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Research Article

Potential Effects of Taif's *Punica granatum* L. Extract on Peripheral Blood Mononuclear Cells from Patients with Rheumatoid Arthritis via Regulation of the NF- κ B Signaling Pathway by the I κ B α Gene

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Abstract

Background and Objective: Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease characterized by joint inflammation and cartilage and bone destruction. Abnormal nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling contributes to the pathogenesis of RA. The I κ B α gene plays an important role as an inhibitor of NF- κ B and proinflammatory gene expression. We investigated the expression of the I κ B α gene in peripheral blood mononuclear cells (PBMCs) from patients with RA, after treatment with Taif *P. granatum* L. (TPG) extract. **Materials and Methods:** The PBMCs from RA patients were treated with 50, 100 and 150 μ g mL⁻¹ TPG and their RNA was extracted after 1, 2, 4, 6, 8 or 18 h. the I κ B α mRNA expression in the samples, determined by polymerase chain reaction, was compared with that in controls, using nonparametric one-way analysis of variance. **Results:** Dimethyl sulfoxide treatment induced no significant change in I κ B α gene expression as compared with that in non-treated control cells; however, there was a significant, dose-dependent increase in I κ B α gene expression in PBMCs treated with 50, 100 or 150 μ g mL⁻¹ TPG as compared with that in control cells ($p < 0.0001-0.05$). Evidently, the potential effect of TPG extract on PBMCs from RA patients can be detected by changes in I κ B α gene expression. **Conclusion:** The TPG extract markedly increases I κ B α gene expression, which might subsequently inhibit NF- κ B activation. Thus, TPG extract could be developed as new, safer drug with fewer side effects for RA treatment.

Key words: I κ B α , NF- κ B signaling pathway, NF- κ B blockade, peripheral blood mononuclear cells (PBMCs), rheumatoid arthritis (RA)

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is a transcription factor that plays a key role in several biological processes, such as cell proliferation, differentiation, apoptosis and immune and inflammatory responses¹. The NF- κ B transcription complex consists of five structurally related members, including NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), RelA (p65), RelB and c-Rel (Rel)². The protein complex is maintained in an inactive form in the cytoplasm through the action of three inhibitor proteins: I κ B α , I κ B β and I κ B ϵ ³. The I κ B α gene has an important role as an inhibitor in the regulation of the expression of the nuclear transcription factor NF- κ B and proinflammatory genes⁴.

Deregulated NF- κ B activation contributes to the pathogenesis of several diseases, including rheumatoid arthritis (RA)⁵. The RA is a chronic, inflammatory autoimmune disease characterized by joint inflammation and the destruction of cartilage and bones⁶. Five main categories of conventional treatment have been used to relieve the symptoms of this disease: Biological and non-biological disease-modifying antirheumatic drugs (DMARDs), non-steroidal anti-inflammatory drugs (NSAIDs), analgesics, glucocorticosteroids and combinations of these therapies⁷. The long-term use of these drugs often results in adverse effects, such as gastrointestinal disorders, elevation of hepatic enzyme levels and bleeding⁸.

Because of the risks and limitations of these conventional therapies, there is an increasing demand for alternative approaches to treat RA, including the use of botanicals, dietary modifications and the use of nutrient supplements⁹. Medicinal plants are considered as sources of potentially effective therapeutic agents for RA¹⁰. *Punica granatum* L. is the most common plant used for treating inflammatory diseases. It produces more than 100 different phytochemicals. Especially, ellagitannins are vital bioactive polyphenols found in *P. granatum* that make this plant an interesting species to be considered for the development of novel therapeutics for RA¹¹. These compounds have been proven to exhibit anti-inflammatory activity by inhibiting the expression of several genes encoding proinflammatory proteins, including those encoding inducible nitric oxide synthase, cyclooxygenase-2 (COX-2) and C-reactive protein and by down regulating NF- κ B and tumor necrosis factor alpha (TNF- α) production¹².

The study hypothesized that *P. granatum* has anti-inflammatory properties and can inhibit NF- κ B activation in RA by upregulating I κ B α expression. To test this, changes in I κ B α mRNA expression in peripheral blood mononuclear

cells (PBMCs) from patients with RA after treatment with Taif *P. granatum* (TPG) extract were examined and were compared with that in the control.

MATERIALS AND METHODS

Preparation of TPG crude extract: *Punica granatum* fruits were collected in June, 2017 during the harvest season in the Taif region of Saudi Arabia and were taxonomically identified and authenticated by the Department of Biotechnology, Taif University, Taif, Saudi Arabia. Fruit peels were air-dried in a shaded room and ground into a fine powder using a blender. TPG extract was prepared by using a Soxhlet apparatus with methanol (HPLC grade, $\geq 99.9\%$) for 4 h at 60°C. After extraction, the methanol evaporated and 500 mg of TPG crude extract was dissolved in 10 mL dimethyl sulfoxide (DMSO) (GC headspace grade, $\geq 99.9\%$) as a stock solution. The stock solution was used to prepare three different concentrations: 50, 100 and 150 $\mu\text{g mL}^{-1}$, using DMSO.

Cell isolation, culture and treatments: Twenty milliliters of blood from patients with RA was collected in collection tubes. The blood samples were immediately transferred into a 50 mL tube and diluted with 20 mL of phosphate-buffered saline (PBS, cell-culture grade), pH 7.4 (Sigma-Aldrich, Dorset, UK). The diluted blood samples were carefully layered on to sterile-filtered Histopaque®-1077 (premium grade; Sigma-Aldrich, Dorset, UK) and centrifuged at 400 $\times g$ at 25°C for 30 min. The opaque interface was carefully transferred with a Pasteur pipet into a clean conical centrifuge tube and washed twice with PBS for 10 min at 250 $\times g$. Then, the cells were suspended in 2 mL of filter-sterilized RPMI-1640 medium (AQmedia™ grade; Sigma-Aldrich). The viability of PBMCs was determined immediately by staining with trypan blue solution (microscopy grade, $\geq 0.4\%$; Life Technologies, Carlsbad, CA, USA). The PBMCs (1×10^7 cells mL^{-1}) were transferred to six-well tissue culture dishes with RPMI-1640 medium containing 1% penicillin/streptomycin and 10% fetal bovine serum (Sigma-Aldrich) and incubated at 37°C and 5% CO₂ for 24 h. Then, the PBMCs were serum-starved for another 24 h before they were exposed 50, 100 or 150 $\mu\text{g mL}^{-1}$ TPG extract for 1, 2, 4, 6, 8 or 24 h under culture conditions. Absolute DMSO was used as negative control.

RNA extraction and reverse transcription (RT)-PCR: Trizol® reagent (molecular biology grade; Life Technologies) was used to extract RNA from the treated PBMCs as described previously¹³. The RNA obtained was dissolved in diethyl pyrocarbonate-treated water (molecular biology grade,

≥97%). Complementary DNA (cDNA) was synthesized from the RNA using the Access RT-PCR System (Promega Corporation, Madison, WI, USA), following the manufacturer's protocol and using specific primers (HPLC grade; Macrogen Inc, Korea). In brief, first-strand cDNA was synthesized by combining the following components in a reaction tube: 10 µL of 1 × AMV/Tfl reaction buffer, 1 µL of 0.2 mM dNTP mix, 1 µL of 1 µM forward primer (for IκB-α: 5'-TAGCCTTCAGGATGGAGTGG-3'; for β-actin: 5'-AAGTCATAGTCCGCCTAGAAGCAT-3'), 1 µL of 1 µM reverse primer (for IκB-α: 5'-TCCTGAGCTCCGAGACTTTC-3'; for β-actin: 5'-GATCTTCGGCAGCCAGCACAAATGAAGATC-3'), 2 µL of 1 mM MgSO₄, 1 µL of 0.1 U µL⁻¹ AMV reverse transcriptase, 1 µL of 0.1 U µL⁻¹ Tfl DNA polymerase, 1 µg RNA sample and nuclease-free water to a final volume of 50 µL. The reaction mixture was mixed and incubated at 45°C for 45 min (reverse transcription). Thereafter, the reaction mixture was incubated at 94°C for 2 min (AMV RT inactivation and RNA/cDNA hybrid denaturation). Second-strand cDNA synthesis and PCR amplification were done using 40 cycles of amplification at 94°C for 30 sec, 60°C for 1 min and 68°C for 2 min, followed by a final extension at 68°C for 7 min. The PCR products were resolved by agarose gel electrophoresis and were visualized with an ultraviolet transilluminator. Gel images were analyzed by using the Gel Pro analysis program (Silk Scientific, Inc. Orem, UT, USA) and band intensities were quantified by densitometry. The IκBα mRNA levels in PBMC samples treated with 50, 100 and 150 µg mL⁻¹ TPG were normalized to β-actin levels and expressed relatively to the levels in control samples (RA patient-derived PBMCs not treated with TPG extract). The samples were analyzed in triplicate, in each PCR run.

Statistical analysis: All data are presented as the Mean ± standard deviation (SD). Nonparametric one-way analysis of variance (ANOVA) was used for comparing the mean of each treatment with the mean of the respective control. The p-values less than 0.05 were considered significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

RESULTS

The IκBα gene expression levels in PBMCs from RA patients were investigated after treatment of the cells with TPG extract at various concentrations and were compared with the levels in control cells. Figure 1 shows that upon treatment with DMSO there was no significant change in the mRNA expression level of IκBα when compared to non-treated control cells. However, IκBα mRNA expression significantly increased in PBMCs

treated with TPG extract at all three concentrations tested when compared to control cells ($p < 0.0001-0.05$).

The mRNA expression of IκBα after 1 h of exposure to the TPG extract at the three concentrations is shown in Fig. 1a. IκBα mRNA expression after treatment with 50 µg mL⁻¹ TPG extract was not significantly different from that in the control cells. However, treatment with 100 or 150 µg mL⁻¹ TPG extract significantly promoted IκBα gene expression ($p < 0.01$). After 2 h of exposure to 50, 100 or 150 µg mL⁻¹ TPG extract, IκBα gene expression significantly increased as compared with that in the control cells (Fig. 1b, 50 µg mL⁻¹: $p < 0.05$, 100 µg mL⁻¹: $p < 0.001$ and 150 µg mL⁻¹: $p < 0.0001$), which suggested that treatment with a low concentration of TPG extract (50 µg mL⁻¹) was effective at this time point.

The IκBα expression levels induced by 50, 100 or 150 µg mL⁻¹ TPG extract were similar after 4 and 8 h of treatment (Fig. 1c, e), while there was a significant difference between these two time points ($p < 0.0001-0.001$), which suggested that treatment with a low concentration (50 µg mL⁻¹) was as effective as that with higher concentrations (100 and 150 µg mL⁻¹). The expression levels after 6 and 12 h of treatment revealed a similar pattern (Fig. 1d, f), with a significant difference as compared to that in the control cells and a similar difference in gene expression (50 µg mL⁻¹: $p < 0.01$; 100 and 150 µg mL⁻¹: $p < 0.0001$).

Overall, changes in IκBα gene expression were noted in treated cells as compared with control cells, overtime. All of the concentrations of TPG extract induced differential gene expression as compared with the DMSO treatment. Treatment with TPG extract increased IκBα gene expression in a dose-dependent manner. The potential therapeutic effect of TPG extract on PBMCs from patients with RA was evident from its effect on IκBα gene expression. The TPG extract might consequently inhibit the expression of NF-κB and proinflammatory genes.

DISCUSSION

In the present study, the effect of TPG extract on the expression level of the IκBα gene, which has a vital role in the regulation of NF-κB, in PBMCs from patients with RA was investigated. When cells were treated with TPG extract at 50, 100 or 150 µg mL⁻¹, this gene was upregulated in a dose-dependent manner. Moreover, at each concentration of TPG extract tested, the difference between the cells treated with the extract and those treated with DMSO alone and non-treated cells was significant. This suggested that TPG extract might be appropriate for inhibiting the expression of NF-κB and proinflammatory genes, even at a low concentration.

Fig. 1(a-f): Analysis of the potential effect of Taif *Punica granatum* L. (TPG) extract on the expression level of $\text{IkB}\alpha$ gene. Peripheral blood mononuclear cells (PBMCs) from patients with rheumatoid arthritis (RA) were treated with the TPG extracts at 50,100 and 150 $\mu\text{g mL}^{-1}$. The RNA was extracted after 1, 2, 4, 6, 8 and 18 h. The samples were examined for changes in $\text{IkB}\alpha$ mRNA expression using PCR and compared with the controls. The $\text{IkB}\alpha$ expression was normalized to that of β -actin. Data were analyzed by one-way nonparametric analysis of variance (ANOVA) to compare the mean of each treatment with the mean of a control column. (a) Treatment after 1 h, (b) Treatment after 2 h, (c) Treatment after 4 h, (d) Treatment after 6 h, (e) Treatment after 8 h and (f) Treatment after 24 h

p-values are shown to be statistically significant as follows; ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05 and ns $\geq$ 0.05 (not significant)

Previous studies have shown the critical involvement of NF- κ B activation in the pathogenesis of RA^{2, 14-15}. The expression of proinflammatory genes is regulated by NF- κ B, which can be inhibited by $\text{IkB}\alpha$ ¹⁶. In non-stimulated cells, $\text{IkB}\alpha$ inhibitor proteins are associated with NF- κ B, which is consequently retained in the cytoplasm in an inactive form¹⁷.

Stimulation by extracellular inducers results in the degradation of $\text{IkB}\alpha$ and activation of NF- κ B, which allows the active NF- κ B to translocate into the nucleus¹⁸. In the nucleus, NF- κ B triggers the expression of multiple target genes by binding to their promoters¹⁹. The $\text{IkB}\alpha$ gene is transcriptionally activated by NF- κ B²⁰. Activation of $\text{IkB}\alpha$ expression leads to association

with NF- κ B and prevents NF- κ B from binding to DNA and in its inactive form, it is retained in the cytoplasm²¹. Therefore, NF- κ B is an attractive therapeutic target for RA²². Blockage of NF- κ B not only relieves inflammation, but also controls hyperplasia in the RA synovium²³. The NF κ B plays a fundamental role as a therapeutic target for RA²⁴. Novel therapeutic strategies, such as gene therapy and enzyme inhibition and activation by small molecules and decoy oligonucleotides have been applied in many experiments to block NF κ B activation²⁵.

A previous study based on mobility shift electrophoresis assay and immuno histochemistry showed that the expression of NF- κ B in patients with RA is significantly high²⁶. Various anti-inflammatory agents that can effectively inhibit NF- κ B in patients with RA, e.g., NSAIDs, DMARDs and glucocorticoids have been used; however, these agents are not selective and can have side effects via NF- κ B-independent toxicity²⁷. The I κ B α expression is induced through the activation of glucocorticoid receptors, resulting in the inhibition of NF κ B activation, which prevents it from stimulating the gene expression of several proinflammatory factors, such as COX-2, TNF α , interleukins 1, 2, 6 and 8²⁸.

Traditional medicine is an alternative method for treating RA. Polyphenol-rich fruits, such as *P. granatum*, have been widely used because of their anti-inflammatory properties²⁹. Ghavipour *et al.*³⁰ investigated the therapeutic effects of *P. granatum* extract in RA patients by treating them with two capsules of 250 mg *P. granatum* extract per day for 2 months. They found a significant increase in glutathione peroxidase concentrations after the 2 month treatment. Their findings suggested that *P. granatum* extract could relieve disease activity and promote several inflammation biomarkers in RA patients³⁰. Another study investigated the effect of oral supplementation with *P. granatum* extract on COX activity in rabbit plasma collected after 2 h of treatment; the plasma samples from treated rabbits showed inhibited COX-2 activity³¹.

Bioavailability and bioactivity of polyphenols existing in an extensive variety of medicinal plants have been investigated widely, including studies on stimuli-induced responses and anti-inflammatory properties in different cell types^{32, 33}.

The anti-inflammatory components of *P. granatum*, such as punicalagin, notably decrease nitric oxide and prostaglandin E2 by blocking the expression of proinflammatory proteins³⁴. *Punica granatum* extract inhibited nitric oxide in lipopolysaccharide-induced RAW 264.7 cells³⁵. Another study revealed that *P. granatum* extract inhibits the secretion of inflammatory cytokines by inhibiting

the NF- κ B and mitogen-activated protein kinase pathways³⁶. Activation of these pathways is linked with upregulation of the expression of genes such as TNF- α , IL-1 and COX-2, which are important mediators in the pathogenesis of RA³⁷. The potential of *P. granatum* for preventing joint damage in RA has been investigated in a mouse model of RA and was found to reduce the incidence and severity of RA and lower inflammatory cytokine levels³⁸.

CONCLUSION

The TPG extract has a potential therapeutic effect on PBMCs from patients with RA, as detected by the increase in the I κ B α gene expression level. Consequently, TPG extract might inhibit NF- κ B activation and proinflammatory genes. The results from this study are important in that they indicate that TPG extract can be further developed as a safer treatment option for RA, with less side effects than conventional treatments, which are limited by long-term use-related risks.

SIGNIFICANCE STATEMENT

This study revealed that TPG extract might be valuable for treating patients with RA. This study will aid researchers in revealing the important aspect of potential effects of TPG extract on the NF- κ B signaling pathway and to explain the health benefits of TPG, which could not be explained to date. Thus, a new theory on the effect of TPG extract on NF- κ B signaling may be arrived at.

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