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Anti-inflammatory Potential of Thymoquinone in Tumor Necrosis Factor-alpha Stimulated SW982 Human Synovial Fibroblasts

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ABSTRACT

Rheumatoid arthritis is a common systemic autoimmune disease characterized by chronic inflammation of the joints that can induce the formation of pannus tissue and ultimately leads to joint destruction. Thymoquinone, the major bioactive constituent of Nigella sativa seed oil has diverse pharmacological properties. Although there are some studies in the literature showing the anti-inflammatory activity of thymoquinone, it is not yet clear whether thymoquinone can prevent inflammation caused by rheumatoid arthritis. The goal of this study was to investigate the potential anti-inflammatory effects of thymoquinone treatment on synovial fibroblasts. In our study, we investigated the effects of thymoquinone on nitric oxide production, interleukine-6 (IL-6), IL-8, and prostaglandin E2 (PGE2) levels, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), tumor necrosis factor receptor-1 (TNF-R1), and TNF-R2 protein expressions, extracellular signal-regulated protein kinases 1 and 2 (ERK1/2), Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and Akt phosphorylation levels in tumor necrosis factor-alpha (TNF-α) stimulated SW982 human synovial fibroblasts. Thymoquinone treatment (0-1 μM) resulted in significant and concentration-dependently reduced the TNF-α stimulated production of nitric oxide, IL-6, IL-8, and PGE2 levels compared to the untreated group ($p<0.05$). Also thymoquinone treatment in high concentrations exerted an anti-inflammatory effect by suppressing iNOS, COX-2, TNF-R1, and TNF-R2 protein expressions and the phosphorylation of JNK, p38 MAPK, ERK1/2 and Akt in SW982 synovial fibroblasts ($p<0.05$). Taken together, these results show that thymoquinone in high concentrations is able to play a beneficial role in TNF-α mediated signaling in rheumatoid arthritis synovial fibroblasts.

Keywords: Thymoquinone, SW982 cell line, human synovial fibroblasts, inflammation, tumor necrosis factor-alpha, mitogen-activated protein kinases

Introduction

Rheumatoid arthritis is a chronic disorder characterized by inflammation of the synovial membrane of peripheral joints and may lead to progressive functional impairment. Common joints involved are the small joints of hands and feet, often asymmetrically resulting in pain, stiffness and loss of function (1). Approximately 1% of the population is affected in the world. Women are 3 times more affected than men, it usually occurs in the age group of 40 and 60 years (2,3). Although the cause is unknown, rheumatoid arthritis is considered to be due to faulty response of the immune system and the complex interaction between genetic and environmental factors (4). Rheumatoid arthritis is characterized by the occurrence of chronic inflammatory changes in the joints (5). The synovial membrane is primarily involved in rheumatoid arthritis. Synovial membrane is a thin lining present between joint capsule and joint cavity. It offers nutrients to the cartilage as well as secretes lubricants like hyaluronic acid, collagen, and fibronectin and contains fibroblasts and macrophages (6).

During inflammation the synovium becomes infiltrated with cluster of differentiation 4 ($CD4^+$) T cells, macrophages and mononuclear cells. The mononuclear cells differentiate into multinucleated osteoclasts (7). Synovial infiltration is accompanied by angiogenesis leading to synovial hypertrophy. This

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hypertrophy is also called as pannus that causes destruction of cartilage (8,9). These inflammatory changes are mediated by pro-inflammatory cytokines, such as interleukine-1 (IL-1), IL-6, IL-8, and tumor necrosis factor-alpha (TNF-α) elaborated by the inflammatory cells which activate osteoclasts causing bone destruction (9-11). In addition, elaboration of proteolytic enzymes such as collagenase, metalloproteinase and stromelysin by the synovial cells and chondrocytes results in further destruction of the cartilage and narrowing of joint space (12,13).

There is no cure for rheumatoid arthritis, but there are several different pharmaceutical options available to treat the symptoms of the disease as well to slow the progression. It has been found that these treatments are most effective when prescribed early in the disease. The pharmaceutical options include disease-modifying antirheumatic drugs, biologics, corticosteroids and nonsteroidal anti-inflammatory drugs (14,15).

There are several herbal treatments that have been shown to have beneficial effects in rheumatoid arthritis patients. *Camellia sinensis*, *Celastrus aculeatus*, *Uncaria rhynchophylla* (cat's claw), *Lepidium meyenii*, *Tripterygium wilfordii* Hook F (thunder god vine), *Perna canaliculus*, *Curcuma longa* (turmeric), *Curcuma phaeocaulis*, *Zingiber officinale* (ginger), and *Semecarpus anacardium* Linn are some of the available herbal medicines used in the complementary medicine for rheumatoid arthritis treatment (16).

Nigella sativa, commonly known as black seed or black cumin, is an annual plant belonging to the family *Ranunculaceae*. Native to western Asia, black seed has been used for many years, dating as far back as ancient Egypt. Black seeds were found in the tomb of Tutankhamun (17). Black seed has been used in the Middle and Far East countries for culinary and medicinal purposes to treat many ailments including asthma, hypertension, diabetes, inflammation, cough, bronchitis, headache, eczema, fever, dizziness, and influenza. The seeds of *N. sativa* or its oils are used as a diuretic, to promote digestion and elimination, to increase lactation, to aid in the elimination of intestinal parasites, and to relieve flatulence and abdominal distension (18). Black seed has been used as a condiment and as a spice in foods. *N. sativa* seeds contain fixed oils, proteins, alkaloids, saponin, and essential oil. The major active compound of *N. sativa* seed is thymoquinone (19,20). Numerous studies have indicated the anti-inflammatory effects of thymoquinone. Houghton et al. (21) found that thymoquinone inhibited cycloxygenase and 5-lipooxygenase pathways of arachidonate metabolism in rat peritoneal leukocytes. Although there are some studies in the literature showing the anti-inflammatory activity of thymoquinone (22,23), it is not yet clear whether thymoquinone can prevent inflammation caused by rheumatoid arthritis.

In view of all this, the goal of this study was to investigate the potential effects of thymoquinone treatment in TNF-α-stimulated SW982 cells (human rheumatoid arthritis-synovial fibroblast cell line) and to investigate in depth their effects on anti-inflammatory signaling pathways. In addition, the production of proinflammatory mediators, protein expression of proinflammatory enzymes and the role of mitogen-activated protein kinases (MAPKs) pathway were examined in the current study.

Materials and Methods

Materials: SW982 cell line was purchased from American Type Culture Collection (ATCC® HTB-93™). All cell culture chemicals were bought either from Sigma-Aldrich or Merck. Primer and seconder antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All the other chemicals were bought either from Sigma-Aldrich, Merck or other standard suppliers.

Culture Media: The SW982 human synovial fibroblasts were cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) supplemented with foetal bovine serum (FBS) (5%) containing penicillin (100 IU/mL)/streptomycin (100 µg/mL) and 1% L-glutamine in 75 cm^2 flasks. The flasks cells were maintained in a humidified incubator (at 37°C, 95% air, and 5% CO_2). Culture medium was changed with fresh RPMI-1640 every 48 hours. Confluent cells were washed with phosphate-buffered saline (PBS) and trypsinized with 0.25% trypsin- ethylenediamine tetraacetic acid (EDTA) solution. RPMI-1640 was added to terminate the action of trypsin. Thereafter, cells were collected in 50 mL sterile centrifuge tubes and centrifuged at 1200 rpm for 5 minutes. The medium was then aspirated, and cells were re-suspended in fresh complete medium. The suspended cells were counted and sub-cultured in a new flask or seeded out at a concentration of 2 x 10^5 cell/mL in various cell culture plates (24). To determine the numbers of cells that needed to be seeded for experiments, 10 µL of the cell suspension was loaded onto a haemocytometer. Cells in the four squares of the haemocytometer were counted, averaged, and multiplied by 10^4 (25).

Preparation of Thymoquinone Solutions In Different Concentrations: To examine the effect of thymoquinone on SW982 human synovial fibroblasts, various concentrations of thymoquinone solutions (0.1, 0.25, 0.5, 1, 2.5, 5, and 10 µM) were prepared.

First, thymoquinone was dissolved in dimethyl sulfoxide (DMSO) to make a 50 mM stock solution and stored at -20°C until use in subsequent experiments. Additional dilutions were performed in RPMI-1640 so that the final DMSO concentration was <0.1%. However, in order to eliminate the possible cytotoxic effect of DMSO used in minimal concentration, the same concentration of DMSO used as a solvent was also applied to the cells that were not treated with thymoquinone.

Cell Viability Assay: To determine the concentrations of thymoquinone that did not exhibit harmful effects on cell viability (≤10% cytotoxicity), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed. This assay is a quantitative colorimetric method that determines cell viability based on the reduction of yellow MTT into purple formazan by nicotinamide adenine dinucleotide (NADH) in the mitochondria of proliferating cells. SW982 human synovial fibroblasts were seeded at 10,000 cells/cm^2 in a 96-well plate and incubated for 24 hours in the cell culture incubator. The media was replaced with fresh culture media containing 0.1-10 μM concentrations of thymoquinone. After 48 hours of incubation, cell viability was determined according to the manufacturer's instruction (Sigma-Aldrich, USA). Briefly, the media was removed and replaced with 100 μL of fresh culture media including 0.5 mg/mL of MTT reagent, and cells were incubated for 3 hours at 37°C in the cell culture incubator. During the incubation period, the yellow MTT reagent was reduced and resulted in the formation of purple formazan precipitation at the bottom of the wells. The media was gently removed and 100 μL dimethyl sulfoxide (DMSO) was added to solubilize the precipitated formazan. Absorbance was measured at a wavelength of 570 nm using VersaMax Microplate Reader (26). All experiments were performed in at least five replicates.

Determination of Nitrite Production: Nitrite concentration in the culture medium was measured using the Griess method to measure the nitric oxide level. The principle of the assay is based on the conversion of sulfanilic acid to a diazonium salt by reaction with nitrite in acid solution. The diazonium salt is then coupled to N- (1-naphthyl) ethylenediamine, forming an azo dye that can be quantified through colorimetry at 540 nm in microplate reader and calculated against a sodium nitrite standard curve (27).

Prostaglandin E_2 (PGE_2) Assay: The concentration of PGE_2 was measured using enzyme immunoassay (EIA) kit (Lifespan Biosciences, Seattle, WA, USA) at 450 nm according to the manufacturer's instructions. The concentration of PGE_2 was calculated compared to the standard curve.

IL-6 and IL-8 Assay: The concentration of IL-6 and IL-8 were measured using enzyme-linked immunosorbent assay (ELISA) kits (Lifespan Biosciences, Seattle, WA, USA) at 450 nm according to the manufacturer's instructions. The concentrations of IL-6 and IL-8 were calculated compared to the standard curve.

Western Blotting Analysis: Cell lysis buffer is used to lyse cells under non-denaturing conditions to study protein levels in the cell. Whole cell lysates were prepared by washing cells with ice-cold PBS, followed by addition of 20 μL cell lysis radioimmunoprecipitation assay buffer (RIPA) (Cell Signaling) which contains 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, St. Louis, MO, USA). The cells were incubated on ice for 10 minutes and sonicated for 1 minute followed by cold centrifugation for 15 minutes at 13500 rpm. The resulting supernatants were collected, quantified and stored at -80 ºC. Total protein concentrations of the supernatant were determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific Pierce).

An equal amount of total protein (20-50 μg) was loaded into sodium dodecyl sulphate polyacrylamide gel electrophoresis and separated by electrophoresis under 80–100 V. After electrophoresis, the protein bands were transferred to polyvinylidene difluoride (PVDF) membrane under 100 V for 1 hour in cold water bath. Then, the PVDF membranes were blocked with phosphate-buffered saline (PBS) containing 5% nonfat milk or BSA for 1 hour and incubated with the primary antibodies against tumor necrosis factor receptor-1 (TNF-R1) (1:1,000) (sc-8436), TNF-R2 (1:1,000) (sc-8041), inducible nitric oxide synthase (iNOS) (1:500) (sc-7271), cyclooxygenase-2 (COX-2) (1:500) (sc-376861), extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) (1:1,000) (sc-514302), p-ERK1/2 (1:1,000) (sc-101760), Jun N-terminal kinase (JNK) (1:1,000) (sc-7345), p-JNK (1:1,000) (sc-6254), p38 MAPK (1:1,000) (sc-81621), p-p38 MAPK (1:1,000) (sc-7973), Akt (1:1,000) (sc-5298), p-Akt (1:1,000) (sc-135650), and β-actin (1: 1,000) (sc-8432) overnight at 4°C. The PVDF membrane was washed 3 times with phosphate-buffered saline containing 0.05% Tween-20 (PBST) and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000) (sc-2357) at 37°C for 1 hour. The PVDF membranes were washed 3 times with PBST again and labeled with the enhanced Pierce™ Fast Western Blot Kit (Thermo Scientific Pierce) for 1 minute at room temperature in dark. Finally, the PVDF membranes were exposed to the imaging system

(ImageJ software, National Institutes of Health, Bethesda, MD, USA) to capture the light signals of protein bands. The protein levels were expressed as a relative value to that of β-actin.

Statistical Analyses: All experiments were performed in at least five replicates. For the 96-well microtiter tissue culture plates, 4 replicate wells were used per category. The data were analyzed by SPSS statistics software (version 15.0, SPSS Inc., Chicago, IL, USA). For significant differences between control and experimental values, the *p* value between groups was determined by one-way analysis of variance followed by Tukey's test. The significance level was set at $p<0.05$.

Results

After 48 hours of treatment with varying doses of thymoquinone (0.1-10 μM), the viability of the synovial fibroblasts was measured using an MTT viability assay. The effects of different concentration of thymoquinone on cell viability were shown in Figure 1. Thymoquinone treatment at concentrations of 0.1-1 μM for 48 hours, this compound had no significant inhibitory effects on cell viability (≤10% cytotoxicity). At the concentration of 2.5-10 μM, thymoquinone showed inhibition activity on cell growth in 48 hours (>10% cytotoxicity) (Figure 1).

The effect of thymoquinone on nitrite production in TNF-α stimulated SW982 synovial fibroblasts was evaluated. Thymoquinone dose-dependently suppressed the release of nitrite following stimulation with in TNF-α ($p<0.01$) (Figure 2(A)). Further investigations using western blotting showed that thymoquinone (0.1-1 μM) produced significant suppression of elevated levels of iNOS protein following TNF-α stimulation ($p<0.01$) (Figure 2(B)). These results suggest that thymoquinone suppressed NO production in TNF-α activated SW982 synovial fibroblasts by reducing the levels of iNOS protein.

The effects of treatment with thymoquinone were tested on PGE_2 production in SW982 synovial fibroblasts which were stimulated with TNF-α. As shown in Figure 3(A), stimulation of SW982 synovial fibroblasts produced elevation of PGE_2 compared to untreated cells in a dose-dependent manner. Besides this, in the presence of thymoquinone at concentrations of 0.5 μM and 1 μM, there was significant reduction in PGE_2 production ($p<0.01$) (Figure 3(A)). Following results showing effects of thymoquinone on PGE_2 production, further experiments were performed to determine whether its actions were mediated by the COX-2 enzyme. Results in Figure 3(B) show an increase in the levels of COX-2 protein in TNF-α stimulated cells, when compared with unstimulated cells. Treatment with 1 μM thymoquinone produced significant reduction in TNF-α stimulated elevation in COX-2 protein expression ($p<0.01$) (Figure 3(B)). These results suggest that thymoquinone inhibits TNF-α stimulated PGE_2 production by suppressing the levels of COX-2 protein in SW982 synovial fibroblasts.

There was a significant decrease in IL-6 production by SW982 synovial fibroblasts at doses of 0.5 μM and 1 μM thymoquinone compared to the untreated TNF-α stimulated cells ($p<0.05$). On the other hand, a dose-dependent decrease in IL-8 production occurred in all concentrations applied to SW982 synovial fibroblasts compared to the untreated TNF-α-stimulated cells ($p<0.001$) (Figure 4).

TNF-R1 and TNF-R2 protein expressions significantly decreased in TNF-α stimulated SW982 synovial fibroblasts after administration of thymoquinone at a concentration of 1 μM ($p<0.05$). The other thymoquinone concentrations didn't alter TNF-R1 and TNF-R2 protein expressions significantly ($p>0.05$) (Figure 5).

In SW982 synovial fibroblasts treated with 0.5 and 1 μM thymoquinone, phosphorylation of JNK and p38 MAPK significantly decreased, besides this only 1 μM thymoquinone treatment significantly decreased the phosphorylation of ERK1/2 and Akt in SW982 synovial fibroblasts that received 30 minute TNF-α stimulation ($p<0.05$) (Figure 6).

Discussion

In recent years, many studies have been carried out to develop effective strategies for the prevention and treatment of rheumatoid arthritis, but only a few of them seem to benefit to the desired extent (14,15). Based on our evaluation in cultured SW982 cells, thymoquinone may have promising potential for the development of a new and effective nutritional supplement for rheumatoid arthritis that suppresses the key proinflammatory mediators of rheumatoid arthritis and cytokines involved in the pathogenesis of rheumatoid arthritis through blockage of MAPKs signalling pathway. In pathogenesis of rheumatoid arthritis, synovial fibroblasts function as a major population of cells to participate in chronic inflammatory responses in the invasive pannus. In the current study, it was aimed to investigate whether thymoquinone inhibits the activation of TNF-α induced SW982 human synovial fibroblasts and to what extent it may be effective in inhibiting the inflammatory response (28,29).

In this study, firstly non-cytotoxic concentrations of thymoquinone were determined by using MTT test.

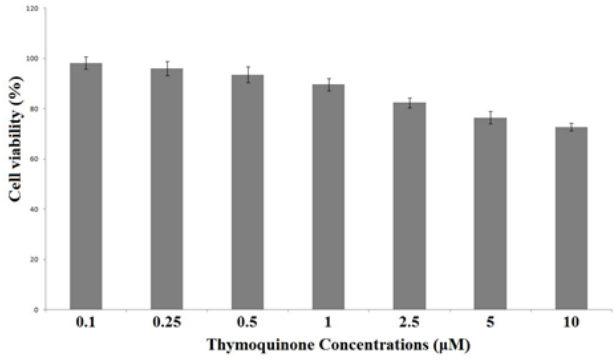

Fig. 1. Effect of thymoquinone on the cell viability of SW982 synovial fibroblasts. Cells were plated in 96 well plate at density of 1×10^4 cells/well. The cells were pretreated with various concentrations of thymoquinone (0-10 µM) for 24 h and cytotoxicity was determined by MTT assay. Data are shown as the means ± SD (n = 5). MTT; 3-(4, 5-dimethythiazol- 2-yl)-2, 5 diphenyl tetrazolium bromide

While thymoquinone at concentrations of 0.1-1 µM did not show cytotoxic effect on SW982 synovial fibroblasts, application of higher concentrations led to an excessive increase in cell death. In our study, we also calculated the IC_{10} value of thymoquinone on SW982 synovial fibroblasts. IC_{10} value of thymoquinone was found as 1.59 µM. This shows that thymoquinone in low concentrations can be safely used without causing too much cell death.

Inflammatory changes in synovial fibroblasts play a vital role in the progression of rheumatoid arthritis. One of the most critical proinflammatory mediator released by TNF-α stimulated synovial fibroblasts is nitric oxide. This molecule has been shown to be an important signaling molecule that regulates a diverse range of physiological processes, including host defense as well as vasodilation and produced by the action of iNOS (30). Nitric oxide is an important mediator in rheumatoid arthritis synovium as it mediates many metabolic events such as cytokine production, signal transduction, mitochondrial functions and apoptosis occurring at the site of synovial inflammation. Many researchers have suggested that experimentally induced arthritis is suppressed by inducible nitric oxide synthase inhibition and so this inhibition has beneficial effects in acute and chronic joint inflammation (31). This study demonstrated that in the presence of thymoquinone, there was a reduction in nitric oxide production caused by TNF-α stimulation of SW982 synovial fibroblasts. It was further shown that suppression of nitrite oxide production by thymoquinone was through reduction of iNOS. These observations provided further evidence on the inhibition of inflammation by thymoquinone, and confirmed recent reports showing iNOS mediated

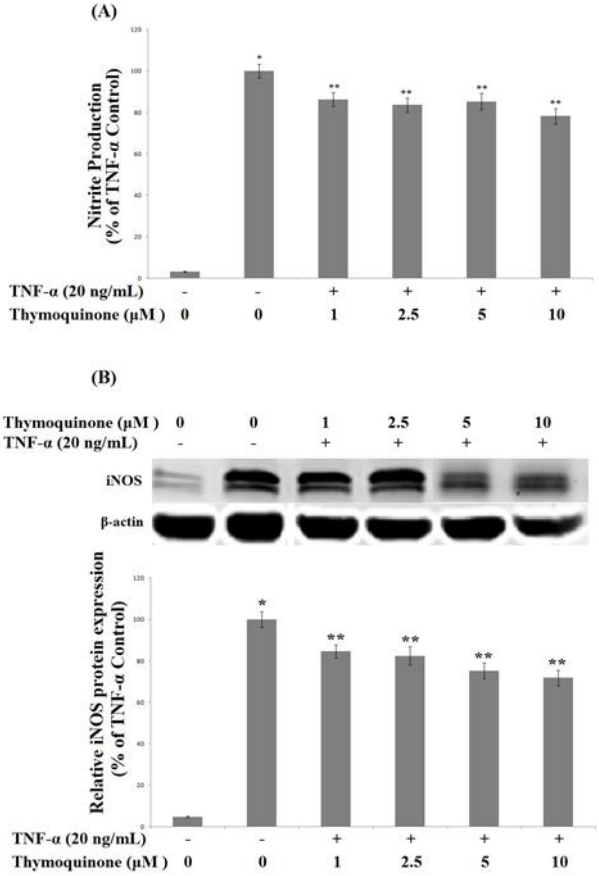

Fig. 2. Effect of thymoquinone on nitrite production (A) and iNOS protein expression (B) in SW982 cells stimulated with TNF-α. SW982 cells were stimulated with TNF-α (20 ng/mL) in the presence or absence of thymoquinone (0.1, 0.25, 0.5, and 1 µM) for 24 hours followed by performing Griess assay for nitrite production and western blot for iNOS protein expression. Data are shown as the means ± SD (n = 5). TNF-α; tumor necrosis factor-alpha, iNOS; inducible nitric oxide synthase. (*p<0.05 in comparison to control cells, **p<0.05 in comparison to TNF-α stimulated cells)

inhibition of nitric oxide production in lipopolysaccharide induced RAW264.7 cells (32).

The role of COX-2-mediated inflammation in rheumatoid arthritis synovium has been well explored. One of the key downstream products of COX-2 is prostaglandin E_2 (PGE_2). COX-2 is upregulated during inflammation in synovial fibroblasts. PGE_2 levels were found to be increased in the synovial fluid of rheumatoid arthritis patients. Thus, PGE_2 is considered as an important marker of the synovial inflammation. Moreover, PGE_2 mediated release of TNF-α, IL-1β, and nitrite may result in fibroblast damage (33). In this study it was shown that thymoquinone suppressed COX-2 protein expression which might explain its ability to reduce PGE_2 production in TNF-α stimulated synovial fibroblasts. A number of studies reported that several

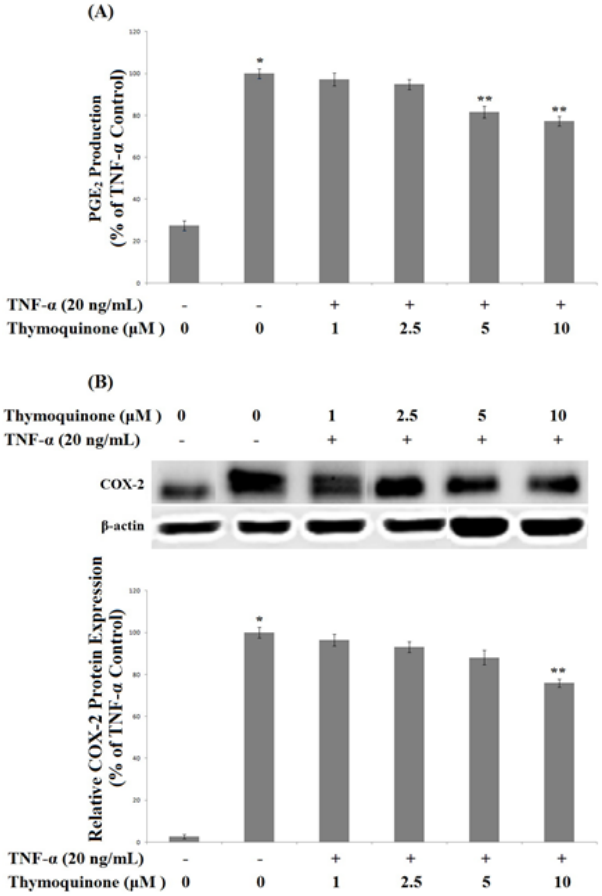

Fig. 3. Effect of thymoquinone on PGE_2 production (A) and COX-2 protein expression (B) in SW982 cells stimulated with TNF-α. SW982 cells were stimulated with TNF-α (20 ng/mL) in the presence or absence of thymoquinone (0.1, 0.25, 0.5, and 1 μM) for 24 hours followed by performing EIA assay for PGE_2 production and western blot for COX-2 protein expression. Data are shown as the means ± SD (n = 5). TNF-α; tumor necrosis factor-alpha, PGE_2; Prostaglandin E_2, COX-2; cyclooxygenase-2. (*p<0.05 in comparison to control cells, **p<0.05 in comparison to TNF-α stimulated cells)

phytochemicals inhibited the expression of COX-2 and reduced PGE_2 levels in synovial fibroblasts. It has also been shown that thymoquinone suppresses COX-2 and PGE_2 in LPS-stimulated mouse macrophage RAW 264.7 cells (34). Another study revealed that thymoquinone reduces spinal cord injury by inhibiting inflammatory response, oxidative stress and apoptosis (35). These outcomes show that thymoquinone appears to exert modulatory activities on COX-2/PGE_2 in diverse models of inflammation (34,35). These results are also consistent with the effects of the compound shown in the current investigation.

This study provides evidence that exposure to thymoquinone at 1 μM concentration may diminish TNF-α mediated TNF-R1 protein expression in

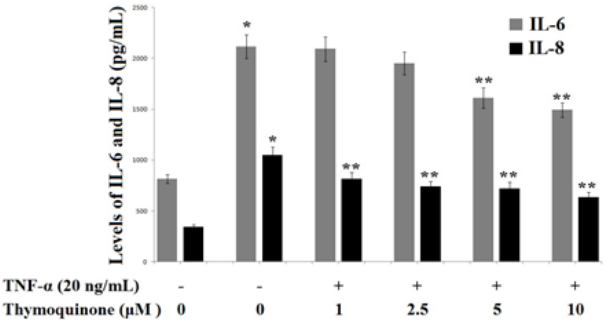

Fig. 4. Effect of thymoquinone on pro-inflammatory cytokines; IL-6 and IL-8 production in TNF-α stimulated SW982 synovial fibroblasts. Cells were stimulated with TNF-α (20 ng/mL) in the presence or absence of thymoquinone (0.1, 0.25, 0.5, and 1 μM) for 24 hours. At the end of the incubation period, supernatants were collected for ELISA measurements. Data are shown as the means ± SD (n = 5). TNF-α; tumor necrosis factor-alpha, IL-6; interleukin-6, IL-8; interleukin-8. (*p<0.05 in comparison to control cells, **p<0.05 in comparison to TNF-α stimulated cells)

synovial fibroblasts. The protein expression of TNF-R2 also reduced in TNF-α stimulated synovial fibroblasts exposed to thymoquinone at a concentration of 1 μM. The reduction in protein expression of TNF-R2, which is known to be useful in the treatment of rheumatoid arthritis by administration of thymoquinone at this dose to SW982 synovial fibroblasts, was more pronounced than the decrease in TNF-R1. This significant decrease in expression levels of TNF-R2 could be the result of potential cleavage of this receptor from the cells, which has also been suggested as a possible mechanism for the beneficial effects of TNF-R2 in rheumatoid arthritis (36). Based on these results, one can argue that thymoquinone treatment in high concentrations may provide a sufficient systemic concentration of thymoquinone to produce beneficial effects in rheumatoid arthritis.

In the current study, we found that the exposure of high concentration of thymoquinone (0.5 and 1 μM) to synovial fibroblasts was more effective than lower thymoquinone concentrations in downregulating TNF-α-stimulated p38 MAPK activation. Our results showed that phosphorylation of JNK was slightly decreased dose-dependently at low concentrations of thymoquinone, but at high thymoquinone concentrations (0.5 and 1 μM), the pJNK expression significantly decreased. It appeared that the maximum effect of thymoquinone on pJNK was reached with the 1 μM concentration of thymoquinone. The decrease seen in JNK phosphorylation with exposure to thymoquinone at high concentrations is encouraging because the JNK pathway has been shown to mediate collagenase gene expression that

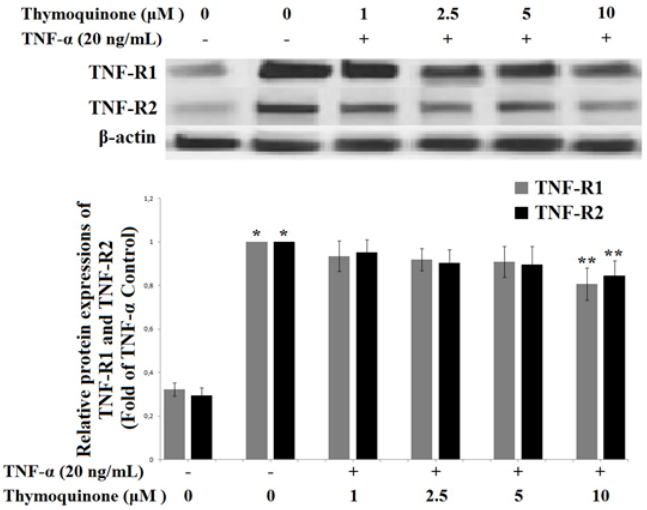

Fig. 5. Effect of thymoquinone on the protein expressions of TNF-R1 and TNF-R2 in SW982 cells stimulated with TNF-α. SW982 cells were stimulated with TNF-α (20 ng/mL) in the presence or absence of thymoquinone (0.1, 0.25, 0.5, and 1 μM) for 24 hours followed by performing western blot for TNF-R1 and TNF-R2 protein expressions. Data are shown as the means ± SD (n = 5). TNF-α; tumor necrosis factor-alpha, TNF-R1; tumor necrosis factor receptor-1, TNF-R2; tumor necrosis factor receptor-2. (*p<0.05 in comparison to control cells, **p<0.05 in comparison to TNF-α stimulated cells)

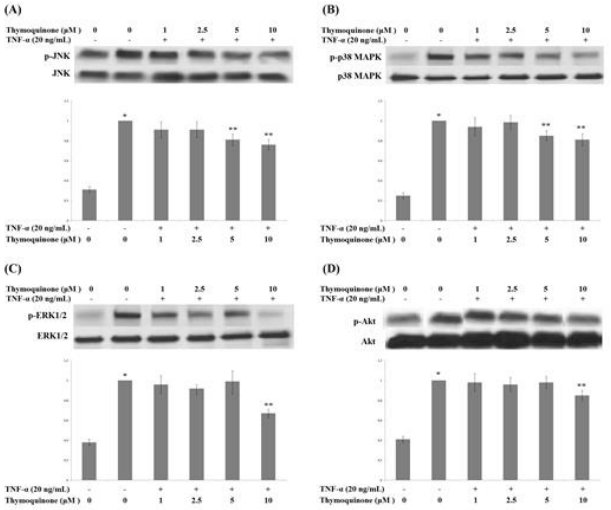

Fig. 6. Effect of thymoquinone on the phosphorylation of JNK (A), p38 MAPK (B), ERK1/2 (C), and Akt (D) in SW982 cells stimulated with TNF-α. SW982 cells were stimulated with TNF-α (20 ng/mL) in the presence or absence of thymoquinone (0.1, 0.25, 0.5, and 1 μM) for 24 hours followed by performing western blot for JNK, p38 MAPK, ERK1/2, and Akt phosphorylation. Data are shown as the means ± SD (n = 5). TNF-α; tumor necrosis factor-alpha, JNK; Jun N-terminal kinase, p38 MAPK; p38 mitogen-activated protein kinase, and ERK1/2; extracellular-signal-regulated kinase 1 and 2. (*p<0.05 in comparison to control cells, **p<0.05 in comparison to TNF-α stimulated cells)

regulates cartilage destruction in rheumatoid arthritis (37). Because of this linkage, a decrease in JNK phosphorylation may result in a protective effect against the destruction of the extracellular matrix in rheumatoid arthritis, slowing the joint destruction resulting from this disease (38). It appeared that in the study in which TNF-α stimulated synovial fibroblasts were exposed with 1 μM thymoquinone, there was a significant decrease in ERK1/2 phosphorylation. As observed in a study done using osteoarthritis chondrocytes by Wang et al. (39), thymoquinone showed inhibitory effect on the phosphorylation of JNK, ERK, and p38 MAPK. These results suggest that inhibition of arthritis by thymoquinone maybe dependent of the MAPKs pathway.

In animal models, inhibition of p38 MAPK activation resulted in reduced inflammation in the animal as well as a decrease in the production of inflammatory cytokines (40). The pro-inflammatory cytokine IL-6 is a major cytokine involved in the progression of rheumatoid arthritis. It promotes MMP production, autoantibody production and the differentiation of Th17 cells. Besides this, synovial fibroblasts also produce IL-8, a pro-inflammatory cytokine, which recruits more T-cells, neutrophils and basophils to the already inflamed joint (10). In this study, our results suggest that high doses of thymoquinone (0.5 and 1 μM) were capable of inhibiting IL-6 and IL-8 production in rheumatoid arthritis synovial fibroblasts. These results further attest to previous publications showing the efficacy of thymoquinone in regulating IL-1β-induced IL-6 production (39). Because IL-6 plays such a major role in rheumatoid arthritis, even a modest decrease in IL-6 levels could be quite beneficial, especially when the fact that this decrease would be coming as an effect of a supplemental therapy (31). The results showing a decrease in both IL-6 and IL-8 production may support a study by Umar et al. (41), in which, inhibition of phosphorylation of the JNK and p38 MAPK in rheumatoid arthritis synovial fibroblasts exposed to thymoquinone resulted in the suppression of IL-6 and IL-8 production. Overall, these results suggest that the decrease in IL-6 and IL-8 production in thymoquinone treated cells, may be at least partially due to the inhibition of p38 MAPK and JNK phosphorylation.

After observing the inhibitory effect that thymoquinone had on JNK and p38 MAPK phosphorylation, we decided to look into what effect, if any, thymoquinone had on the phosphorylation of Akt in TNF-α stimulated synovial fibroblasts. Akt is an important survival protein that helps rheumatoid arthritis synovial fibroblasts in uncontrolled proliferation (42). Fibroblast-like synoviocytes

obtained from rheumatoid arthritis patients expressed higher levels of p-Akt, further incurring synovial hyperplasia and inflammation observed in rheumatoid arthritis patients (43). In our study, only 1 μM thymoquinone application decreased the phosphorylation of Akt protein in TNF-α stimulated SW982 synovial fibroblasts. This data showed us that thymoquinone treatment in only high concentration may sufficiently suppress the phosphorylation of Akt in rheumatoid arthritis.

In conclusion, this study suggest that thymoquinone is able to play a beneficial role in TNF-α mediated signaling in rheumatoid arthritis synovial fibroblasts. Low concentrations of thymoquinone results in no marked change in synovial fibroblasts inflammation. As a result, thymoquinone may be a beneficial supplemental adjunct treatment option for rheumatoid arthritis. However, further studies are required to validate the effect of thymoquinone in *in vivo* rheumatoid arthritis models.

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