Research Article

Effect of Mn₃O₄ Nanoparticles on Lipopolysaccharide-Induced Inflammatory Factors in the Human Tendon Cells and Its Mechanism

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Objective. To investigate the effect of Mn₃O₄ nanoparticles (Mn₃O₄NPs) on inflammatory factors induced by lipopolysaccharide (LPS) in human tendon cells and its mechanism.

Methods. The Mn₃O₄NPs were synthesized by a hydrothermal method. RT-qPCR was used to detect the expression levels of miRNAs related to inflammation in human tendon cells. The expression level of NLRP1 (NOD-like receptor containing pyrin domain 1) was measured by Western blotting. ELISA assay was used to measure the level of TNF-α, IL-1β, IL-4, and IL-10. The relationship between miR-181a-5p and NLRP1 was verified by dual-luciferase reporter assay.

Results. Mn₃O₄NPs produced in this study were brown spherical particles with an average size of 7-10 nm. Mn₃O₄NP treatment significantly reduced the levels of TNF-α and IL-1β but increased the levels of IL-4 and IL-10 in the human tendon cells induced by LPS. In addition, Mn₃O₄NP treatment remarkably increased the expression level of miR-181a-5p. NLRP1 is one of the targets of miR-181a-5p, and miR-181a-5p downregulated its expression. Further study showed that Mn₃O₄NPs could alleviate the inflammatory response of human tendon cells induced by LPS by upregulating miR-181a-5p and thus downregulating the expression of NLRP1.

Conclusion. Mn₃O₄NPs affect the expression of inflammatory cytokines in the human tendon cells induced by LPS by modulating the molecular axis of miR-181a-5p/NLRP1.

1. Introduction

Tendonitis is the inflammation of the tendon caused by strain. It often occurs in the hands, wrists, shoulders, and knees. With the increasing use of computers, the hand controlling the mouse has been the most common hand which affected by tendonitis and its incidence has been increasing in recent years [1]. Recently, nanomaterials with new structures, new properties, and new functions have been widely used in the treatment of a variety of diseases including tumors [2], autoimmune diseases, etc. [3]; studies also shown that nanoparticles have good efficacy in the treatment of inflammation-related diseases [4]. Among them, Mn₃O₄ nanoparticles (Mn₃O₄NPs) have been demonstrated to alleviate inflammation of mouse ears induced by ROS [5]. Lipopolysaccharide (LPS), as a cell wall component of Gram-negative bacteria, has been often used to stimulate cells to produce inflammatory mediators to build a model of cellular inflammation. However, there are no reports about the effects of Mn₃O₄NPs on LPS-induced tendon inflammatory factor levels and their regulatory mechanisms. Therefore, in this study, to provide a new strategy for the treatment of tendonitis, human tendon cells were induced by LPS to form a tendinitis model, and the effect of Mn₃O₄NPs on the expression level of inflammatory factors was explored.

2. Material and Methods

2.1. Cell Lines and Reagents. Human tendon cells HT (Cat. No. PR203) were purchased from Beijing Fubo Biotechnology Co., Ltd. Fetal bovine serum, DMEM medium, penicillin, and streptomycin were purchased from Guangzhou Ruite Biotechnology Co., Ltd. Mn(OAc)₂·4H₂O and TRIzol one-
step RNA extraction reagents were purchased from Shanghai Yanjin Biotechnology Co., Ltd. The reverse transcription kit, total protein extraction kit, and dual-luciferase reporter gene detection kit were purchased from Wuhan Purity Biotechnology Co., Ltd. Lipofectamine 2000 kit was purchased from Shanghai Mingming Biotechnology Co., Ltd. TRIsol one-step method. 2

2.5. RT-qPCR. Total RNA of cells in each experimental group was extracted by TRIzol one-step method. 2 μl of total RNA was reversely transcribed into cDNA according to the reverse transcription kit instructions. The reverse transcription product was placed in a PCR instrument for the reaction.

The 20 μl reaction system contains 0.5 μl reverse transcription product, 1 μl upstream and downstream primers, 10 μl SYBR Premix Ex Taq, and 7.5 μl RNase-FREE water. The reaction conditions were 90°C for 10 min, 95°C for 30 s, and 55°C for 1 min. A total of 50 cycles were performed.

The primer sequences are as follows:

miR-181a-5p upstream primer sequence: 5′-CGGCAA CATTCAACGCTTG-3′;

miR-181a-5p downstream primer sequence: 5′-GTGC AGGGTCGGAGGTATTC-3′. U6 upstream primer sequence: 5′-CITCCGCACGACATATAC-3′; U6 downstream primer sequence: 5′-GAACGCTTCAGAATTTGC-3′.

Quantitative fluorescence detection results were calculated by the 2ΔΔCt method.

2.6. Western Blotting. The total protein of each experimental group was extracted using a total protein extraction kit. The equivalent amount of denatured protein sample was loaded, and SDS-PAGE electrophoresis was performed. After 2 h of electrophoresis, the membrane was transferred at 100 V. After the film is transferred, it was sealed in 5% skimmed milk powder. After 2 h, the membrane was washed, and a primary antibody (anti-NLRP1, 1:1000; anti-GAPDH, 1:1000) was added and incubated at 4°C overnight. The next day, after washing the membrane, a secondary antibody (1:1000) was added and incubated at room temperature for 2 h. After the reaction was complete, the film was washed, and a chemiluminescence developing solution was added for development and photographs. Grayscale analysis of protein bands was performed using ImageJ software.

2.7. ELISA. The cell supernatants of each experimental group were obtained by centrifugation, and the contents of TNF-α, IL-1β, IL-4, and IL-10 were measured according to the instructions of the ELISA kit. In specific, 100 μl of the standard sample and the test sample were added to a 96-well plate and incubated at 37°C for 1 hour and rinsed with a washing solution. 100 μl of the primary antibody working solution was added to each well, mixed, and incubated at 37°C. After 1 h, the plate was washed; 100 μl enzyme-labeled antibody working solution was added and incubated at 37°C. After 30 minutes, the plate was washed again; 100 μl of substrate working solution was added and reacted at 37°C in the dark. After 15 min, 100 μl of reaction stop solution was added and samples were further incubated at 37°C for 10 min. After the reaction was completed, the OD value was measured by a microplate reader, and a standard curve was drawn to determine the contents of TNF-α, IL-1β, IL-4, and IL-10 in the measured samples.

2.8. Double Luciferase Reporter Assay. The 3′-UTR fragments of the wild-type and mutant NLRP1 genes were amplified and inserted into a double luciferase reporter gene plasmid. The reporter gene plasmid and miR-181a-5p mimics were cotransfected into T293 cells and cultured. After 48 h, luciferase activity was measured.

2.9. Statistical Analysis. All experimental data in this study were expressed as x ± s. Analysis of data was performed using
SPSS 22.0. The *t*-test was used to compare the two groups, and the one-way analysis of variance was used to compare the multiple groups. *P* < 0.05 or *P* < 0.01 indicates that the difference is statistically significant.

### 3. Results

#### 3.1. Identification of Mn$_3$O$_4$ Nanoparticles and Detection of Their Cytotoxicity

Observed with a transmission electron microscope, the prepared Mn$_3$O$_4$NPs were spherical particles, as shown in Figure 1(a). The particle size distribution of Mn$_3$O$_4$NPs analyzed by laser particle size analyzer was about 7-10 nm, as shown in Figure 1(b). X-ray diffraction analysis results showed that the prepared Mn$_3$O$_4$NPs diffraction peaks are in good concordance with the Mn$_3$O$_4$ standard chart (JCPDS card No. 24-0734) and do not contain impurity peaks as shown in Figure 1(c), which was used in subsequent experiments. The results of the MTT assay showed that Mn$_3$O$_4$NPs had no significant toxicity to HT cells in the experimental range, as shown in Figure 1(d).

#### 3.2. Effects of Mn$_3$O$_4$ Nanoparticles on Inflammatory Factors in Human Tendon Cells Induced by LPS

ELISA results showed that the expression levels of TNF-$\alpha$ and IL-1$\beta$ in HT cells were significantly increased after LPS induction (*P* < 0.01), and the expression levels of IL-4 and IL-10 were significantly decreased (*P* < 0.01). Compared with the LPS group, Mn$_3$O$_4$NP treatment significantly reduced the expression levels of TNF-$\alpha$ and IL-1$\beta$ in cells (*P* < 0.05), and significantly increased the expression levels of IL-4 and IL-10 (*P* < 0.05) (Figure 2). Therefore, Mn$_3$O$_4$NP treatment significantly inhibited LPS-induced human tendon cell inflammation.

#### 3.3. Effects of Mn$_3$O$_4$ Nanoparticles on the Expression Level of Inflammation-Related miRNA in Human Tendon Cell-Induced LPS

RT-PCR results showed that Mn$_3$O$_4$NPs affected the expression level of inflammation-related miRNAs in HT cells induced by LPS, and the expression level of miR-181a-5p was significantly higher than that of the untreated group, as shown in Figure 3. The mechanism of miR-181a-5p affecting LPS-induced inflammation of human tendon cells was further explored in subsequent experiments.

#### 3.4. Targeting Relationship between miR-181a-5p and NLRP1

The StarBase database was used to predict the binding sites of NLRP1 and miR-181a-5p as shown in Figure 4(a). The results of the double luciferase experiment showed that miR-181a-5p mimics significantly reduced the luciferase activity of the NLRP1 wild-type vector (*P* < 0.05), but had no effect on the luciferase activity of the NLRP1 mutant vector (Figure 4(b)). In addition, Western blotting results showed that overexpression of miR-181a-5p significantly reduced the expression level of NLRP1 protein in LPS-induced human tendon cells.
induced HT cells \( (P < 0.05) \) (Figure 4(c)). From these results, it seemed that there is a targeting relationship between miR-181a-5p and NLRP1 in LPS-induced human tendon cells and miR-181a-5p downregulated the NLRP1 expression.

### 3.5. Mechanism of Mn\(_3\)O\(_4\) Nanoparticles Affecting LPS-Induced Inflammatory Factors in Human Tendon Cells

ELISA results showed that knockout of NLRP1 significantly reduced the expression levels of TNF-\( \alpha \) and IL-1\( \beta \) in HT cells induced by LPS \( (P < 0.01) \) and significantly increased the expression levels of IL-4 and IL-10 \( (P < 0.01) \). Compared with the NLRP1 knockout group, the treatment of NLRP1 knockout with Mn\(_3\)O\(_4\)NPs significantly reduced the expression levels of TNF-\( \alpha \) and IL-1\( \beta \) \( (P < 0.05) \) and at the time significantly increased the expression levels of IL-4 and IL-10 \( (P < 0.05) \). The expression levels of TNF-\( \alpha \) and IL-1\( \beta \) in the si-NLRP1+Mn\(_3\)O\(_4\)NPs+miR-181a-5p inhibitor group were higher than those in the si-NLRP1+Mn\(_3\)O\(_4\)NPs group \( (P < 0.05) \), and the expression levels of IL-4 and IL-10 were lower than si-NLRP1+Mn\(_3\)O\(_4\)NPs group \( (P < 0.05) \). Compared with the si-NLRP1+Mn\(_3\)O\(_4\)NPs+miR-181a-5p inhibitor group, the expression levels of TNF-\( \alpha \) and IL-1\( \beta \) were increased in the Mn\(_3\)O\(_4\)NPs+miR-181a-5p inhibitor group \( (P < 0.05) \), whereas IL-4 and IL expression levels were decreased \( (P < 0.05) \) (Figure 5). From the above experimental results, it seems that Mn\(_3\)O\(_4\)NPs upregulated miR-181a-5p in HT cells induced by LPS to downregulate the expression of NLRP1, thereby inhibiting LPS-induced HT cell inflammation.

### 4. Discussion

Tendonitis can cause abnormalities in the normal biological properties of tendons, such as thickening of the tendon and damage of the synovium, and the pain caused by it severely affects the daily activities of patients [7]. As the hands are the main organ for daily labor, the tendon of the hands is extremely susceptible to cumulative strain; therefore, hand tendinitis is also very common in clinical practice [8]. Local hormone injection is currently the most commonly used method of treating tendinitis, but studies have shown that local hormone injection has the risk of causing necrosis of
tendon collagen [9]. In recent years, with the in-depth study of nanoparticles, its important role in the treatment of inflammation-related diseases has also been confirmed [10].

Mn$_3$O$_4$NPs, as nanomaterials with enzyme-like activity, display good therapeutic potential in the treatment of inflammatory diseases. Studies have confirmed that Mn$_3$O$_4$NPs can functionally mimic superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX), and can significantly remove superoxide anion radicals and peroxidation hydrogen and hydroxyl radicals to protect cells from oxidative damage [11]. ROS imbalances including superoxide anion free radicals, hydrogen peroxide, and hydroxyl free radicals often occurred in inflammation. Therefore, Mn$_3$O$_4$NPs with high active oxygen scavenging activity and high stability have been shown to significantly relieve ROS-induced mouse ear inflammatory response [12]. However, there are few reports about Mn$_3$O$_4$NPs in the treatment of inflammation, and the research on its mechanism of action is lacking. The results of this study indicate that Mn$_3$O$_4$NPs significantly reduced the levels of proinflammatory-related proteins in human tendon cells induced by LPS and increased the levels of anti-inflammatory-related proteins to alleviate the inflammatory response.

MicroRNAs (miRNAs), as a class of noncoding single-stranded small RNAs composed of 19 to 22 nucleotides, can target mRNAs to degrade them or inhibit the translation

![Figure 3: Mn$_3$O$_4$ nanoparticles effect on the expression of inflammatory-related miRNA in human tendon cells induced by LPS.](image)

![Figure 4: Relationship between miR-181a-5p and NLRP1. (a) Binding sites between miR-181a-5p and NLRP1 predicted by StarBase. (b) The interaction of miR-181a-5p and NLRP1 determined by dual-luciferase reporter assay. (c) NLRP1 expression level measured by western blotting. *P < 0.01 vs. NC group.](image)
process, thereby regulating the physiological processes of cells [13]. Many studies have confirmed that the occurrence and development of inflammatory-related diseases are closely associated with miRNAs [14]. For instance, studies have shown that the expression level of miRNA-146a is positively correlated with the severity of the inflammatory response and can be used as an indicator of the inflammatory response disease activity [15]. Studies by Marques-Rocha et al. [13] showed that continuously upregulated miR-155 can also lead to sustained inflammatory responses. Among them, miR-181a has also been shown to be associated with inflammation. Studies have shown that miR-181a can significantly inhibit the expression of inflammatory factors IL-1β, IL-6, and TNF-α in macrophages induced by LPS [16]. In addition, miR-181a can also affect the myometrial inflammatory response by regulating the expression levels of ER-α and c-Fos [17]. Studies have also illustrated that miR-181a participates in the homeostatic response to inflammatory stimuli by regulating the TLR-4 signaling pathway [18]. The experimental results of this study also confirmed that miR-181a-5p is involved in regulating the expression of inflammatory factors in human tendon cells induced by LPS.

miRNAs play an important role in the occurrence and development of inflammatory diseases by binding to target genes and regulating the expression of target proteins related to inflammation. The association of NLRP1 with inflammation has been demonstrated [19]. NLRP1 is widely present in T cells, B cells, macrophages, and dendritic cells. When not stimulated, NLRP1 leucine-rich repeat-rich domains bind to the central nucleotide-binding oligomerization regions (NACHT), self-oligomerization is inhibited and in an inactive state. Upon stimulation, its domain changes, and it binds to proteins such as apoptosis-related speckle-like protein (ASC) and semi-aspartase (caspase-1) to form a protein complex called an inamasome, which can regulate the inflammatory response [20]. The proven proinflammatory factors including tumor necrosis factor-α (TNF-α) produce monocyte macrophages function in immune regulation, participate in fever and inflammation, and can further induce the production of other cytokines [21]. Interleukin-1β (IL-1β), as a pleiotropic factor, is the main mediator of the host’s response to infection or tissue damage [22]. And the immune factors, especially the imbalance of Th1/Th2 immune response, often occur in the inflammatory response, among which interleukin-4 (IL-4) and interleukin-10 (IL-10) produced by the Th2 subset of CD4+ T cells. IL-10 has been shown to increase expression levels in the inflammatory response [23]. Studies have demonstrated that in rat

**Figure 5:** Mechanism of MnO2 nanoparticles effect on the inflammatory factors levels in human tendon cells induced by LPS. (a) TNF-α level measured by ELISA. (b) IL-1β level measured by ELISA. (c) IL-4 level measured by ELISA. (d) IL-10 level measured by ELISA. **P < 0.01 vs. NC group; #P < 0.05 vs. si-NLRP1 group; △P < 0.05 vs. si-NLRP1+MnO2NPs group; ▽P < 0.05 vs. si-NLRP1+MnO2NPs+miR-181a-5p inhibitor group.
tendon cell inflammation models, stimulating factors can promote the activation of NLRP inflammasomes by disaggregating cytoskeleton F-actin, thereby increasing the expression and release of inflammatory factors TNF-α, IL-6, and IL-1β to aggravate the development of inflammation [24]. The results of this study indicate that miR-181a-5p downregulated NLRP1 expression in human tendon cells induced by LPS, and knocking out NLRP1 significantly alleviated the inflammatory response of human tendon cells induced by LPS.

The results of this study indicate that Mn₃O₄NP treatment can significantly reduce the levels of proinflammatory factors TNF-α and IL-1β in human tendon cells induced by LPS and increase the levels of IL-4 and IL-10. Further research confirmed that Mn₃O₄NPs upregulated miR-181a-5p in human tendon cells induced by LPS, and by doing that downregulated the expression of NLRP1 thus alleviating the LPS-induced human tendon cell inflammation.

Data Availability

All the data are available with the handwritten notebook documented in our lab.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

Acknowledgments

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