Integration Traditional Chinese Medicine and Nibble Debridement and Dressing Method Reduce Thrombosis and Inflammatory Response in the Treatment of Thromboangiitis Obliterans

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Research

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Abstract

Background: Thromboangiitis obliterans (TAO), also known as Buerger's disease, is an occlusive arterial disease. However, the pathogenesis of TAO is still unclear. Research has shown that traditional Chinese medicine (TCM) has significant advantages in the treatment of TAO. Our purpose is to explore the underlying roles of TCM in combination with nibble debridement and dressing method (NDDM) in TAO rat model.

Methods: 10 mg/ml sodium laurate was utilized to establish a TAO rat model, and then the TAO mode rats were treated with notoginseng powder (NP), maifusheng (MFS) or the combination of NP or MFS and NDDM. Ganrene's classification and, blood rheology was evaluated; the pathological characteristics of rat limbs were examined by H&E staining and Masson staining; CD3+ and CD20+ levels were measured by immunohistochemistry and flow cytometry. In addition, inflammation-associated cytokines were analyzed by RT-qPCR, Western blot and ELISA assays.

Results: ntegration NP or MFS and NDDM dramatically reduced the ganrene's classification and affected blood rheology parameter of TAO model rats compared with NP and MFS alone. Meanwhile, NP or MFS in combination with NDDM decreased CD3+CD20+T cells, reduced thrombosis and inflammatory cell infiltration, and dramatically decreased the levels of inflammation-associated cytokines.

Conclusion: Our results suggested that integration NP or MFS and NDDM could relieve the symptoms of TAO model rats induced by sodium laurate, which might provide new management strategy for TAO.

Introduction

Thromboangiitis obliterans (TAO) is a chronic occlusive disease characterized by segmental, non-suppurative inflammation, and intravascular thrombus formation[1]. TAO mainly invades the limbs, especially the small and medium arteries and veins of the lower limbs, and then causes the ischemic changes in the distal side of the affected limb[2]. At present, TAO mainly presents as limb ischemia, pain, intermittent claudication, migrating phlebitis, extremity gangrene, secondary infection, and even disabling[3, 4]. Studies have indicated that the pathogenesis of TAO is associated with a number of factors, such as smoking, infection, immune response, genetic inheritance, antiphospholipid antibody, hyperhomocysteinemia, cold, trauma, and malnutrition, etc[5–7]. Current treatments for TAO include application of vasodilators, improvement of microcirculation, antibiotics, glucocorticoids, traditional Chinese medicine (TCM), surgical or interventional surgery, etc[8–10]. However, the treatment of TAO is ineffective and recurrent. Although the short-term curative effect of surgical treatment is good, the surgery can only improve certain blood circulation in the limbs and cannot change the process of arterial autoimmune response, so re-occlusion will occur with the extension of postoperative time. Therefore, it is urgent to explore the effective therapies for TAO.

Notoginseng powder (NP) is a representative medicine of blood circulation drugs[11]. Modern pharmacology researches indicated that NP has multiple clinical effects, such as hemostasis (small dosage), anticoagulant (large dosage), blood tonic (long-term medication), suppression of inflammatory cells, cholesterol-lowing, treatment of arrhythmia, inhibition of tissue and organ fibrosis, tumor adjuvant treatment, and prevention of aging, etc[11–13]. NP also has an important pharmacological property, that is, long-term moderate oral administration without any drug toxicity[11]. However, the underlying therapeutic effect and mechanism of NP on TAO remain largely undetermined.

Maifusheng (MFS), serves as a Chinese herbal water preparation, often is used to treat arteriosclerosis obliterans[14]. Among them, beautiful millettia root, prepared rhizome of rehmannia, caulis spatholobi have the effects of reinforcing kidney, nourishing the blood and promoting blood circulation; oldenlandia, Anemone raddeana Regel and Shinyleaf Pricklyash Root have the effects of clearing away heat and toxic materials, expelling wind-damp and activating collaterals[15]. Modern pharmacological studies also confirmed that Caulis Spatholobi can reduce blood lipid, promote total phosphorus metabolism in kidney and resist atherosclerosis; oldenlandia has the functions of immunoregulation, anti-tumor, anti-bacteria and anti-oxidation; the triterpenoid saponins in Anemone raddeana Regel has the effects of anti-tumor, anti-inflammation, anti-rheumatism, antipyretic analgesia, sedation, anti-convulsion[14]. However, the therapeutic effect of MFS on TAO remains unclear.

Researches increasingly suggested that nibble debridement and dressing method (NDDM) can effectively promote the healing of ulcer surface[16–19]. Studies have indicated that NDDM in combination with traditional Chinese medicine (TCM) could significantly improve the syndrome of diabetic foot ulcer[20]. However, it is not entirely clear for the role of integration NP or MFS and NDDM on the treatment of TAO.

In the present study, sodium laurate was used to induce TAO model, and the mechanism and therapeutic effects of NP or MFS in combination with NDDM on the symptoms of TAO were further investigated.

Methods

Animals

A total of 48 healthy male Wistar rats were provided by Guangzhou Hospital of Traditional Chinese Medicine, Guangzhou (Guangdong, China). According to the national standard, all rats were raised for 7 days before the experiment, and the appropriate environment were as follows: enough food and water ad libitum, temperature of 20-24°C, humidity of 50-60%, lights period from 8:00 AM to 8:00 PM. All the animal protocols have also been approved by the Animal Ethics Committee of Guangzhou Hospital of Traditional Chinese Medicine.

Establishment of TAO model

As described in previous research[21], 48 male Wistar rats were randomized into two groups: sham group (n=8) and TAO model group (n=40). The rats were anesthetized by intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg, Sigma-Aldrich, Cat# P-010). After the rats were fixed in the supine position, the inner femur of the left lower limb was shorn and skinned to expose femoral artery. Blood flow was blocked by artery clipping, the femoral artery in the sham group was injected with 0.1 ml normal saline, and the femoral artery in the TAO model group was injected with 0.1 ml sodium laurate solution (10 mg/ml). After 20 mins of injection, the artery clamp was removed and the wound has been stitched up.

Grouping

The rats were divided into sham group (n=8), TAO group (n=8), TAO+NP group (n=8), TAO+MFS group (n=8), TAO+NP+NDDM group (n=8) and TAO+MFS+NDDM group (n=8). Sham group (healthy rats, administered saline alone, 2 ml/day for 7 d); TAO group (TAO model rats, dministered saline alone, 2 ml/day for 7 d); NP group (TAO model rats, notoginseng powder, 2 ml/day for 7 d, lavage administration; the ulcer was washed and bandaged with 0.2% Anerdian); MFS group (TAO model rats, maifusheng, 4 ml/day for 7d, lavage administration; the ulcer was washed and bandaged with 0.2% Anerdian); NP+NDDM group (TAO model rats, notoginseng powder, 2 ml/day for 7 d, lavage administration; the ulcer was treated by nibble debridement and dressing method); MFS+NDDM group (TAO model rats, maifusheng, 4 ml/day for 7 d, lavage administration; the ulcer was treated by nibble debridement and dressing method). Rats were fed and observed for 14 days after gavage, blood was collected from the orbits on day 1, day 7 and day 14, at the same time, partial serum was isolated on the 14th day. The experimental rats were put to death in a CO_2 euthanasia chamber, all rats were killed and got the muscles on the inside of hind legs at 14 day. The tissues were fixed with 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany, Cat. #1.04005).

Classification of gangrene

According to the grading standard, level 0: normal; level Ⅰ, the ganrene is confined to the toenail; level Ⅱ: the ganrene is confined to the toes; level Ⅲ: the ganrene is confined to the podoculi; level Ⅳ, the ganrene exceeds the ankle joint[22]. Three time points (1 day, 7 days and 14 days) were recorded after intragastric administration.

Hemorheology detection

Blood samples were collected from the eye socket of rats at day 1, day 7, and day 14 after intragastric administration, and put into EDTA-containing Vacutainer (BD, Franklin Lakes, NJ), respectively. Blood platelet count (BPC), white blood cell count (WBC), fibrinogen, plasma viscosity (PV), Red blood cell count (RBC), hemoglobin (Hb), neutrophil counts (NC) and erythrocyte sedimentation rate (ESR) levels were measured using automatic biochemistry analyzer (Roche, cobas c 311) and Hemorheology system (HLIFE, Shandong, China, LB-2A).

RNA extraction and real-time quantitative PCR (RT-qPCR) assay

Total RNAs were extracted from the muscles on the inside of the right hind limbs using TRIzol reagent (#9109, Takara, Japan). The quality of total RNAs was examined using NanoDro2000c (Thermo Scientific). 2 μg RNAs were used to synthesize cDNA using the Reverse Transcription kit (Takara, Japan). The PCR amplification was performed using SYBR GREEN PCR Master Mix (Applied Biosystems). The results were counted by 2^{-DDCt} method [23]. The sequences of primers were shown in Table 1.

Western blot assay

The muscles on the inside of the right hind limbs were added a little liquid nitrogen and quickly crushed. Proteins were extracted using RIPA buffer (Beyotime, Cat. No. P0013B) including PMSF (Genebase, #329-98-6/ #115-39-9). Bicinchoninic acid (BCA) kit (Thermo Scientific) was utilized to determine the concentrations of proteins. Total proteins (30 μg) were separated using 10% SDS-PAGE, transferred onto nitrocellulose membranes (Millipore, USA). After blocking with 5% skimmed milk (BD Biosciences), the membranes were incubated with primary antibodies overnight at 4˚C. next day, the membranes were incubated with HRP Goat anti-Rabbit IgG (1: 20000, BOSTER, cat#BA1054) or HRP Goat anti-Mouse IgG (1: 20000, BOSTER, cat# BA1051) for 40 mins. Finally, the membranes were treated with the enhanced chemiluminescent reagents (MILLIPORE, WBKLS0500), he results were observed by X-ray films (XBT-1, Eastman Kodak Company, NY, USA), and the data were analyzed by Image-Pro Plus 6.0. The primary antibodies included IL-17 (1:1000, Abcam, ab77171), IFN-γ (1:1000, Abcam, ab77246), IL-1β (1:1000, Abcam, ab2105), IL-6 (1:1000, Abcam, ab208113), TNF-α (1:1000, Abcam, ab6671) and GAPDH (1:2000, Abcam, ab9482).

Enzyme-linked immunosorbent assay (ELISA) detection

After 14 days, serum was isolated from all the rats blood. According to the protocols of manufacturer, the concentrations of IL-17, IFN-γ, IL-β, IL-6 and TNF-α were measured using commercial ELISA kits as per the instructions of manufacturers. The ELISA kits included IL-1β (Cusabio, CSB-E08055r), IL-6 (Cusabio, CSB-E04640r), IL-17 (Cusabio, CSB-E07451r), IFN-γ (Cusabio, CSB-E04579r) and TNF-α (Cusabio, CSB-E11987r).

Immunohistochemistry (IHC) assay

The samples were embedded with paraffin, and cut into 4 μm section. After dewaxing and hydration, the antigen retrieval was performed with sodium citrate buffer at 95˚C. The sections were treated with 3% hydrogen peroxide for 10 mins. And then the sections were blocked with 3% BSA at 37˚C for 30 mins and incubated with anti-CD3 (1:150, Abcam, ab16669) and anti-CD20 (1:400, Bioss, bs-20639R) overnight at 4˚C. Next day, the sections were incubated with goat Anti-Rabbit IgG H&L (HRP, 1:1000, Abcam, ab6721) for 50 mins at room temperature. And then the sections were treated with diaminobenzidine (DAB) for 5 mins, hematoxylin for 3 mins and 1% acid alcohol for a few seconds. Finally, the sections were observed under a light microscope.

Haematoxylin and eosin (HE) staining

The medial muscles of the right hind limbs were fixed with 4% paraformaldehyde and dehydrated with alcohol gradient. Then 4-μm sections were stained with H&E solution (H8070, Solarbio, China). The pathology results were obtained under a microscope (Nikon Eclipse Ci, Japan).

Masson staining

The sections were treated with dimethylbenzene and dehydrated with graded ethanol. And then the sections were stained using Masson staining kit (G1006). After staining, the sections were treated with 1% glacial acetic acid for 1 min. finally, the sections were treated with graded ethanol and dimethylbenzene. The results were obtained using a microscope (Nikon Eclipse CI).

Statistical analysis

The results were calculated by GraphPad (Ver. Prism 7, GraphPad Prism Software, La Jolla, CA, USA). All data are expressed as mean ± standard deviation (SD). Differences between groups were analyzed by one-way analysis of variance or Student's t-test. $P < 0.05$ was considered as significant.

Results

The combination of NP or MFS and NDDM reduced the gangrene's classification of TAO rats

To investigate the role of integration TCM and NDDM on the treatment of TAO, TAO mode rats were treated with NP, MFS or the combination of NP or MFS and NDDM. The results of gangrene's classification showed that rats in the sham group showed normal performance; pathological changes of the limbs of TAO model rats were all concentrated in grade IV ganrenes; pathological changes of rats in the NP or MFS group mainly concentrated in grade II and III ganrenes; pathological changes of rats in the NP or MFS plus NDDM group mainly concentrated in grade I and II ganrenes. Overall, the manifestations of rats in TAO model group all were grade IV ganrenes, and the ganrenes were observably reduced in NP or MFS group compared with TAO model group, the ganrenes were also dramatically reduced in NP or MFS in combination with NDDM groups compared with NP or MFS alone group. In addition, our results revealed that

along with the increase of the drug treatment time, the ganrenes gradually relieved, especially, the combined treatment group and the single treatment group on the 14th day after intragastric administration (Figure 1).

The impacts of NP or MFS in combination with NDDM on hemorheology index

Next, we examined the effects of NP or MFS in combination with NDDM treatments on hemorheology indexes. The results indicated that BPC, RBC, WBC, fibrinogen and PV levels were significantly increased in the TAO model group relative to the sham group, and NP and MFS alone or in combination with NDDM could decrease BPC, RBC, WBC, fibrinogen and PV levels. While, Hb, NC and ESR levels were not affected by NP, MFS or in combination with NDDM (Table 2).

NP or MFS in combination with NDDM decreased CD3+CD20+T cells

To identify the changes of CD3+CD20+T cells in TAO model rats after NP or MFS in combination with NDDM, we determined CD3 and CD20 levels. The results from IHC assay indicated that CD3 expression was dramatically decreased in the TAO model group relative to the sham group, was markedly increased in the NP or MFS group compared with the TAO model group, and was memorably increased in the NP or MFS in combination with NDDM groups in comparison to the NP or MFS alone group; while CD20 expression was dramatically increased in the TAO model group relative to the sham group, was markedly decreased in the NP or MFS group compared with the TAO model group, and was memorably decreased in the NP or MFS in combination with NDDM groups in comparison to the NP or MFS alone group (Figure 3A). In addition, using flow cytometry, we found that compared with sham group, CD3+CD20+T cells were markedly increased in TAO model group. Meanwhile, NP or MFS treatment decreased CD3+CD20+T cells in rats of the TAO model group, NP or MFS in combination with NDDM further decreased CD3+CD20+T cells (Figure 3B).

NP or MFS in combination with NDDM reduced thrombosis and inflammatory infiltration of rat limbs

The pathological section of muscle tissues in the sham group showed smooth tunica interna, well-ranged muscle fibers, no thrombus and inflammatory cell infiltration; The pathological section of muscle tissues in the medial side of the right hind limb in the TAO model group showed large amounts of thrombosis, disordered arrangement of muscle fibers, no atrophy, fibrosis or calcification in tunica media, thickened tunica externa without fibrosis and many infiltrating inflammatory cells; while NP or MFS treatment could improve the pathological histology of rat limbs, and NP or MFS in combination with NDDM further recovered the pathological histology of rat limbs (Figure 2A). In addition, Masson staining was performed to assess the degree of vascular fibrosis. Plentiful new blood vessels and few thrombi were observed in the sham group, while histopathology in the TAO model group indicated the accumulation of collagen fibers and obvious thrombus organization. We found that compared with the sham group, the degrees of collagen deposition and vascular fibrosis were increased in the TAO model group; and NP or MFS treatment markedly attenuated the degrees of collagen deposition and vascular fibrosis of rats in the TAO model group, NP or MFS in combination with NDDM further decreased the degrees of collagen deposition and vascular fibrosis of rats in the TAO model group (Figure 2B).

NP or MFS in combination with NDDM inhibited inflammation-associated cytokines

To further explore the influence of NP or MFS in combination with NDDM on inflammation-associated cytokines in TAO model rats, RT-qPCR, Western blot and ELISA assays were utilized to analyze IL-17, IFN-γ, IL-1β, IL-6 and TNF-α levels. As shown in Figure 4, IL-17, IFN-γ, IL-1β, IL-6 and TNF-α levels were higher in the TAO model group than the sham group, were lower in the TAO model rats with NP or MFS treatment than the TAO model rats; IL-17, IFN-γ, IL-1β, IL-6 and TNF-α expressions were memorably decreased in the TAO model rats with

NP or MFS in comparison to NDDM treatments compared with NP or MFS treatment alone.

Discussion

Lauric acid is a free saturated fatty acid in serum[24]. After a large amount of lauric acid is injected into the blood vessels, the blood vessels deposited in the corresponding blood vessels, which will cause vascular endothelial cells and vascular wall injury, induce thrombosis, cause local vascular circulation disorder, and eventually form the ischemic state of the local organism[25, 26]. At present, lauric acid has been widely used to induce TAO model[21]. In our study, we also adopted 0.1ml sodium laurate solution (10 mg/ml) to inject the femoral artery of rats. According to the ganrene's classification, rats in the sham group showed normal performance, rats in TAO model group were all concentrated in grade IV ganrenes, suggesting that TAO model was successfully constructed. In addition, we demonstrated that NP or MFS alone treatment could reduce the degree of limb injury in rats, and the injuries mainly concentrated in grade II and III ganrenes; the combination of NP or MFS and NDDM could dramatically alleviate the degree of limb injury in rats, and the

injuries mainly concentrated in grade I and II ganrenes. Therefore, we suggested that integration NP or MFS and NDDM observably reduced the ganrene's classification of TAO rats.

TAO is one of the common chronic periarterial ischemia disorders in China, and the etiology and pathogenesis have not been elucided[27, 28]. At present, researches have shown that autoimmune mechanisms, especially abnormal erythrocyte immune function and hemorheology changes, play essential roles in the occurrence and development of TAO[29, 30]. Studies have suggested that blood rheology was closely associated with a variety of diseases, such as chronic heart failure[31], sickle cell anemia[32], angina pectoris[33], diabetic retinopathy[34], etc. In our study, we proved that NP or MFS in combination with NDDM could decrease BPC, WBC, fibrinogen and PV levels. Therefore, we indicated that integration TCM and NDDM could affect blood rheology.

CD20 is a human leukocyte differentiation antigen, which is expressed on the surface of B cells at all stages of cell development and differentiation except plasma cells, and is recognized as the surface marker of B cells[35, 36]. CD3 is the co-differentiation antigen of mature T cells, expressed on the surface of all mature T cells[37]. CD3 and T cell receptor (TCR) can form CD3-TCR complex through salt bridge, which can transfer the antigen stimulation signal received by TCR into cells. Therefore, CD3 is a crucial role in the process of T cell immunity[38]. Previous studies also have confirmed that a group of CD3 T cells also express CD20 molecules in the body apart from B cells, which have similar and unique biological characteristics with conventional CD3 T cells[39, 40]. Studies have demonstrated that CD3+CD20+ T cells play an important role in multiple diseases[41], such as rheumatoid arthritis[42, 43], multiple sclerosis[41], psoriasis[44], lymphoproliferative disorder[45] and cancers[46, 47]. In our study, we further indicated that NP or MFS treatment decreased CD3+CD20+T cells in TAO model rats, which could be further decreased in the combined effects between NP or MFS and NDDM. In addition, we verified that NP or MFS in combination with NDDM treatment could improve thrombus and inflammatory cell infiltration of rat limbs, and decreased the degrees of collagen deposition and vascular fibrosis of rats in the TAO model group. Therefore, we further proved that the combination of NP or MFS and NDDM could relieve the symptoms of TAO.

Studies have shown that TAO, as an inflammatory disease, is associated with the inflammatory reaction of endothelial cells, platelets, leukocytes and sensory neurons[48]. Inflammation is significantly correlated with thrombogenesis, and thrombosis and vasculitis are major ganrenes in many systemic inflammatory diseases, such as TAO, antineutrophil cytoplasmic antibody (ANCA)-related vasculitis, Takayasu's arteritis, systemic lupus erythematosus, antiphospholipid syndrome (APS) and inflammatory bowel disease, etc[49, 50]. Plenty of researches also showed that inflammation-associated cytokines, such as interleukin (IL)-1β, IL-4, IL-17, IL-23, IL-6 and Tumor necrosis factor-α (TNF-α) were associated with TAO[51-54]. In our study, we also certified that NP or MFS in combination with NDDM dramatically decreased IFN-γ, IL-1β, IL-6 and TNF-α levels, indicating that he combination of NP or MFS and NDDM inhibited inflammation-associated cytokines.

Conclusions

We have demonstrated that NP or MFS combination with NDDM could reduce the ganrene's classification and change blood rheology of TAO rats. In addition, the combination NP or MFS and NDDM could improve thrombus, inflammatory cell infiltration, muscle fiber and collagen fiber of rat limbs. We also proved that the combination could dramatically decrease CD3+CD20+ T cells and inhibited inflammation-associated cytokines. Therefore, we suggested that the combination NP or MFS and NDDM might be Potential treatment for TAO.

Abbreviations

TAO: Thromboangiitis obliterans. Abstract, Paragraph 1.

TCM: traditional Chinese medicine. Abstract, Paragraph 1.

NDDM: nibble debridement and dressing method. Abstract, Paragraph 1.

NP: notoginseng powder. Abstract, Paragraph 2.

MFS: maifusheng. Abstract, Paragraph 2.

BPC: Blood platelet count. Methods, Paragraph 10.

WBC: white blood cell count. Methods, Paragraph 10.

PV: plasma viscosity. Methods, Paragraph 10.

RBC: Red blood cell count. Methods, Paragraph 10.

Hb: hemoglobin. Methods, Paragraph 10.

NC: neutrophil counts. Methods, Paragraph 10.

ESR: erythrocyte sedimentation rate. Methods, Paragraph 10.

RT-qPCR: real-time quantitative PCR. Methods, Paragraph 11.

BCA: Bicinchoninic acid. Methods, Paragraph 14.

ELISA: Enzyme-linked immunosorbent assay. Methods, Paragraph 15.

IHC: Immunohistochemistry. Methods, Paragraph 17.

DAB: diaminobenzidine. Methods, Paragraph 18.

HE: Haematoxylin and eosin. Methods, Paragraph 19.

SD: standard deviation. Methods, Paragraph 24.

TCR: T cell receptor. Discussion, Paragraph 3.

IL: interleukin. Discussion, Paragraph 4.

TNF-a: Tumor necrosis factor-a. Discussion, Paragraph 4.

ANCA: antineutrophil cytoplasmic antibody. Discussion, Paragraph 4.

APS: antiphospholipid syndrome. Discussion, Paragraph 4.

Declarations

Ethics approval and consent to participate

All the animal protocols have also been approved by the Animal Ethics Committee of Guangzhou Hospital of Traditional Chinese Medicine.

Consent to publish

Not application.

Availability of data and materials

The data used in this research are available form the correspinding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

All authors have contributed significantly. JHL, JFZ and CFH carried out the studies. JWG and ARL analyzed the data. JHL wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1. The sequences of primers in this study

ID	Sequence (5’- 3’)	Product Length (bp)
GAPDH F	CCTCGTCTCATAGACAAGATGGT	169
GAPDH R	GGGTAGAGTCATACTGGAACATG	
TNF-a F	CTTCTCATTCCTGCTCGTGG	201
TNF-a R	TCCGCTTGGTGGTTTGCTA	
IL-1 F	TTTGAGTCTGCACAGTTCCC	96
IL-1 R	AACTATGTCCCGACCATTGC	
IL-6 F	ACCACCCACAACAGACCAGTA	232
IL-6 R	CAGAGCAGATTTTCAATAGGCA	
IL-17 F	CCTCAGACTACCTCAACCGT	142
IL-17 R	ATGTGGTGGTCCAACTTCCC	
IFN-γ F	GTCATCGAATCGCACCTGAT	103
IFN-γ R	GGATCTGTGGGTTGTTCACC	

Table 2. Hematological and hemorheological parameters in Sham, TAO, NP, MFS, NP+NDDM and MFS+NDDM rat

	BPC (10^8/ml)	RBC (10^8/ml)	Hb (g/l)	WBC (10^8/ml)	NC (10^8/ml)	Fibrinogen (g/l)	ESR (mm/h)	PV (mPa.s)
Sham								
1 day	7.15±0.12	6.40±0.06	125.64±3.83	4.61±0.31	1.06±0.03	2.46±0.08	1.02±0.04	1.48±0.07
7 day	7.2±0.09	6.38±0.29	126.90±5.24	4.51±0.25	1.09±0.05	2.42±0.08	1.08±0.04	1.33±0.07
14 day	7.15±0.08	6.40±0.11	131.39±10.01	4.54±0.26	1.07±0.08	2.85±1.18	1.08±0.04	1.49±0.15
TAO								
1 day	8.97±0.92*	6.06±0.10**	126.04±5.19	5.08±0.06	1.06±0.07	2.77±0.13*	1.06±0.05	2.46±0.31*
7 day	9.59±0.76*	5.83±0.13**	115.52±6.97	5.05±0.10*	1.12±0.07	2.91±0.13*	1.12±0.03	2.66±0.32*
14 day	11.24±1.07*	5.60±0.21**	111.93±8.24	5.23±0.06*	1.22±0.09	3.06±0.11*	1.18±0.07	3.55±0.79*
NP								
1 day	8.80±0.71	6.21±0.10	130.10±4.70	5.07±0.05	1.11±0.08	2.64±0.20	1.05±0.10	2.18±0.16#
7 day	9.04±0.55	6.02±0.17	123.44±8.63	5.05±0.09	1.05±0.09	2.85±0.29	1.09±0.13	2.20±0.32#
14 day	8.93±0.11#	6.76±0.12#	119.00±10.19	5.15±0.12	1.14±0.15	2.74±0.09#	1.28±0.37	3.01±0.34#
MFS								
1 day	9.06±1.07	6.24±0.15	128.45±4.16	5.08±0.04	1.05±0.06	2.75±0.12	1.04±0.07	2.21±0.17#
7 day	8.98±0.63#	6.21±0.49	119.95±7.58	5.04±0.11	1.10±0.05	2.88±0.13	1.10±0.04	2.05±0.18#
14 day	8.82±0.52#	6.75±0.17#	118.92±8.89	5.15±0.09	1.18±0.09	2.80±0.10#	1.15±0.05	3.18±0.89#
NP+NDDM								
1 day	8.55±0.56	6.42±0.36	126.19±12.77	5.06±0.11	1.10±0.13	2.58±0.25	1.05±0.13	2.50±0.08$
7 day	9.30±0.66	6.14±0.37	124.19±5.97	5.08±0.10	1.13±0.08	2.55±0.15	1.07±0.18	2.15±0.16
14 day	9.31±0.13$	7.15±0.13$	119.81±6.82	4.81±0.13$	1.16±0.10	2.56±0.06$	1.12±0.15	2.34±0.22$
MFS+NDDM								
1 day	7.83±0.56	6.23±0.16	126.03±2.77	4.67±0.31	1.06±0.04	2.51±0.26	1.01±0.04	1.65±0.24&
7 day	7.46±0.26&	6.29±0.11	126.86±3.31	4.78±0.23	1.18±0.18	2.60±0.21	1.09±0.04	1.86±0.21&
14 day	7.61±0.14&	6.38±0.06&	131.30±6.68	4.67±0.28&	1.05±0.11	2.54±0.16&	1.15±0.09	1.99±0.19&

BPC, Blood platelet count; RBC, red blood cell count; Hb, hemoglobin; WBC, white blood cell count; NC, Neutrophil counts; ESR, erythrocyte sedimentation rate; PV, Plasma viscosity; TAO, thromboangiitis obliterans; NP, notoginseng powder; MFS, maifusheng; NDDM, nibble debridement and dressing method. Values were expressed as means ± SD. *P<0.05, **P<0.01 vs. Sham group; #P<0.05 vs. TAO group; $$P$<0.05 vs. NP group; &$P$<0.05 vs. MFS group;

Figures

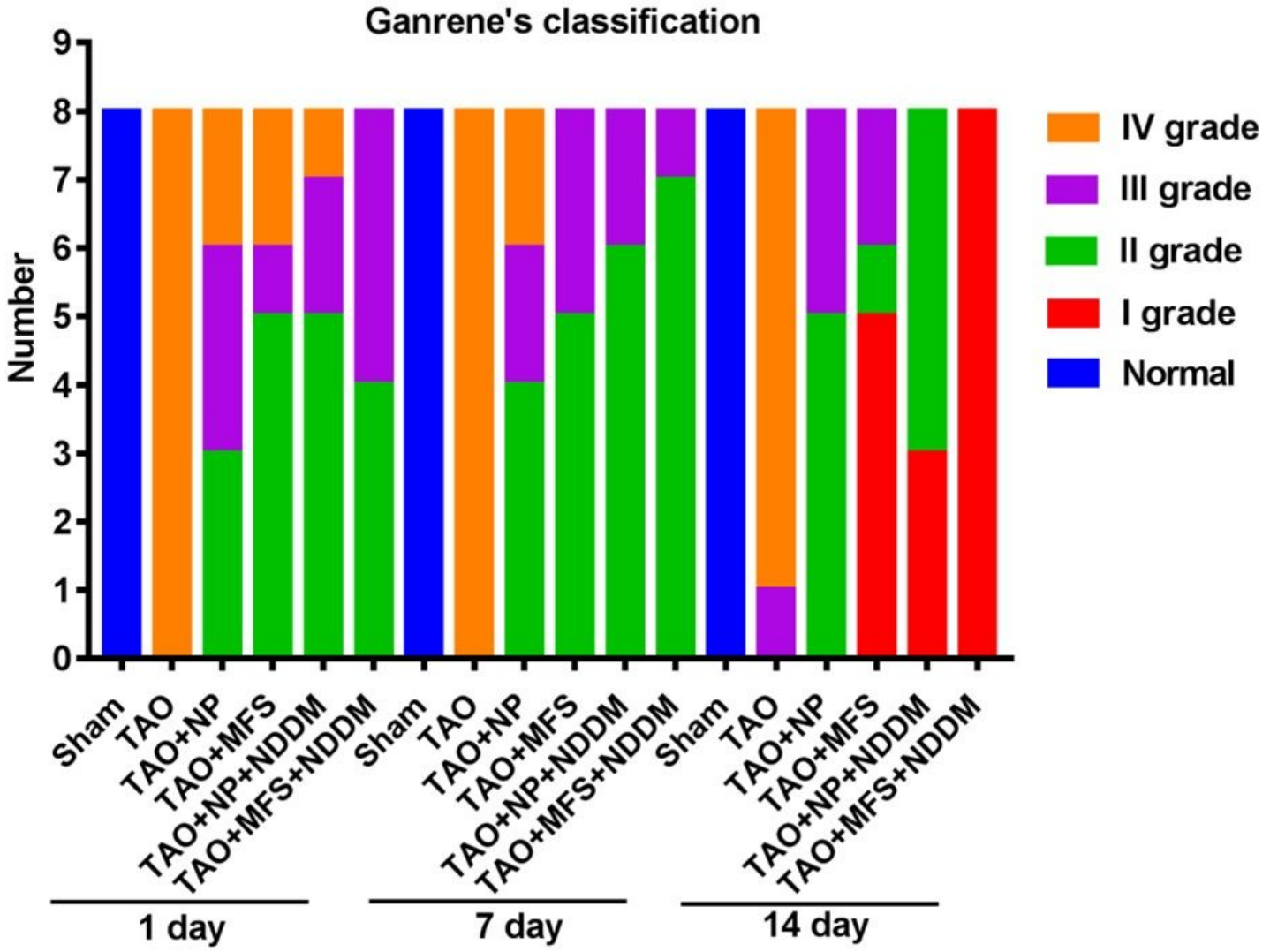

Figure 1

Effects of NP, MFS or NDDM on the ganrene's classification of TAO rats. TAO Rats were administrated with NP or MFS for 7 day, and ulcers were given NDDM treatment. The extent of the ganrenes was ranked by the Grade Evaluation of the Gangrene at 1st, 7th and 14th day. TAO, thromboangiitis obliterans; NP, Notoginseng powder; MFS, Maifusheng; NDDM, nibble debridement and dressing method.

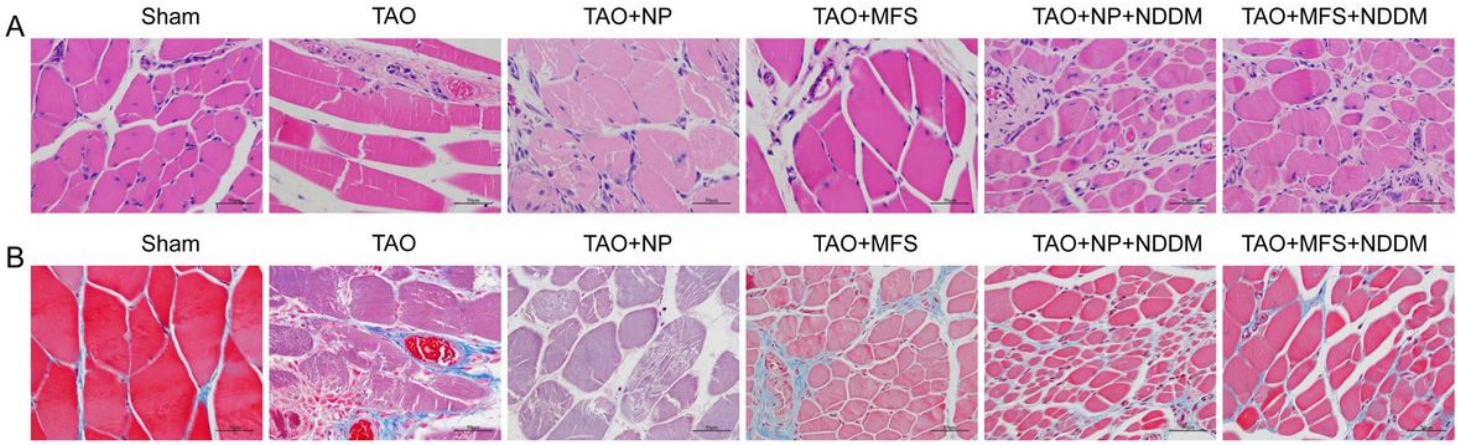

Figure 2

NP or MFS in combination with NDDM reduced thrombosis and inflammatory infiltration of rat limbs. TAO Rats which were administrated with NP or MFS, and ulcers were given NDDM. (A) The degree of vascular damage in rats was assessed by H&E staining, magnification, ×400; Scale bar=50μm. (B) The degree of vascular fibrosis in rats was evaluated by Masson staining, magnification, ×400; Scale bar=50μm. TAO, thromboangiitis obliterans; NP, Notoginseng powder; MFS, Maifusheng; NDDM, nibble debridement and dressing method.

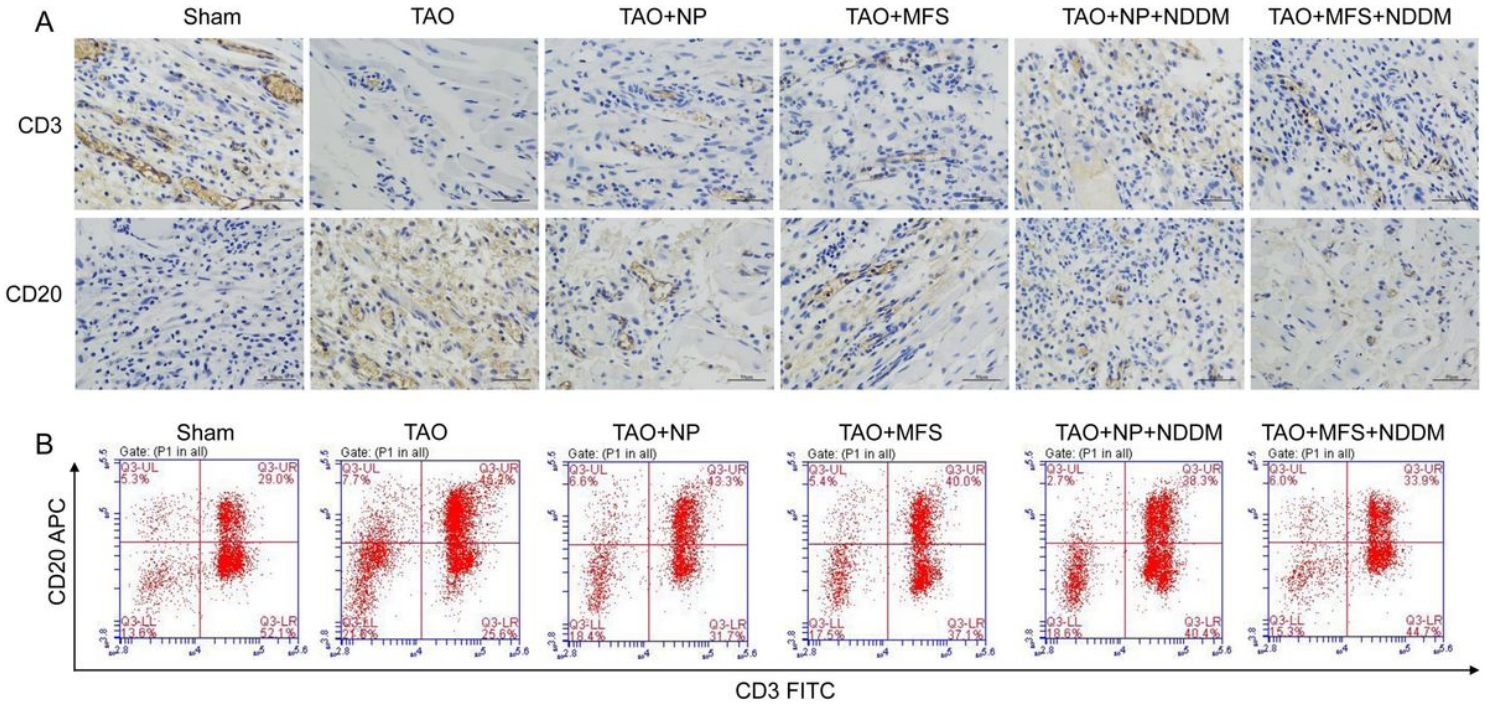

Figure 3

The influences of NP or MFS in combination with NDDM on CD3+CD20+T cells. TAO Rats which were treated with NP or MFS, and ulcers were given NDDM. (A) The CD3+ and CD20+ levels were examined by IHC assay in each group, respectively. Magnification, ×200, Scale bars=50 μm. (B) The peripheral blood which were extracted from rats in each group were dyed with isotype controls, anti-CD20 and anti-CD3 antibodies, and the level of CD3+CD20+T cells was analyzed by flow cytometry. TAO, thromboangiitis obliterans; NP, Notoginseng powder; MFS, Maifusheng; NDDM, nibble debridement and dressing method.

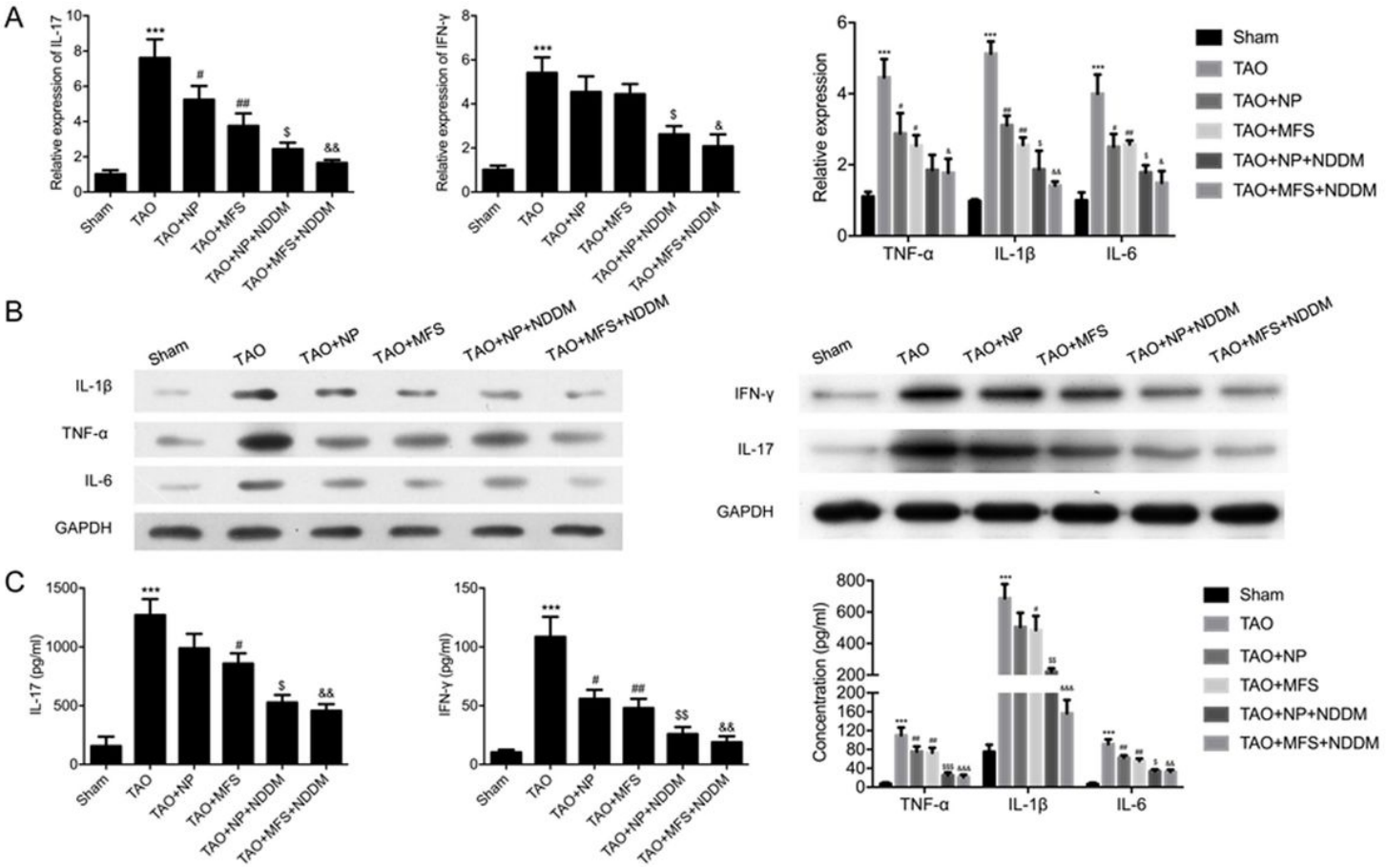

Figure 4

Effects of NP or MFS combination with NDDM on Inflammation-associated cytokines. TAO Rats were administrated with NP or MFS, and ulcers were given NDDM treatment. (A) Expression levels of IL-17, IFN-γ, IL-1β, IL-6 and TNF-α were assessed by RT-qPCR assay in each group. (B) Western blot assay was carried out to examine IL-17, IFN-γ, IL-1β, IL-6 and TNF-α expressions. GAPDH was used as a reference. (C) The relative concentrations of IL-17, IFN-γ, IL-1β, IL-6 and TNF-α were analyzed by ELISA assay in the serum of rats in each group. TAO, thromboangiitis obliterans; NP, Notoginseng powder; MFS, Maifusheng; NDDM, nibble debridement and dressing method. ***P<0.001 vs. the sham group; #P<0.05, ##P<0.01 vs. the TAO group; $P<0.05,

$$P < 0.01,$$

$P<0.001 vs. the TAO+NP group; &P<0.05, &&P<0.01, &&&P<0.001 vs. the TAO+MFS group.