SPANDIDOS PUBLICATIONS

In vitro study of inhibitory millimeter wave treatment effects on the TNF-α-induced NF-κB signal transduction pathway

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Abstract. Abnormal activation of the nuclear factor-κB (NF-κB) in chondrocytes initiates the transcription of inflammatory mediators, promotes their generation and release, and amplifies initial inflammatory signals. This results in the release of chondral matrix-degrading enzymes and accelerates the degeneration of articular cartilage. As a non-pharmaceutical and non-invasive physical therapy regimen, millimeter wave treatment has been successfully used for the treatment of osteoarthritis. In this study, chondrocytes were derived from the cartilages of knee joints of 4-week-old male Sprague-Dawley rats and were mechanically digested by collagenase type II treatment for further culture *in vitro*. The third-passage chondrocytes were stained with toluidine blue and treated with a gradient of tumor necrosis factor-α (TNF-α) for various times. Chondrocytic activity was measured by MTT assay, and the apoptotic rate of the chondrocytes was determined with Hocehst 33342 staining to identify effective treatment concentrations and durations and to establish an apoptosis model for the chondrocytes in response to TNF-α. Using this model, the chondrocytes were randomly divided to receive millimeter wave treatment for various times. The apoptotic rate of the chondrocytes was measured by Annexin V-FITC staining and the protein expression levels of RIP, TAK1, IκB kinase (IKK)-ß, IκB-α and NF-κB, were determined by Western blotting. Chondrocytic structure was examined by transmission electronic microscopy. The apoptotic rates were significantly lower at 4 and 8 h of treatment than at 0 and 2 h. The expression levels of RIP, TAK1, IKK-ß and NF-κB were also significantly lower at 4 and 8 h than at 0 and 2 h, whereas that of IκB-α was significantly higher at 4 and 8 h than at 0 and 2 h. Therefore, we can conclude that millimeter wave treatment can inhibit the activation of the TNF-α-mediated NF-κB signal transduction pathway through the down-regulation of RIP, TAK1, IKK-ß and NF-κB, and the up-regulation of IκB-α, in chondrocytes.

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Introduction

The morphological changes of osteoarthritis manifest mainly as synovitis and chondral destruction of various severities, usually caused by abnormal stress on the knee joints. The wear of articular cartilages stimulates the inflammatory reaction in synovial cells and chondrocytes. The local increase in inflammatory stimuli and mediators in the articular tissues leads to secondary synovial proliferation, articular effusion and cartilage malnutrition, resulting in articular cartilage degeneration (1-4). As one of the pivotal inflammatory factors in the pathogenesis of osteoarthritis, tumor necrosis factor-α (TNF-α) functions to induce inflammation, promote the release of bone matrix-degrading enzymes, and mediate fibroblastic hyperplasia and histological damage (5,6). Nuclear factor-κB (NF-κB) signalling is one of the critical pathways that regulate the inflammatory response and chondrocytic apoptosis in osteoarthritis induced by TNF-α (7).

NF-κB, a key transcriptional regulator largely present in the cytoplasm, is involved in the inflammatory and immune responses by regulating the expression of cytokines, chemoattractants, growth factors, adhesion molecules and enzymes. It also plays an important role in the regulation of cellular proliferation, differentiation and apoptosis (8,9). When inactive, NF-κB forms a trimeric complex of p50 and p65 subunits and IκB. Various cellular stimuli activate the ubiquitin-dependent IκB kinases (IKKs) to phosphorylate two conserved serine residues in the N-terminus of trimeric IκB, which allows for the covalent conjugation of multiple ubiquitins to lysines 21 and 22 via ubiquitin ligases. The phosphorylation and ubiquitination events alter the configuration of IκB, which is consequently degraded by ATP-dependent 26S proteasomes, exposing the nuclear localization signal of the NF-κB (Rel) protein. This activation of NF-κB causes its rapid intranuclear translocation and binding to the κB sites of the target genes, thus controling the transcription and expression of multiple genes involved in inflammatory

and immune responses, as well as cellular proliferation, differentiation and apoptosis (10,11).

The millimeter wave is a high-frequency electromagnetic wave. It has a wavelength of 1-10 mm and a frequency of 30-300 GHz. Living organisms generate $0.5x10^{10}$-$3x10^{12}$ coherent oscillations during metabolism, which is within the frequency of a millimeter wave. Therefore, the energy of the millimeter wave can be absorbed by biological tissues through resonance and produces a non-thermal biological effect that interferes with signal transduction and effectively and dynamically regulates cellular metabolism (12,13). As a physical therapeutic regimen, millimeter wave treatment differs from conventional medical regimens in its convenience, safety and non-invasiveness. Our pilot study previously demonstrated that millimeter wave treatment down-regulated the expression and activation of P-p38 and p53 in apoptotic chondrocytes and suppressed the activity of caspase-3, a key player in chondrocytic apoptosis, thus leading to the inhibition of chondrocytic apoptosis (14). As a rapidly responding nuclear transcription factor, a large amount of NF-κB is involved in cell proliferation and immune response. Various intracellular and extracellular signals stimulate the abnormal activation of NF-κB and initiate the gene transcription of multiple inflammatory mediators such as TNF-α and IL-6. The increased production and release of inflammatory mediators that amplify initial inflammatory signals, boost the release of chondral matrix-degrading enzymes and accelerate articular cartilage degeneration, thus playing a key role in the development and progression of osteoarthritis (15,16). It has been hypothesized that the effective regulation of NF-κB signaling could be used as a novel target for the prevention and treatment of osteoarthritis. Thus, we established chondrocytic colonies *in vitro* from the knee joint cartilages of Sprague-Dawley rats and used TNF-α to induce NF-κB signaling in order to identify the effects of millimeter wave treatment on the regulation of NF-κB signaling.

Materials and methods

Instruments and equipment. KFA-100A millimeter wave therapeutics were manufactured by Zhongcheng Kangfu Technology Co., Ltd. (Beijing, China) at wavelengths of 7.5-10.0 mm, a power density of 4 mw/cm^2, and a radiation area of 15.1 cm^2 (45.6±4 mm long and 33.2±3 mm wide).

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and trypsin, were purchased from Hyclone (Carlsbad, CA, USA). MTT and collagenase type II were from Sigma (NJ, USA), and toluidine blue was from Sinopharm Chemical Reagents Co., Ltd. (Shanghai, China). The annexin V-FITC kit was from Jingmei Biotech Co., Ltd. (Shenzhen, China) and the total protein extraction kit was from KeyGen Biotech Co., Ltd. (Nanjing, China). RIP, TAK1, IKK-ß, IκB-α, NF-κB and ß-actin antibodies were from Santa Cruz (Santa Cruz, CA, USA), horseradish peroxidase-labeled rabbit anti-goat IgG was from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China), the immnoblotting chemiluminescence kit was from the Beyotime Institute of Biotechnology (Nantong, China), and PVDF membranes were from Millipore (NJ, USA).

Laboratory animals. Four-week-old, male Sprague-Dawley (SD) rats (n=30) were purchased from Slac Laboratory Animal Co., Ltd. (Shanghai, China; certification no. SCXK(SH)2007-0005). The use and care of laboratory animals was in compliance with the regulations of the Guidelines for Laboratory Animal Welfare established by the Ministry of Science and Technology, China (17).

In vitro culture and characterization of chondrocytes. SD rats were anesthetized with 10% chloral hydrate (0.3 ml/100 g) and the bilateral knee joints were isolated in a sterile manner. Superficial cartilages were harvested and rinsed in 1X PBS buffer containing penicillin and streptomycin and then washed in DMEM three times. The cartilages were sectioned into 1 mm^3 pieces and incubated in 0.2% collagenase type II at 37˚C. Supernatants were collected every 60 min and centrifuged at 1,000 rpm for 5 min. Cell pellets were harvested and the digestion solution was refreshed four times. The cells were reconstituted in DMEM containing 10% FBS and supplemented with 50 mg/l vitamin C, 100 U/ml penicillin and 100 U/ml of streptomycin, and filtered through a 200-micron stainless steel screen. The cells were adjusted to a density of $3x10^5$/ml using a hemocytometer and incubated at 37˚C at an atmosphere of 5% CO_2 for the primary culture. Cells were passaged at 80% confluency as determined by microscopy. Third-passage chondrocytes were harvested and characterized by toluidine blue staining.

Establishment and characterization of chondrocyte apoptosis model. The third-passage chondrocytes were seeded into 96-well plates at a density of $1x10^4$/ml in a final volume of 200 μl and incubated in DMEM with 10% FBS for 72 h. Cells were equally and randomly divided into four groups of six wells each, which were supplemented with 10% FBS DMEM containing TNF-α at final concentrations of 0, 10, 20 and 40 ng/ml for 2, 4 and 8 h. The activity of the chondrocytes was examined by MTT assay, and their apoptotic profiles were characterized using Hoechst 33342 staining.

Millimeter wave treatment protocol of chondrocytes. The third-passage chondrocytes were seeded into 6-well plates at a density of $1x10^5$/ml and incubated in 10% FBS-DMEM for 72 h. Cells were equally and randomly divided into four groups and treated with millimeter wave treatment for 0, 2, 4 and 8 h. Cells were continuously incubated in 10% FBS-DMEM containing 10 ng/ml TNF-α for the total duration of 8 h. The millimeter wave irradiation was achieved by ultraviolet light for 30 min and suspension 30 mm away from the cells in the incubator. Chondrocytes were placed around the probe within a 20-mm radius, and the treatment was effective at 33.2±3 x 45.6±4 mm. Following millimeter wave treatment, the early apoptotic rate of the chondrocytes was examined by flow cytometry using Annexin V-FITC/PI. The protein expression levels of RIP, TAK1, IKK-ß, IκB-α and NF-κB were determined by Western blotting. Chondrocytic ultrastructures were analyzed using a transmission electronic microscope.

Toluidine blue staining of chondrocytes. The third-passage chondrocytes were plated onto cover slips, rinsed in 1X PBS,

4% paraformaldehyde for 30 min and stained with ine blue for 30 min. The sections were rinsed briefly in absolute ethanol, dried, mounted, and were then examined by microscopy.

MTT assay of chondrocytic activity. The treated third-passage chondrocytes were collected and rinsed in 1X PBS. The rinse buffer was discarded and 20 μl of 0.5% MTT solution were added to each well and incubated at 37˚C for 4 h. MTT was discarded and replaced by 150 μl DMSO, and the mixture was vortexed for 10 min. The optical density (OD) value was analyzed for each group by using a spectrometer at λ=570 nm, and the means were calculated.

Hoechst 33342 staining of apoptotic chondrocytes. The treated third-passage chondrocytes were collected and fixed in 4% paraformaldehyde for 15 min. The fixation solution was discarded, and the cells were rinsed in 1X PBS for 5 min. The buffer was discarded, and the cells were incubated in 10 μM Hoechst 33342 solution in the dark at 37˚C for 15 min. The Hocehst 33342 solution was discarded, and the stained cells were rinsed in 1X PBS and supplemented with fresh 1X PBS buffer. Stained cells were examined under an inverted fluorescent microscope for cellular morphology and nuclear profiles. Ten visual fields were randomly selected for each group to count the number of apoptotic chondrocytes.

Annexin V-FITC assay of apoptotic rate of chondrocytes. The treated third-passage cells were digested, centrifuged and collected into 1X PBS buffer at 4˚C, and labeled using the Annexin V-FITC/PI kit. Rinsed cells were reconstituted with binding buffer at a density of $1x10^6$/ml. The cell suspension (100 μl) was added into a 5-ml dry flow tube supplemented with 5 μl Annexin V-FITC and 10 μl PI (20 μg/ml). The cells were vortexed and incubated in the dark at room temperature for 15 min. An additional 400 μl of 1X PBS were added, and cells were sampled by flow cytometry to determine the apoptotic fraction of the chondrocytes.

Western blotting of RIP, TAK1, IKK-ß, IκB-α and NF-κB. Following the manufacturer's instructions, total proteins were isolated from treated third-passage chondrocytes, and protein concentrations were determined using the BCA method. Samples of 25 μl, containing 20 μg total protein, were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto PVDF membranes. The membranes were incubated with blocking solution (48 mM Tris, 39 mM glycine, 0.05 % w/v SDS, 5 % v/v methanol) at room temperature for 2-4 h. Proteins were detected with rabbit anti-RIP, -TAK1, -IKK-ß, -IκB-α, -NF-κB and -ß-actin antibodies at 4˚C overnight and anti-rabbit secondary antibodies at 37˚C for 2 h. The membranes were rinsed in 1X PBS, labeled with ECL substrates, and exposed to X-ray. Protein bands were analyzed using the Fluor-S Gel Imaging Analysis System and normalized to ß-actin in the sample.

Transmission electron microscopy of chondrocytes. The treated third-passage chondrocytes were digested and collected. The cells were pre-fixed in 3% glutaraldehyde and 1.5% paraformaldehyde at 4˚C for 24 h, post-fixed in 1% osmic acid and 1.5% potassium hexacyanoferrate (II) for 1.5 h, and rinsed in 1X PBS. Rinsed cells were stained with uranyl acetate-saturated 70% ethanol, dehydrated in a gradient of ethanol and acetone, and embedded in epoxide resin 618. The solidified blocks were ultrasectioned into 80-nm sections and further stained with uranyl acetate and lead citrate for 5 min. The stained ultrasections were examined using a transmission electronic microscope.

Statistical analysis. All the data were processed with SPSS 3.0. The quantitative data were expressed as the means ± standard deviation. The differences among the four groups were compared by using one-way analysis of variance (ANOVA), and multiple comparisons were performed with the SNK-q test. P-values of <0.05 were considered to be statistically significant.

Results

Morphology and characterization of chondrocytes in vitro. The *in vitro* cultured chondrocytes were small, round cells floating in the medium. On day 2 of culture, the chondrocytes became adherent to the culture flask and extended pseudopod-like projections, whereas their nuclei were mainly round or oval in shape (Fig. 1A). On day 4, the cells were mainly polygonal in shape, and most of the nuclei were round or oval and located in the centers of the cell bodies (Fig. 1B). On day 8, the cells became confluent and formed a monolayer structure (Fig. 1C). Both second- and third-passage cells grew mostly in irregular shapes of variable sizes, most with round or ovoid nuclei (Fig. 1D and E). In order to characterize the chondrocytes, the cells were examined by toluidine blue staining. On day 3 of culture, the third-passage chondrocytes exhibited reddish-purple metachromatic granules inside the cytoplasm, and they were also surrounded by a few such granules. The nuclei were mainly round or oval in shape and dark blue in color (Fig. 1F).

Activity of TNF-α stimulated chondrocytes. Prior to treatment, the OD values of the chondrocytes did not vary significantly between the groups. At 2 h after TNF-α induction, the OD values were significantly lower in the cells treated with 20 or 40 ng/ml (final concentration) TNF-α, than those in the cells treated with no TNF-α (P=0.041, P=0.015). At 4 h, the OD values of the cells treated with 10, 20, or 40 ng/ml TNF-α, were significantly lower than those of the untreated cells (P=0.036, P<0.001). The OD values were also significantly lower in the 20 or 40 ng/ml groups than in the 10 ng/ml group (P=0.023, P=0.001). At 8 h, the OD values of the 10, 20, or 40 ng/ml groups were significantly lower than those of the untreated cells (P<0.001) and the OD values in the 20 or 40 ng/ml treatment groups were also significantly lower than those in the group exposed to 10 ng/ml TNF-α (P=0.015, P<0.001). In addition, the OD values of the cells treated with 40 ng/ml TNF-α were significantly lower than those of the cells treated with 20 ng/ml (P=0.033) (Table I, Fig. 2).

Characterization of TNF-α-induced apoptosis. The Hoechst 33342 assay was performed to determine the apoptotic rate of the chondrocytes treated with 0, 10, 20, or 40 ng/ml for 2, 4

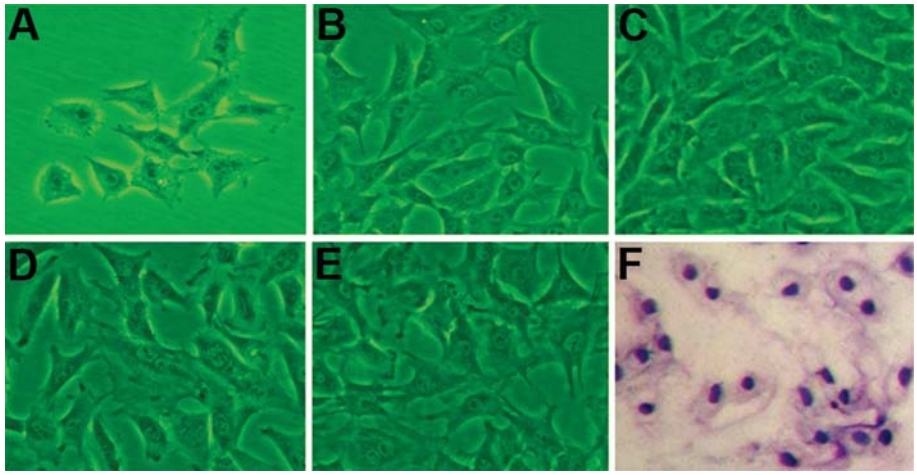

Figure 1. Morphology and characterization of chondrocytes *in vitro*. (A) primary culture on day 2, (B) primary culture on day 4, (C) primary culture on day 8, (D) second-passage culture on day 3, (E) third-passage culture on day 3, (F) third-passage culture on day 3 stained with toluidine blue.

Table I. MTT activities of chondrocytes treated with TNF-α.

TNF-α concentration (ng/ml)	OD values of chondrocytes			
	Pre-treatment	2 h of treatment	4 h of treatment	8 h of treatment
0	0.47±0.04	0.50±0.02	0.51±0.03	0.53±0.04
10	0.50±0.03	0.48±0.03	0.46±0.03[b]	0.42±0.03[a]
20	0.48±0.04	0.45±0.05[b]	0.40±0.04[a,d]	0.36±0.04[a,d]
40	0.49±0.03	0.44±0.04[b]	0.37±0.05[a,c]	0.31±0.04[a,c,e]

[a]P<0.01 compared to 0 ng/ml TNF-α, [b]P<0.05 compared to 0 ng/ml TNF-α, [c]P<0.01 compared to 10 ng/ml TNF-α, [d]P<0.05 compared to 10 ng/ml TNF-α, and [e]P<0.05 compared to 20 ng/ml TNF-α.

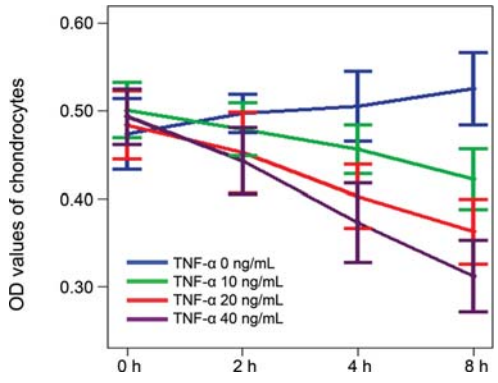

Figure 2. Activity trends of chondrocytes treated with various concentrations of TNF-α for 0, 2, 4 and 8 h.

and 8 h. The untreated chondrocytes did not show marked apoptosis, whereas the TNF-α-treated cells exhibited varying degrees of apoptosis (Fig. 3). Most of the untreated cells had round or oval and light blue nuclei, whereas the apoptotic cells had round or oval and bright blue nuclei, indicating nuclear condensation. At 2 h of TNF-α treatment, the apoptotic rates of the chondrocytes treated with 20 or 40 ng/ml TNF-α were significantly higher than those of the untreated cells (P≤0.001) or the cells treated with 10 ng/ml TNF-α (P=0.015, P<0.001). At 4 and 8 h, the apoptotic rates of the cells treated with 10, 20 or 40 ng/ml were significantly higher than those in the untreated groups (P=0.008, P<0.001). The rates significantly increased with increasing doses of TNF-α (Table II, Fig. 4).

Inhibitory effects of millimeter wave treatment on TNF-α-induced apoptosis. Flow cytometry of annexin V-FITC/PI stained cells was used to analyze the inhibitory effects of millimeter wave treatment on chondrocytic apoptosis induced by TNF-α. The percentages of normal chondrocytes were significantly higher when treated with millimeter wave therapy for 4 or 8 h than when treated for 0 or 2 h (P<0.001). The percentage of early and late apoptotic cells was significantly higher after 4 or 8 h of treatment than it was in the untreated cells (P≤0.001) or after 2 h of treatment (P=0.008, P=0.002). The percentage of dead cells was also significantly lower after 4 or 8 h treatment than it was in the untreated groups (P=0.003, P=0.019) (Table III, Figs. 5 and 6).

Expression of RIP, TAK1, IKK-β, IκB-α and NF-κB in millimeter wave-treated chondrocytes. Upon 8 h of TNF-α

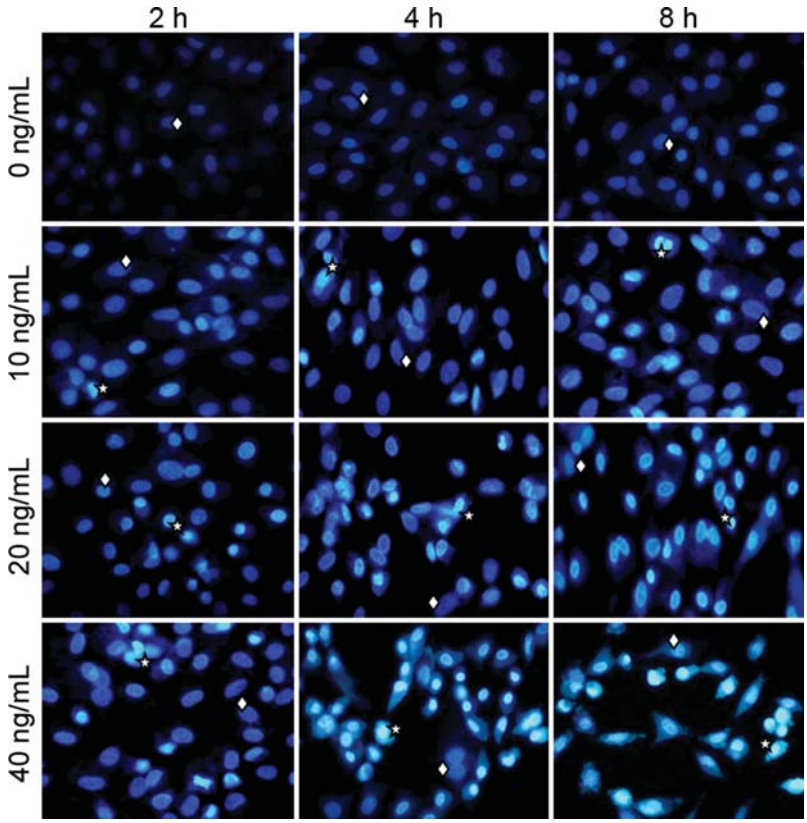

Figure 3. Characterization of TNF-α-induced chondrocyte apoptosis. The apoptotic rate of the chondrocytes was closely associated with TNF-α dosage and treatment duration. Treatment with TNF-α at a higher concentration for the same duration (2, 4 and 8 h) increased the apoptotic rate in a dose-dependent manner, increasing from 10 and 20 to 40 ng/ml. Additionally, the treatment with TNF-α for longer times at the same concentration (10, 20 and 40 ng/ml) also increased the apoptotic rate progressively from 2 and 4 to 8 h (☆, normal chondrocytes and ◇, apoptotic cells).

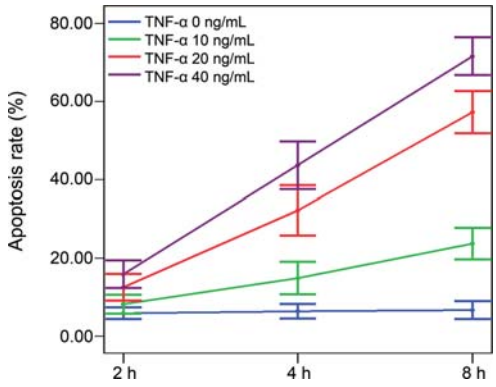

Figure 4. Apoptotic rates of TNF-α-treated chondrocytes.

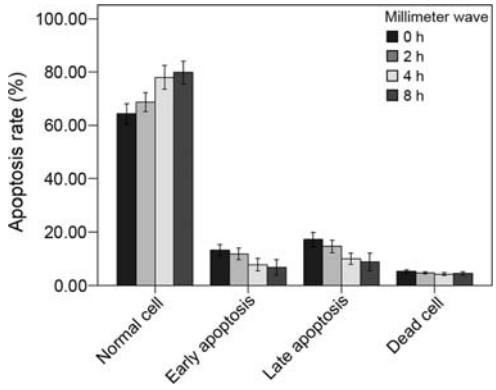

Figure 5. Millimeter wave treatment inhibited the apoptosis of chondrocytes treated by TNF-α.

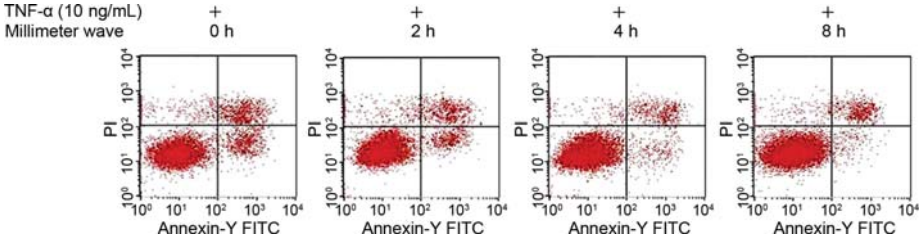

Figure 6. Millimeter wave treatment inhibited the apoptotic rate (%) of chondrocytes treated by TNF-α. Millimeter wave treatment was performed for 0, 2, 4 and 8 h in the TNF-α-treated (10 ng/ml) cells for a total of 8 h. After 4 and 8 h of treatment, the percentage of live cells increased significantly, whereas the number of cells in the early and late stages of apoptosis, as well as the number of dead cells, significantly decreased. Lower left quadrant, normal cells; lower right quadrant, early apoptotic cells; upper right quadrant, late apoptotic cells; upper left quadrant, dead cells.

Table II. Apoptotic rates of TNF-α-treated chondrocytes.

TNF-α concentration (ng/ml)	Apoptotic rate of chondrocytes (%)		
	2 h of treatment	4 h of treatment	8 h of treatment
0	5.83±1.47	6.33±1.86	6.67±2.25
10	8.17±2.40	14.83±4.17[a]	23.67±4.08[a]
20	12.50±3.39[a,c]	32.17±6.40[a,b]	57.17±5.42[a,b]
40	15.83±3.54[a,b]	43.67±6.09[a,b,d]	71.50±4.85[a,b,d]

[a]P<0.01 compared to 0 ng/ml TNF-α, [b]P<0.01 compared to 10 ng/ml TNF-α, [c]P<0.05 compared to 10 ng/ml TNF-α, and [d]P<0.01 compared to 20 ng/ml TNF-α.

Table III. Millimeter wave treatment inhibited apoptosis of chondrocytes treated by TNF-α (%).

Group	Normal cells	Early apoptotic cells	Late apoptotic cells	Dead cells
0 h of treatment	64.27±3.78	13.24±2.09	17.23±2.62	5.26±0.53
2 h of treatment	68.76±3.59	11.88±2.15	14.69±2.37	4.67±0.40
4 h of treatment	78.02±4.48[a,c]	7.78±2.33[a,c]	9.95±2.10[a,c]	4.25±0.51[a]
8 h of treatment	79.86±4.25[a,c]	6.83±2.93[a,c]	8.82±3.38[a,c]	4.49±0.63[b]

[a]P<0.01 compared to 0 h of treatment, [b]P<0.05compared to 0 h of treatment, and [c]P<0.01 compared to 2 h of treatment.

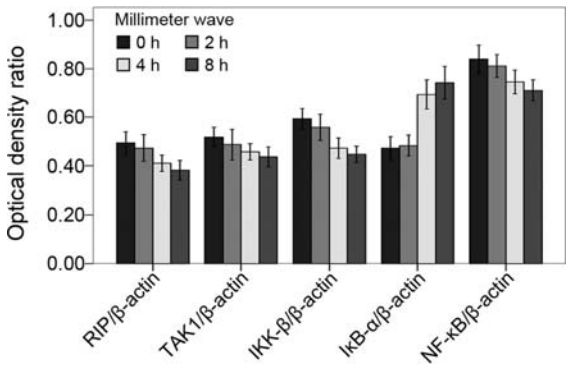

Figure 7. Expression profiles of RIP, TAK1, IKK-ß, IκB-α and NF-κB in millimeter wave-treated chondrocytes.

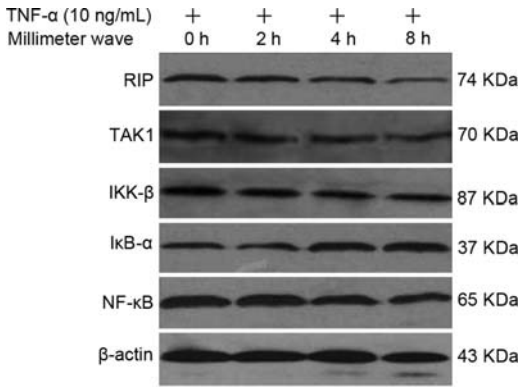

Figure 8. Expression levels of RIP, TAK1, IKK-ß, IκB-α and NF-κB in millimeter wave-treated chondrocytes. Millimeter wave treatment was applied for 0, 2, 4 and 8 h in the TNF-α-treated (10 ng/ml) cells for a total of 8 h. At 0 h of treatment, RIP, TAK1, IKK-ß, IκB-α and NF-κB proteins were highly expressed in the chondrocytes, whereas IκB-α was expressed at a lower level. At 4 and 8 h of treatment, RIP, TAK1, IKK-ß, IκB-α and NF-κB proteins were present at lower levels, in contrast to the significantly increased expression of IκB-α.

stimulation, RIP, TAK1, IKK-ß and NF-κB were highly expressed in the millimeter wave untreated chondrocytes, whereas IκB-α was expressed at a low level. RIP expression levels were lower after 4 or 8 h of treatment than after 2 h (P=0.025, P=0.002) or no treatment at all (P=0.004, P<0.001). TAK1 protein levels were lower in the 4- or 8-h treatment groups than in the untreated cells (P=0.032, P=0.006). IKK-ß levels were also significantly lower after 4 or 8 h of treatment than in the untreated (P<0.001) or 2-h treatment groups (P=0.003, P<0.001). IκB-α levels were significantly higher when treated for 4 or 8 h than when not treated (P<0.001) or treated for 2 h (P<0.001). Finally, NF-κB levels were lower when treated for 4 or 8 h than when treated for 0 h (P=0.004, P<0.001) or 2 h (P=0.034, P=0.002) (Table IV, Figs. 7 and 8).

Ultrastructure of TNF-α-induced apoptotic chondrocytes treated by millimeter wave. At 8 h of TNF-α treatment, most of the untreated chondrocytes became apoptotic, manifesting apoptotic bodies. When treated for 2 h, more apoptotic cells

RIP, TAK1, IKK-ß, IκB-α and NF-κB expression levels in millimeter wave-treated chondrocytes.

Group	RIP/ß-actin	TAK1/ß-actin	IKK-ß/ß-actin	IκB-α/ß-actin	NF-κB/ß-actin
0-h treatment	0.49±0.05	0.52±0.04	0.59±0.04	0.47±0.05	0.84±0.06
2-h treatment	0.47±0.06	0.49±0.06	0.56±0.05	0.48±0.04	0.81±0.05
4-h treatment	0.41±0.03[a,c]	0.46±0.03[b]	0.47±0.04[a,c]	0.69±0.06[a,c]	0.75±0.05[a,c]
8-h treatment	0.38±0.04[a,d]	0.44±0.04[a]	0.45±0.03[a,c]	0.74±0.07[a,c]	0.71±0.04[a,d]

[a]P<0.01 compared to treatment of 0 h, [b]P<0.05 compared to treatment of 0 h, [c]P<0.01 compared to treatment of 2 h, and [d]P<0.05 compared to treatment of 2 h.

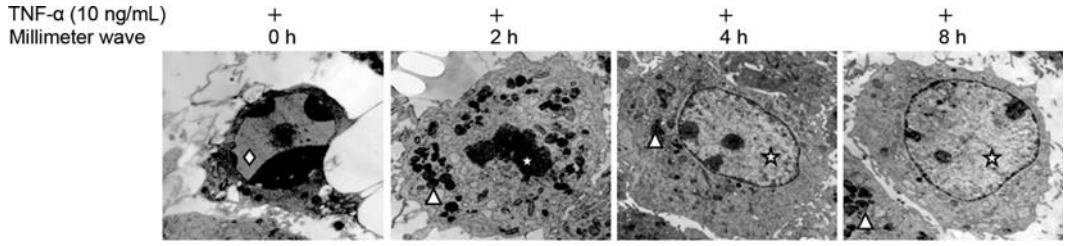

Figure 9. Ultrastructures of apoptotic chondrocytes treated with TNF-α and millimeter wave treatment (magnification, x8000). Chondrocytes exhibited apoptotic bodies after 8 h of TNF-α (10 ng/ml) stimulation. After 2 h of millimeter wave treatment, more lysosomes and condensed nuclei were present in the chondrocytes. At 4 and 8 h of millimeter wave treatment, significantly fewer apoptotic cells were visible and the intracellular lysosomes were also significantly reduced (Δ, apoptosis body, ☆, nuclei and ◇, lysosome).

were visible and a few intracellular lysosomes were also present. When treated for 4 or 8 h, however, only a few cells became apoptotic, and there were significantly fewer lysosomes. The nuclei were mainly round or oval in shape, with uniformly distributed chromatin and nucleoli with clear margins (Fig. 9).

Discussion

Millimeter wave treatment has been used as a non-medicinal, non-invasive physical therapeutic regimen for the treatment of osteoarthritis. We treated TNF-α-induced chondrocytes with millimeter wave therapy with the aim of further investigating the potential mechanisms of action through which millimeter wave treatment regulates chondrocytic functions.

Osteoarthritis is mainly characterized by articular cartilage degeneration and chondral matrix degradation, in which the alteration of chondrocytic function plays a vital role. Such an alteration is primarily associated with the complex network of interactions among inflammatory factors, resulting in synovial inflammation and chondrocytic disturbance (18,19). As one of the key inflammatory factors responsible for the articular cartilage degeneration, TNF-α is involved in the synovial inflammatory response, the initiation of chondrocytic apoptosis, the imbalance between chondral damage and repairs and the eventual acceleration of articular cartilage degeneration (20). In order to study the effects of millimeter wave treatment on TNF-α-induced cell death, we established chondrocyte culture *in vitro* by using stepwise 0.2% collagenase type II digestion and then we treated the chondrocytes with 0, 10, 20 and 40 ng/ml TNF-α for 2, 4 and 8 h. As expected, we found an evident decreasing trend in the activities of chondrocytes by MTT assay with time and TNF-α dosage. Chondrocytes also exhibited apoptosis to various extent by Hoechst 33342 staining in a time- and dose-dependent manner. We found that the 10 ng/ml TNF-α treatment for 8 h was optimal for the induction of chondrocyte apoptosis, and this dose was used for further studies.

A positive feedback cycle is formed by the interaction between inflammatory factors and activated NF-κB to enhance the progression of inflammation (21). NF-κB is a homogeneous or heterogeneous protein dimer consisting of p50 and p65 Rel family proteins (22). The affinity of NF-κB for DNA is regulated by a group of inhibitory proteins of the IκB family, including IκB-α, IκB-ß, IκB-γ, IκB-δ, IκB-ε and BCL-3. Upon binding to NF-κB, IκB blocks its translocation from the cytoplasm to the nucleus and suppresses its regulation of gene transcription (23). The IKK complex is a key factor regulating the activity of IκB, including that of the catalytic subunit IKKα, IKKß and the regulatory subunit IKKγ (NF-κB essential modulator). IKKα and IKKß have Ser/Thr protein kinase activity, and their conformation can be regulated by IKKγ to adjust the kinase activity (24). IKKß and IKKγ have been shown to be required for the activation of NF-κB by inflammatory factors, and NF-κB can be activated by the IKKα-free IKK complex. The IKKα-dependent organ-derived chemoattraction optimizes adaptive immunity through the secondary lymphoid organs and tissues over the course of a few hours, whereas the IKKα-dependent degradation of IκB-α

only takes a few minutes. IKKß is mainly involved in inflammatory and innate immune responses, mediating the rapid response of recruited immune cells to inflammation and injury through NF-κB signalling (25). In response to TNF-α, TAK1 recruits TNFR1 through RIP to construct the fusion protein, TAK1-DD, through the binding of TAK1 to the RIP death domain, which in turn activates the IKK complex. IκB is degraded in the presence of activated IKK, releasing NF-κB to regulate the expression of multiple inflammatory factors. We therefore examined the critical signal molecules, RIP, TAK1, IKK-ß, IκB-α and NF-κB (p65), to identify the targets of millimeter wave treatment in TNF-α-mediated NF-κB signalling.

Our results show that the 8-hour treatment of TNF-α can effectively activate NF-κB signaling and results in chondrocytic apoptosis, whereas the millimeter wave treatment efficiently suppresses apoptosis through the down-regulation of RIP, TAK1, IKK-ß, IκB-α and NF-κB (p65), and the up-regulation of IκB-α. These results suggest that millimeter wave treatment could decrease the apoptosis of chondrocytes through the inhibition of NF-κB signaling, leading to the suppression of articular cartilage degeneration.

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