Article

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Effects of TNF-α on Behaviour and Inflammation in Rats with Rotator Cuff Injury through NGF

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Objective: Rotator cuff injury is a common injury that includes inflammation, partial tearing, or complete tearing of the rotator cuff tendon. In cases of rotator cuff tears (RCTs), Tumor Necrosis Factor-alpha (TNF-α) can trigger the release of nerve growth factor (NGF). TNF-α is an important inflammatory mediator that affects rotator cuff activity and increased NGF expression is observed in RCTs. Therefore, this study aimed to investigate whether inhibition of TNF-α could reduce behavioural responses and inflammation levels in rats through NGF.

Methods: A rat RCT model was established, and the CatWalk gait analysis system was used for behavioural assessment. Immunohistochemistry was used to detect NGF protein levels in tendon tissue. Hematoxylin eosin (HE) staining was used to observe histopathological changes. The expressions of Interleukin-1beta (IL-1β) and Cyclooxygenase-2 (COX2) were detected by western blotting (WB) and quantitative real-time polymerase chain reaction (qRT-PCR). The expression of apoptosis protein Bcl-2-associated X (Bax), B-cell lymphoma 2 (Bcl-2), and Cysteine-aspartic acid protease-3 (Caspase-3) were detected using WB. Oxidative stress markers, namely Reactive Oxygen Species (ROS), Malondialdehyde (MDA), and Superoxide Dismutase (SOD) were quantified in tissues using an ELISA kit.

Results: In the RCT model, elevated NGF protein expression, noticeable atrophy in the supraspinatus muscle tissue, and substantial fat infiltration were observed. The levels of IL-1β, COX2, apoptosis, and oxidative stress were all increased. TNF-α inhibition resulted in decreased NFG expression, decreased tissue fibrosis, and improved tendon atrophy. Moreover, when TNF-α was inhibited, the expressions of IL-1β and Cox2 were reduced and both apoptosis and oxidative stress were decreased. The results showed that inhibiting TNF-α had the potential to reduce inflammation levels and behavioural responses in rats.

Conclusion: TNF-α can affect behaviour and inflammation in rats with RCTs through NGF, and TNF-α inhibition can improve rotator cuff injury.

Keywords: TNF-α; NGF; pathway; rotator cuff tears; rat behaviour; inflammation

Introduction

According to statistics, the incidence of rotator cuff injury can reach 31% in people aged 60 to 69 years, while it is expected to be as high as 65% in people over 80 years old [1]. Rotator cuff injury causes shoulder pain, limited motor function, and reduced quality of life, significantly impacting activities of daily living (ADLs). Rotator cuff injury is a common problem in shoulder diseases, affecting the quality of life of millions of people worldwide. This injury usually involves the four major tendons of the rotator cuff, including the supraspinatus, subscapularis, teres minor, and infraspinatus [2]. These tendons are subjected to significant daily stress, making them prone to acute or chronic injuries. In the pathogenesis of rotator cuff injury, inflammatory mediators play a pivotal role, particularly the increased presence of inflammatory cytokines lead to tissue destruction and chronic pain [3].

Tumor Necrosis Factor-alpha (TNF-α) serves a dual role as a major pro-inflammatory cytokine and anti-tumor agent. Many studies have demonstrated that TNF-α acts as an inflammatory mediator [4,5], and plays a key role in inflammation, immune responses, and apoptosis. TNF-α is frequently expressed at elevated levels in the subacromial bursae tissue and worn tendon tissue of patients with rotator cuff injuries [6,7]. The overexpression of TNF-α in rotator cuff injuries may lead to an increased inflammatory response, leading to more severe tissue damage and pain.

Nerve growth factor (NGF) is a neurotrophic factor, a protein that is essential for nerve cell growth, survival, and differentiation. Recent studies have established that NGF also plays an important role in inflammation and pain [8]. NGF can increase pain perception and regulate a variety of inflammation-associated cells and factors [9]. In conditions like chronic pain and inflammatory diseases, NGF expression and release are both significantly increased [10,11]. Recent research indicates that TNF-α can regulate NGF ex-

pression and activity, subsequently affecting pain and inflammation [12]. However, the specific effect of TNF-α on rotator cuff tears (RCTs) through NGF remains unclear.

Etanercept is a chimeric monoclonal antibody that operates primarily through the specific binding of TNF-α, resulting in TNF-α inhibition [13]. TNF-α is an inflammatory mediator involved in the occurrence and development of many inflammatory diseases [14]. Enaxil typically overbinds to TNF-α, preventing its binding to cell surface receptors, thereby inhibiting TNF-α-mediated inflammatory responses [15]. Specifically, etanercept forms a stable complex with both dissolved and membrane-bound TNF-α [16], preventing TNF-α from binding to its receptors, such as TNF-α receptors I and II, thus preventing signal initiation [17]. TNF-α inhibition can reduce the activity of inflammatory cells and reduce the release of inflammatory mediators, positively impacting the treatment of various autoimmune diseases, inflammatory bowel disease, and other inflammatory diseases [18].

When rotator cuff tissue is damaged via tearing or wear, cells at the injury site release inflammatory mediators, including TNF-α, Interleukin-1beta (IL-1β), and Cyclooxygenase-2 (COX2), as a self-protection mechanism [19,20]. These mediators trigger an acute inflammatory response, causing classic inflammatory symptoms such as swelling, redness, pain, and heat, altering the body to the injured area. Inflammatory symptoms also encourage immune cells and repair mechanisms to treat the injury [21]. Simultaneously, inflammatory mediators can interact with apoptosis [22]. In cases of RCTs, excessive or prolonged inflammatory responses may lead to increased apoptosis of tendon cells, which can further impair tissue structure and function. In conclusion, inflammatory mediators play a dual role in RCTs, as part of self-protection and repair [23], and may also lead to pain and tissue destruction. Therefore, studying and regulating the expression and activity of these mediators is crucial for the treatment and rehabilitation of RCTs.

To better understand the influence of TNF-α and NGF in rotator cuff injury, this study aimed to explore whether TNF-α affects the behaviour and inflammatory response of rats with RCTs through NGF. The objective of this study is to offer fresh insights into the pathophysiological mechanisms of rotator cuff injury and provide a valuable reference for future treatment strategies.

Experimental Design and Methods

Animals and Materials

15 male SD rats aged 8–10 weeks and weighing 280.12 $\pm$ 20.12 g were purchased from Hunan Sleek Jingda Experimental Animal Co., Ltd. The rats were fed with rat growth and breeding feed (SPF-F01-001, SiPeiFu, Beijing, China) normally, and the ambient temperature was maintained at 20~25 °C with a relative humidity of 50%~65%.

Animal Modelling

All rats were anaesthetized with sevoflurane (2%) inhalation. The skin was disinfected, shoulder hair was shaved, and a longitudinal incision of about 2 cm was made in the shoulder joint to expose the deltoid muscle. The deltoid muscle was cut open to expose the unilateral supraspinatus muscle. The tendon of the unilateral supraspinatus muscle tendon was sharply cut at the top of the greater tubercle, and a 0.5 cm $\times$ 0.5 cm section of the tendon was removed. After suturing the wound, the model was established and CatWalk gait analysis was used to evaluate the model to ensure successful establishment [24]. Subsequently, all animals were randomly assigned to two different groups: Sham group (only the deltoid muscle was cut to expose the supraspinatus tendon, and the deltoid muscle and skin were immediately closed without excision of the rotator cuff), Model group, Model + EN (model + subcutaneous injection of Etanercept (inhibitors of TNF-α, 0.25 μg/mL, once/d)), with five rats in each group.

After grouping, the rats were fed normally for one week, and all rats were allowed unrestricted movement in their cages. After 7 days, the CatWalk gait analysis system (Noldus Information Technology, Beijing, China) was used to collect free-walking evaluation data, including stride length, standing width, and paw area, for all three groups of rats. Subsequently, rats received an intraperitoneal injection of pentobarbital (150 mg/kg) and tissue was collected. All experimental procedures were approved by the Institutional Animal Protection and Use Committee of The Affiliated Nanhua Hospital, Department of Sports Medicine, Hengyang Medical School, University of South China (Ethical Approval Number: 202203004).

NGF in Tissues Detected by Immunohistochemistry

Supraspinatus tendon tissues were taken and fixed overnight with 4% paraformaldehyde. Each specimen was washed with PBS and dehydrated with xylene gradient alcohol. The tissue was embedded in paraffin wax, sliced into 3-μm thick slices, and pasted on the slide. Tissues were treated with EDTA repair solution (1 mM EDTA/10 mM Tris, pH 9.0) for 10 minutes, 3% hydrogen peroxide aqueous solution for 15 minutes, and 10% normal goat IgG (Beyotime, Shanghai, China) for 20 minutes. Following incubation with NGF antibody (AC909, 1:200, Beyotime, Shanghai, China) and PBS washing, a biotin-labeled sheep anti-rabbit secondary antibody (A0277, 1:200, Beyotime, Shanghai, China) working solution was applied. Horseradish peroxidase (HRP) treatment was then performed, and AEC (36304ES01, Yeasen, Shanghai, China) was color rendering. Then PBS washing, dehydration, sulfite solution sealing. The expression of the antigen in the brown area was observed under an optical microscope (Nikon Eclipse Ci, Shanghai, China). Statistical analysis of the area proportion of positive signals was performed using Image J software (V1.8.0.112, NIH, Madison, WI, USA).

Table 1. Primer sequence of *IL-1β* and *COX2*.

Gene	F(5′-3′)	R(5′-3′)
IL-1β	ATGGCAGAAGGAGATCACCTC	TACCAGTTGGGGAACTCTGC
COX2	TGGAGTCTGGAAGACACCCA	GGGAAATTCTGCTCGTCTCA
GAPDH	AGGTCGGTGTGAACGGATTG	TGTAGACCATGTAGTTGAGGTA

IL-1β, Interleukin-1beta; *COX2*, Cyclooxygenase-2; GAPDH, Glyceraldehyde-3-Phosphate Dehydrogenase.

Tendon Tissue Observed by Hematoxylin Eosin (HE) Staining

The supraspinatus tendon tissue was fixed with 4% formaldehyde, dehydrated with ethanol, stained for 3–5 minutes, dehydrated with alcohol for 5 minutes, and stained again for 2–3 minutes. The slides were dehydrated, then made transparent by xylene, and finally sealed with neutral glue. The optical microscope was used to observe and take pictures of the samples.

IL-1β and COX2 were Detected by Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Tendon tissues were homogenized using Trizol reagent (B511311, Sangon Biotech, Shanghai, China) following the manufacturer's instructions to extract total RNA from the supraspinatus muscle tendon tissues. Reverse transcription was performed on qualified RNA using the reverse transcription kit (B639277-0050, Sangon Biotech, Shanghai, China). According to the specified procedures, cRNA Synthesis was carried out by Sangon Biotech, Shanghai, China. RNA *IL-1β* and *COX2* expression levels were determined by SYBR Green PCR assay kit (Beyotime, Shanghai, China), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) was used as the internal control. After the reaction, the melting curve was analyzed, and the Ct value was obtained. The expression of primers in cells was calculated by the $2^{-\Delta\Delta Ct}$ formula. The primer sequence is shown in Table 1.

IL-1β, COX2, Bcl-2-Associated X (Bax), Cysteine-Aspartic Acid Protease-3 (Caspase-3), B-Cell Lymphoma 2 (Bcl-2), and NGF were Detected by Western Blotting (WB)

Total protein was extracted from tendon tissue using RIPA lysis buffer (P0013B, Beyotime, Shanghai, China) and 100 mM PMSF (ST507-10ml, Beyotime, Shanghai, China) according to the manufacturer's instructions. The protein was quantified using the BCA protein detection kit (P0012S, Beyotime, Shanghai, China). Equal amounts of protein were taken from each sample and boiled at 100 °C for 5 minutes with protein loading buffer, then separated in a 10–12% SDS-PAGE gel kit (P0012A, Beyotime, Shanghai, China) and transferred to a PVDF membrane (FFP36, Beyotime, Shanghai, China) for electrophoresis. The membrane was combined with IL-1β (12242S, 1:1000, Cell Signaling Technology, Shanghai, China), COX2 (12282S, 1:1000, Cell Signaling Technology, Shanghai, China), Bax (89477S, 1:1000, Cell Signaling Technology, Shanghai, China), Bcl-2 (15071S, 1:1000, Cell Signaling Technology, Shanghai, China), Caspase-3 (9662S, 1:1000, Cell Signaling Technology, Shanghai, China), and NGF (2046S, 1:1000, Cell Signaling Technology, Shanghai, China) was placed at 4 °C for two hours and incubated with the corresponding secondary antibody (A0208, 1:1000, Beyotime, Shanghai, China) at room temperature for one hour the following day. After washing with PBS, BeyoECL Star (P0018AS, Beyotime, Shanghai, China) was added for development detection and photo analysis.

Reactive Oxygen Species (ROS), Malondialdehyde (MDA), Superoxide Dismutase (SOD) and NGF were Detected by ELISA

The serum was extracted and centrifuged at high speed to obtain the supernatant. ROS (SP13358, Spbio, Wuhan, China), SOD (SP12914, Spbio, Wuhan, China), NGF (SP12266, Spbio, Wuhan, China), and MDA (SP30131, Spbio, Wuhan, China) were detected via an ELISA kit following the kit instructions. The OD value of each well was measured at a wavelength of 450 nm with an enzyme labeling instrument (800TS, Biotek, ANTPEDIA, Shanghai, China). Contents of the ROS, MDA, and SOD in the serum were calculated according to the standard curve.

Statistical Analysis

Student *T*-tests were used for two-group comparisons while Analysis of variance (ANOVA) was used for multiple-group comparisons using GraphPad Prism 9 (Dotmatics, Boston, MA, USA). Post hoc comparisons were conducted using the sequential Bonferroni test for data with significant differences. Data are expressed as mean ± standard deviation, and significance was established at $p < 0.05$.

Results

Establishment of Rotator Cuff Tear Model and Behavioural Evaluation

Rotator cuff tear modeling was performed on rats ($p < 0.05$) (Fig. 1A–E), and correlation coefficient analysis showed that the step length, standing width and foot area of rats were reduced after 1 week of modeling compared with those of Sham group. After taking EN, step length,

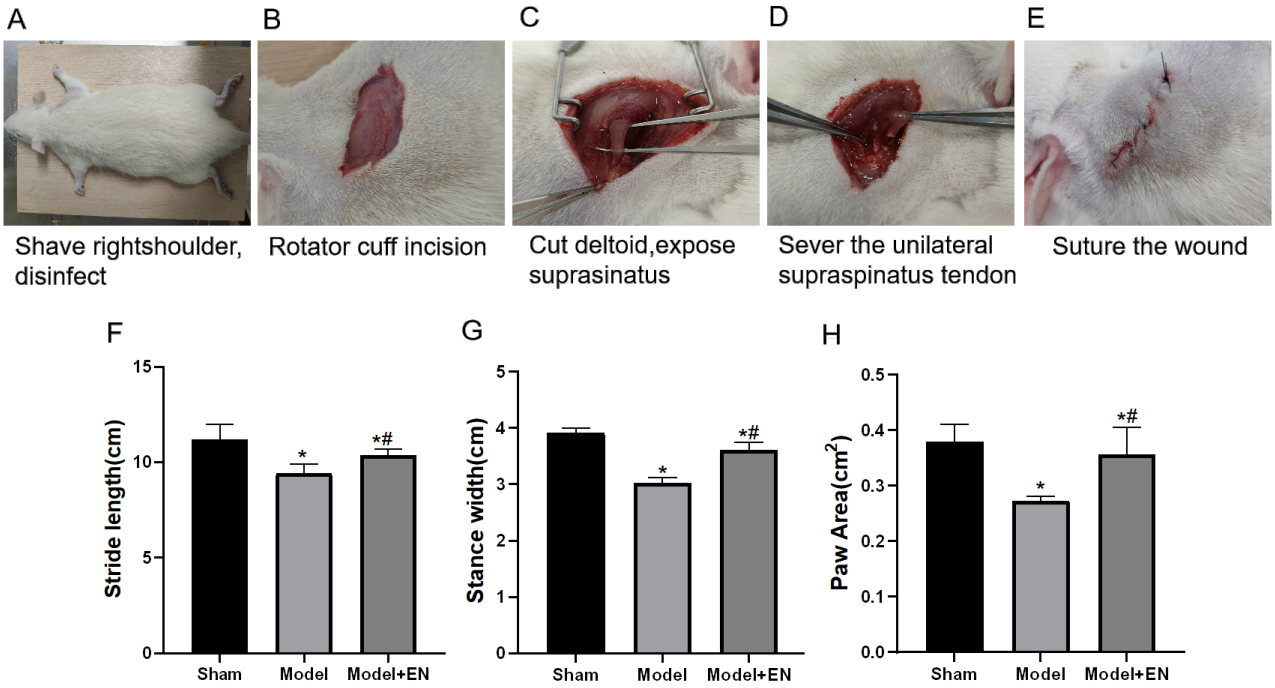

Fig. 1. Establishment of rotator cuff tear model and behavioural evaluation. (A–E) Surgical procedure in generating large-scale rotator cuff tears (RCTs) rats. (F–H) Behavioural (stride length, stance width, and paw area) assessment in rats. *$p < 0.05$ vs Sham; *#$p < 0.05$ vs Model. n = 5.

standing posture width, the foot area of rats increased and the pain was relieved to some extent ($p < 0.05$) (Fig. 1F–H). The results showed that inhibition of TNF-α could alleviate motor dysfunction in rats.

Influence of TNF-α on NGF Expression in Rotator Cuff Tear

The results showed that the expression, content, and positive rate of NGF in the Model group were increased compared with the Sham group. Compared with the Model group, the content, expression level, and positive rate of NGF in the Model + EN group were decreased ($p < 0.05$) (Fig. 2A–E). These results indicate that TNF-α inhibition can decrease the expression of NGF in rats with rotator cuff injury.

Influence of TNF-α on Histopathological Morphology of Rotator Cuff Tear through NGF

Hematoxylin eosin (HE) staining results showed a reduction in cell number and significant muscle atrophy with fat infiltration in the Model group compared to the Sham group. In the Model + EN group, cell nuclei exhibited overlap with dense arrangement, and there was a significant improvement in atrophic muscle tissue compared to the Model group ($p < 0.05$) (Fig. 3).

Effect of TNF-α on the Expression of Inflammatory Factors in Rotator Cuff Tear through NGF

The results showed that IL-1β and COX2 levels were increased in the Model group compared with the Sham group. IL-1β and COX2 levels were decreased in the Model + EN group compared with the Model group ($p < 0.05$) (Fig. 4A–E). These results indicate that TNF-α inhibition can reduce the expression of inflammatory factors through NGF, thereby reducing the inflammation of tendon tissue.

Effect of TNF-α on the Expression of Apoptotic Protein and Oxidative Stress in Rotator Cuff Tear through NGF

The results showed that Bax and Caspase-3 expression levels were increased, and Bcl-2 expression levels were decreased in the Model group compared with the Sham group. Additionally, the Model + EN group exhibited decreased expression levels of Bax and Caspase-3 and increased levels of Bcl-2 compared to the Model group ($p < 0.05$) (Fig. 5A–D). Compared with the Sham group, ROS and MDA levels increased while SOD levels decreased in the Model group. In the Model + EN group, ROS and MDA levels decreased, and SOD levels increased compared to the Model group ($p < 0.05$) (Fig. 5E–G). These results demonstrate that TNF-α inhibition can inhibit apoptosis and reduce oxidative stress damage in tissues, thereby reducing inflammation and promoting tissue repair.

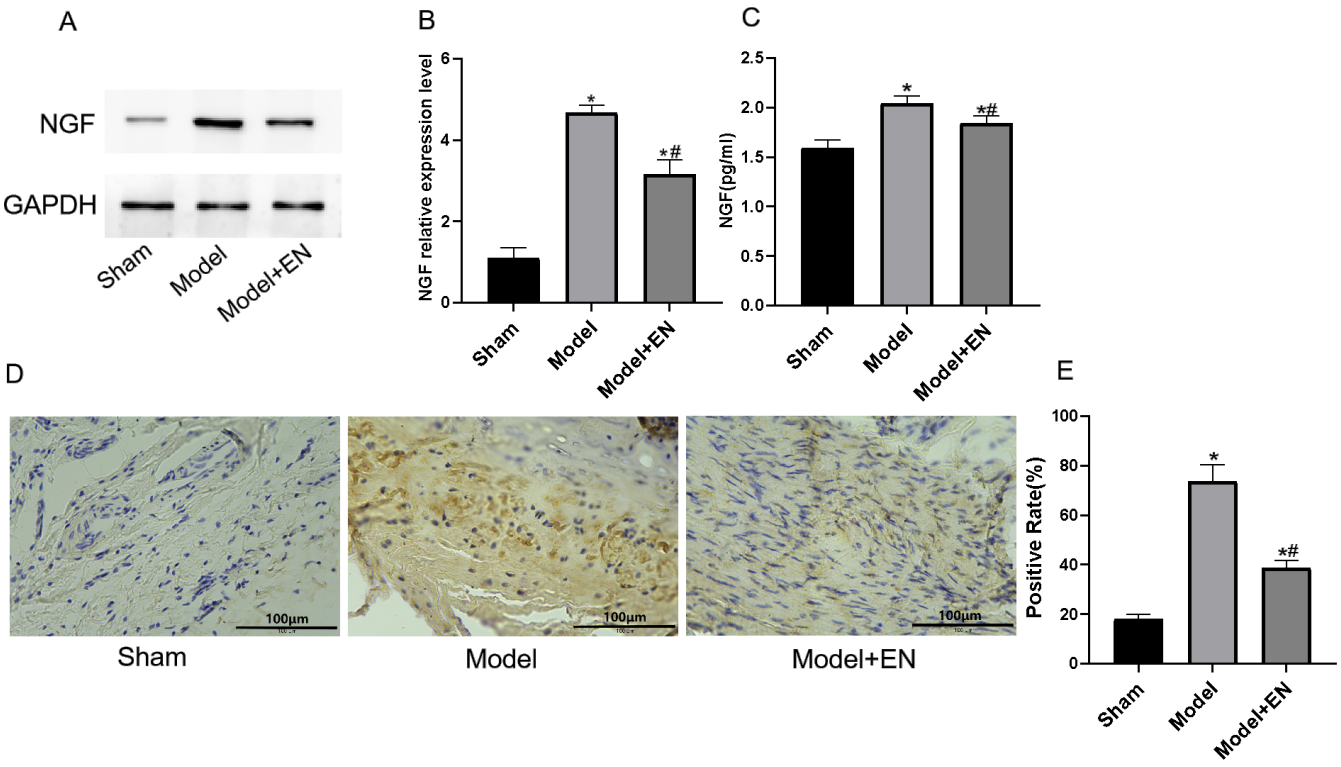

Fig. 2. Influence of Tumor Necrosis Factor-alpha (TNF-α) on nerve growth factor (NGF) expression in rotator cuff tear. (A,B) NGF expression was detected by western blotting (WB). (C) NGF content was detected by ELISA. (D,E) NGF expression detected by IHC. Scale bars indicate 100 μm for 400× magnification. Data are shown as the mean ± standard deviation. *$p < 0.05$ vs Sham; *#$p <$ 0.05 vs Model. n = 5.

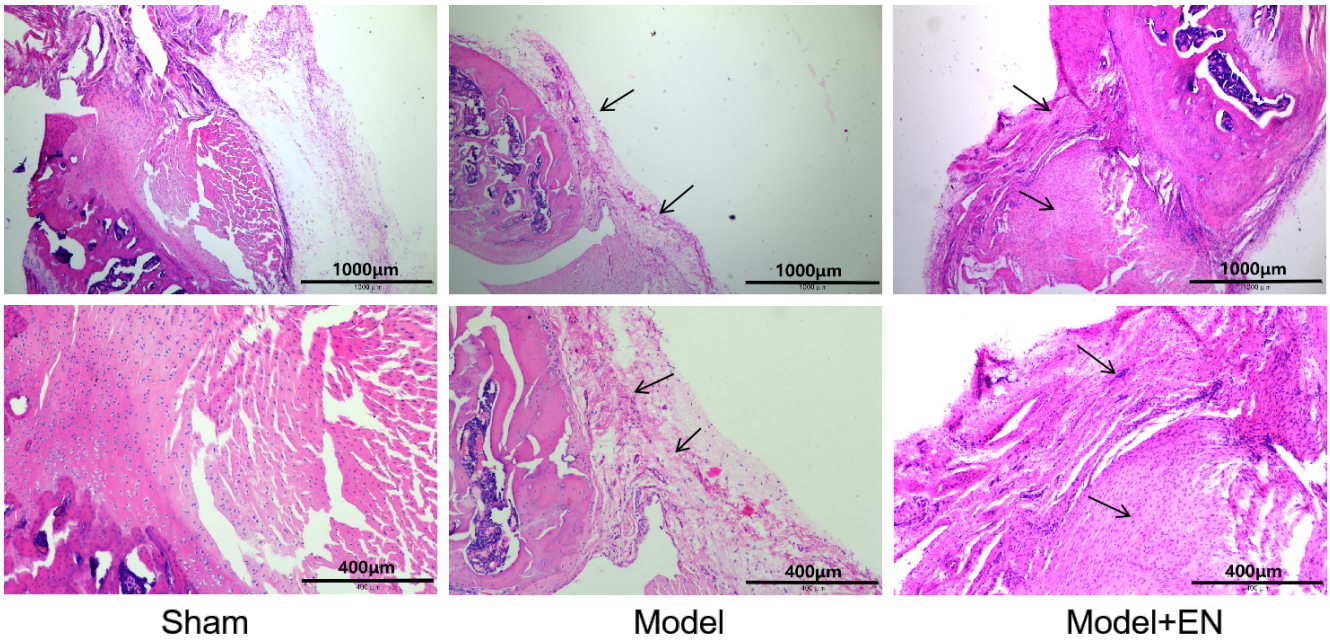

Fig. 3. Influence of TNF-α on histopathological morphology of rotator cuff tear through NGF. Hematoxylin eosin (HE) staining was used to observe tendon tissue. Scale bars indicate 400 μm for 100× magnification and 1000 μm for 40× magnification. The arrows represent sites of muscle tissue atrophy and fat infiltration.

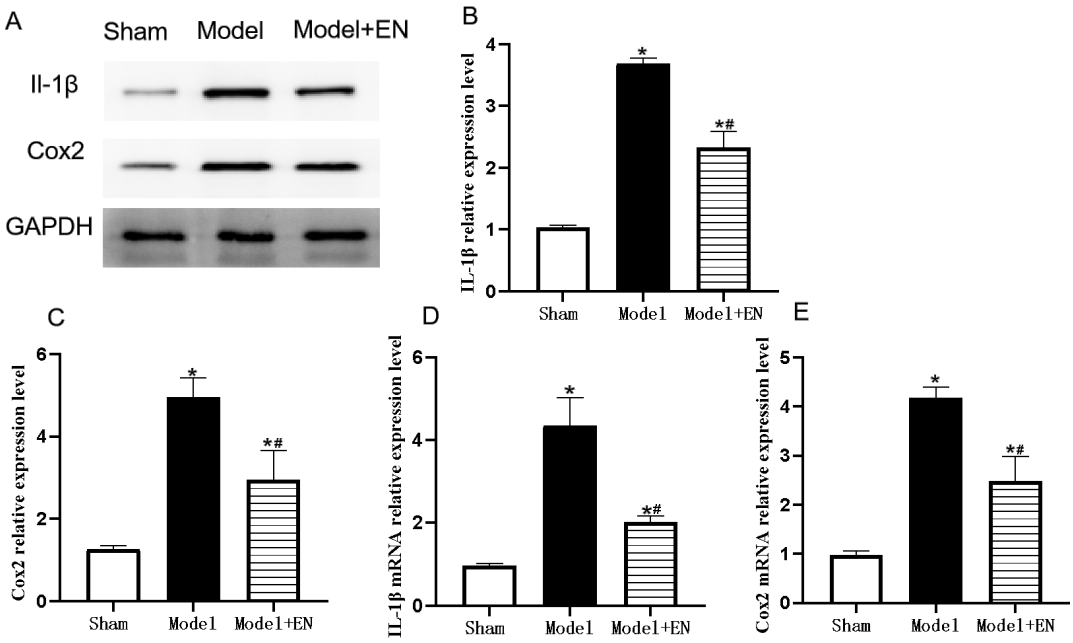

Fig. 4. Effects of etanercept (EN) on inflammatory factors in RCTs. (A–C) WB was used to detect the expression of inflammatory factors. (D,E) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the mRNA levels of IL-1β and COX2. *$p < 0.05$ vs Sham; *#$p < 0.05$ vs Model. n = 5.

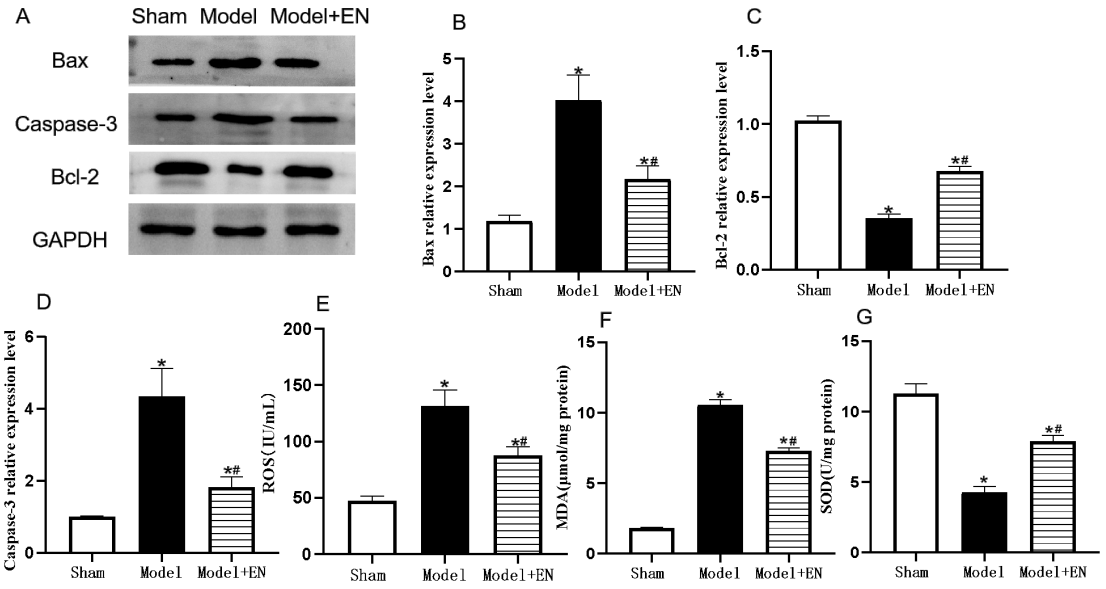

Fig. 5. Effects of EN on apoptotic proteins and oxidative stress in rotator cuff tissue. (A–D) WB was used to detect the expression of apoptosis protein. (E–G) ELISA was used to detect the level of oxidative stress factor. *$p < 0.05$ vs Sham; *#$p < 0.05$ vs Model. n = 5.

Discussion

In this study, we constructed a rotator cuff tear model by severing the supraspinatus tendon. Consequently, we observed the progression of muscle atrophy, fatty infiltration, and fibrosis after rotator cuff tenotomy. Additionally, apoptotic proteins and oxidative stress levels were quantified using a kit. Behavioural analysis showed that TNF-α passed through NGF increases pain signal transmission, increasing sensitivity to painful stimuli in rats. When EN was used to inhibit TNF-α, pathological rotator cuff tissue exhibited reduced degeneration and corresponding inflammatory signs, ultimately reducing pain and inflammation in rats.

Previous studies have shown a relationship between TNF-α and RCTs [25–27]. These studies suggest that TNF-α may interact with other inflammatory mediators and growth factors [28,29]. These interactions may influence the pathophysiology of RCTs, especially pain perception and the degree of inflammation [30]. TNF-α is an inflammatory mediator that plays a key role in tissue damage and inflammation. In the context of rotator cuff injury, inflammation is usually accompanied by injury of the rotator cuff tendon, resulting in local pain, swelling, and tissue alterations [31]. The release of TNF-α can trigger and exacerbate these inflammatory responses, resulting in more intense pain in affected patients [32]. Although NGF plays a positive role in promoting neuronal growth [33], it can also impact tissue repair and regeneration, potentially hindering tendon and muscle tissue recovery during rotator cuff injury healing [34]. Additionally, NGF can increase inflammatory cell migration and cytokine release, further promoting the inflammatory process [35,36]. Inflammatory responses and cell damage in damaged tissues release oxygen-free radicals that trigger oxidative stress [37,38]. High levels of oxidative stress may adversely affect motor function in rats, leading to dyskinesia and coordination problems, impairing muscle function, and ultimately reducing mobility [39].

Etanercept (EN) is highly effective in inhibiting TNF-α and has been approved for the treatment of arthritis and ankylosing spondylitis [40]. TNF-α plays an important role in many inflammatory diseases, including inflammatory bowel disease, ankylosing spondylitis, and Crohn's disease [41]. The inflammatory cytokine TNF-α passes through TNFR2, triggering a signal that induces NGF production. TNF-α signaling promotes NGF-dependent survival through TNFR2 [42]. NGF contributes to the maintenance of inflammation and mainly exerts its effect by binding two receptors: p140TrKA and P75NTR. TNF-α signaling and NGF signaling regulate each other, and maintaining a balance between them is critical for normal cellular responses [43]. In this study, we injected EN into rats to inhibit TNF-α expression, resulting in a decrease in NGF. WB analysis revealed decreased expression levels of IL-1β, COX2, Bax, and Caspase-3, indicating a reduction in the body's inflammatory response. Subsequent ELISA analysis of ROS and MDA levels demonstrated a significant decrease in oxidative stress after EN treatment. These results indicate that TNF-α aggravates the inflammatory response, increases tissue damage, and induces pain by activating the NGF signaling pathway.

There were some limitations in the study design. We used sharp amputation of a unilateral (right) supraspinatus muscle tendon to construct a rotator cuff tear model, which may have influenced the inference of causality and interpretation of the findings. There are many biological differences between rats and humans, including metabolism, immune system, drug metabolism, and physiology. As a result, rat models may not be able to fully replicate the features and responses of human disease. Despite these limitations, this study provides a preliminary understanding of the behavioural and inflammatory effects of TNF-α via NGF in rats with rotator cuff injury. Future studies consider treatments directly applicable to human rotator cuff tissue to address these limitations and better understand the role of TNF-α on human RCTs.

In this study, we investigated the effects of TNF-α and NGF on the behaviour and inflammation of rats with RCTs, providing context on the mechanism of pain associated with injury. Future experimental research can involve grouping EN with other drugs to compare their efficacy, offering insights into pain management, facilitating the development of more effective pain relief strategies, guiding clinical drug guidelines, and identifying targets for innovative treatment approaches.

Conclusion

By regulating the expression of the NGF signaling pathway, TNF-α enhances apoptosis and oxidative stress, prolongs healing time, and decreases motor function in rats with RCTs.

Availability of Data and Materials

The data used and/or analyzed during the current study are available from the corresponding author.

Author Contributions

LX and HLD designed the research study. HLD and SPZ performed the research. LX provided help and advice on the experiments. SPZ analyzed the data. All authors were involved in drafting and critical revision of the manuscript. All authors have read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were approved by the Institutional Animal Protection and Use Committee of The Affiliated Nanhua Hospital, Department of Sports Medicine, Hengyang Medical School, University of South China (Ethical Approval Number: 202203004).

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Conflict of Interest

The authors declare no conflict of interest.

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