NICORANDIL MITIGATES ARTHROGENIC CONTRACTURE INDUCED BY KNEE JOINT EXTENSION IMMOBILIZATION IN RATS: INTERFERENCE WITH RHOA/ROCK SIGNALING AND TGF-β1/SMAD PATHWAY

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Abstract

Objective: Prolonged immobilization often results in myogenic and arthrogenic contractures, with capsular fibrosis being the critical aspect of the latter. This study aimed to determine whether nicorandil can mitigate joint contracture and fibrosis by inhibiting the RhoA/ROCK signaling pathway, thereby affecting the TGF-β1/Smad signaling pathway.

Materials and Methods: This study used a rat model of knee extensor joint immobilization to assess the impact of nicorandil on arthrogenic contracture and joint capsule fibrosis. Sixty Sprague–Dawley rats were divided into control, immobilization, and nicorandil treatment groups with varying durations of immobilization. The extent of arthrogenic contracture was assessed using joint mobility metrics, while joint capsule fibrosis was quantified using Masson staining, α-SMA immunohistochemistry, and H&E staining. The protein expression levels of the TGF-β1/Smad and RhoA/ROCK signaling pathways were analyzed by western blotting. Additionally, lysophosphatidic acid (LPA), a specific ROCK activator, was used to further investigate the involvement of RhoA/ROCK signaling in TGF-β1/Smad pathway modulation.

Results: This study revealed a positive correlation between the duration of immobilization and the severity of arthrogenic contracture, joint capsule fibrosis, and inflammatory infiltration. Nicorandil administration effectively reduced these immobilization-induced changes and concurrently inhibited the activation of TGF-β1/Smad and RhoA/ROCK signaling pathways. Application of LPA counteracted the inhibitory effects of nicorandil on these pathways.

Conclusion: Nicorandil significantly alleviated arthrogenic contracture and joint capsule fibrosis resulting from knee extension immobilization. The underlying mechanism appears to involve inhibition of the TGF-β1/Smad signaling pathway, mediated by RhoA/ROCK signaling pathway suppression.

Keywords: nicorandil, arthrogenic contracture, fibrosis, TGF-β1/Smad signaling pathway, RhoA/ROCK signaling pathway.

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Introduction

Regardless of whether the injury is sports-related or traumatic, the knee is often treated according to the POLICE principle: Protest, Optimal Loading, Ice, Compression, and Elevation. Prolonged braking of the knee is one of the most important causes of joint contractures. In the early stages of immobilization, myogenic contractures occur first, followed by arthrogenic contractures, which are contractures of the joint capsule, articular cartilage, ligaments, and other tissues (Zhang et al., 2023). It is usually more difficult to intervene effectively in arthrogenic contractures. The pathological mechanism of arthrogenic contracture involves fibrosis of the joint capsule. Fibrosis has been described as excessive proliferation of fibroblasts and myofibroblasts, deposition of extracellular matrix, increased content of collagen fibers, and in some studies, the presence of lymphocyte infiltration (Huang et al., 2021).

Myofibroblasts are activated after tissue injury. These cells produce an extracellular matrix that facilitates repair. If they do not undergo apoptosis after successful repair, myofibroblasts persistently produce excess matrix, leading to extensive scarring and fibrosis. Tissue fibrosis can be effectively alleviated by inhibiting myofibroblast activation.
(Yu et al., 2021). Therefore, chronic fibrotic lesions are characterized by the persistence of myofibroblasts. Rho-GTPase and ROCK are major signaling molecules in the Rhoa/ROCK pathway. ROCK activation leads to the formation of F-actin stress fibres, decreasing the abundance of G-actin and exposing the nuclear localisation sequence of Myocardin-Related Transcription Factor (MRTF). This leads to nuclear accumulation of MRTF, where it can bind to serum response factor (SRF) and induce gene expression (Tsou et al., 2014). Whereas multiple target genes of MRTF/SRF are known drivers of fibrosis (Hanna et al., 2009; Luchinger et al., 2011; Mack et al., 2001). Studies have shown that the absence of ROCK2 in cardiac fibroblasts leads to cardiac hypertrophy and reduced fibrosis (Shimizu et al., 2017).

ROCK1 was shown to play a more important pro-fibrotic role than ROCK2 in knockout ROCK1 and haploid ROCK1 mic (Sunamura et al., 2014). Previous literatures have shown that inhibition of the Rhoa/Rock signalling pathway can attenuate pulmonary and renal fibrosis (Jiang et al., 2012; Komers, 2013). Lyosphosphatidic acid (LPA) promotes tissue fibrosis by activating TGF/β1 via a Rhoa/ROCK-dependent pathway (Zhou et al., 2021), leading to the release of TGF-β and subsequent activation of downstream signalling pathways. It has been demonstrated that the expression levels of TGF-β1, Rhoa, and ROCK1 are all significantly increased in rats with uterine adhesions and decreased in human Wharton’s glial MSCs. This also confirms that the Rhoa/ROCK signaling pathway is involved in fibrosis of uterine epithelial cells (Li et al., 2021).

Nicorandil (2-nicotinamideethyl nitrate) is an ATP-sensitive potassium channel opener, and it has been proven safe in animal experiments (Garnier-Raveaud et al., 2002; Lee et al., 2008). In rats with myocardial infarction, Lee et al. (Lee et al., 2008) found that nicorandil significantly slowed cardiomyocyte hypertrophy and the area of cardiac fibrosis. In addition to inhibiting myocardial fibrosis, the antifibrotic effects of nicorandil have been discovered in other tissues, such as lung tissues (El-Kashef, 2018; Kseibati et al., 2020) and hepatic tissues (Abdel-Sattar et al., 2020). Mohamed et al., 2018). The molecular mechanism by which nicorandil attenuates fibrosis has not been elucidated. In a rat model of myocardial infarction, nicorandil inhibited Rhoa translocation and inactivated ROCK, and it enhanced the activation of M2 macrophages, which reduced the infiltration of myofibroblasts in the infarcted area and collagen accumulation (Lee et al., 2018). Given the functions of nicorandil established in previous studies, we hypothesized that nicorandil treatment effectively attenuates and slows the progression of arthrogenic fibrosis by inhibiting the Rhoa/ROCK signaling pathway and modulating the TGF-β1/Smad signaling pathway.

Methods

Animals and Grouping

Sixty skeletally mature male Sprague-Dawley rats (Experimental Animal Center of Anhui Medical University, Hefei, China, 3 months old, weighing 250-300 g) were used in this experiment. The rats were housed in standard cages (room temperature 24 °C-25 °C, 12 h light/dark cycle) for 2 weeks, with four rats per cage, and the rats had free access to food and water. The experimental protocol was divided into two parts. In the first part, test a, 42 rats were randomly assigned to a control group (group C, rats without any intervention, n = 6), a nicorandil group (groups 1-1w(a), 1-2w(a), and 1-4w(a) for 1, 2, and 4 weeks of immobilization, respectively, with saline gavage during the immobilization period, n = 6), and a nicorandil-treated group (groups N-1w(a), N-2w(a), and N-4w(a), respectively, were immobilized for 1, 2, and 4 weeks of nicorandil gavage during immobilization, n = 6) using random number table method. In the second part, test b, 18 rats were randomly assigned to an immobilization group (1-4w(b) group, 4 weeks of immobilization; n = 6), a nicorandil-treated group (N-4w(b) group, using nicorandil by gavage for 4 weeks of immobilization, n = 6), or a nicorandil-treated combined LPA group (N+LPA group, using nicorandil by gavage and concurrently injecting LPA intraperitoneally for 4 weeks of immobilization; n = 6) using random number table method (Fig. 1a,b).

For the experimental procedure, the rats were anesthetized in batches by intraperitoneal injection of 40 mg/kg sodium pentobarbital. Subsequently, the left knee joint of the rats was placed in a fully extended position and immobilized using the support method previously used in the experimental group (Zhou et al., 2023) (Patent No. 02120470158.0) (Fig. 2A). The immobilization time was determined as indicated in the previous grouping instructions. The rats were observed daily for loosening of the immobilization device and health status to make timely adjustments. During the immobilization period, the N-1w(a), N-2w(a), N-4w(a), and N-4w(b) groups were subjected to nicorandil gavage at 3 mg/kg once a day. For comparison, groups 1-1w(a), 1-2w(a), 1-4w(a), and 1-4w(b) were subjected to immobilization for the corresponding period, and saline gavage was used. To further verify the involvement of Rhoa/ROCK in the fibrotic process, rats in the N+LPA group received LPA, a ROCK activator, 40 µg/kg intraperitoneally (Davies et al., 2017) via once-daily gavage of nicorandil (3 mg/kg) (Mohamed et al., 2018).

Measurement of Joint Contracture

Rats in each group were euthanized by intraperitoneal injection of 10 % chloral hydrate 400 mg/kg after an adequate time of immobilization. In test a, the left hind limb of the rats was disarticulated and completely excised from the left hip joint. As in previous studies (Zhou et al., 2023), we measured knee range of motion (ROM) at this point using a knee measurement tool (Patent No. ZL20212099643.1).
Fig. 1. Experimental Groups and Intervention Methods. (a) Design of trial a in this study. (b) Design of test b in this study.

Subsequently, the muscles around the knee joint were excised, the intact knee capsule structure was preserved, and ROM was measured again. Two study members without knowledge of subgroups conduct the measurements of ROM. The degree of knee contracture was defined using the following formula (Fig. 2B,C):

Total contracture angle = (control knee) pre-myotomy ROM – (contracted knee) pre-myotomy ROM.

Arthrogenic contracture angle = (control knee) post-myotomy ROM – (contracted knee) post-myotomy ROM.

Tissue Preparation

At the end of the experimental cycle, the rats were euthanized. The excised anterior capsule of the rat knee was cut into small portions and used to prepare the joint capsule homogenates and histopathological sections. A portion of the anterior joint capsule tissue was homogenized at 1000 × g, centrifuged at 4 °C for 15 min, and the supernatant was collected and stored at -80 °C for determination of TGF-β1, RhoA, ROCK1, and p-MLC. Some of the anterior joint capsule samples were immediately fixed in 10% neutral buffered formalin solution in saline for 24 h. They were rinsed with tap water and then dehydrated with graded concentrations of ethanol (50% ~ 100%). The specimens were cleared in xylene and embedded in paraffin in a hot air oven at 56 °C for 24 h. Waxy paraffin nectar tissue blocks were prepared using a slide slicer and cut to a thickness of 4-5 µm.

Masson Staining

The degree of joint capsule fibrosis was assessed using Masson staining. Staining was performed using a Masson Staining Solution Kit (Servicebio, Wuhan, Hubei, China). Paraffin sections were dewaxed, dehydrated, immersed in Masson A solution overnight, rinsed under running tap water, placed in an equal mixture of Masson B and Masson C solutions, washed with water, and then differentiated using a differentiation solution. Massons D and E were used for staining and rinsing, respectively. Masson F was used for staining. The sections were dehydrated with anhydrous ethanol, made transparent with xylene for 5 min, and sealed with neutral gum. The sections were observed under a microscope (BX43F; Olympus, Tokyo 163-0914, Japan) at 400× magnification, and six fields of view were randomly selected and photographed. Collagen deposition was expressed as a percentage of the blue area and analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Rockville, MD, USA).

Immunohistochemistry

The degree of joint capsule fibrosis was evaluated using immunohistochemistry. The paraffin sections were de-waxed and dehydrated for antigenic autoclave repair. Next, 2 L of citrate repair solution (Zsbio, Beijing, China) was prepared, and the sections were placed in an autoclave and boiled for 2 min for hot repair. The sections were incubated for 20 min at room temperature by drawing a circle around the tissue with an immunohistochemistry pen and adding 3% H2O2 dropwise to the tissue. Sections were rinsed three
Fig. 2. **Animal model and joint mobility of rats.** (A) Animal model. The left knee joint of the rat was immobilized in full extension using a shaped aluminum splint. (B) The left hind limb of each rat was immobilized (before muscle incision) on the arthrometry meter. The handle was then turned (maintaining a constant torque of 0.053 Nm) and the range of motion of the left knee joint before and after myotomy was read from the scale on the dial. (C) The left hind limb of each rat was immobilized (after myotomy) on the arthrometry measuring instrument. The handle was then turned (maintaining a constant torque of 0.053 Nm) and the range of motion of the left knee joint before and after myotomy was read from the scale on the dial. (D) Total joint range of motion and arthrogenic contracture of rats in each group. (E) Comparison of total joint mobility of rats in each group. (F) Comparison of arthrogenic contracture in rats in each group.

\( \text{Total contracture}^{(*)} \quad \text{Arthrogenic contracture}^{(*)} \)

<table>
<thead>
<tr>
<th>Grouping</th>
<th>Quantity</th>
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<th>Arthrogenic contracture</th>
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<tr>
<td>C</td>
<td>6</td>
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<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>I-W(a)</td>
<td>6</td>
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<td>N-W(a)</td>
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<tr>
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<td>20.42 ± 1.49**</td>
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<tr>
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<td>43.75 ± 2.14**</td>
<td>16.06 ± 1.82**</td>
</tr>
<tr>
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<td>6</td>
<td>77.44 ± 17.86*</td>
<td>47.94 ± 2.86**</td>
</tr>
<tr>
<td>N-4W(a)</td>
<td>6</td>
<td>68.63 ± 2.39**</td>
<td>41.36 ± 2.64**</td>
</tr>
</tbody>
</table>

\( ^{*} p < 0.05 \text{ vs. group C}, \) \( ^{(*)} p < 0.05 \text{ vs. group N-1w(a)}, \) \( ^{**} p < 0.05 \text{ vs. group N-2w(a)}, \) \( ^{***} p < 0.05 \text{ vs. group I-1w(a)}, \) \( ^{****} p < 0.05 \text{ vs. group I-2w(a)}, \) \( ^{*****} p < 0.05 \text{ vs. group I-4w(a)} \)

times using PBS, after which the sections were shaken dry and incubated overnight at 4 °C with rabbit anti-α-SMA antibody (1:100; Affinity Biosciences). The sections were washed and incubated with secondary antibodies at 37 °C for 30 min. DAB colorant was added, the color development time was controlled under a microscope (with positive termination of color development), and the sections were rinsed in distilled water and stained with hematoxylin and lithium carbonate. It was then made transparent with xylene and sealed with neutral gum. Observations were performed under a microscope (BX43F; OLYMPUS, Tokyo 163-0914, Japan) at 400× magnification, and five fields of view were randomly selected and photographed. Finally, the percentage of positive cells in the entire field of view was calculated for each picture using Image-Pro Plus 6.0 software as an indicator of their positive expression.

**H&E Staining**

The total cell count and inflammatory cell infiltration of the joint capsule were assessed by H&E staining. Paraffin sections were deparaffinized and dehydrated using hematoxylin (Baso, Zhuhai, Guangdong, China) staining solution for 3-5 minutes and blued using dilute lithium carbonate for 30 s. The sections were dehydrated with ethanol and stained using eosin staining solution (Baso, Zhuhai, Guangdong, China) for 10-30 seconds. It was made transparent using xylene and sealed with neutral gum. Observations were performed under a microscope (BX43F; Olympus, Tokyo 163-0914, Japan) at 400× magnification, and six fields of view were randomly selected and photographed. The cells in the joint capsule consist mainly of myofibroblasts and inflammatory cells, which are distinguished by morphological identification. We counted these different types of cells together to briefly show the inflam-
mation and fibrosis of the joint capsule, and the cells were analyzed using Image-Pro Plus 6.0.

Western Blotting

The protein expression levels of TGF-β1, Smad2/3, p-Smad2/3, α-SMA, Collagen I, RhoA, ROCK1, and p-MLC were analyzed using western blotting. The supernatant of the prepared joint capsule homogenate was used to determine the protein concentration using a bicinchoninic acid (BCA) protein quantification kit (Beyotime, Shanghai, China). Next, equal amounts of protein from each pair of samples were separated by SDS–PAGE on a 10% polyacrylamide gel and electrotransferred to a polyvinylidene difluoride microporous membrane, which was then blocked in 5% skim milk before being dissolved in TBST and incubated for 2 h at room temperature. Membranes were incubated overnight at 4°C with various primary antibodies. On the second day, the membranes were incubated with secondary antibody for 45 min at room temperature and then washed three times with TBST solution. Finally, ECL chemiluminescent droplets were added to the target band, and the signal was detected with a digital imaging device. The density of each band was quantified using Image-Pro Plus software (version 6.0). TGF-β1, Smad2/3, p-Smad2/3, α-SMA, Collagen I, RhoA, ROCK1, and p-MLC proteins were calculated by comparing them with the amount of GAPDH as a loading control.

Data Analysis

The results are expressed as the mean ± standard deviation. Statistical analyses were performed using IBM SPSS Statistics version 26 (IBM Corp., Armonk, NY, USA). In test a, One-way ANOVA was used for within-group comparisons in groups N(N-1w(a), N-2w(a), and N-4w(a)) or I(I-1w(a), I-2w(a), and I-4w(a)). T-test was used to compare the immobility groups and nicorandil-treated groups with the control group. At the same time point during immobilization, T-test were used for intergroup comparisons between the groups N and groups I. For test b, between-group comparisons were performed using an unpaired t test. Statistical significance was set at p < 0.05.

Results

None of the rats died during the immobilization period. No prolonged edema or acute inflammation was observed in the hind limbs of the rats.

Arthrogenic Contracture

The results of test a showed that the total degree of contracture and arthrogenic contracture increased with increasing immobilization time in groups N(a) and I(a) (p < 0.05) (Fig. 2D-F). At the same time point, there was a significant increase in arthrogenic contracture in group I(a) compared to group N(a) (p < 0.05, Fig. 2F). Thus, nicorandil had a good therapeutic effect on immobilization-induced arthrogenic contractures.

H&E Staining of the Joint Capsule

To investigate the anti-inflammatory effect of nicorandil, we performed H&E staining of the joint capsule. The results of H&E staining and quantitative analysis showed that the total cell number and inflammatory cell infiltration tended to increase with increasing immobilization time in both group I(a) and group N(a) (p < 0.05). In test a, at the same time point, the total cell count and inflammatory cell infiltration were significantly reduced in group N(a) compared to those in group I(a) (p < 0.05) (Fig. 3A,B). These results suggest that nicorandil attenuates the immobilization-induced inflammatory response.

Joint Capsule Fibrosis

To study the effect of nicorandil on fibrosis of the joint capsule, we performed Masson staining and western blotting for collagen I on the joint capsule. As shown in the figure, the collagen area and collagen I level tended to increase significantly in both group I(a) and group N(a) as the immobilization time increased (p < 0.05). During the 4-week immobilization period, the collagen area and collagen I increased to different degrees in both group I(a) and group N(a). At the same time point, the increased area of collagen and elevated collagen I were more obvious in group I(a) than in group N(a) (p < 0.05) (Fig. 4A,B, Fig. 5A,C). To further determine the severity of fibroblast differentiation into myofibroblasts in the joint capsule, we examined the immunohistochemical and protein levels of α-SMA in the joint capsule. As shown, α-SMA expression tended to increase significantly in both the I(a) and N(a) groups with increasing immobilization time (p < 0.05). During the 4-week immobilization duration, the percentage of cells expressing α-SMA increased to different degrees in both group I(a) and group N(a); at the same time point, the percentage of cells expressing α-SMA was more pronounced in group I(a) than in group N(a) (p < 0.05, Fig. 6A,B). The results of the WB assay of α-SMA protein in the rat joint capsule showed the same results as above (Fig. 5A,B). In conclusion, the oral administration of nicorandil has a protective effect against immobilization-induced fibrosis in rats.

The Expression Level of the TGF-β1/Smad Signaling Pathway

The TGF-β1/Smad signaling pathway includes TGF-β1, Smad2/3, p-Smad2/3, collagen I, and α-SMA. The expression levels were increased in both the N-4w(b) and I-4w(b) groups compared to the control group C (p < 0.05) (Fig. 5D-H). But the expression level of the TGF-β1/Smad signaling pathway was significantly reduced in the N-4w(b) group compared with the I-4w(b) group (p < 0.05) (Fig. 5D-H). However, the TGF-β1/Smad signaling pathway expression level was significantly higher in the N+LPA group.
Fig. 3. H&E staining. (A) Results of H&E staining of the joint capsule. The scale bars indicate 50 µm (left panels) and 20 µm (right panels). (B) Quantitative analysis of total cell number in each group. Data are expressed as mean ± standard deviation. (*p < 0.05 vs. group C, †p < 0.05 vs. group N-1w(b), ‡p < 0.05 vs. group N-2w(a), §p < 0.05 vs. group I-1w(a), ‡p < 0.05 vs. group I-2w(a), *p < 0.05 vs. group I-1w(a), †p < 0.05 vs. group I-2w(a), ‡p < 0.05 vs. I-4w(b) group).

than in the N-4w(b) group. In summary, nicorandil inhibited the protein expression level of the TGF-β1/Smad signaling pathway in the joint capsule to a certain extent, whereas LPA counteracted the inhibitory effect on the TGF-β1/Smad signaling pathway in the joint capsule.

The Expression Level of the RhoA/ROCK1 Signaling Pathway

The RhoA/ROCK signaling pathway includes the RhoA, ROCK1, and p-MLC proteins. Total RhoA protein did not show significant changes in any of the groups (Fig. 5D,J). The expression levels of ROCK1 and p-MLC proteins were increased in both the N-4w(b) and I-4w(b) groups compared with the control group C (p < 0.05) (Fig. 5D,I,K). However, ROCK1 and p-MLC protein expression levels were suppressed in the N-4w(b) group compared with those in the I-4w(b) group (p < 0.05) (Fig. 5D,I,K). This indicated that nicorandil inhibited activation of the RhoA/ROCK signaling pathway in the joint capsule during immobilization. However, ROCK1 and p-MLC proteins were significantly higher in the N+LPA group than in the N-4w(b) group (p < 0.05) (Fig. 5D,I,K). Thus, LPA effectively reversed RhoA/ROCK activation induced by nicorandil in the joint capsule during immobilization.
Fig. 4. Masson staining. (A) Masson staining results of the joint capsule, field of view. The scale bars indicate 50 µm (left panels) and 20 µm (right panels). (B) Quantitative analysis of the percentage of collagen deposition (blue area) in each group. Data are expressed as mean ± standard deviation. (*p < 0.05 vs. group C, †p < 0.05 vs. group N-1w(a), ‡p < 0.05 vs. group N-2w(a), §p < 0.05 vs. group I-1w(a), ¶p < 0.05 vs. group I-2w(a), ††p < 0.05 vs. group I-1w(a), †‡p < 0.05 vs. group I-2w(a), †§p < 0.05 vs. group I-2w(a), †¶p < 0.05 vs. I-4w(a) group).

Discussion

In the present study, we investigated the effect of nicorandil on arthrogenic contracture induced by immobilization of the rat knee at several levels, including morphological, histological, and molecular levels. Recent studies have shown that nicorandil inhibits TGF-β1-induced collagen secretion and ECM deposition during chronic fibrosis in a variety of tissues (Abdel-Aziz et al., 2023; El-Kashef, 2018; Kseibati et al., 2020). Here, we demonstrated that nicorandil can inhibit arthrofibrosis via the RhoA/ROCK-mediated TGF-β1/Smad signaling pathway during knee immobilization (Fig. 7), suggesting that nicorandil has therapeutic potential as a new therapeutic agent for arthrogenic contractures.

Joint immobilization is often unavoidable as the most common treatment after an injury occurs. Immobilization usually takes one month or more after a fracture or ligament injury. During the first period of immobilization, atrophy and fibrosis of the periarticular muscles occur, followed by ligament shortening, collagenous hyperplasia and fibrosis (Huang et al., 2021; Zhou et al., 2023). Previous studies have confirmed that 4 weeks of free movement after the re-
lease of joint immobilization does not completely reverse joint mobility restriction (Liu et al., 2022). Further studies have found that residual joint contractures after free movement are dominated by contractures of arthrogenic origin (Hu et al., 2023). Therefore, the application of new therapeutic strategies during immobilization is of great significance in improving joint contractures.

We evaluated arthrogenic mobility of the rat knee joint in the functional assessment of this study. We observed that the joint mobility of rats in group I(a) was less than that of rats in group C, which is consistent with the results of other studies in our group (Wang et al., 2023b). The joint capsule is an important component of the joint and is crucial for maintaining stability during joint movement (Wang et al., 2023a). Therefore, thickening of the joint capsule due to contracture can lead to reduced joint mobility. We observed that at the same time point during immobilization, knee mobility was higher in all N(a) groups than in the I(a) groups. Thus, nicorandil reduced the degree of capsular contracture caused by joint immobilization. In the histological evaluation, the results of Masson staining and immunohistochemistry for SMA further confirmed these functional
Fig. 6. Immunohistochemistry of α-SMA. (A) Immunohistochemical results of joint capsule α-SMA. The scale bars indicate 50 µm (left panels) and 20 µm (right panels). (B) Quantitative analysis of the percentage of α-SMA positive cells in each group. Data are expressed as mean ± standard deviation. (*p < 0.05 vs. group C, †p < 0.05 vs. group N-1w(a), ‡p < 0.05 vs. group N-2w(a), †′p < 0.05 vs. group I-1w(a), ‡′p < 0.05 vs. group I-2w(a), a*p < 0.05 vs. group I-1w(a), b*p < 0.05 vs. group I-2w(a), c*p < 0.05 vs. group I-4w(a))

results. Masson staining showed that the area of collagen fibers was larger in groups I(a) and N(a) than in group C. The collagen fiber area increased significantly over time in the I-2w(a) and I-4w(a) groups. However, after intervention treatment with nicorandil, the collagen fiber area in group N(a) was smaller than that in group I(a) at the same time point of immobilization. This suggests that nicorandil slows the development of fibrosis by inhibiting the excessive deposition of fibrous capsular connective tissue. This is consistent with the findings of Deng et al. (Deng et al., 2022). As expected, histological assessment of α-SMA immunohistochemistry showed similar results. The proportion of α-SMA in group I(a) was significantly higher than that in group C and showed a time-dependent progression to 4 weeks after immobilization. After intervention with nicorandil, the proportion of α-SMA gradually decreased and reached its maximum at four weeks post-immobilization. This is in general agreement with the results of previous studies (Abdel-Aziz et al., 2023; Abdel-Sattar et al., 2020).

Nicorandil also has potent anti-inflammatory and antioxidant properties. As in a previously conducted study (Kseibati et al., 2020), the levels of lymphocytes, neu-
Fig. 7. Schematic diagram of nicorandil to attenuate immobilization-induced joint capsule fibrosis.

trophils, malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH) were significantly increased in the lung tissues of rats that had been orally administered bleomycin (BLM). Nicorandil significantly attenuated the activity of these compounds. In the unilateral ureteral obstruction (UUO) rat model of renal fibrosis, the levels of TNF-α and IL6 were significantly elevated in renal tissues. However, these levels were attenuated after treatment with nicorandil (Abdel-Aziz et al., 2023). In another study, silica was used to induce lung fibrosis in rats. They found that nicorandil effectively resolved oxidant/antioxidant disorders by downregulating iNOS (El-Kashef, 2018). We observed that the number of inflammatory cells was higher in rats in group I(a) than in those in group C, and nicorandil significantly attenuated the increase in inflammatory cells. Chronic inflammation in tissues ultimately leads to the proliferation and activation of fibroblasts into myofibroblasts, excessive deposition of collagen fibers, interstitial fibrosis, and the appearance of scar tissue (Della Latta et al., 2015).

Our results showed that TGF-β1/Smad signaling was significantly enhanced in the articular capsule of the rat knee joint after immobilization. Activation of TGF-β1/Smad pathway signaling leads to fibrosis of the knee joint capsule. Nicorandil intervention reduced the activation of the above pathway. TGF-β1 is a cytokine that maintains homeostasis of the immune system. It is involved in coordinating tissue repair after tissue injury. When intracellular mechanical resistance is elevated to a certain level, TGF-β1 is further activated and promotes fibroblast activation in myofibroblasts (Lodyga and Hinz, 2020). Smad2/3 is a major downstream target. Smad signaling is a key mechanism involved in this process. The p-Smad2/3 complex enters the nucleus to regulate downstream proteins. Consistent with our expectations, we found that the levels of both TGF-β1 and p-Smad2/3 were elevated in I-4w(b) compared to those in Group C. After nicorandil treatment, TGF-β1 and p-Smad2/3 levels were significantly lower in group N-4w(b).

The integration of molecular signaling between TGF-β1/Smad and Rhoa/ROCK has been studied. Previous studies have found that TGF-β1 induces profound activation of RhoA, which further generates actin stress fibers and promotes α-SMA expression. Interestingly, the RhoA inhibitor Y27632 inhibited TGF-β1-induced α-SMA expression but had no effect on Smad signaling (Sandbo et al., 2011). Li et al. (2021) found that TGF-β1 promotes migration and induces cytoskeletal remodeling of primary rat hepatic stellate cells (HSCs) through chemotaxis and haptotaxis mechanisms, which are based on the mechanism of increased RhoA activity (Li et al., 2012). TGF-β1 also regulates F-actin formation through Rhoa/ROCK signaling. Inhibition of the Rhoa signaling pathway with Y27632, a ROCK inhibitor, resulted in a significant decrease in the expression of α-SMA and Collagen I in TGF-β1-treated HSC-T6 cells (Moon et al., 2019). Downregulation of ROCK expression also reduces cardiac fibrosis (Sawada and Liao, 2014). The pharmacological effects of nicorandil may be mediated through Rhoa/ROCK signaling. It has been demonstrated (Lee et al., 2017) that nicorandil
stimulates macrophage differentiation toward the M2 phenotype through RhoA/ROCK signaling and ultimately attenuates cardiac fibrosis. Considering the involvement of RhoA/ROCK signaling, we evaluated the molecules in the RhoA/ROCK signaling pathway and their phosphorylation levels. In the present study, no significant difference in total RhoA levels was observed between the groups in test b. However, the protein levels of ROCK1 and p-MLC, the downstream products of the RhoA/ROCK pathway, were higher in group I-4w(b) than in group C, suggesting that RhoA/ROCK signaling was activated after immobilization, whereas the levels of ROCK1 and p-MLC were significantly lower in group N-4w(b) than in I-4w(b).

To further verify whether nicorandil exerted an antifibrotic effect through the RhoA/ROCK signaling pathway, we conducted additional experiments. In this trial, we treated rats with LPA (a ROCK activator), which effectively promoted RhoA/ROCK activation and reversed the antifibrotic effects of nicorandil. The protein expression level of the TGF-β1/Smad signaling pathway was inhibited to some extent in the N+LPA group compared with the I-4w(b) group. However, inhibition of the TGF-β1/Smad signaling pathway was much weaker in the N+LPA group than in the experimental group using nicorandil alone. Previous studies have demonstrated that nicorandil attenuates cardiac, pulmonary, hepatic, and renal fibrosis (Abdel-Sattar et al., 2020; Deng et al., 2022; Harb et al., 2021; Harb et al., 2023) by lowering TGF-β1 and/or inhibiting Smad2/3 phosphorylation. In N-4w(b), we found that various fibrosis indicators, such as collagen proliferation area, α-SMA, and TGF-β1, were significantly inhibited. After LPA intervention, all the above indicators were reversed to different degrees. However, we did not evaluate the expression levels of fibrosis-related genes due to the limitations of the experimental conditions.

It is necessary to acknowledge the limitations of this study. Before the experiment officially began, we allowed six normal rats to take nicorandil orally for up to 4 weeks, during which time there was no significant decrease in blood pressure (BP-2010A, Softron Biotechnology, Beijing, China) (Fig. 8). But we did not monitor blood pressure in immobilized rats. In addition, we did not test the bioavailability, pharmacokinetics, and toxicity of nicorandil. These will be the research directions of our team in the future. In subsequent studies, we will conduct further research on the above aspects.

**Conclusion**

In summary, treatment with nicorandil can significantly attenuate extension immobilization-induced arthrofibrosis, and the mechanism may be related to Rhoa/ROCK signaling-mediated inhibition of the TGF-β1/Smad signaling pathway. Therefore, in diseases requiring long-term joint immobilization, nicorandil may be used to prevent arthrofibrosis.
List of Abbreviations

LPA, lysosphatidic acid; MRTF, Myocardin-Related Transcription Factor; SRF, serum response factor; ROM, range of motion; BCA, bicinchoninic acid; MDA, malondialdehyde; SOD, superoxide dismutase; GSH, glutathione; BLM, bleomycin; UUO, unilateral ureteral obstruction; HSCs, hepatic stellate cells.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the first author on reasonable request. All data generated or analyzed during this study are included in this published article. The manuscript, including related data, figures and tables have not been previously published and are not under consideration elsewhere.

Author Contributions

XML conceived the study, participated in its design and coordination, and drafted the manuscript. KW participated in the design, assisted with animal management, performed animal experiments, and helped draft the manuscript. ML performed animal experiments and molecular studies, and assisted with data statistics and analysis, participated in the drafting of the manuscript. QBZ performed the molecular studies, organized the data, and assisted in the drafting of the manuscript. YZ participated in the design and co-sequencing, and reviewed the final manuscript. All authors read and approved the final manuscript.

Ethics Approval and Consent to Participate

Animal care and experimental procedures were performed in accordance with the Guidelines for Animal Experimentation of Anhui Medical University and were approved by the Institutional Animal Care and Use Committee (LLSC20221126).

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Conflict of Interest

The authors declare no conflict of interest.

Appendix

See Fig. 8.

References


**Editor’s note:** The Scientific Editor responsible for this paper was Denitsa Docheva.