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ORIGINAL ARTICLE

Therapeutic Effect of Total Saponins from *Dioscorea nipponica* Makino on Gouty Arthritis Based on the NF-κB Signal Pathway: An *In vitro* Study

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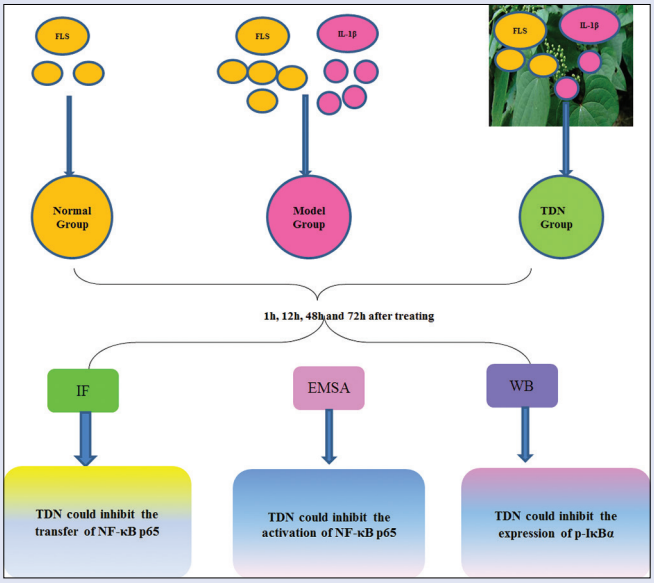
ABSTRACT

Objective: *Dioscorea nipponica* Makino is one of the most common used traditional Chinese drugs which are used to treat gouty arthritis (GA). Nuclear factor-κB (NF-κB) pathway plays an important role during this process. In the present study, we investigated the effects of total saponins from *D. nipponica* Makino (TDN) on NF-κB pathway in interleukin-1β (IL-1β) induced fibroblast-like synoviocytes (FLS). **Materials and Methods:** FLS were divided into three groups: Normal group, model group, which was given 10 μg/L IL-1β to induce the proliferation, and TDN group (10 μg/L IL-1β +100 μg/L TDN). 1 h, 24 h, 48 h, and 72 h after treating, immune fluorescence method was used to detect the cell location of NF-κB p65. Electrophoretic mobility shift assay was used to detect the activation of NF-κB p65. Western blot method was used to detect the protein expressions of NF-κB p65, IκBα, and p-IκBα. **Results:** TDN could inhibit the activation and transfer of NF-κB p65. As time went on, the expression of NF-κB p65 in the cytoplasm was decreased while it was increased in the nucleus. The expression of p-IκBα was increased, whereas the expression of IκBα was not changed. TDN could regulate these abnormal expressions. **Conclusion:** TDN may treat GA by regulating NF-κB signal pathway.

Key words: *Dioscorea nipponica* Makino, gouty arthritis, IκBα, nuclear factor-κB p65, p-IκBα

SUMMARY

- TDN could inhibit the transfer of NF-κB p65.
- TDN could inhibit the activation of NF-κB p65.
- TDN could inhibit the expression of p-IκBα.



Abbreviations used: TDN: Total saponins from *Dioscorea nipponica* Makino, GA: Gouty arthritis, FLS: Fibroblast-like synoviocytes, IL-1β: Interleukin-1 beta, IF: Immune fluorescence, EMSA: Electrophoretic mobility shift assay, WB: Western blot.

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INTRODUCTION

Gouty arthritis (GA) is a relatively common inflammatory arthritis that is typically known to occur in middle-aged men. It is characterized by severely intense pain and can greatly impact patient quality of life.[1] It is an inflammatory disorder caused by the deposition of monosodium urate (MSU) crystals in articular and periarticular tissues.[2] In recent years, knowledge about pathogenesis, pathophysiology, and differential diagnosis of gout has advanced on a broad front.[3] When serum uric acid concentrations are lowered below MSU saturation point, the crystals dissolve and gout can be cured.[4] Interleukin 1 beta (IL-1β) is an important regulatory factor in attack of GA. It has become a major target in treating GA.[5] IL-1β mediates proliferation and activation of fibroblast-like synoviocytes (FLS). It promotes FLS to secrete many kinds of adhesion molecules, proinflammatory cytokines, chemotactic factors, matrix degrading proteases, and so on. In this way, it will lead

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to accumulation of inflammatory cell in the joint, bodiness of synovial tissues, destroy and degradation of substrate extracellular. As a significant mediator of inflammation, IL-1β mediates inflammatory responses of synoviocytes.[6,7] Activation of nuclear factor-κB (NF-κB) is mainly regulated by NF-κB inhibitory protein IκB and IκB kinase IKK. NF-κB and IκB are coupled in resting cells so that the transfer of NF-κB to the nuclear is inhibited. IL-1β, tumor necrosis factor alpha (TNFα), and other inflammatory cytokines act on corresponding receptors. In this way, IKK is activated and IκB is phosphated, so that IκB is departed from NF-κB. NF-κB transfers to the nuclear and combines to κB enhancers to promote expressions of relate genes. NF-κB controls many biosynthesis of regulatory substances that relate to inflammation such as IL-1β, IL-2, IL-6, and other chemokines.[8] Inflammatory responses are amplified as a result.

Dioscorea nipponica Makina belongs to *Dioscoreaceae*. It is a common perennial liane herb and considered as one of the most famous region drugs in Heilongjiang province, PR China. In the Chinese Pharmacopoeia, the effects of *D. nipponica* Makino are removing wind, dehumidification, analgesia, relaxing muscles, and tendons, invigorating the circulation of blood, relieving cough, and preventing asthma.[9] In modern pharmacological researches, *D. nipponica* Makino has the effects of anti-inflammation and is usually used to treat all kinds of arthritis. The authentication of *D. nipponica* Makino was based on both its macroscopic and microscopic characteristics. It was externally irregular and longitudinally furrowed, bearing spinous remains of roots and protuberant stem scars on one side, cork layers readily detached. Cortex emitting dark blue fluorescence was found based on microscopic characteristics of transverse sections of the herb. The starch granules are large, approximately 20 μm in diameter, without a black cross (or not obvious) when viewed under polarized light-based on microscopic characteristics of the powder of the herb.[10] The contents of total saponins are high in *D. nipponica* Makino and the main components are dioscin, gracillin, methyl protodioscin, pseudoprotodioscin, etc.[11] The formation of diosgenin by desugarization was the main pathway by which steroidal glycosides were metabolized. Diosgenin was one of the main metabolites commonly found in plasma and feces from all groups receiving total saponins, as well as individual saponins. This is likely to be one of the bioactive constituents playing an essential role in treating GA.[12] It was also known that *D. nipponica* Makino was used as a source for the steroidal sapogenin diosgenin.[13] Extracts of *D. nipponica* Makina and its monomer have the abilities of anti-inflammation, antitumor, and immunoregulation.[14] However, its concrete mechanisms are still need to be explored.

Our previous studies confirmed the anti-inflammatory and lysosomal membrane stabilizing effects of total saponins from *D. nipponica* Makino (TDN) on adjuvant-induced arthritis in rats.[15,16] There was also good effect of TDN in reducing the uric acid concentration in the blood serum, and it was confirmed that it could regulate the renal organic ion transporters in hyperuricemic animals.[17] This promoted us to study the effect of TDN in treating GA from the NF-κB signal pathway point of view. The proliferation of FLS is a feature of GA, which is induced by inflammation. In this study, FLS was incubated with IL-1β to promote its proliferation. Control group, IL-1β-induced group, and TDN intervened groups were all detected of the cell location of NF-κB p65 by immune fluorescence (IF) method. Electrophoretic mobility shift assay (EMSA) was used to detect the activation of NF-κB p65 and Western blot (WB) method was used to detect the protein expressions of NF-κB p65, IκBα, and p-IκBα.

MATERIALS AND METHODS

Reagents

Nonimmune goat serum was purchased from Fuzhou Maixin Biotech. Co., Ltd., (China). Anti-NF-kB p65 antibody was purchased from Abcam (Saudi Arabia). Anti-Rabbit IgG (H + L), F (ab') 2 Fragment was purchased from Cell Signaling Technology (USA). The EMSA kit was purchased from Thermo Fisher Scientific (USA). The natural protein extraction kit was purchased from MERCK company (German).

Plant material and extraction

The purchase and identification as well as the extraction of TDN had been published. The drug-extract ratio was 4.97% (w/w) according to Ho *et al.*[14] The same batch had been analyzed and tested here.

Determination of the content of total saponins from *Dioscorea nipponica* Makino

The content of TDN was calculated according to the content of dioscin. According to Zhou *et al.*,[17] the content of TDN in the extraction was approximately 55.9%.

Ultra performance liquid chromatography/mass spectrometry analysis

To standardize the herbal extract chemically, ultra performance liquid chromatography/mass spectrometry (UPLC/MS) analysis was performed. UPLC was performed on a Waters Acquity™ UPLC system (Waters Corporation, Milford, MA, USA), which is equipped with an on-line degasser, binary solvent delivery system, autosampler, column oven. An Acquity UPLC BEH C18 column (50 mm × 2.1 nm, 1.7 μm) was used for all chromatographic separations. The mobile phase was A) acetonitrile and B) water-formic acid (100:0.01, v/v). The UPLC eluting conditions were as follows: 0–7 min, 20–38% A; 7–11 min, 38–100% A. The flow rate was 0.3 mL/min. The column temperature was 40°C, and the sample injection volume was 5 μL. The RDN was dissolved in methanol, and the concentration of the sample was 50 μg/mL. UPLC-MS/MS analysis was carried out using an Acquity UPLC system connected online to a Micromass Quattro Premier XE triple-quadrupole mass spectrometer (Micromass, Manchester, UK) equipped with an electrospray ionization source operated in negative ion mode. The cone voltage was set at 100 V and the capillary voltage, at 1.5 kV; the source temperature was 110°C; and the desolvation temperature was 35°C. The desolvation gas was nitrogen, and its flow rate was 750 L/h; the flow rate of the cone gas was 20 L/h. An m/z range of 100–1500 was scanned. Mass-Lynx 4.1 software (Waters) was used for system control and data processing. Three major compounds in TND were identified. They were dioscin, protodioscin, and pseudoprotodioscin.[17]

Animals

About 10 male Wistar rats (200 ± 20 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd., China. They were allowed at least 1 week to adapt to their environment before used for experiments. Animals were housed 5 per cage (600 mm × 450 mm × 280 mm) under a normal 12-h/12-h light/dark schedule with the lights on at 07:00 a.m. They were housed at room temperature (23°C ± 2°C) with relative humidity (55% ± 5%) and given a standard chow and water *ad libitum* for the duration of the study. The ethical approval for the experiments was followed by the legislation on the protection of animals used for experiment purposes (Directive 86/609/EEC).

Preparation of fibroblast-like synoviocytes

Synovial membrane samples were obtained from the knee joint of rats. FLS were prepared according to Lu *et al.*[18]

Cytotoxicity assay

According to Lu *et al.*,[18] compared with that in control group ($P < 0.05$), the cell viability in 10, 100, and 1000 μg/L TDN was decreased. The cell

viability in 0.01, 0.1, and 1 μg/L TDN were also less than that in control group, but there was no significant difference. Hence, 100 μg/L TDN was chosen for this experiment.

Effects of total saponins from *Dioscorea nipponica* Makino on synovial cells proliferation by MTT analysis

According to Lu *et al.*,[18] compared with that in control group ($P < 0.05$), the cell viability in model group was increased by 12.8%. Compared with the cell viability in model group ($P < 0.05$), that in TDN group was decreased by 9.8%. There was no significant difference within control and TDN group.

Immune fluorescence assessment

The cell culture medium was spilled, and the cells were washed with 1x PBS buffer (0.01M, pH 7.4) for 3 times. 2–4% methanol was used to fix the cells for 15 min. 1x PBS buffer (0.01M, pH 7.4) was used to wash the cells for 3 times, for 5 min each time. Non-immune goat serum sealing fluid containing 0.3% Triton X-100 was dripped to cover the liquid level for 2–3 mm. It was stayed at room temperature for 60 min and excess liquid was thrown. Anti-NF-kB p65 antibody was dropped and incubated at 4°C passing the night. 1x PBS buffer (0.01M, pH 7.4) was used to wash the cells for 3 times, for 5 min each time. About 50 μL anti-rabbit IgG (H + L), F (ab') 2 fragment (1:200) was dripped and incubated at room temperature for 60 min. 1x PBS buffer (0.01M, pH 7.4) was used to wash the cells for 3 times, for 5 min each time. They were observed under a fluorescence microscope, and the pictures were taken.

Electrophoretic mobility shift assay

According to the manufacture' protocol, activation of NF-κB was examined using a commercial nonradioactive EMSA kit. NF-κB double chain probe sequence was 5'-AGT TGA GGG GAC TTT CCC AGG C-3'. The purified marked oligonucleotide probe was obtained by ethanol precipitation. Negative control and positive control were all prepared. The samples were subjected to electrophoretic separation, and the gels were exposed to a radiography film for 6–18 h at −70°C with intensifying screens. Software was used to analyze the electrophoretic band, and the activities of NF-κB p65 was represented as the product of the absorbance and the area of the electrophoretic bands.

Western blot analysis

Pre-cooled protease inhibitor cocktail Set 1 was dissolved in 100 μL sterilized water and stored at −20°C. All the proteins should be extracted on ice to keep the stability of the proteins. The cells were collected into the Eppendorf (EP) tubes, and liquid nitrogen was added to grind as soon as possible to make them into the powders.

Appropriate amount of powders was transferred into 1.5 mL EP tubes and 150 μL NucBuster reagent 1 was added. They were vortex vibrated at high speed for 15 s, incubated on ice for 15 min, and vortex vibrated at high speed for 15 s once again. They were centrifuged at speed of 16,000 g for 5 min at 4°C. The supernatant (plasmosin) was removed and 500 μL pre-cooled 1x PBS was added to wash out the redundant plasmosin. About 1 μL 100x protease inhibitor cocktail, 1 μL 100 mM DTT, and 75 μL NucBuster extraction reagent 2 were added into the floc precipitation. They were vortex vibrated at high speed for 15 s, incubated on ice for 15 min and vortex vibrated at high speed for 15 s once again. They were centrifuged at speed of 16,000 g for 5 min at 4°C. The supernatant (nucleoprotein) was transferred to another tube and stored for further detection. The proteins were mixed with 5x loading buffer and heated at 100°C for 3 min to denature. They were loaded into the wells and the voltage used was 200 V. The loading quantity of the sample was 25 μL per well. The proteins were electrophoretically transferred for 1 h to a nitrocellulose filter (NC) membrane (83 mm × 75 mm). The NC membrane was put into 5% dried skimmed milk to seal off and stayed over at 4°C. The specific antibodies used were shown in Table 1. The

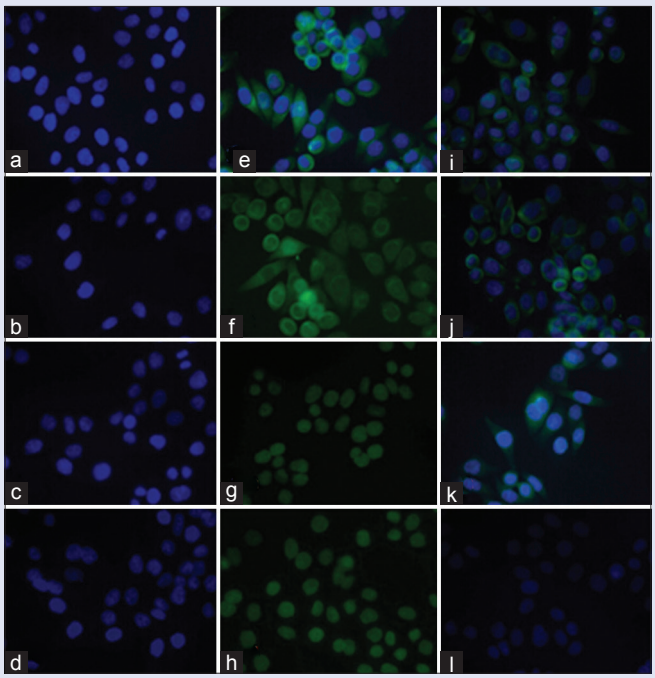

Figure 1: Effects of total saponins from *Dioscorea nipponica* Makino on the nuclear transfer of nuclear factor-κB p65 by immune fluorescence assessment. (a) 1 h after the experiment; (b) 1 h after inducing with interleukin-β; (c) 1 h after treating with total saponins from *Dioscorea nipponica* Makino; (d) 24 h after the experiment; (e) 24 h after inducing with interleukin-β; (f) 24 h after treating with total saponins from *Dioscorea nipponica* Makino; (g) 48 h after the experiment; (h) 24 h after inducing with interleukin-β; (i) 24 h after treating with total saponins from *Dioscorea nipponica* Makino; (j) 2 h after the experiment; (k) 24 h after inducing with interleukin-β; (l) 24 h after treating with total saponins from *Dioscorea nipponica* Makino

Table 1: Summary of antibodies used in western blot analysis

First antibody	Dilution	Company	Code number	Second antibody	Dilution
Rabbit NF-κB p65 antibody	1:500	Abcam	ab31481	Goat anti-rabbit IgG/HRP	1:50,000
Rabbit NF-κB p-p65 antibody	1:500	CST	#3033	Goat anti-rabbit IgG/HRP	1:50,000
Rabbit IκB alpha antibody	1:5000	Abcam	ab32518	Goat anti-rabbit IgG/HRP	1:50,000
Rabbit IκB alpha (phosphorylation S32 + S36) antibody	1:1000	Abcam	ab5682	Goat anti-rabbit IgG/HRP	1:50,000
Rabbit histone H2 antibody	1:400	CST	#2595	Goat anti-rabbit IgG/HRP	1:40,000
Mouse GAPDH antibody	1:800	SANTA	SC-365062	Goat anti-mouse IgG/HRP	1:80,000

NF-κB: Nuclear factor-κB; HRP: Horseradish peroxidase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

NC membrane was dried and attached on the glass paper. The substrate was added, and the chemiluminescence was achieved so that the film was gotten. The film with higher background was put into X-ray film background deleting liquid, and good result was observed. The reaction was ended, and the background, which was not needed, was wiped out.

Statistical analysis

All the data were expressed as the mean ± standard error of the mean, and the statistical analysis was performed using independent samples *t*-test to determine the levels of significance. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of total saponins from *Dioscorea nipponica* Makino on the nuclear transfer of nuclear factor-κB p65 by immune fluorescence assessment

The effect of TDN on nuclear transfer of NF-κB p65 in FLS induced with IL-1β 1 h, 24 h, 48 h, and 72 h after treating was assessed. As it was shown in Figure 1, TDN could inhibit the inflammation by inhibiting the nuclear transfer of NF-κB p65.

Effects of total saponins from *Dioscorea nipponica* Makino on the activation of nuclear factor-κB p65 by electrophoretic mobility shift assay

The translocation of the transcription factor NF-κB p65 to the nucleus in FLS was determined by EMSA. As shown in Figure 2a and b, the DNA banding activity of NF-κB p65 in the normal group was not changed with the time development. However, the DNA banding activities in the model groups were increased significantly when compared with the normal groups ($P < 0.05$, $P < 0.01$, and $P < 0.01$). TDN could decrease these DNA binding activities compared with the model groups ($P < 0.05$, $P < 0.01$, and $P < 0.01$).

Effects of total saponins from *Dioscorea nipponica* Makino on protein expressions of IκBα, p-IκBα as well as nuclear factor-κB p65 both in the cytoplasm and nucleus by Western blot analysis

As shown in Figures 3 and 4, compared with the normal group, the amounts of IκBα were decreased in the model group significantly after 48 h and 72 h inducing with IL-1β ($P < 0.01$, $P < 0.01$) [Figure 4a]. However, the amounts of p-IκBα were increased significantly 12 h, 48 h, and 72 h after inducing with IL-1β ($P < 0.01$, $P < 0.01$, and $P < 0.01$) [Figure 4b]. It had been well demonstrated that the activation of IκBα correlates with proteolytic degradation of IκBα and its phosphorylation. TDN could reregulate these abnormal expressions of both IκBα ($P < 0.01$, $P < 0.01$) and p-IκBα ($P < 0.01$, $P < 0.01$) [Figure 4a and b].

As it was shown in Figures 3 and 4, compared with the normal group, the amounts of NF-κB p65 in the cytoplasm were decreased significantly after 48 h and 72 h inducing with IL-1β ($P < 0.01$, $P < 0.01$) [Figure 4c]. However, the amounts of NF-κB p65 in the nucleus were increased significantly 1 h, 24 h, 48 h, and 72 h after inducing with IL-1β ($P < 0.01$, $P < 0.01$, $P < 0.01$, and $P < 0.01$) [Figure 4d]. It was well recognized that the activation of NF-κB p65 is accompanied with its translocation into the nucleus. TDN could reregulate these abnormal expressions of both NF-κB p65 both in the cytoplasm ($P < 0.01$, $P < 0.01$) and nucleus ($P < 0.01$, $P < 0.01$, and $P < 0.01$) [Figure 4c and d].

DISCUSSION

GA is an inflammatory condition associated with debilitating clinical symptoms, functional impairments, and a substantial impact on quality of life. This condition is initially triggered by the deposition of MSU crystals into the joint space. This causes an inflammatory cascade resulting in the secretion of several pro-inflammatory cytokines and neutrophil recruitment into the joint.[19] Recent researches have made greater insight into the mechanism of pain and inflammation of GA, which makes the development of new approaches to treat the disease possible.

NF-κB proteins are a family of transcription factors that play essential roles in cell adhesion, immune, and pro-inflammatory responses. The mammalian NF-κB family consists of five members, including RelA (also named p65), RelB, c-Rel, NF-κB1 p50, and NF-κB2 p52, all of which form homo- or hetero-dimers in the cytoplasm.[20] NF-κB proteins are normally bound to inhibitory molecules of the IκB family of proteins as an inactive complex. Inflammatory cytokines, such as IL-1β and TNFα, can activate the canonical NF-κB pathway, start the phosphorylation and degradation of the IκB protein, particularly IκBα, and cause the onset of NF-κB translocation to the nucleus and target gene transcription.[21] In this way, NF-κB signal pathway is activated and the inflammatory

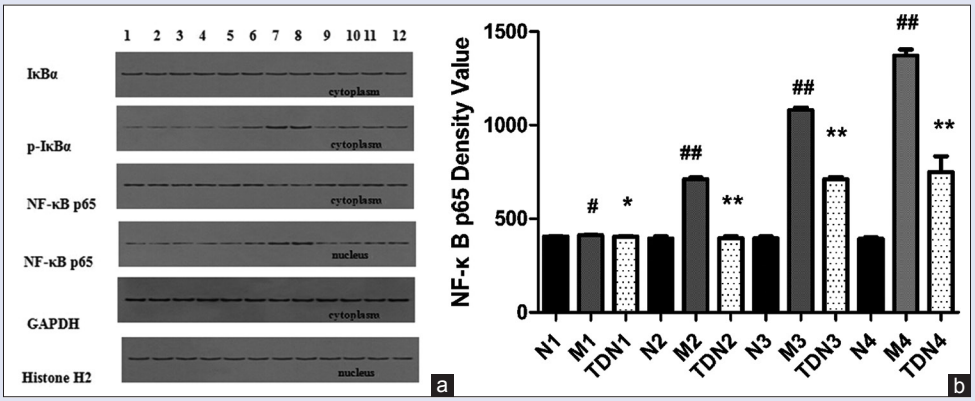

Figure 2: Effects of total saponins from *Dioscorea nipponica* Makino on inhibition of nuclear factor-κB p65 activity. (a) Shifted band of nuclear factor-κB p65 DNA complexes. Lane 1: positive control, lane 2: negative control, lane 3: cold competition assay, lane 4: 1 h after the experiment, lane 5: 1 h after inducing, lane 6: 1 h after treating, lane 7, 24 h after the experiment, lane 8: 24 h after inducing, lane 9: 24 h after treating; lane 10, 48 h after the experiment, lane 11: 48 h after inducing, lane 12: 48 h after treating, lane 13: 72 h after the experiment, lane 14: 72 h after inducing, lane 15: 72 h after treating. (b) Amounts of nuclear factor-κB p65 DNA complexes. The data represent the values of the mean ± standard error of the mean for five treatments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group (independent samples *t*-test); $^{*}P < 0.05$, $^{**}P < 0.01$, versus model group (independent samples *t*-test)

cytokines, such as IL-1β and TNFα, will be produced and released continuously. The inflammatory reaction will be amplified.

In this study, 10 μg/L IL-1β was used to induce the proliferation of FLS and it was found by IF assessment that, with the development of time, the NF-κB p65 was transferred into the nucleus. TDN could inhibit the nuclear transfer of NF-κB p65. Further, EMSA method was used to detect the activation of NF-κB p65. The DNA banding ability of NF-κB p65 was increased in the model groups 1 h, 24 h, 48 h, and 72 h after inducing while TDN could decrease this ability. At last, WB method was used to detect the protein expressions of IκBα, p-IκBα as well as NF-κB p65 both in the cytoplasm and nucleus. It was found that the expressions of IκBα were decreased while the expressions of p-IκBα were increased with the course of time in the model groups. It was a good feature that IκBα was activated. At the same time, the expressions of NF-κB p65 in the cytoplasm were decreased, whereas its expressions in the nucleus were increased. They were in accordance with the fact that the NF-κB signal pathway was activated. Obviously that TDN could regulate these abnormal expressions. All these could explain that TDN may treat GA by regulating the NF-κB signal pathway.

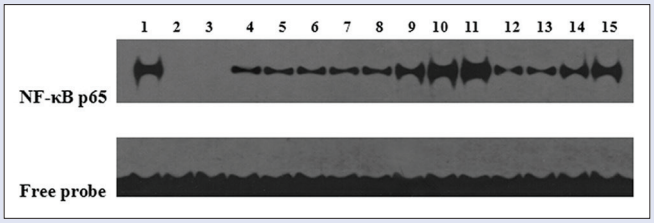

Figure 3: Effects of total saponins from *Dioscorea nipponica* Makino on inhibition of IκBα and nuclear factor-κB p65. Lane 1: 1 h after the experiment, lane 2: 24 h after the experiment, lane 3: 48 h after the experiment, lane 4: 72 h after the experiment, lane 5: 1 h after inducing, lane 6: 24 h after inducing, lane 7: 48 h after inducing, lane 8: 72 h after inducing, lane 9: 1 h after treating, lane 10: 24 h after treating, lane 11: 48 h after treating, lane 12: 2 h after treating

D. nipponica Makino are widely used in clinical field to treat all kinds of arthritis in China. However, the mechanisms are still under investigation. We focus on studying the mechanism of TDN in treating GA. It was the first time that IF assessment, EMSA, and WB method were all used to illustrate the treating mechanism of TDN. In the study of Gao, it was found that TDN containing serum could inhibit the DNA banding ability of NF-κB p65 in IL-12 and TNFα-induced rat synovial cell strain RSC-364; thus, it illustrated that TDN may regulate the production of the key factors involving angiogenesis by influencing NF-κB signal pathway and it is the mechanism treating rheumatoid arthritis.[22] It was in accordance with our research to explain the mechanism of TDN in treating GA. As they were both inflammation reactions of arthritis, they were in common to a certain degree. Similarly, the study of Yu showed that TDN containing serum could inhibit the production of IL-6 in lymphocyte of spleen induced with LPS.[23] They were all evidence that TDN could regulate the production of proinflammatory cytokines.

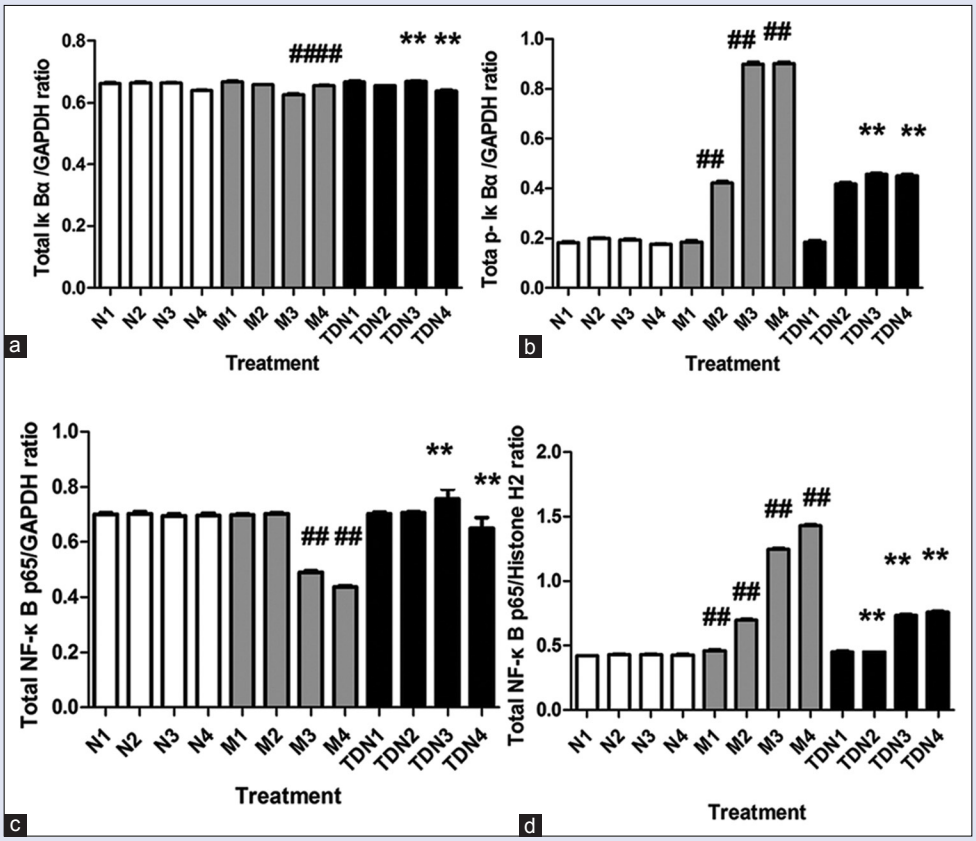

Figure 4: Amounts of IκBα and nuclear factor-κB p65. (a) Represents the amount of IκBα in the endochylema, (b) represents the amount of p-IκBα in the endochylema, (c) represents the amount of nuclear factor-κB p65 in the endochylema, (d) represents the amount of nuclear factor-κB p65 in the nucleus. The data represent the values of the mean ± standard error of the mean for five treatments. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, versus normal group (independent samples *t*-test); $^{*}P < 0.05$, $^{**}P < 0.01$, versus model group (independent samples *t*-test)

Recently, the study of blocking agents of IKK/IκB/NF-κB signal pathway has made great progress. Traditional Chinese medicine has the feature of working as multiple pathways and targets. There is also the advantage of little side effect of *D. nipponica* Makino and it is well recommended, especially for chronic phase of GA. In this paper, the mechanism of TDN in treating GA was well studied. However, there were still some limitations such as it was only an *in vitro* study. Further studies using animal models were necessary.

CONCLUSION

It was revealed in this study that TDN could treat GA by inhibiting the NF-κB signal pathway and influence the production of proinflammatory factors.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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