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Effects of RANKL on the proliferation and apoptosis of fibroblast-like synoviocytes in rheumatoid arthritis through regulating the NF-κB signaling pathway

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Abstract. – OBJECTIVE: The aim of this study is to investigate the regulatory effects of receptor activator of nuclear factor-kappa B ligand (RANKL) on the proliferation and apoptosis of fibroblast-like synoviocytes (FLS) in rheumatoid arthritis (RA), and to explore its regulatory mechanism.

MATERIALS AND METHODS: Synoviocytes were primarily cultured in rats of recognized collagen-induced arthritis (CIA) model. Meanwhile, they were induced into FLS models by lipopolysaccharides (LPS). All cells were divided into three groups, including blank group, model group and RANKL inhibitor group. The levels of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) in the cells were detected by enzyme-linked immunosorbent assay (ELISA). The proliferation and apoptosis of FLS were detected *via* 3-(4,5)-dimethylthiazol (-z-y1)-3,5-diphenytetrazoliumromide (MTT) assay and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, respectively. Reverse transcription-polymerase chain reaction (RT-PCR) was conducted to measure the messenger ribonucleic acid (mRNA) expression levels of nuclear factor-kappa B ligand (NF-κB) and Caspase-3 in FLS. Furthermore, Western blotting was adopted to detect the protein expression levels of NF-κB and Caspase-3 in FLS.

RESULTS: Compared with the blank group, the expression levels of TNF-α and IL-1β in the cells of the model group increased significantly. Cell proliferation rate increased significantly, whereas the cell apoptosis rate decreased remarkably in the model group. Meanwhile, the mRNA and protein levels of NF-κB and Caspase-3 in FLS were significantly up-regulated. Compared with the model group, the levels of TNF-α and IL-1β in cells of RANKL inhibitor group notably declined. Similarly, cell proliferation rate was significantly reduced, whereas the cell apoptosis rate increased significantly. Furthermore, the mRNA and protein levels of NF-κB and Caspase-3 in FLS were evidently down-regulated.

CONCLUSIONS: RANKL inhibitors can inhibit the proliferation and promote the apoptosis of FLS in RA. In addition, its mechanism may be related to the inhibition of NF-κB signaling pathway.

Key Words:
RANKL, NF-κB, Rheumatoid arthritis (RA), Fibroblast-like synoviocyte, Proliferation, Apoptosis.

Introduction

Rheumatoid arthritis (RA) is a chronic, symmetrical and poly-synovial arthritis. It belongs to autoimmune inflammatory disease. RA seriously reduces life quality of patients regardless of age or sex, requiring lifelong treatment[1,2]. It is known to all that RA involves joint synovial membranes, articular cartilages, bone tissues, etc. The pathological manifestation of RA includes hyperplasia of membranous tissue. Abnormal proliferation of RA synovial fibroblasts (RASFs) can be observed in a tumor-like manner. This may lead to a destruction of articular cartilages and loss of working ability of patients, eventually endangering life by involving other organs[3,4]. Fibroblast-like synoviocytes (FLS) secrete synovial fluid under physiological conditions to provide joint nutrition and buffer vibration. Under pathological conditions, FLS proliferate excessively, leading to the release of a large number of inflammatory factors. This further stimulates the proliferation

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of FLS, causing chronic inflammatory of joints, as well as aggravating the occurrence and development of RA. At present, there is no cure for RA in the clinic. Furthermore, the treatment of the disease can only delay inflammation and reduce bone erosion. The discovery of osteoclast activator of nuclear factor-kappa B ligand (RANKL) brings hope for the treatment of RA. RANKL is a nuclear factor-kappa B (NF-κB) activated receptor ligand, whose gene is located on human chromosome 13q14. It contains 316 amino acid peptide chains with three subtypes, which are known as a type I transmembrane proteins. The homology between human and mouse is about 70%[5,6]. RANKL acts as a necessary cytokine in the osteoclast process. Meanwhile, its abnormal expression plays an important role in regulating bone resorption around the joint. If RANKL can inhibit the abnormal proliferation of FLS and the release of inflammatory factors, it is of great significance for the prevention and treatment of RA[7]. In this study, recognized collagen-induced arthritis (CIA) rat models[8] were selected. Synovial tissue cells were isolated from CIA rat synovial tissues and primarily cultured *in vitro*. FLS inflammatory models were induced and constructed using lipopolysaccharides (LPS). In addition, the regulatory effects of RANKL on the proliferation and apoptosis of RA FLS, as well as its regulatory mechanism, were explored[9].

Materials and Methods

Reagents

Tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) enzyme-linked immunosorbent assay (ELISA) kits were purchased from Wuhan Cusabio Co., Ltd. (Wuhan, China), phosphate-buffered saline (PBS), agaroses, LPS, 1640 medium, fetal bovine serum (FBS), double-antibody and pancreatins from Gibco (Rockville, MD, USA), TRIzol lysate and primers (NF-κB, Caspase-3 and β-actin) from Invitrogen (Carlsbad, CA, USA), diethyl pyrocarbonate (DEPC)-treated water from Sigma-Aldrich (St. Louis, MO, USA), NF-κB, Caspase-3, and β-actin primary antibodies and secondary antibodies from CST (Danvers, MA, USA), and culture plate from Corning (Corning, NY, USA).

Instruments

Electrophoresis apparatus, enzyme labeling apparatus and polymerase chain reaction (PCR) apparatus were purchased from Bio-Rad (Hercules, CA, USA), first strand complementary deoxyribonucleic acid (cDNA) synthesis kit from Thermo Fisher Scientific (Waltham, MA, USA), CO_2 incubator from Changzhou Henglong Co., Ltd. (Changzhou, China), and gel imager from Shanghai Tanon Technology Co., Ltd. (Shanghai, China).

Laboratory Animals

Sprague Dawley (SD) rats [Beijing Vital River Laboratory Animal Technology Co., Ltd., Animal Certificate No.: SCXK (Beijing, China) 2012-0001], weighing (210±20) g, were kept under constant temperature and humidity. All rats were given free access to food and water. This study was approved by the Animal Ethics Committee of Nanjing Medical University Animal Center (Nanjing, China).

Primary Culture of FLS

CIA model rats were anesthetized by intraperitoneal injection of 10% chloral hydrate and fixed in supine position. Under sterile conditions, the knee joints of rats were cut to expose the joints. Ligaments were stripped, and synovial tissues in the inner layer were exposed. After washing with precooled PBS for 3 times, blood samples and adipose tissues on the synovial surface were removed to obtain relatively pure white synovial tissue mass. Subsequently, synovial tissues were cut into 1 mm^3 tissue pieces and centrifuged, and the supernatant was discarded. After re-suspension with FBS, the tissues were paved into a 25 cm^3 culture bottle. After culture for 1 h, 1640 complete medium containing 15% FBS was added, followed by incubation in an incubator with 5% CO_2 at 37°C. The medium was replaced once about every 3 days. Cell passage was conducted when the density of cells reached 80%[10].

The Levels of TNF-α and IL-1β in FLS by ELISA

Cells were divided into three groups, namely, blank group (non-treatment group), model group (LPS model group), and RANKL inhibitor group (treatment group). The supernatant of FLS in each group was aspirated for the experiment according to the instructions of ELISA. Briefly, 100 μL standards and samples were added to each well. After sealing with a sealing film, they were incubated at 37°C for 2 hours. Next, the liquid was sucked off, and 100 μL biotin antibodies were added to each well and sealed with sealing

film. Subsequently, after 1 hour of incubation was carried out, the mixture was washed for three times, and the liquid in wells was spin-dried. 100 μL horseradish peroxidase (HRP)-labeled solution was added for 1 hour of incubation, followed by washing for three times. 90 μL TMB was then added for incubation for another 20 minutes. Finally, 50 μL stop buffer was added to terminate the reaction. The absorbance at 450 nm was detected, and the levels of TNF-α and IL-1β were finally calculated.

The Proliferation of FLS via 3-(4,5)-Dimethylthiazol (-z-y1)-3,5-Diphenytetrazoliumromide (MTT) Assay

FLS were first seeded into 96-well plates at a density of 5×10^3 cells/well. After overnight culture, 10 μL MTT solution (5 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, followed by culture for another 4 hours. Then, the culture medium was discarded, and 150 μL dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added to each well to terminate the culture. Subsequently, the plate was shaken on a shaker for 10 minutes to dissolve crystals. Finally, the absorbance at 490 nm was measured, and the proliferation rate was calculated.

The Apoptosis of FLS via Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) Assay

Cells were first fixed with 4% paraformaldehyde, permeabilized and washed with PBS for 3 times. 50 μL TdT enzyme reaction solution was added to each well, followed by 1 hour of reaction in the dark at 37°C. Later, they were washed with PBS for 3 times. Subsequently, 50 μL streptavidin-TRITC-labeled solution was added for 30 minutes of reaction in the dark, followed by washing with PBS for 3 times. The nucleus was re-stained with 4',6-diamidino-2-phenylindole (DAPI) staining solution, and the cells were incubated at room temperature for 15 minutes. DAPI staining solution was washed off. Finally, the staining was observed under a microscope.

The Messenger Ribonucleic Acid (mRNA) Levels of NF-κB and Caspase-3 in FLS via Reverse Transcription-PCR (RT-PCR)

Cells in each group were first collected and lysed with TRIzol lysate (Invitrogen, Carlsbad, CA, USA). Chloroform and isopropanol were added, followed by centrifugation at 12000 rpm for 5 minutes. The supernatant was discarded, and the precipitate was retained and gently washed with 75% ethanol. After naturally air-dried, the cells were re-suspended with 0.01% diethyl pyrocarbonate (DEPC)-treated water. The concentration of RNA was measured, and extracted RNA was reverse transcribed into complementary deoxyribose nucleic acids (cDNAs) according to the instructions of the first strand kit. PCR amplification was carried out after the reaction. The primer sequences are shown in Table I. Specific reaction conditions were as follows: denaturation at 95°C for 0.5 minutes, annealing at 58°C for 0.5 minutes and extension at 72°C for 1 minute, for a total of 35 cycles. PCR amplification products were subjected to agarose gel electrophoresis and photographed under a gel imager. The relative gray value of bands was analyzed by Image J software (NIH, Bethesda, MD, USA).

The Protein Levels of NF-κB and Caspase-3 in FLS via Western Blotting

Cells in each group were first collected, lysed with 1×radioimmunoprecipitation assay (RIPA) lysate (Beyotime, Shanghai, China) and centrifuged. The supernatant was preserved, and the protein concentration was quantified. Subsequently, 30 μg proteins were separated by electrophoresis under 100 V and transferred onto membranes. After sealing with 5% skim milk powder solution for 1 hour, the membranes were incubated with primary antibodies of NF-

Table I. Primer sequences.

Gene name	Category	Sequence
NF-κB	Forward	5' TGC CGA GTG AAC CGA AAC 3'
	Reverse	5' GCT CAG GGA TGA CGT AAA GG 3'
Caspase-3	Forward	5' AGA TAC CGG TGG AGG CTG ACT 3'
	Reverse	5' TCT TTC GTG AGC ATG GAC ACA 3'
β-actin	Forward	5' GGG AAA TCG TGC GTG ACA 3'
	Reverse	5' TCA GGA GGA GCA ATG ATC TTG 3'

Table II. Levels of TNF-α and IL-1β.

Group	TNF-α (pg/mL)	IL-1β (pg/mL)
Blank group	28.37 ± 3.89	42.56 ± 5.72
Model group	135.15 ± 6.26**	152.42 ± 7.93**
RANKL inhibitor group	78.16 ± 7.25$^{\#}$	89.43 ± 9.04$^{\#}$

Note: Model group *vs*. blank group, $^{**}p < 0.01$ and $^{*}p < 0.05$. RANKL inhibitor group *vs*. model group, $^{\#}p < 0.05$.

κB, Caspase-3 and β-actin at 4°C overnight. On the next day, the membranes were washed with Tris-Buffered Saline and Tween-20 (TBST) solution, followed by incubation with corresponding secondary antibodies at room temperature for 1 hour. Then, the membranes were washed again with TBST. Finally, diaminobenzidine (DAB) color developing solution was added for color development.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 software (SPSS Inc., Chicago, IL, USA) was adopted for all statistical analysis. Experimental data were expressed as mean ± standard deviation. One-way analysis of variance was used to compare the differences among different groups, followed by Post-Hoc Test Least Significant Difference (LSD). A *t*-test was applied to compare the difference between the two groups. *p*-values < 0.05 were considered statistically significant.

Results

RANKL Inhibitors Inhibited the Release of TNF-α and IL-1β

Compared with the blank group, the levels of TNF-α and IL-1β in cells of the model group increased significantly ($^{**}p<0.01$, $^{*}p<0.05$). Compared with the model group, the levels of TNF-α and IL-1β in cells of the RANKL inhibitor group were notably reduced ($^{\#}p<0.05$, $^{\#}p<0.05$) (Table II). The results indicated that RANKL inhibitors could inhibit the release of TNF-α and IL-1β.

RANKL Inhibitors Suppressed the Proliferation of FLS

The results of MTT assay revealed that the cell proliferation rate in the model group was increased compared with that in the blank group ($^{**}p<0.01$). Compared with that in the model group, the cell proliferation rate in RANKL inhibitor group was obviously decreased ($^{\#}p<0.05$) (Figure 1), indicating that RANKL inhibitors can suppress the proliferation of FLS.

RANKL Inhibitors Promoted the Apoptosis of FLS

TUNEL staining results (Figure 2A) revealed that the red TUNEL-positive cells indicated cell apoptosis. It was found that cell apoptosis rate in the model group declined significantly when compared with the blank group ($^{*}p<0.05$). Compared with model group, cell apoptosis rate in RANKL inhibitor group increased significantly ($^{\#}p<0.05$) (Figure 2B). The above results indicated that the RANKL inhibitors promoted the apoptosis of FLS.

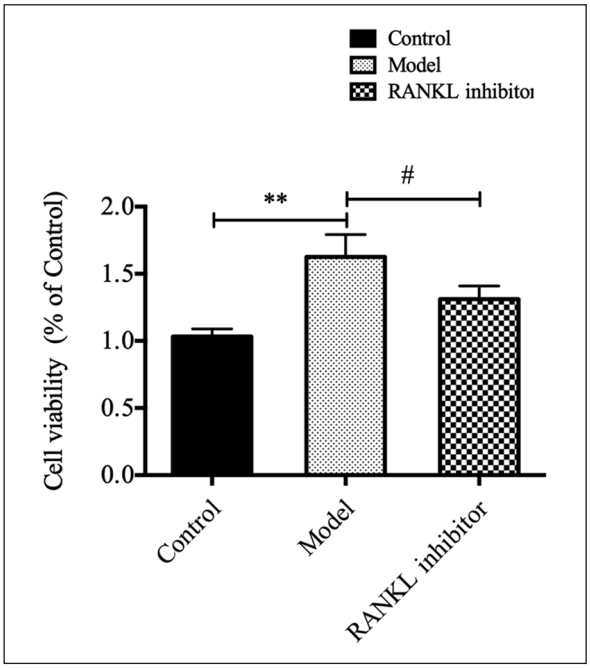

Figure 1. Proliferation of FLS in each group ($^{**}p<0.01$, $^{\#}p<0.05$).

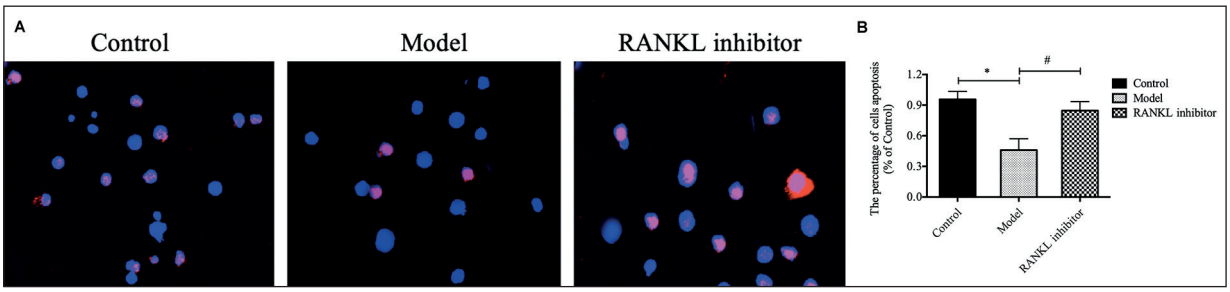

Figure 2. Apoptosis of FLS in each group. **A,** TUNEL staining graphs. **B,** Statistical charts of cell apoptosis (20×, *p<0.05, $^{\#}p$<0.05).

RANKL Inhibitors Inhibited the mRNA levels of NF-κB and Caspase-3 in FLS

RT-PCR bar graphs (Figure 3A) showed that compared with the blank group, the mRNA levels of NF-κB and Caspase-3 in the model group were markedly up-regulated (*p<0.05, *p<0.05). Meanwhile, the mRNA levels of NF-κB and Caspase-3 in RANKL inhibitor group were significantly lower than those of the model group ($^{\#}p$<0.05, $^{\#}p$<0.05) (Figure 3B). The above results suggested that RANKL inhibitors reduced the mRNA levels of NF-κB and Caspase-3 in FLS.

RANKL Inhibitors Suppressed the Protein Levels of NF-κB and Caspase-3 in FLS

Western blotting bar graphs (Figure 4A) illustrated that compared with blank group, the protein levels of NF-κB and Caspase-3 in model group increased significantly (*p<0.05, *p<0.05). Meanwhile, the protein levels of NF-κB and Caspase-3 in RANKL inhibitor group declined remarkably when compared with the model group ($^{\#}p$<0.05, $^{\#}p$<0.05) (Figure 4B). The above results illustrated that RANKL inhibitors suppressed the protein levels of NF-κB and Caspase-3 in FLS.

Discussion

RA is an autoimmune disease with long onset cycle. The average prevalence rate of RA is about 1% worldwide[11]. In the late stage, various degrees of joint deformity and dysfunction may occur in patients. Therefore, RA is a disease with high disability rate, bringing burdens to society and seriously reducing the life quality of patients[12]. Currently, there is no radical cure method for RA. Commonly used therapeutic drugs include non-steroidal anti-inflammatory drugs and glucocorticoids. However, these drugs can only alleviate the development of inflammation, with no effect of radical cure[13]. Among the pathogenesis of RA, abnormal proliferation of synoviocytes is the most recognized feature. They release a large number of inflammatory factors, exacerbating inflammatory responses[14,15]. NF-κB is one of the major transcription factors expressed in inflammatory cells. At present, studies have shown that NF-κB plays an important role in two aspects of RA[16]. On the one hand, activated NF-κB activates the release of pro-inflammatory factors and promotes the proliferation of FLS. On the other

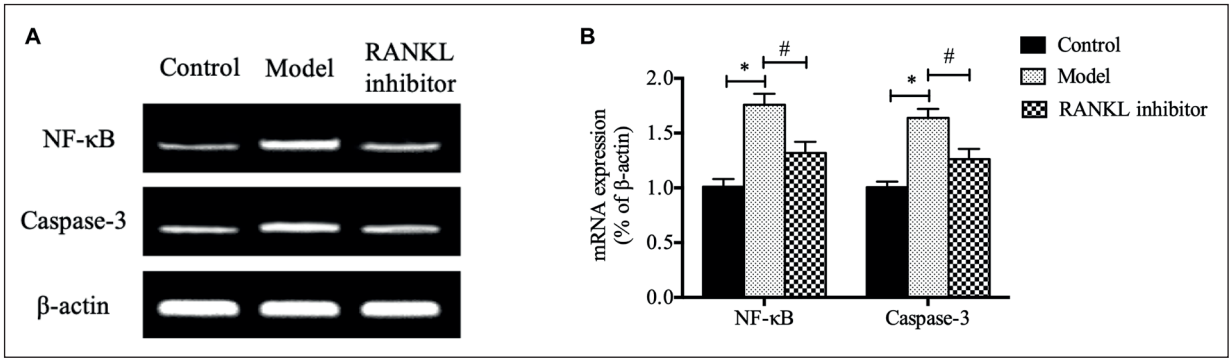

Figure 3. mRNA levels of NF-κB and Caspase-3 in each group of FLS. **A,** RT-PCR bar graphs with β-actin as an internal reference. **B,** RT-PCR statistical charts (*p<0.05, $^{\#}p$<0.05).

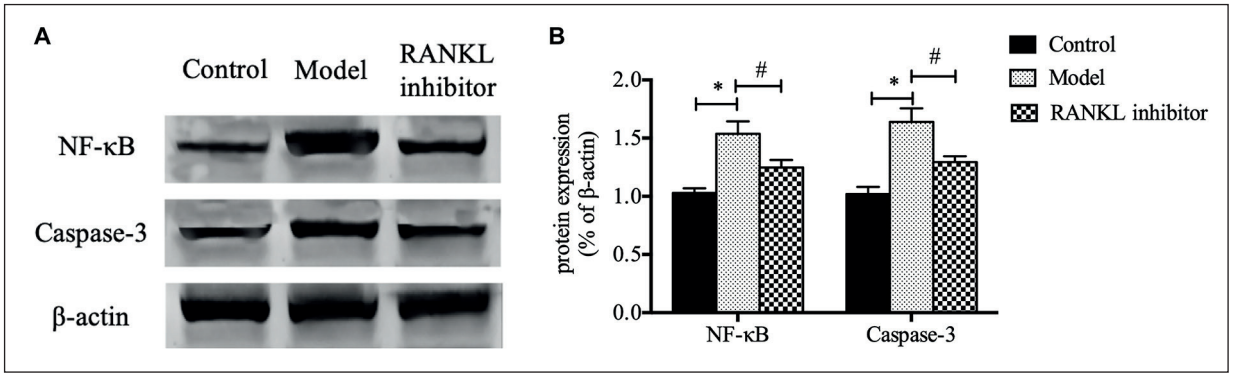

Figure 4. Protein levels of NF-κB and Caspase-3 in each group of FLS. **A,** Western blot bar graphs with β-actin as an internal reference. **B,** Western blotting statistical charts (*p<0.05, #p<0.05).

hand, multiple inflammatory factors further lead to the activation of NF-κB, as well as inhibit the apoptosis of FLS. Qi et al[17] induced FLS models with TNF-α. After treatment with heparin, the expression level of NF-κB decreased significantly when compared with that in models. Meanwhile, the abnormal proliferation of FLS is inhibited. These findings indicate that NF-κB plays a key role in the pathogenesis and development of RA. The discovery of RANKL has brought new ideas for the treatment of RA. RANKL is a receptor activator ligand of NF-κB, which is expressed in various tissues, such as brain, intestine, skeletal muscle, and kidney. It has been confirmed that its expression is the most significant in bone tissues. When RANKL is over-expressed, it will cause femoral injury and bone destruction. This is characterized by an increase in bone absorption and inhibition of bone formation. Eventually, it will lead to bone erosion and bone loss around bone joints. Researches have revealed that RANKL exerts crucial effects in both bone tissues and immune system. Chiu et al[18] have found that corticosteroids can reduce bone erosion activity and inhibit RANKL expression in a targeted way. However, side effects may also appear. Modified methotrexate is safe and effective in treatment, indicating that RANKL is the target of RA treatment. The existing drugs are not very ideal. However, reduced toxicity and increased efficiency can be achieved through the modification of drug structures, which provides new ideas for treatment. Through the study of a small peptide chain, Sims et al[19] have indicated that small peptide chain can block the binding of NF-κB receptors to ligands in a targeted way, inhibit bone absorption, and promote bone formation, and cartilage protection. This shows good therapeutic effect, suggesting that RANKL may be a potential target for the treatment of RA. In this study, synoviocytes were collected from synovial tissues of rats in CIA model and primarily cultured *in vitro*. LPS-induced FLS were used to investigate the effects of RANKL on the proliferation and apoptosis. LPS, as bacterial endotoxins, can stimulate the release of inflammatory factors after entering the body. Meanwhile, they are widely applied in cell experiments *in vitro* to establish cell inflammation models[20]. The levels of TNF-α and IL-1β in cells of each group were detected *via* ELISA. The results showed that RANKL inhibitors remarkedly reduced their expressions, indicating that RANKL inhibitors could inhibit the release of inflammatory factors. Subsequently, MTT and TUNEL assays verified that RANKL inhibitors could suppress the proliferation, while promoting the apoptosis of FLS. To further study the mechanism of RANKL inhibitors, the expressions of NF-κB and Caspase-3 were examined at mRNA and protein levels. It was found that RANKL inhibitors could evidently reduce their expressions. Our findings suggested that RANKL inhibitors might inhibit the proliferation and promote the apoptosis of FLS by inhibiting NF-κB signaling pathway, thus providing a new research idea for RA.

Conclusions

RANKL inhibitors can inhibit the proliferation and promote the apoptosis of FLS in RA. The possible underlying mechanism may be related to the inhibition of NF-κB signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Acknowledgements

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