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Research Article

Investigation of The Response Of THE NF-κB Signalling Pathway Of Peripheral Blood Mononuclear Cells (PBMCs) From Rheumatoid Arthritis (RA) Patients And Healthy Control Treated With Taif Pomegranate Peel Extract.

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ABSTRACT

Rheumatoid arthritis (RA) is a common autoimmune disease that affects approximately 0.8% of adults globally, affecting 2-3 times more women than men, which usually develops between 30-50 years old. It is a chronic condition that affects the joints causing swelling, stiffness and pain, which may result in loss of function, sever joint damage and disability.

Currently the exact disease pathogenesis has not been determined; however, there is evidence free radicals play a role in disease progression, and that the proliferation of synovicytes can produce pannus formation leading to bone and cartilage damage.

Treatment for RA currently relies on disease modifying antirheumatic drugs (DMARDs), glucocorticoids, and nonsteroidal anti-inflammatory drugs, but due to the long term side effects of these treatments, new drugs are being investigated. Another approach to RA treatment is alternative medicine, especially as some medicinal plants are already being investigated to develop novel drugs. In this study the effect of methanolic pomegranates peel extract pomegranates juice on mRNA expression activity of genes involved in NF-κB pathway was investigating. Pomegranate extracts showed displayed anti-inflammatory activity, and we found their effects on decreasing certain NF-κB genes.

INTRODUCTION:

Rheumatoid arthritis (RA) is defined as a progressive inflammatory disorder that involves uncontrolled inflammation and proliferation of the synovial membrane, which causes chronic destructive polyarthritis [1]. RA usually begins as systemic arthritis affecting many large and small joints, and can also cause systemic inflammations symptoms including fever, articular stiffness, fatigue and anorexia [2] [3].

In adults RA is currently the most common of the inflammatory forms of arthritis [4]. The high prevalence within the population and its destructive nature mean that the patients are affected by progressive disability, a high level of morbidity and accelerated mortality, which can have long ranging effects not only on the patients but also on their families and society in general [5]. If there is no effective treatment, then the expected course for RA results in progressive disability [6], resulting in the lack of ability to work, which has been found by comprehensive studies conducted in diverse countries [7]. There was a consistent relationship between work disability and disease duration found in a systematic review of the literature, with many of the studies highlighting how RA is a costly disease [8] [9].Currently the mechanisms underlying the pathophysiological causes of RA have not been fully elucidated, which make optimization of treatment difficult [10]. Treatment strategies are reliant on nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and disease modifying antirheumatic drugs (DMARDs), all of which produce serious side effects if used long term, including gastrointestinal and hepatic disorders, meaning safer new drugs are continuously being investigated to improve treatment outcomes [11]. The potential therapeutic effects of micronutrients found in natural products have been investigated in recent studies, and the results suggest they may have a role in the treatment of chronic inflammatory diseases, which is further supported by the use of plants and plant-derived formulations to treat inflammatory disorders by various cultures worldwide since ancient times [12] [13].

Studies have shown that nutritional compounds that have antioxidant or anti-inflammatory effects can be used to prevent oxidative stress induced injury, for example different plant compounds have been studied within the literature and can show anti-inflammatory activities including pomegranate fruit extract [14]. The investigation of pomegranate fruit extract and the compounds derived from it by Ahmed *et al*. (2005) showed that, in osteoarthritis, it could inhibit cartilage degradation, suggesting future use as a nutritional supplement to help maintain joint function and integrity [15]. The results of this study also showed *in vitro* suppression of nuclear factor-kappaB and mitogen-activated protein kinases activation in human chondrocytes, which led to inhibition of matrix metalloproteinase expression induced by interleukin (IL)-1β. It also showed that there was a dose-dependent inhibition of tumor necrosis factor α (TNF-α) production, via NF-κB inhibition, by the methanol pomegranate extract in lipopolysaccharide (LPS) stimulated BV2 microglia cells [15]. Further investigation of the benefits of pomegranate extract in arthritis has been carried out in mice by Shukla *et al*. (2008). They found there was a reduction in the incidence and severity, and a delay in onset, of collagen-induced arthritis in the mice fed hydrolysable tannins-rich pomegranate extract; they had reduced inflammatory cell infiltrates in their joints, and lower levels of cartilage and bone destruction [16]. The results also indicated there were significantly decreased levels of IL-6 within the mice fed with pomegranate extract, and multiple signal transduction pathways and downstream mediators important in RA pathogenesis were eliminated in mouse macrophages treated with pomegranate extract [16]. Studies of the mode of action of the pomegranate fruit extract suggest the high levels of polyphenols and related compounds may produce the anti-inflammatory effects in human osteoarthritis

chondrocytes by inducing p38α-MAPK, MKK-3, transcription factor RUNX-2, and inhibiting IL-1β [17]. Overall the results of these studies suggest that pomegranate extract, and its derived compounds, can induce pharmacological actions which have the potential to be developed into inflammatory inhibitors for use in treatment of inflammatory diseases.

In the present study the effect of methanolic pomegranates peel extract and pomegranates juice were investigated on mRNA expression activity of genes involved in NF-κB pathway using peripheral blood mononuclear cells (PBMCs) from rheumatoid arthritis (RA) patients and healthy control.

MATERIAL AND METHODS

Cell isolation and Culture:

Human blood (20 ml) from healthy control and patients with Rheumatoid arthritis (RA) was collected in Vacutainer® collection tubes containing sodium heparin as an anticoagulant (Becton Dickinson, Mississauga, ON). Blood was processed immediately after collection and peripheral blood mononuclear cells (PBMC) were separated as previously described [18] . Briefly, 20 ml of PBS solution (Sigma- Aldrich, UK) was added into the tubes and the diluted blood samples were carefully layered and isolated by density centrifugation on Histopaque ® gradients (Sigma- Aldrich, UK) at 900 x g for 20 minutes. The white buffy coat layer was carefully collected and washed out twice by Hank's buffered saline (Sigma-Aldrich, UK) for 5minutes at 750g then resuspended in 2ml RPMI-1640 medium (Sigma- Aldrich, UK) and the cell viability was determined after using trypan blue dye(Invitrogen Life Technologies Corp). After isolation, the PBMCs pellet was suspended in RPMI-1640 medium at 1 x 10^6 cells/ml and were seeded into 6-well tissue culture dishes (Falcon; Becton Dickinson) at 37°C in 5% CO2. The experiments conformed to the principles set out in the WMA Declaration of Helsinki and the NIH Belmont report.

Plant Material and Preparation of Pomegranate Extract:

The pomegranate (*Punica granatum* L.) fruits were collected in May 2016 during the harvest season from Al Shifa local farm (Taif-Saudi Arabia). The fruits were taxonomically identified and authenticated by Biotechnology Department, Taif University. Fresh fruit were washed well with distilled water and a peel was removed and minced into small pieces. The total methanolic extracts was prepared by Soxhlet extraction method. Fresh peel parts were extracted with 100% methanol at ratio of 8:1. The process of extraction carries on for 6 hours at 60°C. The crude methanol extract was obtained after evaporated the methanol to dryness at 40°C under reduced pressure. The crude extract was collected and filtered through Whatman No. 1 filter paper and the filtrate was stored at -20°C for further studies, and the percentage yield of the extract was calculated. 100% Dimethyl sulfoxide (DMSO) was used later to dissolve the dried extracts. Pomegranate juice was prepared from fresh fruit.

Cell Treatments:

After 24 hours of culturing PBMCs, cells were serum starved for additional 24 before they were treated with 100 µg/ml of pomegranates peel extract and pomegranate juice separately then were incubated for 24 and and 48 hours hours at 37°C in a humidified incubator with 5% CO2.

RNA Extraction

Total RNA was extracted directly from treated PBMCs cells by using the Trizol® method according to the manufacturer's instructions (Invitrogen Life Technologies Corp., Carlsbad, Calif). Briefly, After isolation, the cells pellet was suspended in 500µl Trizol® RNA isolation reagent. The homogenized samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Samples were centrifuged to remove cell debris and the supernatants were transferred to new tubes. 100µl of chloroform was added per 500µl of Trizol reagent. Samples were vigorously vortexed for 15 seconds and incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4^0C.

Following centrifugation, the mixture separates into lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The RNA was precipitated from the aqueous phase by mixing with 250µl isopropyl alcohol for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4ºC. the supernatant was removed completely and the RNA pellet was wash once with 75% ethanol and air-dried for 5 minutes. The RNA was dissolved in DEPC-treated water. The quality and quantity of extracted total RNA were estimated by UV spectrophotometry and electrophoresis on 1.0% agarose gel and visualized by UV transilluminator (Biometra UV star 15).

Oligonucleotides for PCR

Semi-quantitative reverse transcription PCR (sqPCR) using gene-specific primers was performed using the GAPDH transcript level as housekeeping standard gene in order to determine the expression patterns of P65, P50 and A20 genes in PBMCs . Specific primers were designed for each gene (Bio Basic Canada Inc.) (Table1).

Table 1: *Primers sequence and PCR conditions*

Gene product			Primer Sequence	Fragment size (bases)	Annealing temperature, time (cycle)
1	**p65**	Forward	GCGAGAGGAGCACAGATACC	250	58ºC, 30 sec. (40)
		Reverse	CTGATAGCCTGCTCCAGGTC		
2	**p50**	Forward	CCTGGATGACTCTTGGGAAA	174	58ºC, 30 sec. (40)
		Reverse	TCAGCCAGCTGTTTCATGTC		
3	**A20**	Forward	TGCAGCATATCTGCCTTTTG	175	58ºC, 30 sec. (40)
		Reverse	AGCATTTCTTCCTGCTTCCA		
4	**GAPDH**	Forward	GAGTCAACGGATTTGGTCGT	238	58ºC, 30 sec. (40)
		Reverse	TTGATTTTGGAGGGATCTCG		

REVERSE TRANSCRIPTION-PCR

Total RNA was used to synthesize complementary DNA (cDNA) using the Access RT-PCR System (Promega Corporation) according to the manufacturer's protocols. Briefly, first-strand cDNA was synthesized by combining the 1 µg RNA sample, 10mM dNTP mixture, 1X AMV/*Tfl* 5X reaction buffer, 1 µM oligonucleotide specific primers, 25mM MgSO4, 5u/µL AMV reverse transcriptase, 5u/µl *Tfl* DNA Polymerase. The reaction mixture tubes were gently mixed. After the vortex, the tubes were centrifuged and incubated 1cycle at 45°C for 45 minutes, and 1cycle at 94°C for 2 minutes. Second strand cDNA synthesis PCR amplification was generated by 40 cycles at 94°C for 30 seconds, 60°C for 1 minute, and 68°C for 2 minutes followed by 1 cycle at 68°C for 7 minutes and 1 cycle at 4°C for 5 minutes. The PCR products were analyzed by 2% agarose gel electrophoresis after electrophoresis the gel is illuminated with an ultraviolet lamp. Gel Pro analysis program (Version 1.33, USA) was used to analyzed the

gel image and generated densitometry data for band intensities.

STATISTICAL ANALYSIS

Statistical analysis was completed using GraphPad Prism. One-way analysis of variance (ANOVA) was use to analyze data. P value was considered significant if it is less than 0.05.

RESULTS AND DISCUSSION

The results from this study showed that the methanolic pomegranates peel extract pomegranates juice significantly decreased p65, p50 and A20 mRNA expression level ($P < 0.05$) after 24 h when compared to untreated control, In contrast, pomegranates extract had no effect after 48 hr as shown in Figure 1 and Figure 2.

Inflammation in RA is linked with development of an autoimmune response that progresses to a sustained, self-perpetuated inflammation. Evidence indicates that NF-κB activation plays an essential role both at the stage of initiation and the stage of perpetuation of inflammation in RA. Therefore, in this study we were fascinated in investigating the *in vitro* effects of pomegranates extracts, which have been described to possess anti-inflammatory activity [21]. We chose the peripheral blood mononuclear cells (PBMCs) for this study because it has been shown to be used to study various parameters that might contribute to disease severity, including the innate and adaptive immune response. These responses could be measured at the transcriptional level [22].

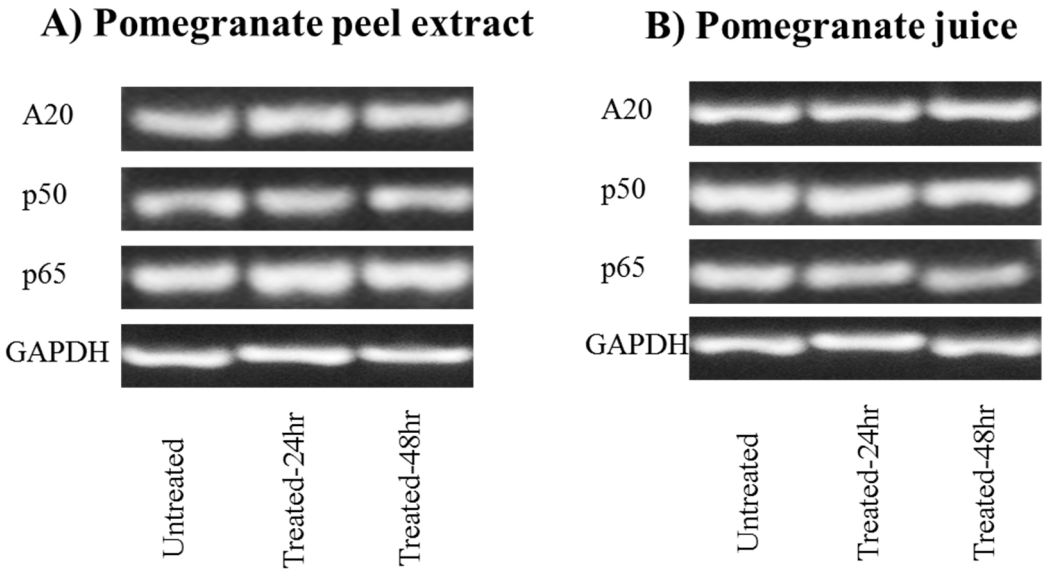
Extracts of all parts of the pomegranate fruit show therapeutic potentials and could be used to treat a variety of diseases including cancer, diabetes, Alzheimer's disease, and aging [19]. Even though pomegranate's wide therapeutic benefits might be attributed to a number of molecular mechanisms, researchers have determined its anti-inflammatory, anticarcinogenic, and antioxidant properties [20].

This study investigates the effect of methanolic pomegranates peel extract and pomegranates juice on NF-κB signalling pathway by analyses gene expression in PBMC cells using semi-quantitative reverse transcription PCR (sqPCR) after treating PBMC cells isolated from RA patients with methanolic pomegranates peel extract and pomegranates juice for 24 h and 48h.

In this study we investigating the effect of methanolic pomegranates peel extract pomegranates juice on mRNA expression activity of genes involved in NF-κB pathway, which consisted of P65, P50 and A20, we found that pomegranates extract decreased mRNA expression of these gene after 24 hr when compared to 48hr and pomegranates juice showed the highest activity to decrease the expression of P65 gene when compared to pomegranates peel extract. In inflammation, NF-κB has been reported to be expresenesed at levels higher than those in normal cells based on immunohistochemistry [23].The anti-inflammatory effect of pomegranates on an in vitro intestinal model was initially described in 2006 in a study examining the molecular mechanisms underlying its anti-tumoral activties [24], researcher in this study was demonstrated that treatment of a colon cancer cell line with 50 μg/mL pomegranates juice, 30–200 μg/mL total pomegranate tannins, and 25–200 μg/mL punicalagin found to inhibite COX-2 expression, NF-κB activation, and AKT activity [24].

Pomegranates juice exhibited the highest activity on these factors with regard to total pomegranate tannins and the pure component, indicating that greater than a single bioactive compound contributes to the therapeutic activity utilized by the pomegranate extract. Using a different cellular model proved that the pretreatment with aqueous extract from pomegranate peels reduced several inflammatory mediators [25].Studies showed that consumption pomegranate extract was shown to reduce severity collagen induced arthritis in model mouse, and suggested that inhibition of a variety of signal transduction pathways and the downstream pathogenic cellular response by pomegranate or compounds derived from it might be a valuable method for the prevention of attacked and

severity of inflammatory arthritis [16]. Furthermore, the pilot study involved patients with rheumatoid arthritis who were given pomegranate extract exhibited that the extract decreases the composite disease activity index (DAS28) of arthritis by 17% and reduced the tender joint count by 62%. Furthermore, pomegranate extracts significantly reduced serum oxidative status, demonstrating a decrease in the inflammatory response [26].



These novel pharmacological actions of pomegranate provide new suggestion that pomegranate or pomegranate-derived compounds might be attractive therapeutic approach for the treatment and prevention of inflammatory diseases by suppressing NF-κB activation [27].

Figure 1: *The effect of methanolic pomegranates peel extract and pomegranates juice on the expression of mRNA transcripts of P65,050 and A20 NF-κB signaling network biomarkers in PBMCs cells isolated from RA patients and treated for 24 hr and 48hr with100 µg/ml of pomegranates and analyzed by semiquantitative RT-PCR. (A&B) Representative 2% agarose gel electrophoresis of RT-PCR products showing corresponding mRNA transcripts of P65, P50, A20 and GAPDH after treatment for 24 h and 48 h with methanolic pomegranates peel extract and pomegranates juice.*

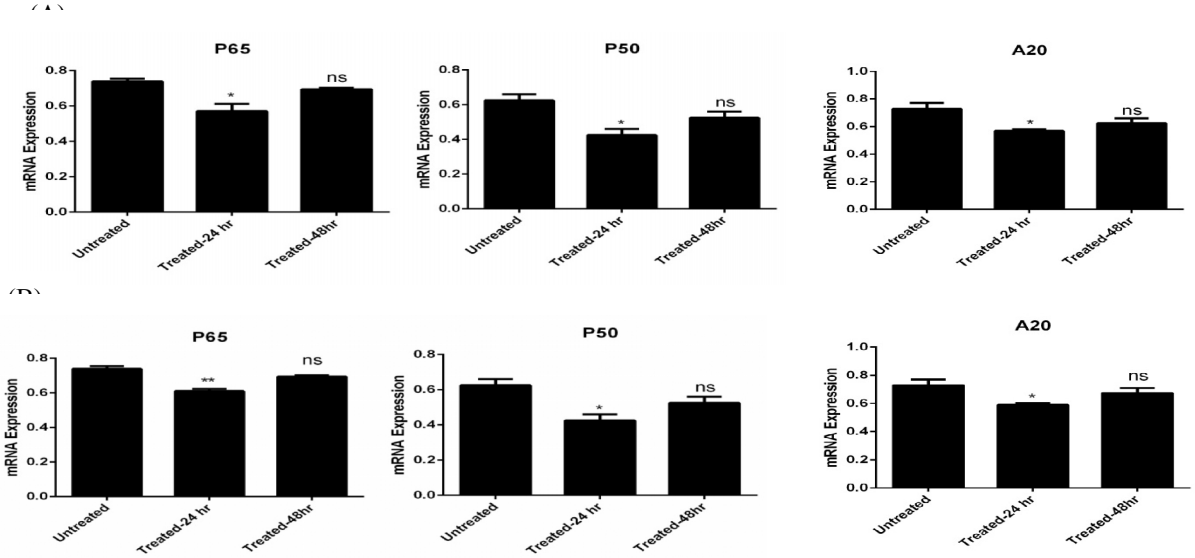


Figure 2: *Average fold expression of of p65, p50 and A20 genes in PBMCs cells from RA patients treated with (A) methanolic pomegranates peel extract and (B) pomegranates juice compared with untreated cells. The data are expressed as mean ± SEM from three independent experiments. The symbol, *, indicates the statistical difference of mRNA expression between pomegranates extract treated cells and untreated control cells ($p < 0.05$).*

CONCLUSION

Results of this study provide strong evidence to support further molecular studying of pomegranate extracts for the prevention of rheumatoid arthritis. In this study pomegranate extracts showed displayed anti- inflammatory activity, and we found their effects on decreasing certain NF-κB genes. Consequently, it must be noted that the identity of the active constituents in pomegranate extracts must be investigated. In the future, a discovery of active compounds in pomegranate extracts affecting mRNA and NF-κB signaling pathway genes should be completed. Combination of pomegranate compounds derived from several plants might also be needed because target the molecules of NF-κB signalling pathway for not only safer, but better in treatment of rheumatoid arthritis. Nevertheless, this need be mentioned that to our knowledge no clinical trials in rheumatoid arthritis patients have yet been achived to document the safety and efficacy of pomegranate extracts.

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