Therapeutic Effect and Mechanism of Jipei Dilong Ointment on Acute Soft Tissue Injury in Rats

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Abstract [**Objectives**] This study was conducted to observe the therapeutic effect of Jipei Dilong Ointment on rats with acute soft tissue injury caused by heavy objects and to explore its action mechanism. [**Methods**] Thirty six rats were randomly divided into six groups (control group, model group, high-dose Jipei Dilong Ointment group (JP-H), medium-dose Jipei Dilong Ointment group (JP-M), low-dose Jipei Dilong Ointment group (JP-L) and diclofenac group). Except for the Control group, other groups were subjected to modeling of acute soft tissue injury by the weight impact method. All administration was performed once a day for nine consecutive days. The local appearance score and activity disorder score were determined after soft tissue injury in rats. HE staining was used to detect the pathological changes of injured soft tissues in rats. RT-PCR was used to detect the relative mRNA expressions of Bax, Bcl-2, MMP-9 and TIMP-1 in injured soft tissues of rats. Western Blot was used to detect the protein expressions of MMP-9, TIMP-1, TLR4, MyD88 and NF-κB p65 in injured soft tissues of rats. Results were statistically analyzed. [**Results**] Compared with the model group, Jipei Dilong Ointment could significantly improve the appearance symptoms such as swelling and ecchymosis in the injured area and the movement function of the affected limb ($P<0.05$). It could also improve the infiltration of inflammatory cells and widening of the intermuscular space caused by injury. Among them, the JP-H group and the diclofenac group had more significant curative effects. After 9 d of administration, each administration group could significantly up-regulate the ratio of Bcl-2/Bax mRNA expression level ($P<0.05$ or $P<0.01$), and the ratio of MMP-9/TIMP-1 mRNA expression level showed a downward trend ($P>0.05$). The expression level of NF-κB p65 protein in each administration group was significantly decreased ($P<0.01$). The protein expression levels of TLR4 and MyD88 and the ratio of MMP-9/TIMP-1 protein expression level in each administration group decreased to varying degrees. Among them, the JP-H group and diclofenac group significantly decreased ($P<0.05$). [**Conclusions**] Jipei Dilong Ointment has the functions of relieving pain, swelling and inflammation. It could improve the local appearance, functional activity and tissue morphology of affected limbs in rats, and has a therapeutic effect on acute soft tissue injury in rats. Its mechanism of action might be related to the inhibition of TLR4/MyD88/NF-κB p65 signaling pathway and the regulation of Bcl-2/Bax and MMP-9/TIMP-1 balance.

Key words Jipei Dilong Ointment; Acute soft tissue injury; NF-κB p65; TLR4; MyD88

DOI:10.19759/j.cnki.2164-4993.2023.05.008

Acute soft tissue injury is a common disease in which muscles, tendons and other tissues are subjected to direct or indirect violence, resulting in symptoms such as local injury, edema, and bleeding in the body[1-4]. After soft tissue injury, microcirculation disorders and aseptic inflammation[5-6] occur, causing local swelling and pain, seriously affecting people's quality of life, and it has become a widely existing public health problem. At present, Western medicine often uses oral non-steroidal anti-inflammatory drugs and other methods to treat acute soft tissue injury. Although the therapeutic effect is significant, there are often side effects such as gastrointestinal dysfunction and hepatorenal toxicity, which limit the clinical application of Western medicine. However, Shui ethnic medicine has significant clinical efficacy due to its unique advantages. The classic formula of Shui ethnic medicine, Jipei Dilong Ointment, is a commonly used folk medicine in the treatment of acute soft tissue injury in ethnic minority areas. Initially, it was widely used in clinical practice at Aishan Hospital in Sandu Shui Autonomous County. It is composed of 10 herbs, namely chicken embryos, earthworms, Dipsaci Radix, Drynariae Rhizoma, Herba seu Radix Gaultheriae Yunnanensis, root bark of *Toricellia angulata* Olinv, Fructus Gardeniae, Giant Knotweed Rhizome, preparead Strychni Semen, and borneol. Jipei Dilong Ointment has the effects of promoting blood circulation and resolving blood stasis, reducing swelling and pain, relaxing muscles and activating collaterals, and is widely used in clinical practice for diseases such as soft tissue injury[8]. However, research on its pharmacodynamics is still relatively blank. Therefore, this study aimed to observe the therapeutic effect of Jipei Dilong Ointment on acute soft tissue injury at the animal level, and explore its specific molecular mechanism, hoping to provide experimental basis for the clinical treatment of acute soft tissue injury with Jipei Dilong Ointment.

Received: July 25, 2023 Accepted: September 27, 2023

Supported by National Key R&D Plan (2019YFC1712500); Guizhou Provincial Science and Technology Planning Project (QKHHBZ [2020] 3003); On-campus Project of Guizhou University of Traditional Chinese Medicine (2018YFC170810520).

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Materials and Methods

Materials and instruments

Animals Thirty six healthy SPF SD rats, weighing 180 - 220 g,

half male and half female, were provided by Changsha Tianqin Biotechnology Co., Ltd., under production license number: Scxk (Xiang) 2019-0013. The rats were subjected to adaptive feeding for one week, and kept in animal feeding rooms that complied with relevant regulations. The animals were kept in separate cages by male and female, at room temperature of 18 – 25 ℃ and relative humidity of 50% – 70%. The feeding rooms had sufficient lighting, normal ventilation, and regular disinfection and replacement of bedding.

Medicines Jipei Dilong Ointment (composed of 10 herbs, namely chicken embryos, earthworms, Dipsaci Radix, Drynariae Rhizoma, Herba seu Radix Gaultheriae Yunnanensis, root bark of *T. angulata* Olinv, Fructus Gardeniae, Giant Knotweed Rhizome, preparead Strychni Semen, and borneol), provided by Aishan Hospital Co., Ltd. of Sandu Shui Autonomous County, batch number: 20200901; Diclofenac Cream (batch number: B82X/SV3R, GSK Consumer Healthcare Schweiz AG).

Main reagents RIPA lysis buffer (Strong) (batch number: 21062202, Biosharp); prestained protein Maker (batch number: 01062055, Thermo); HiScript II One Step qRT-PCR SYBR Green Kit (batch number: 7E220E8, Vazyme); SDS-PAGE gel parparation kit (batch number: 35421), BCA protein assay kit (batch number: 32521), HRP/Rabbit (batch number: 03802/33620), SDS-PAGE loading buffer (batch number: 01411), high-sensitivity ECL detection kit (batch number: 35521), Ultrapure RNA Kit (batch number: 50250), all purchased from Cowin Bio.; bovine serum albumin (batch number: 1218Q054, Beijing Solarbio Science & Technology Co., Ltd.); TLR4 (batch number: 20211210) and TIMP-1 (batch number: 20211210), both purchased from Bioswamp company; MyD88 (batch number: GR3356289-12), NF-κB p65 (batch number: GR3275776-13), GAPDH (batch number: GR3316865-11) and MMP9 (batch number: GR3399016-12), all purchased from abcam company.

Main instruments Rotary cutting machine (Leica 2016, Leica, Germany); microplate reader (ZS-2, Beijing Xinfeng Electromechanical Company); Desktop high-speed freezing centrifuge (Thermo ST16R, Thermo Fisher, USA); upright fluorescence microscope (DM500, Leica, Germany); microplate plate constant temperature oscillator (ST60-4, Hangzhou Miulab Instrument Co., Ltd.); real time fluorescence quantitative analyzer (QuantStudio 1, Applied Biosystems, USA); electrophoresis apparatus (EPS-300, Shanghai Tanon Technology Co., Ltd.); protein electrophoresis tank (Mini PROTEAN Tetra Electrophoresis System) and protein transfer system (Mini Trans Blot), both purchased from BIO-RAD company; gel imager (4600SF, Shanghai Tanon Technology Co., Ltd.).

Methods

Modeling and grouped administration Rats were randomly divided into a blank control group (control), a model control group (model), high- (JP-H), medium- (JP-M) and low-dose (JP-L) Jipei Dilong Ointment groups, and a diclofenac group, with 6 rats in each group. Except for the control group, other groups were subjected to modeling of acute soft tissue injury caused by striking with heavy objects.

Before modeling, the hind limbs of rats were depilated with a shaver and 8% sodium sulfide solution. After anesthetizing rats with chloral hydrate (0.3 g/kg), each rat's right hind leg was fixed on a striking platform. A PVC hollow tube with a height of 140 cm and an inner diameter of 2.8 cm was vertically placed above the muscle-abundant part of the right hind leg (the bottom of the hollow tube was about 1 – 2 cm away from the striking part), and the striking part was covered with a layer of gauze. A 50 g weight was placed on the top of the hollow tube, which then fell freely to strike for 8 times continuously. After the models were established, the injured part showed ecchymosis, swelling and scattered bleeding points, but fracture and skin damage were not observed, indicating that models were successfully made.

After successful modeling, from the next day, the control group and model group were given 70% ethanol (containing 5% glycerol) onto the affected part, and the JP-H group, JP-M group and JP-L group were given 4.5, 1.5 and 0.5 g crude drug/kg of Jipei Dilong Ointment, respectively, and the diclofenac group was given 1.0 g/kg of diclofenac cream. Each drug was given once a day and fixed with a bandage, 90 min each time, for 9 d consecutively.

Sample collection After 9 d of administration, each rat was euthanized by taking blood from the abdominal aorta. The damaged central tissue was taken and divided into two parts, one of which was fixed with 4% paraformaldehyde, and the other was stored in an ultra-low temperature refrigerator at –80 ℃ for later use.

Scoring on local appearance after soft tissue injury in rats The local conditions of rats after injury were observed on the first day and the ninth day after modeling, and scored for comparison. The scoring criteria were as follows: 4 points for surface bleeding, congestion or edema greater than 2 cm^2, 3 points for 1 – 2 cm^2, 2 points for 0 – 1 cm^2, and 1 point for complete recovery and normal tissue.

Scoring on activity disorder after soft tissue injury in rats The movement degree of rats after injury was observed on the first day and the ninth day after modeling, and scored for comparison. The scoring criteria were as follows: 4 points for obvious limp, 3 points for non-obvious limp and obvious changes in movement state, 2 points for slight changes in movement state, and 1 point for good movement condition.

HE staining detection of histopathological changes in rats with soft tissue injury due to Jipei Dilong Ointment Injured soft tissues of rats were soaked in 4% paraformaldehyde and fixed for at least 24 h. After tissue fixation, dehydration, wax impregnation, embedding and sectioning were performed. The sections were put into xylene, and gradient ethanol sequentially for dewaxing. HE staining, gradient ethanol dehydration, xylene transparentizing, air drying and neutral balsam sealing were performed in sequence, and histological morphological changes were observed under a microscope and photographed.

RT-PCR detection of relative mRNA expression levels of Bax, Bcl-2, MMP-9 and TIMP-1 in rats with soft tissue injury Frozen damaged soft tissues from rats were taken and weighed for an appropriate amount of each tissue, which was added with TRIzon Reagent, and homogenized with a homogenizer to extract total RNA from the injured soft tissues. According to the instructions of the kit, total RNA was reverse transcribed into cDNA, which was subjected to RT-PCR reaction. Reaction conditions were as follows: reverse transcription at 55 ℃ for 5 min, pre-denaturation at 95 ℃ for 5 min, and 45 cycles of 95 ℃ for 10 s and 60 ℃ for 30 s. Data were monitored and recorded using a real-time fluorescence quantitative PCR instrument. With GAPDH as the internal reference, the relative mRNA expression level of each target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers are shown in Table 1.

Table 1 Primers and base sequences used in RT-PCR

Primer	Forward (5'-3')	Reverse (5'-3')	Size of product//bp
Bax	GGTGGTTGCCCTTTTCTACTTTGC	GCTCCCGGAGGAAGTCCAGTG	113
Bcl-2	GGGCTACGAGTGGGATACTGGAG	CGGGCGTTCGGTTGCTCT	101
MMP9	CCCACTTACTTTGGAAACG	GAAGATGAATGGAAATACGC	228
TIMP-1	GCCATGGAGAGCCTCTGTGG	GCAGGCAGGCAAAGTGATCG	310
GAPDH	CAAGTTCAACGGCACAG	CCAGTAGACTCCACGACAT	138

Western blot detection of protein expression of MMP-9, TIMP-1, TLR4, MyD88 and NF-κB p65 in rats with soft tissue injury Frozen damaged soft tissues from rats were taken and weighed for an appropriate amount of each tissue, which was added into an EP tube, which was then added with a lysis solution according to a ratio of 1 ∶ 10 (g/ml). Each mixture was homogenized in an ice bath and centrifuged at 4 ℃ and 10 000 rpm for 20 min. The supernatant was extracted and measured for protein concentration by the BCA method, and prepared into a protein sample. Next, preparation of gel (10% separation gel, 5% concentration gel), loading, electrophoresis and membrane transfer were performed in sequence, and the products were incubated with 5% BSA at room temperature for 60 min. Next, TLR4(1 ∶ 1 000), MyD88(1 ∶ 1 000), NF-κB p65(1 ∶ 10 000), MMP-9(1 ∶ 1 000) and TIMP-1(1 ∶ 1 000) primary antibodies were added, and incubation was conducted at 4 ℃ overnight, followed by washing with TBS-T for three times. Next, anti-Rabbit HRP-linked secondary antibody (1 ∶ 200) was added for incubation at room temperature for 60 min, followed by washing with TBS-T for 3 times. An ECL chemiluminescent solution was developed, and a gel imaging system was used for observation and photographing. With GAPDH as the internal reference, the grayscale values of protein bands were calculated using Gel-Pro analyzer4 image analysis software.

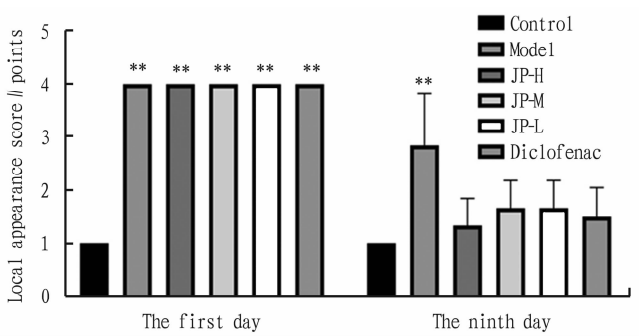
Relative expression level of each target protein = Grayscale value of target protein band/Grayscale value of GAPDH band

Statistical processing The data were expressed as mean ± standard deviation, and statistical analysis was conducted using SPSS 26.0 statistical software. The comparison of means between groups was conducted using one-way analysis of variance (ANOVA). If normality was not observed, non-parametric analysis was used. $P<0.01$ or $P<0.05$ stood for statistical significance. Bar charts were drawn using GraphPad Prism 8.0.1 software.

Results and Analysis

Effects of Jipei Dilong Ointment on local appearance score after soft tissue injury in rats

According to the experimental results, on the first day, compared with the control group, the local appearance scores of various groups significantly increased ($P<0.01$); and compared with the model group, other treatment groups showed no significant differences in ($P>0.05$). On the ninth day, the local appearance score of the model group ($P<0.01$) was still significantly higher than that of the control group; and compared with the model group, each administration group showed a downward trend ($P>0.05$). Overall, with the increase of drug intervention and administration days, the local appearance of each group gradually improved. By the ninth day, except for the model group, there were no significant differences between other groups and the control group, as shown in Fig. 1.



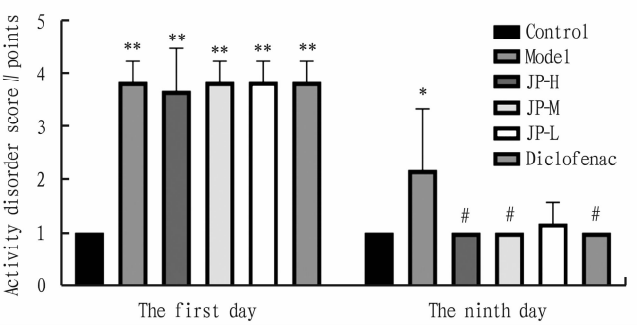
Compared with the Control group, $^{*}P<0.05$, $^{**}P<0.01$; compared with the Model group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

Fig. 1 Effects of Jipei Dilong Ointment on local appearance score after soft tissue injury in rats ($n=6$)

Effects of Jipei Dilong Ointment on activity disorder score after soft tissue injury in rats

From the experimental results, it could be seen that on the first day, compared with the Control group, the scores of activity disorder in various groups were significantly higher ($P<0.01$); and compared with the Model group, there were no significant differences in other treatment groups ($P>0.05$). On the ninth day, the activity disorder score of the model group was still significantly higher than that of the Control group ($P<0.05$); and compared with the Model group, various administration groups all showed a downward trend, with significant reductions in the JP-H

group, JP-M group and diclofenac group ($P < 0.05$). Overall, with the increase of drug intervention and administration days, the activity disorder in each group gradually improved. By the ninth day, except for the model group, there were no significant differences between other groups and the control group, as shown in Fig. 2.



Compared with the Control group, $^{*}P < 0.05$, $^{**}P < 0.01$; compared with the Model group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$.

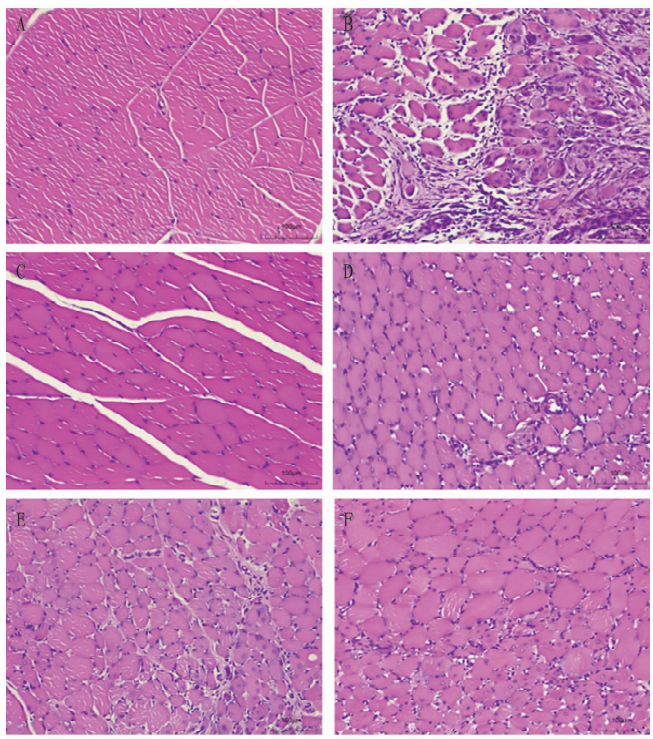
Fig. 2 Effects of Jipei Dilong Ointment on activity disorder score after soft tissue injury in rats ($n = 6$)

Effects of Jipei Dilong Ointment on histopathology of soft tissue injury in rats

From the tissue sections, it could be seen that after 9 d of administration, the cells in the Control group were regular and tidy, with consistent intermuscular space, and no significant pathological changes were observed. In the Model group, a large number of inflammatory cells were infiltrated and the intermuscular space was significantly widened. Compared with the Model group, various administration groups were improved to different degrees, especially in the JP-H group and diclofenac group, of which the JP-H group only showed a small amount of infiltrated inflammatory cells, and the intermuscular space was basically normal, as shown in Fig. 3.

Effects of Jipei Dilong Ointment on relative mRNA expression levels of Bax, Bcl-2, MMP-9 and TIMP-1 in injured soft tissues of rats

The experimental results showed that after 9 d of administration, compared with the Control group, the relative mRNA expression level of Bax in the Model group increased significantly ($P < 0.05$), while the relative mRNA expression level of Bcl-2 and the ratio of Bcl-2/Bax decreased significantly ($P < 0.01$); the relative mRNA expression level of MMP-9 increased and that of TIMP-1 decreased ($P > 0.05$); and the ratio of MMP-9/TIMP-1 increased significantly ($P < 0.05$). Compared with the model group, various drug administration groups showed a downward trend in the relative mRNA expression level of Bax ($P > 0.05$); the relative mRNA expression level of Bcl-2 increased significantly in the JP-H group and diclofenac group ($P < 0.01$), and it also increased in the JP-M group and JP-L group ($P > 0.05$); the ratio of Bcl-2/Bax increased significantly in various administration groups ($P < 0.05$ or $P < 0.01$); the relative mRNA expression level of MMP-9 and the ratio of MMP-9/TIMP-1 both showed a downward trend in various administration groups ($P > 0.05$); and the expression level of TIMP-1 mRNA exhibited no significant differences in various drug administration groups ($P > 0.05$), but an increasing trend was observed in the JP-H group, JP-M group, and diclofenac group. The details are shown in Fig. 4.

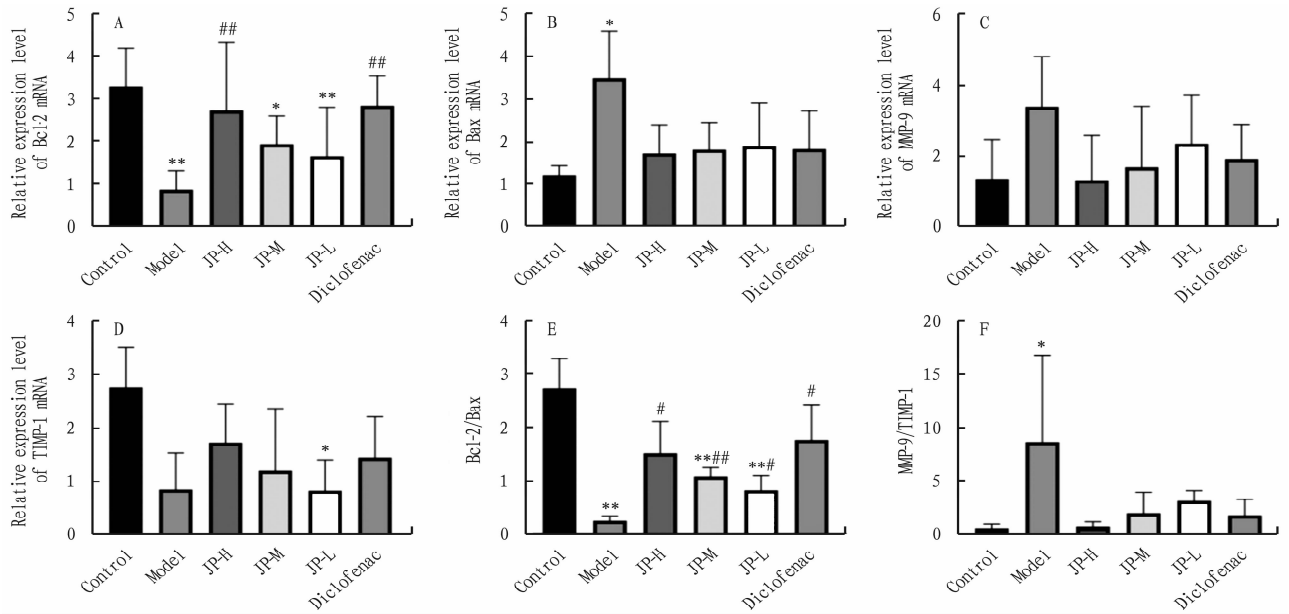


A: Control group; B: Model group; C: JP-H group; D: JP-M group; E: JP-L group; F: Diclofenac group.

Fig. 3 HE staining results of sections from injured soft tissues in rats ($\times 200$)

Effects of Jipei Dilong Ointment on protein expression levels of MMP-9, TIMP-1, TLR4, MyD88 and NF-κB p65 in injured soft tissue of rats

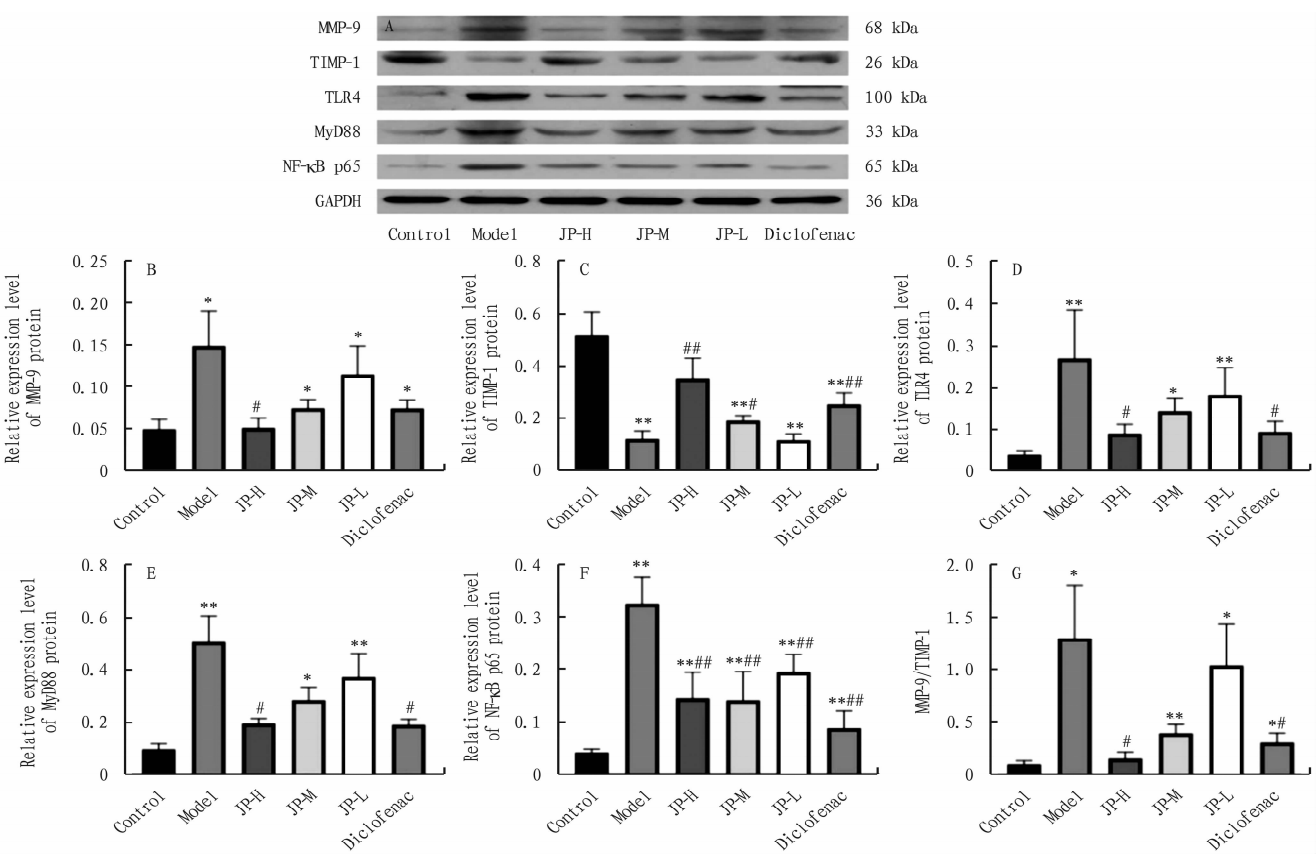
According to the experimental results, after 9 d of administration, compared with the Control group, the relative protein expression levels of MMP-9, TLR4, MyD88 and NF-κB p65 and MMP-9/TIMP-1 ratio in the Model group significantly increased ($P < 0.05$ or $P < 0.01$), while the relative expression level of TIMP-1 significantly decreased ($P < 0.01$). Compared with the model group, the JP-H group and diclofenac group showed significantly-decreased relative protein expression levels of TLR4 and MyD88 ($P < 0.05$), and those in the JP-M group and JP-L group showed a downward trend ($P > 0.05$); the relative protein expression level of NF-κB p65 decreased significantly in various drug administration groups ($P < 0.01$); the relative protein expression level of MMP-9 in the JP-H group decreased significantly ($P < 0.05$), and other drug administration groups showed a downward trend ($P > 0.05$); the relative protein expression levels of TIMP-1 in the JP-H group, JP-M group and diclofenac group increased significantly ($P < 0.05$ or $P < 0.01$), but there was no significant change in the JP-L group ($P > 0.05$); and the values of MMP-9/TIMP-1 in the JP-H group and diclofenac group significantly decreased ($P < 0.05$), and those in the JP-M group and JP-L group showed a downward trend ($P > 0.05$). The results are shown in Fig. 5 in detail.



A: Relative expression level of Bcl-2 mRNA; B: Relative expression level of Bax mRNA; C: Relative expression level of MMP-9 mRNA; D: Relative expression level of TIMP-1 mRNA; E: Bcl-2/Bax ratio; F: MMP-9/TIMP-1 ratio.

Compared with the control group, $^{*}P<0.05$, $^{**}P<0.01$; compared with the model group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

Fig. 4 Effects of Jipei Dilong Ointment on relative expression levels of Bax, Bcl-2, MMP-9 and TIMP-1 mRNA in injured soft tissues of rats ($n=6$)



A: Protein expression bands of MMP-9, TIMP-1, TLR4, MyD88 and NF-κB p65 in injured soft tissues of rats in various groups; B: relative expression level of MMP-9 protein; C: relative expression level of TIMP-1 protein; D: relative expression level of TLR4 protein; E: relative expression level of MyD88 protein; F: relative expression level of NF-κB p65 protein; G: MMP-9/TIMP-1 ratio.

Compared with the control group, $^{*}P<0.05$, $^{**}P<0.01$; compared with the model group, $^{\#}P<0.05$, $^{\#\#}P<0.01$.

Fig. 5 Effects of Jipei Dilong Ointment on expression levels of MMP-9, TIMP-1, TLR4, MyD88 and NF-κB p65 proteins in injured soft tissue of rats ($n=6$)

Conclusions and Discussion

Acute soft tissue injury is a common clinical disease of bone injury, which often occurs in daily life, and the incidence rate is on the rise with the accelerated pace of life and improper exercise[9]. Its pathological process is closely related to inflammatory reactions[10]. After soft tissue injury, aseptic inflammatory reactions will occur, and there will be increased release of inflammatory mediators such as histamine and accumulation of acid products, which will make local microcirculation change, capillary permeability increase, vascular tension decrease, and blood flow slow down and even stagnate, which further aggravates inflammatory reaction[11-14], and the course of disease also develops from initial local edema to inflammatory adhesion and fibrous tissue proliferation, eventually leading to fibrosis[15]. Clinically, the main manifestations are local swelling, ecchymosis, pain and dysfunction[1-4]. Jipei Dilong Ointment, a classic prescription of Shui ethnic medicine, is a commonly used medicine for the treatment of acute soft tissue injury in minority areas, and clinical research shows that it has a definite effect[8]. It is composed of 10 herbs, namely chicken embryos, earthworms, Dipsaci Radix, Drynariae Rhizoma, Herba seu Radix Gaultheriae Yunnanensis, root bark of Toricellia angulata Olinv, Fructus Gardeniae, Giant Knotweed Rhizome, preparead Strychni Semen, and borneol. Jipei Dilong Ointment has the effects of promoting blood circulation and resolving blood stasis, reducing swelling and pain, relaxing muscles and activating collaterals[8]. Among the herbs, Drynariae Rhizoma can improve the injury of bones and muscles and blood stasis[16], and has anti-inflammatory effects[17]; the root bark of *T. angulata* Olinv has the effects of promoting blood circulation and removing blood stasis, and can improve subcutaneous bleeding and blood stasis[18]; earthworms can promote blood circulation, remove blood stasis and relieve swelling and pain[19], and have anti-inflammatory and anti-fibrosis activity[20]; Giant Knotweed Rhizome can remove blood stasis and relieve pain[21], and has anti-inflammatory, anti-oxidation and angiogenesis-promoting activity[22]; Fructus Gardeniae can relieve swelling and pain[23] and has anti-inflammation activity[24]; borneol has anti-inflammatory activity[26]; and chicken embryos, a commonly used medicine in the Shui nationality, can replenish qi and essence and promote granulation[27], and they can lead the medicines directly to affected areas and play a role[8]. In this study, rats with acute soft tissue injury caused by heavy impact showed symptoms such as edema, congestion, and claudication, which were consistent with the clinical manifestations of acute soft tissue injury; and the tissue sections showed pathological changes such as inflammatory cell infiltration and enlargement of intermuscular space. Compared with the model group, after treatment with Jipei Dilong Ointment, the affected limbs of rats showed significant relief in congestion, edema, limb function, and pathological changes such as inflammatory cell infiltration and enlarged intermuscular space were also significantly improved. It indicated that Jipei Dilong Ointment had the effects of improving the local appearance, functional activity and tissue morphology at the affected part of acute soft tissue injury.

Current research has shown that after tissue damage, endogenous molecules such as extracellular matrix degradation products, fatty acids and heat shock proteins (damage-related molecular patterns) will be released[28]. TLRs are a type of pattern recognition receptors located on the surface of cells, which can participate in innate and adaptive immunity[29]. Meanwhile, TLRs can recognize pathogen-related molecular patterns and damage-related molecular patterns, thereby mediating MyD88-dependent or non-dependent signaling pathways and playing an important role in regulating inflammatory response[30-31]. MyD88 is a bridging protein that can mediate the signaling of TLRs and activate downstream NF-κB[32]. NF-κB is an important transcription factor involved in the expression of various inflammatory mediators. After soft tissue injury, TLRs are stimulated and can bind to MyD88 to activate NF-κB, so that NF-κB protein (such as NF-κB p65) becomes free and activated and transfers from the cytoplasm to the nucleus to bind with corresponding inflammatory genes and initiate the transcription of inflammatory cytokines, to thereby release a series of pro-inflammatory factors, exacerbate the inflammatory response of damaged tissues and lead to tissue swelling and increased pain. Yan *et al.*[33] found that Huoluo Xiaoling Dan could inhibit the expression of TLR2 and NF-κB protein in injured soft tissue of rats, down-regulate the expression of pro-inflammatory factors such as IL-1, up-regulate the expression of IL-10, and improve local symptoms of rats. In addition, studies have shown that TLR4 plays an important role in the initiation of inflammation after peripheral tissue damage[34]. Therefore, inhibiting the expression of TLRs, MyD88 and NF-κB can regulate the occurrence and development of inflammatory reaction and relieve the symptoms of acute soft tissue injury. The results of this study showed that Jipei Dilong Ointment could significantly inhibit the expression levels of TLR4, MyD88 and NF-κB p65 proteins in injured soft tissues of rats, suggesting that Jipei Dilong Ointment might alleviate the symptoms of acute soft tissue injury in rats by inhibiting TLR4/MyD88/NF-κB p65 signaling pathway.

The course of acute soft tissue injury is also closely related to the excessive apoptosis of damaged cells[35]. Bax and Bcl-2 are closely related to apoptosis, as the former promotes apoptosis and the latter inhibits apoptosis, and they are antagonistic to each other. Studies have shown that the ratio of Bcl-2/Bax can measure whether cells are apoptotic or not, and the increase of the ratio of Bcl-2/Bax can inhibit cell apoptosis, and while the opposite promotes cell apoptosis[36]. Xiao[37] found that Zhengshi Fumigation and Washing Medicine I could increase the content of Bcl-2 and reduce the content of Bax, thereby inhibiting cell apoptosis, promoting proliferation and facilitating the reconstruction and repair of injured local tissues in rats with acute soft tissue injury. The results of this study indicated that Jipei Dilong Ointment could increase Bcl-2 mRNA expression, inhibit Bax mRNA expression, and significantly increase Bcl-2/Bax ratio, suggesting that Jipei Dilong Ointment might promote the repair of injured tissues by inhibiting excessive cell apoptosis.

MMPs are a type of proteases that can degrade extracellular matrix and basement membrane, with MMP-9 mainly degrading type IV collagen fibers[38]. TIMPs are natural specific inhibitors of MMPs, of which TIMP-1 can bind to MMP-9 and specifically

inhibit its activity[39]. Muscle fibers are wrapped in a layer of basement membrane composed of type IV collagen as an important component[40-41]. Under the action of MMP-9, the basement membrane and extracellular matrix degrade, and the integrity of muscle fibers is disrupted, leading to an increase in inflammatory cell infiltration and exacerbation of inflammatory reactions. In addition, studies have shown that an imbalance of MMP-9/TIMP-1 can cause metabolic disorders in the extracellular matrix, and an increase in MMP-9/TIMP-1 ratio promotes extracellular matrix degradation, while the opposite inhibits extracellular matrix degradation[42]. Therefore, regulating the balance of MMP-9/TIMP-1 may help alleviate inflammatory symptoms of acute soft tissue injury. The results of this study showed that Jipei Dilong Ointment could downregulate the expression level of MMP-9 protein, upregulate the expression level of TIMP-1 protein, and significantly reduce the ratio of MMP-9 protein expression level/TIMP-1 protein expression level; and there was also a corresponding regulatory trend towards the expression levels of MMP-9 and TIMP-1 mRNA, and the ratio of MMP-9 mRNA expression level/TIMP-1 mRNA expression level. It indicated that Jipei Dilong Ointment might regulate the balance of MMP-9/TIMP-1 by inhibiting the overexpression of MMP-9, thereby reducing inflammatory cell infiltration and promoting the repair of damaged tissues.

In summary, Jipei Dilong Ointment has analgesic, anti-inflammatory and other effects, which can improve the local appearance, functional activity of affected limb, and tissue morphology in rats. It has a therapeutic effect on acute soft tissue injury in rats, and its mechanism of action may be related to the inhibition of TLR4/MyD88/ NF-κB p65 signaling pathway and the regulation of the balance of Bcl-2/Bax and MMP-9/TIMP-1.

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Editor: Yingzhi GUANG Proofreader: Xinxiu ZHU

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