while sulforaphane treatment significantly improved PWT and PWL ($p<0.01$, $n=6$), suggesting alleviated pain.

Sulforaphane inhibits ectopic endometrial tissue growth in sciatic endometriosis rats

To further examine the effects of sulforaphane in sciatic endometriosis, we compared the volume of lesion in endometrium with and without sulforaphane treatment. As shown in Figure 2A, ENDO rats with sulforaphane treatment had prominently lower lesion volume

than those without treatment ($p<0.001$). Due to the key role of VEGF in endometriosis, we also compared the serum and tissue VEGF levels in different groups. As shown in Figure 2B-E, the upregulation of serum and lesion tissue VEGF levels were markedly alleviated by sulforaphane treatment, as revealed by ELISA assay (Figure 2B-C) and Western blot (Figure 2D-E).

Anti-inflammatory effects of sulforaphane on sciatic endometriosis

Since inflammation is one of the most important factors driving sciatic endometriosis, we assessed whether sulforaphane treatment reduced inflammation in superficial dorsal horn tissues. ELISA assay (Figure 3A-C) and qRT-PCR (Figure 3D-E) were used to analyze tissue IL-6, IL-1β and TNF-α levels. Expectedly, compared to the Sham rats, ENDO rat demonstrated significantly higher levels of IL-6, IL-1β and TNF-α, which were partially decreased by sulforaphane treatment. This evidence indicated that sulforaphane is capable of suppressing inflammation in endometriosis rats.

To further validate the anti-inflammatory role of sulforaphane in endometriosis, we quantified COX2 and iNOS levels in the superficial dorsal horn tissues after 4 weeks of sulforaphane treatment. We observed that while EDNO rats showed higher COX2 and iNOS levels, in agreement to the elevated inflammation, sulforaphane effectively inhibited COX2 and iNOS upregulation, as revealed by both qRT-PCR (Figure 4A-B) and Western blot (Figure 4C-E).

Sulforaphane activates Keap1-Nrf2 signaling

To understand the mechanism of sulforaphane in treating sciatic nerve endometriosis, the status of Keep1-Nrf2 signaling was analyzed and our results indicated that both Keap1 and Nrf2 were activated under sulforaphane treatment (Figure 5A-C), restoring the normal levels of both proteins. Since Keap1 and Nrf2 are crucial players of inflammation

inhibition, this data demonstrated the anti-inflammatory effects of sulforaphane.

Discussions

While inflammation and chronic pain are two major symptoms of sciatic nerve endometriosis, current treatments have yet to efficiently attenuate these symptoms. In addition, current drugs, such as non-steroidal anti-inflammatory drugs, opioid drugs and hormone drugs, are of considerable side effects, limiting their application in the disease [6]. The use of dietary compounds, such as sulforaphane reported in the present study, for pain and inflammation attenuation is highly desirable. Emerging studies have developed dietary compounds for the treatment of endometriosis [9]. Our data have suggested that sulforaphane exerts anti-nociceptive effects in a rat model of sciatic nerve endometriosis, as revealed by the Von Frey filament and thermal hyperalgesia tests. In this model, rats with sciatic nerve endometriosis had markedly lower PWT and PWL than those in the sham group and sulforaphane treatment successfully improved PWT and PWL. This finding is in line with previous reports on the use of sulforaphane in managing chronic neuropathic pain. Here we used a dose of 30 mg/kg/day, which is also a dose used by previous studies, and our results suggested that the dose of 60 mg/kg/day exerted comparable anti-nociceptive effects in this model. Meanwhile, sulforaphane was shown to reduce endometriosis lesion size in ENDO rats, which concurred with a decrease in the serum and tissue VEGF levels. Endometriosis lesion is mainly comprised of endometriotic epithelial and stromal cells, the growth of which has been found to be stimulated by inflammatory factors and pain [14]. Hence, the reduction of inflammation and pain presumably contributes to shrinkage of lesions. Sulforaphane also attenuates neuro-angiogenesis [2], which is an important pathogenic factor in endometriosis. The

decrease in VEGF, a key factor in angiogenesis, in both serum and tissue levels implicates the reduction of neuro-angiogenesis. Such two effects synergistically contribute to the shrinkage of lesion size, which further unveils the ameliorating effects of sulforaphane in this disease.

The anti-inflammatory effects of sulforaphane was demonstrated by analyzing levels of pro-inflammatory cytokines, including IL-6, IL-1β and TNF-α. We observed a dramatic upregulation of pro-inflammatory cytokines in ENDO rats, which is one of the characteristics of endometriosis models, and sulforaphane prominently downregulated levels of these cytokines. Here, the tissue we studied was the superficial dorsal horn, which is the first synaptic site between the central nervous system and the peripheral afferent nerves, and it plays a critical role in regulating pain. In addition, the levels of iNOS and COX2, which are two markers of inflammation [15], were evaluated, and a reduction of iNOS and COX2 concurred with the downregulation of pro-inflammatory cytokines. Our data are consistent with previous reports showing that sulforaphane attenuated inflammation in superficial dorsal horn [3, 7, 20], which supports the effects of sulforaphane in alleviating pain. Further, this data are also consistent with previous reports on the use of sulforaphane as an anti-inflammatory drug, a desirable property that has been exploited for the management of cancer, sepsis, and ischemia-reperfusion injury [3, 7, 20].

As a mechanistic study, we investigated the effect of sulforaphane in activating the Keap1-Nrf2 pathway. We documented a significant decrease of Keap1 and Nrf2 levels at the superficial dorsal horn in ENDO rats, in agreement with elevated inflammation in the tissue. Treatment with sulforaphane restored the Keap1 and Nrf2 levels, in line with the activated cell defense against inflammation and pain. Nrf2 is an important target in

regulating inflammation and endometriosis, as well as in treating cancer, neuropathic pain and inflammation. The effects of sulforaphane are mainly mediated through the Keap1-Nrf2 signaling [10, 11, 17, 20].

Conclusions

In summary, here we have constructed a sciatic nerve endometriosis model in rats and evaluated the therapeutic effects of sulforaphane. We have showed that sulforaphane successfully alleviated pain and inflammation, reducing lesion size. The effects of sulforaphane are mediated by activation of Keap1-Nrf2 signaling. Our study supports further development of sulforaphane as a drug to treat sciatic nerve endometriosis without considerable side effects.

Credit author statement

Yan Liu: Data curation, Writing, Original draft preparation. Zhiwei Zhang: Data curation. Xiaofen Lu: Data curation. Jian Meng: Data curation. Xuying Qin: Data curation. Jie Zhang: Conceptualization, Supervision, Writing.

Conflict of interest

None.

Funding

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Figure legends

Figure 1. Anti-nociceptive effects of sulforaphane (SFN) on sciatic endometriosis induced mechanical hypersensitivity and thermal hyperalgesia. 30 mg/kg per day sulforaphane was administrated to sciatic endometriosis rats from 3 days pre-surgery and lasted until 4 weeks post-surgery. Von Frey filament test (A) and thermal hyperalgesia test (B) were carried out. Data are presented as mean ± SD. N=6 for each group. ***p < 0.001 compared to sham, ##p < 0.01 compared to ENDO group. Two-way ANOVA analysis followed by a Tukey's post hoc test.

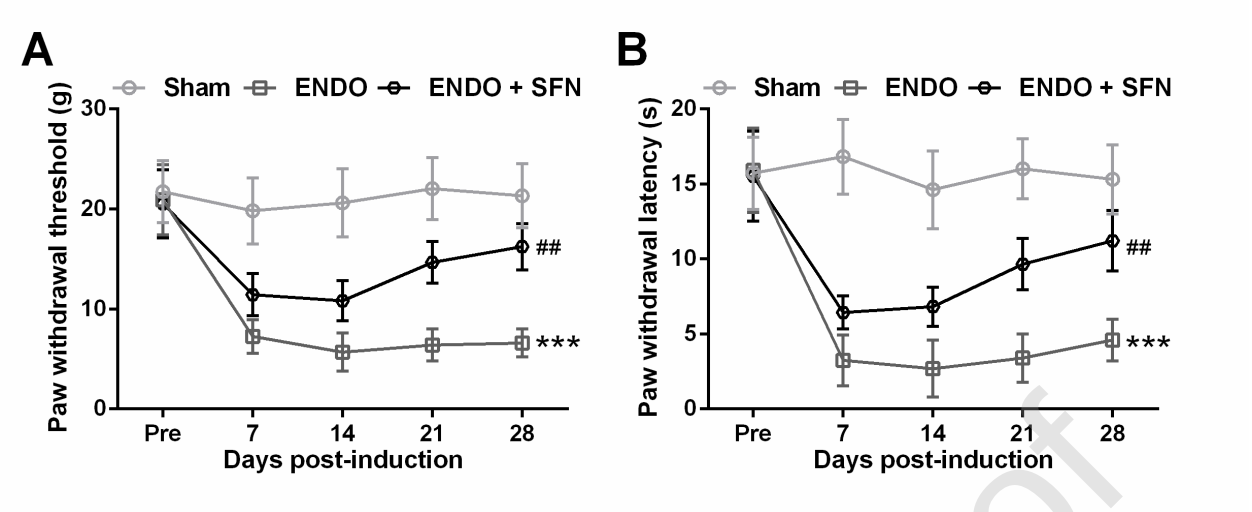
Figure 2. Sulforaphane (SFN) inhibited ectopic endometrial tissue growth in sciatic endometriosis rat. (A) Comparison of the volume of the lesion in each group after 4 weeks of treatment. VEGF levels in serum (B) and ectopic endometrial tissue (C) were measure by ELISA. (D) Western blotting was used to measure the protein expression of VEGF in ectopic endometrial tissue and relative expression was presented in (E). Data are presented as mean ± SD. N=6 for each group. ***p < 0.001 compared to sham, ##p < 0.01, ###p < 0.001 compared to ENDO group.

Figure 3. Anti-inflammatory effects of sulforaphane (SFN) on sciatic endometriosis induced inflammatory in superficial dorsal horn (L4–L6) tissues. 30 mg/kg per day sulforaphane was administrated to sciatic endometriosis rats from 3 days pre-surgery and lasted until 4 weeks post-surgery. ELISA and qRT-PCR were used to measure the expression of IL-6, IL-1β and TNF-α in superficial dorsal horn (L4–L6) tissues. Data are presented as mean ± SD. N=6 for each group. **p < 0.01, ***p < 0.001 compared to sham, #p < 0.05, ##p < 0.01 compared to ENDO group.

Figure 4. mRNA and protein levels of COX-2 and iNOS in superficial dorsal horn (L4–L6) tissues of sciatic endometriosis rats after 4 weeks treatment of sulforaphane (SFN). (A, B) COX2 and iNOS mRNA levels were determined by q-PCR. GAPDH serves as an internal control. (C-E) COX2 and iNOS protein levels were analyzed by Western blotting. Data are presented as mean ± SD. N=6 for each group. **p < 0.01, ***p < 0.001 compared to sham, #p < 0.05, ##p < 0.01 compared to ENDO group.

Figure 5. Sulforaphane (SFN) activated Keap1-Nrf2 signaling in superficial dorsal horn (L4–L6) tissues of sciatic endometriosis rats after 4 weeks treatment (A-C) Keap1 and Nrf2 protein expressions were measured by western blotting and relative expressions were normalized to sham. Data are presented as mean ± SD. N=6 for each group. **p < 0.01, compared to sham, #p < 0.05, ###p < 0.001 compared to ENDO group.

Fig 1



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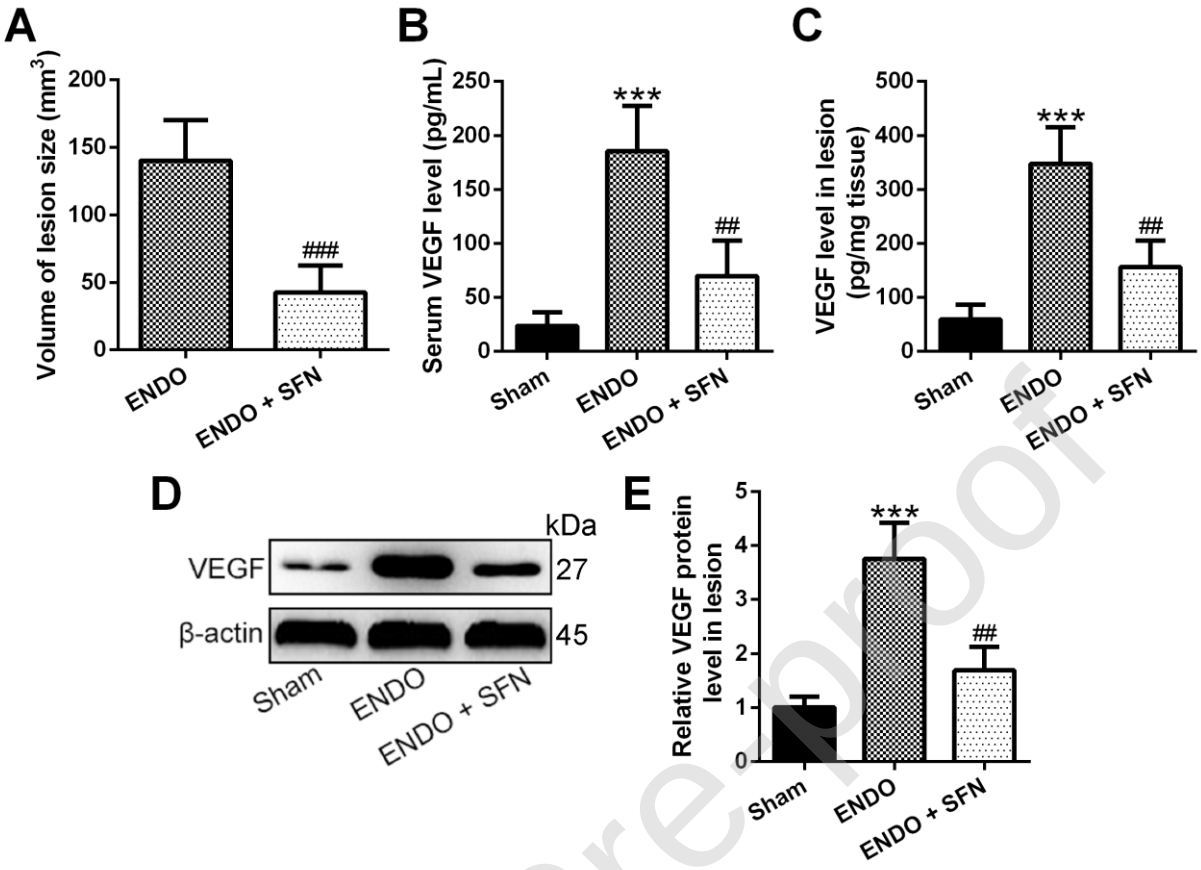

Fig 3

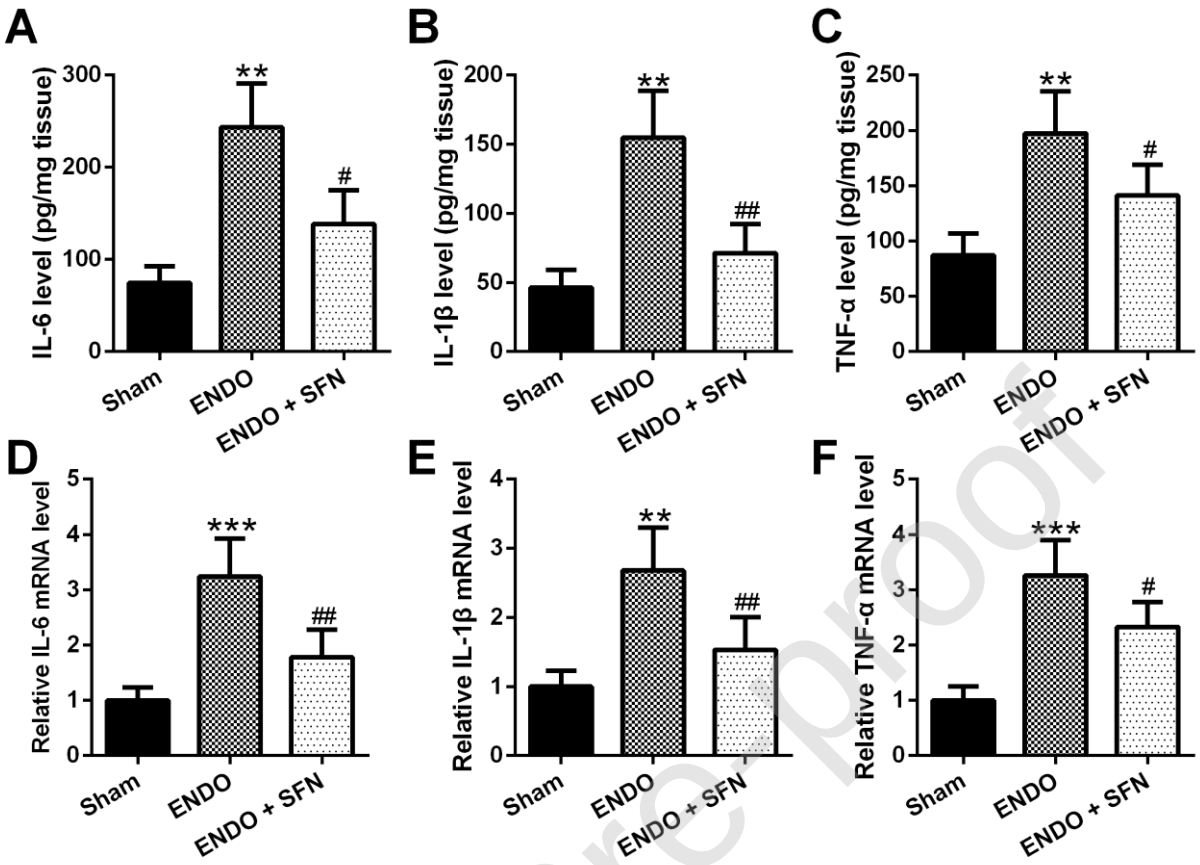

Fig 4

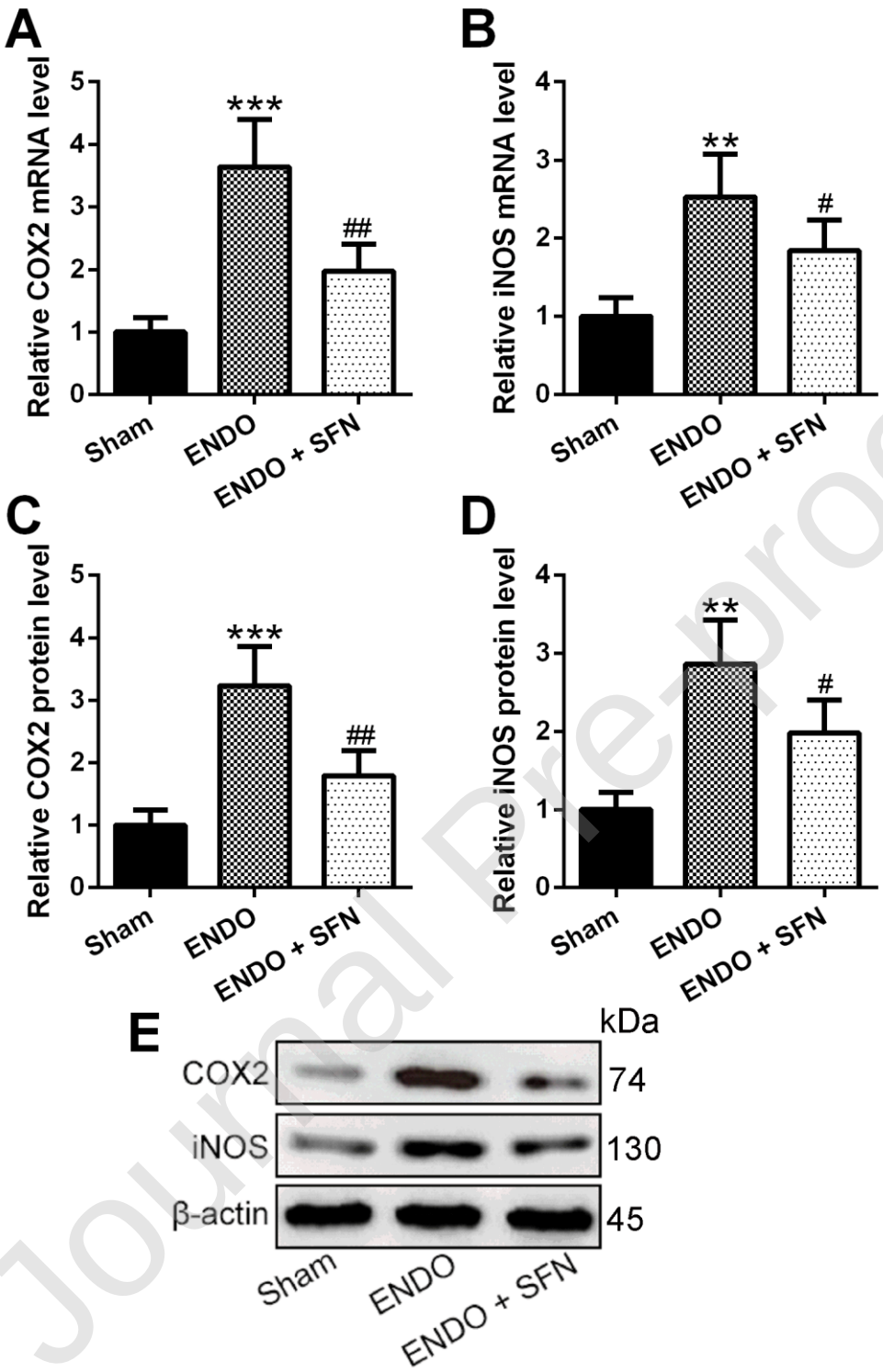

Fig 5