Journal of Clinical and Nursing Research, 2021, Volume 5, Issue 3
http://ojs.bbwpublisher.com/index.php/JCNR
ISSN Online: 2208-3963
ISSN Print: 2208-3685

Effect of Zhuang Medicine Feilongzhangxue on the Expression of HMGB1-TLR4 / RANKL-NF-κB Signaling Pathway Related Factors in Osteoclasts

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Funding: Supported by the open foundation of Key Laboratory of Ethnomedicine (Minzu University of China), Ministry of Education, No.: KLEM-KF2018Z02; Project for Improving Basic Capabilities of Middle-aged and Young Teachers in Guangxi Institutions of Higher Learning (No.: 2020KY07016); Guangxi Health Commission Key Laboratory of Applied Fundamental Research of Zhuang Medicine (Guangxi University of Chinese Medicine) [Gui Wei Ke Jiao Fa (2020) No.17]; Training Program under "139" Plan for Developing High-level Medical Talents in Guangxi [Gui Wei Ke Jiao Fa (2020) No.15]; Traditional Chinese Medicine -- Guangxi first-class discipline [No. Gui Jiao Ke Yan(2018)12].

[Abstract] ***Objective:*** To study the regulatory effect of feilongzhangxue on the levels of HMGB1-TLR4 / RANKL-NF-κB signaling pathway related factors HMGB1, RANKL, rank, TRAF-6 and NF-κBp65 in osteoclasts, so as to explore the mechanism of feilongzhangxue intervention in RA; ***Methods:*** The osteoclasts with good activity were randomly divided into blank group, methotrexate control group and Zhuang medicine feilongzhang blood containing serum treatment group, which were divided into OC + blank group, OC + methotrexate control group, OC + Zhuang medicine feilongzhang blood containing serum group; The expression of HMGB1, RANKL, rank, TRAF-6 and NF-κBp65 mRNA was detected by RT-PCR; The protein expressions of HMGB1, RANKL, RANK, TRAF-6 and NF-κBp65 were detected by immunofluorescence. ***Results:*** PCR results showed that: Compared with the blank group, feilongzhangxue could effectively inhibit the expression levels of HMGB1, RANKL, rank, TRAF-6 and NF-κ B p65 mRNA in OC cells, and the inhibitory effect was stronger than methotrexate. Immunofluorescence test results showed that: Compared with the blank group and methotrexate group, feilongzhangxue could effectively inhibit the protein expression of HMGB1, RANKL, rank, TRAF-6 and NF - κ B p65 in OC cells. ***Conclusion:*** The effect of Zhuang medicine feilongzhangxue on hmgb1-tlr4 / rankl-nf - κ B signaling pathway of osteoclasts is through the regulation of related factors HMGB1, RANKL, rank, TRAF-6 and NF-κBp65, which may be the key mechanism of Zhuang medicine feilongzhangxue on rheumatoid arthritis.

Key words: Zhuang medicine Feilongzhangxue; Osteoclasts; HMGB1; RANK; NF-κB

Publication date: May, 2021; ***Publication online:*** 31 May, 2021

1 Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease

characterized by chronic destructive joint disease. Some data show that the prevalence and disability rate of RA in China is increasing year by year, which has seriously affected people's life and health[1]. The progression of RA is often accompanied by bone loss and increased risk of fracture. Its inflammatory state is closely related to osteoclast (OC) - mediated bone destruction, and OC is the main executor in this erosion process[2]. Some studies suggest that RANKL-RANK-NF-κB[3] and hmgb1-TLR4-MYD88-NF-κB[4] pathways are important promoters of inflammatory signals in the pathogenesis of RA. NF-κB, as a common downstream factor of the two key pathways of RA, is undoubtedly the key in the key. Zhuang medicine longzuantongbi prescription is a Zhuang medicine empirical prescription for the treatment of active rheumatoid arthritis. Existing clinical studies show that Zhuang medicine longzuantongbi prescription can significantly reduce the symptoms of RA patients, and the clinical effective rate can reach 85.0%[5]. Feilongzhangxue is the main drug of this prescription. Therefore, feilongzhangxue was selected to carry out the research on the disintegration of this prescription, in order to reveal the mechanism of longzuantongbi prescription and its effective components, and to provide scientific basis for the clinical application of longzuantongbi prescription and the effective prevention and treatment of RA.

2 Reagents and materials

α - MEM medium (GIBCO, 41500083), FBS (Beijing ZHENGBO, ytys1050), penicillin / streptomycin (GIBCO, 15140-122), T25 cell culture bottle (jet biofil, t25-011-050), cell culture plate (6 wells) (jet biofil, t25-011-050) Biofil, tcp010096), PBS (Beijing solab, p1020), tartrate resistant acid phosphatase staining solution (Beijing solab, g1492), cytotoxicity test kit (Beijing solab), RNA extract (Tiangen biochemical technology (Beijing) Co., Ltd., dp424), isopropanol (Sinopharm Chemical Reagent Co., Ltd., 80109218), anhydrous ethanol (Sinopharm Chemical Reagent Co., Ltd., 80109218) RT-PCR Kit (thermo, # k1622) was used; The primers were synthesized by Wuhan Qingke innovation Biotechnology Co., Ltd. as shown in the table below. Other experimental consumables were provided by the Key Laboratory of Guangxi University of traditional Chinese medicine.

Table 1. Primer sequence

Primer name	Primer sequence (5 '- 3')
GAPDH-F	AGGTCGGTGTGAACGGATTTG
GAPDH-R	GGGGTCGTTGATGGCAACA
HMGB1-F	GGCGAGCATCCTGGCTTATC
HMGB1-R	GGCTGCTTGTCATCTGCTG
RANK-F	GGACGGTGTTGCAGCAGAT
RANK-R	GCAGTCTGAGTTCCAGTGGTA
TRAF6-F	AAAGCGAGAGATTCTTTCCCTG
TRAF6-R	ACTGGGGACAATTCACTAGAGC
NF-κBp65-F	ATGGCAGACGATGATCCCTAC
NF-κBp65-R	TGTTGACAGTGGTATTTCTGGTG
RANKL-F	CAGCATCGCTCTGTTCCTGTA
RANKL-R	CTGCGTTTTCATGGAGTCTCA

3 Methods

3.1 Preparation of medicated serum

Thirty SD rats (female) were randomly divided into blank control group, methotrexate group (0.18mg / 200g) and feilongzhang blood group (1.2g / 200g), with 10 rats in each group. The rats were gavaged continuously for 7 days. Two hours after the last gavage, the blood was collected from abdominal aorta under anesthesia, centrifuged, packed, filtered and stored in refrigerator at - 20 ℃.

3.2 Isolation and induction of osteoclasts

Fifteen C57BL / 6 mice (female) aged 4-6 weeks were selected; Femur and tibia were separated under aseptic conditions. The bone marrow cavities of femur and tibia

were washed with appropriate amount of α-MEM medium. The suspension of washed bone marrow cells was collected. The suspension of bone marrow cells was filtered and centrifuged, and the supernatant was discarded. The obtained cells were washed twice with sterile PBS, and the final concentration was 20ng / ml. The cells were seeded in a culture dish and incubated overnight in a 5% CO2 incubator at 37 ℃. After 3 days of culture, the cells adhered to the wall and were regarded as osteoclast precursors. The culture medium was changed into differentiation medium (20 ng / ml M-CSF, 100 ng / ml RANKL, 10% FBS, 1% PS α - MEM medium) and cultured in 5% CO_2 incubator After 6 days of culture, mature osteoclasts were induced.

3.3 Tartrate resistant acid phosphatase (TRAP) staining

(1) After the cells were induced to mature osteoclasts, the culture medium was sucked and washed twice with PBS.

(2) Natural drying, trap fixative at 4 ℃ for 30 S-3 min, in most cases 30-60 s.

(3) Wash with water and dry slightly.

(4) The slices were incubated in trap medium, placed in 37 ℃ incubator, and stained in dark for 45-60min, then washed with water.

(5) Re dyeing: Hematoxylin staining for 5 minutes or methyl green staining for 2-3 minutes.

(6) Washing, drying and microscopic examination.

3.4 Experimental group

The cells were divided into three groups: OC + blank group, OC + methotrexate control group and OC + feilongzhangxue group.

3.5 Experimental administration

Each group was given 10% serum + 90% α - MEM medium, and the operation process was strictly in accordance with the operation technology of cell experiment. The cells were seeded on a 6-well plate at a density of 2 × 105 cells / ml overnight. After the cells adhered to the wall, the medium containing the drug was changed. After 24 hours, the cells were harvested.

3.6 Data detection

After harvest, the expression levels of HMGB1, RANKL, RANK, TRAF-6 and NF - κ bp65 were detected by RT-PCR; The protein expressions of HMGB1, RANKL, RANK, TRAF-6 and NF - κ bp65 were detected by immunofluorescence.

3.7 Statistical analysis

SPSS 20.0 statistical software was used. The measurement data were expressed in the form of mean ± standard deviation (x±s). *T*-test was used. Analysis of variance was used for comparison between groups. $P < 0.05$ means significant difference, $P < 0.01$ means extremely significant difference.

4 Results

4.1 Identification of osteoclasts

The osteoclasts were isolated and induced according to the operation technique, and then stained with trap to confirm that they were mature and full of vitality. The results are as follows, as shown in Figure 1.

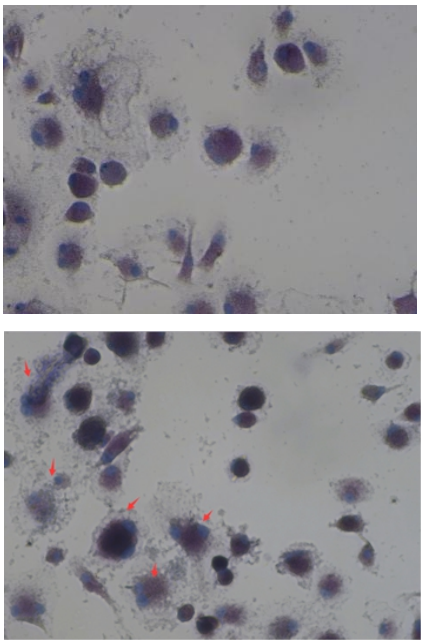

Figure 1. Staining results of osteoclasts

4.2 RT-PCR detection

After the cells were isolated and induced into mature osteoclasts, the cells were treated according to groups, and the RNA was extracted. The expression levels of HMGB1 mRNA, RANKL mRNA, rank mRNA, TRAF-6 mRNA and NF - κ bp65 mRNA were detected by RT-PCR. The results are as follows: The expression of HMGB1 mRNA in blank group was higher than that in methotrexate group and feilongzhang blood group ($P < 0.05$), and that in methotrexate group was higher than that in feilongzhang blood group, but there was no statistical significance; The expression of RANKL mRNA showed that: 1Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$), methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of rank mRNA showed that: Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$),

methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of TRAF-6 mRNA showed that: Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$), methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of NF-κBp65 mRNA showed that the expression of NF-κBp65 mRNA was significantly higher than that of NF - κ bp65 Mrna. Blank group was higher than methotrexate group and feilongzhang blood group ($P < 0.05$), methotrexate group was higher than feilongzhang blood group, but there was no statistical significance. The results showed that dragon palm blood could effectively inhibit the expression levels of HMGB1, RANKL, rank, TRAF-6 and NF - κ B p65 mRNA in OC cells, and the inhibitory effect was stronger than that of methotrexate ($P < 0.05$). See Figure 2.

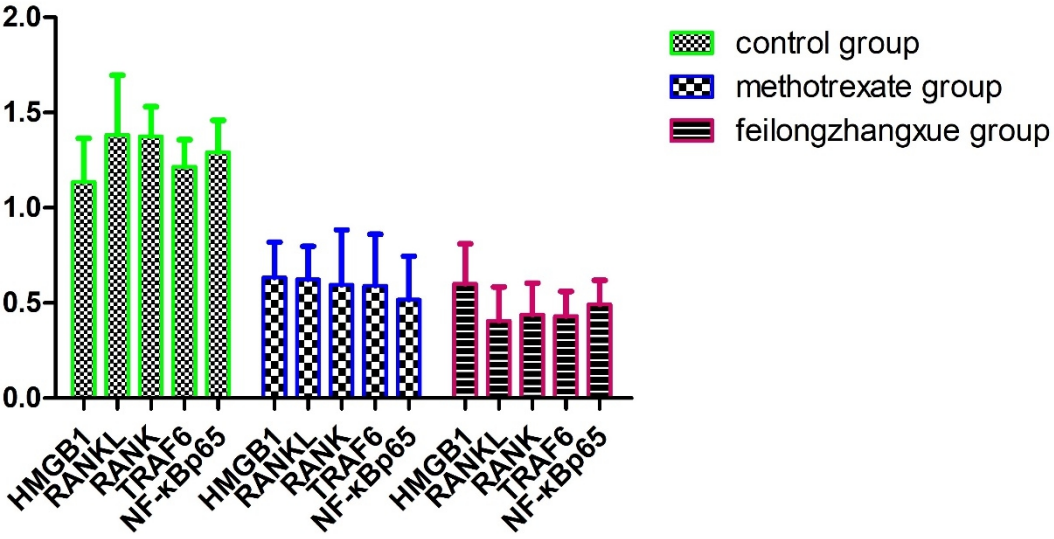

Figure 2. RT-PCR results

4.3 Immunofluorescence staining

After the cells were isolated and induced into mature osteoclasts, the cells were treated according to the above groups and collected for immunofluorescence staining to detect the protein expression of HMGB1, RANKL, rank, TRAF-6 and NF - κ bp65; The experimental results show that: The expression of HMGB1 protein in OC cells showed that the expression of HMGB1 protein in OC cells was higher than that in OC cells. Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$), methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of RANKL protein in OC cells was observed. Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$), methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of rank protein in OC cells was as follows. Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$), methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of TRAF-6 protein in OC cells showed that: 1Blank group was higher than methotrexate group and feilongzhangxue group ($P < 0.05$), methotrexate group was higher than feilongzhangxue group ($P < 0.05$); The expression of NF - κ bp65 protein in OC cells showed that the expression of NF - κ bp65 protein in OC cells was higher than that in OC cells. Blank group was higher than methotrexate group and feilongzhang blood group ($P < 0.05$), methotrexate group was higher than feilongzhang blood group ($P < 0.05$). The results are as follows: Feilongzhangxue could effectively inhibit the expression of HMGB1, RANKL, rank, TRAF-6 and NF - κ bp65 in OC cells; See Figure 3.

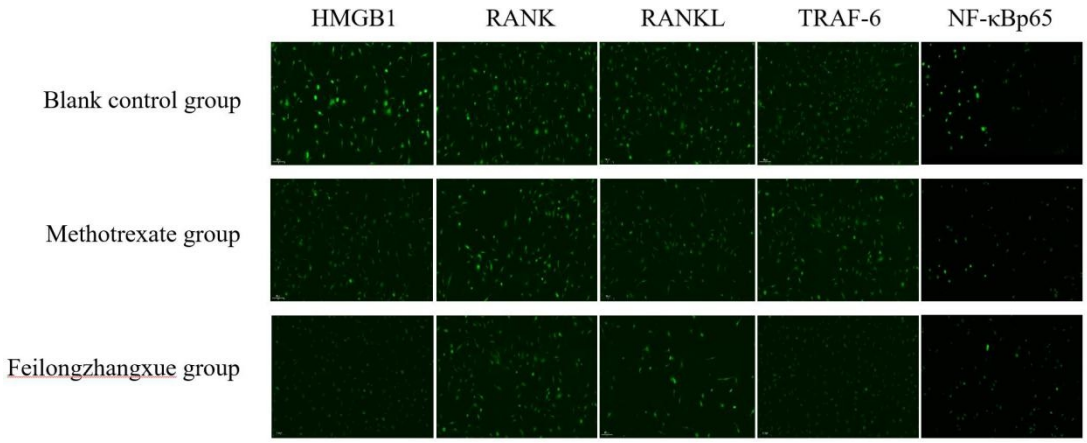

Figure 3. Results of immunofluorescence staining

5 Discussion

Bone erosion is the main feature of rheumatoid arthritis. OC is the only cell in vivo that has the ability to dissolve bone tissue. OC mediated bone destruction is achieved through cell-cell interaction and a variety of cytokine regulation, which is the main cause of RA joint destruction [2]. High mobility group box chromosomal protein 1 (HMGB1) is a cytokine with two-way function. As an endogenous risk signal molecule, once released out of the cell, it can start the natural and acquired immune process and mediate the formation and development of RA[6]. HMGB1 promotes the activation of NF - κ bp65 by binding to the main receptor TLR4, forming HMGB1-TLR4-MYD88-NF - κ bp65 pro-inflammatory pathway. Nuclear factor kappa B receptor activator ligand (RANKL) is the only cytokine that can directly stimulate the development and activation of OC. In the presence of macrophage colony-stimulating factor (M-CSF), RANKL can activate the signal transduction pathway, promote the formation, differentiation and maturation of OC, inhibit the apoptosis of osteoclasts and prolong their survival[7]. And rank can transmit stimulation to NF - κ B through tumor necrosis factor related receptor 6 (TRAF6). Rankl-rank-traf6-nf - κ B pro-inflammatory pathway was formed. These two pathways affect the activity of OC and play an important role in the occurrence and development of RA.

Zhuang medicine, as a national medicine, has a unique understanding of the occurrence and development of RA in long-term medical practice. It is believed that it belongs to the category of "Fawang" (Zhuang name: Fungcaep) of Zhuang medicine, and its pathogenesis is essentially "network block causing bizi", "two routes".(i.e. long road, fire road) is blocked and heavy, pain, for a long time can cause dysfunction, joint deformity, even disability[8]. Under the guidance of Zhuang medicine theory, longzuantongbi prescription is a Zhuang medicine empirical prescription used in the treatment of active rheumatoid arthritis. It is composed of feilongzhangxue, Qingfengteng, jiulongteng, dazan, bajiaofeng, Zanthoxylum nitidum, wuzhimaotaogen, jixueteng and other Zhuang drugs.In order to treat RA, all kinds of herbs act on Longlu and Huolu, dredge the blood stasis of Longlu and Huolu, directly remove pathogenic factors and go out, and restore the synchronous operation of heaven, man and earth. The existing clinical studies show that longzuantongbi prescription can significantly improve the morning stiffness, joint pain and other symptoms of RA patients, and the clinical effective rate can reach 85.0%.At the same time, the experimental study also confirmed that the formula can increase the serum levels of anti-inflammatory factors IL-4 and IL-10 in arthritis model rats, and can down regulate the levels of inflammatory factors IFN - γ, TNF - α and MMP-13[9-11]. Feilongzhangxue, as the main medicine of this prescription, can promote blood circulation, disperse blood stasis, detumescence and relieve pain. Modern pharmacological research found that feilongzhang blood has anti-inflammatory and analgesic, hemostatic and coagulation, antibacterial, antioxidant, anti-tumor, treatment of cardiovascular disease and other effects[12]. It is widely used in the treatment of rheumatoid. It has been found that feilongzhangxue can treat acute gouty arthritis, and its effect may be related to the reduction of IL-1 β, IL-6 and TNF - α mRNA and NF - κ bp65 in peripheral blood and synovial tissue[13].

In this study, we found that the effect of feilongzhangxue on HMGB1-TLR4 / RANKL-NF-κB signaling pathway in OC cells is through the regulation of related factors HMGB1, RANKL, rank, TRAF-6 and NF - κ bp65.The expression of related genes was detected: Feilongzhangxue can effectively inhibit the expression of HMGB1, RANKL, RANK, TRAF-6 and NF-κBp65 mRNA in OC cells, and its inhibitory effect on RANKL, RANK and TRAF-6 mRNA is better than methotrexate. The protein expression of each group showed that: Feilongzhangxue could effectively inhibit the protein expression of HMGB1, RANKL, RANK, TRAF-6 and NF - κ bp65 in OC cells; Its inhibitory effect is stronger than that of methotrexate. Therefore, feilongzhangxue, a Zhuang medicine, may achieve therapeutic effect on RA by inhibiting the expression of HMGB1, RANKL, RANK, TRAF-6 and NF - κ bp65, which are related factors of HMGB1-TLR4 / RANKL-NF - κ B signaling pathway in OC cells. Combined with the current research, the Zhuang medicine longzhuangtongbi prescription has a strong inhibitory effect on RANKL-RANK-NF - κ B[3] pathway and HMGB1-TLR4-MYD88-NF - κ B[4] pathway. It can be seen that other drugs in the prescription also play a role in this aspect, and may also play an anti-inflammatory role from other aspects, which is also a direction of follow-up research.

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