

Elucidation of Molecular Mechanisms Underlying the Protective Effects of Thymoquinone Against Rheumatoid Arthritis

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ABSTRACT

Thymoquinone (TQ) is the major active compound derived from the medicinal *Nigella sativa*. A few studies have shown that TQ exhibits anti-inflammatory activities in experimental models of rheumatoid arthritis (RA) through mechanisms that are not fully understood. The aim of this work was to evaluate the *in vitro* and *in vivo* effects of TQ and to investigate its influence on the major signalling pathways involved in pathophysiological RA changes. We used isolated human RA fibroblast-like synoviocytes (FLS) and a rat adjuvant-induced arthritis model of RA. In isolated RA FLS, TQ (0–10 μ M) was not cytotoxic and inhibited slightly lipopolysaccharide (LPS)-induced FLS proliferation and strongly H₂O₂-induced 4-hydroxynonenal (HNE) generation. By studying different inflammatory and catabolic factors, we determined that TQ significantly abolished LPS-induced interleukin-1beta (IL-1 β), tumour necrosis factor-alpha (TNF α), metalloproteinase-13, cyclooxygenase-2, and prostaglandin E₂. Furthermore, LPS-induced the phosphorylation of p38 mitogen-activated protein kinase, extracellular-regulated kinases 1/2, and nuclear factor-kappaB-p65 were also blocked by TQ in time-dependent manner. In our experimental RA model, the oral administration of TQ 5 mg/kg/day significantly reduced the serum levels of HNE, IL-1 β and TNF α as well as bone turnover markers, such as alkaline phosphatase and tartrate-resistant acid phosphatase. The protective effects of TQ against RA were also evident from the decrease in arthritis scoring and bone resorption. In conclusion, the fact that TQ abolishes a number of factors known to be involved in RA pathogenesis renders it a clinically valuable agent in the prevention of articular diseases, including RA. *J. Cell. Biochem.* 112: 107–117, 2011.

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KEY WORDS: THYMOQUINONE; PROLIFERATION; OXIDATIVE STRESS; INFLAMMATION; CATABOLISM; BONE; RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a chronic, systemic, inflammatory disease accompanying extensive destruction of articular cartilage and bone. Joint cartilage destruction manifests as radiological joint space narrowing that is followed by the destruction of underlying bone visible as geodes [Scott, 2000]. Many histological studies have established that joint destruction occurs mainly when the pannus, a tumour-like proliferation of rheumatoid synovial tissue, is in contact with intra-articular

cartilage and bone [Polzer et al., 2008]. The inflammatory process is characterized by inflammatory cell infiltration into the joints, leading to the proliferation of fibroblast-like synoviocytes (FLS) and the destruction of cartilage and bone [Polzer et al., 2008]. In RA synovial tissue, infiltrating cells consist of macrophages, T cells, B cells, plasma cells, neutrophils, and natural killer cells. FLS and inflammatory cells, such as macrophages and T cells, produce pro-inflammatory cytokines, such as interleukin-1beta (IL-1 β) and

Abbreviations used : AIA, adjuvant-induced arthritis; ALPase, alkaline phosphatase; COX-2, cyclooxygenase-2; ERK, extracellular-regulated kinases; FLS, fibroblast-like synoviocytes; HNE, 4-hydroxynonenal; IL-1 β , interleukin 1beta; JNK, c-Jun N-terminal kinases; LPO, lipid peroxidation; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MMP-13, metalloproteinase-13; NF-kB, nuclear factor kappaB; PGE₂, prostaglandin E₂; RA, rheumatoid arthritis; TNF α , tumour necrosis factor-alpha; TQ, thymoquinone; TRAP, tartrate-resistant acid phosphatase.

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tumour necrosis factor- α (TNF α), which play key roles in the pathogenesis of RA. In response to these cytokines, FLS produce chemokines, metalloproteinases (MMPs), prostaglandin E₂ (PGE₂) and cyclooxygenase-2 (COX-2) which further promote inflammation, hyperplasia and cartilage destruction [Chabaud et al., 2000; Hammaker et al., 2007]. The contribution of these cells to bone destruction has also been reported. These cells are able to promote osteoclastogenic activity by stimulating the receptor activator of nuclear factor- κ B ligand (RANKL), a member of the TNF family of cytokines [Ishida et al., 2009]. RANKL is a key molecule involved in the differentiation of osteoclasts in the presence of macrophage colony-stimulating factor. Local and systemic inflammation also favours generalized osteopenia or osteoporosis. Osteoclasts are considered to be the principal cell type responsible for focal bone resorption in RA [Redlich et al., 2002]. Gravalles et al. [1998] first described tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells in resorption lacunae at the bone-pannus interface in patients with juvenile arthritis [Gravalles et al., 1998]. Several lines of evidence have since confirmed the role of osteoclasts in bone destruction during RA. In RA patients, TRAP-positive cells in the pannus (cartilage edge and subchondral bone marrow) contribute to cartilage erosion [Tsuboi et al., 2003].

Other key modulators of joint inflammation in RA are reactive oxygen species (ROS) and lipid peroxidation (LPO) end-products. Large amounts of ROS and LPO products have been detected in the serum and synovial fluid of inflamed rheumatoid joints, and this production can be induced by cytokine stimulation [Remans et al., 2005; Pasupathi et al., 2009]. LPO products, mainly 4-hydroxynonenal (HNE), form adducts with proteins and make them highly immunogenic. Proteins modified in this manner have been found to induce pathogenic antibodies in a variety of diseases, including RA. Additionally, the advanced glycation end-product (AGE) pentosidine and AGE-modified immunoglobulin G (IgG) have been shown to correlate with RA disease activity [Kurien and Scofield, 2008].

Although several highly effective therapies are available to treat RA at present, they all have serious secondary effects. The available treatments include methotrexate, anti-TNF and non-steroidal anti-inflammatory drugs [Ma et al., 2010]. However, these drugs are often associated with side-effects, some of which can be quite serious. To avoid them, many are now turning to natural anti-inflammatory options to alleviate their arthritis pain. Among them, *Nigella sativa* seeds and oil have been used traditionally in the Middle East, Northern Africa and India to treat RA, diabetes and cancer patients [Ali and Blunden, 2003; Salem, 2005]. Thymoquinone (TQ) is an abundant component of black seed oil extract. Its beneficial effects are related to anti-oxidant, anti-infective, anti-tumour and anti-inflammatory properties [Ragheb et al., 2009]. In an experimental model of RA, Tekeoglu et al. [2007] reported that TQ suppressed adjuvant-induced arthritis (AIA) in rats, an action similar to that of methotrexate. However, scientific data are almost uniformly lacking concerning the anti-arthritis efficacy and mechanism of TQ effect.

The primary aim of this study was to extend our knowledge of the molecular mechanism of TQ in RA. We investigated the ability of TQ to prevent cell proliferation, LPO, catabolic and inflammatory responses in isolated FLS from RA patients. Secondly, we examined

whether TQ attenuates the biomarker expression of LPO, inflammation and bone destruction in an experimental model of RA.

MATERIALS AND METHODS

CELL PREPARATION

Synovial membranes were obtained from 11 RA patients (7 women, 4 men, mean age 62.4 ± 3.8 years) undergoing total knee replacement. Informed consent had been obtained from patients with RA for the use of their tissues for research purposes. All patients were evaluated, and the diagnosis was based on criteria of the American College of Rheumatology [Altman et al., 1986]. The experimental protocols and use of human tissues were approved by the Research Ethics Board of Hôpital du Sacré-Cœur de Montréal. To isolate FLS, finely-cut synovium was digested at 37°C in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Burlington, ON, Canada) with 0.25% (w/v) trypsin (Invitrogen) for 1 h, followed for 6 h by 2 mg/ml of type IV collagenase (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 10% heat-inactivated foetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin/100 µg/ml streptomycin (Invitrogen) [Chabane et al., 2009]. After synovial digestion, isolated FLS were seeded at high density in culture flasks until confluence in 10% FBS/DMEM at 37°C in a humidified atmosphere of 5% CO₂/95% air. At confluence, the cells were detached and passaged twice, then seeded at 10^5 cells/cm² in tissue culture plates in the above media. Twenty-four hour prior to the experiment, the medium was replaced by fresh medium containing 1% FBS and studies were performed in this medium supplemented with the factors under investigation.

AIA IN RATS

A total of 24 female Lewis rats (Charles River, Montreal, QC, Canada) weighing between 220 and 240 g were used. Animals were all conditioned and manipulated according to Canadian Council on Animal Care guidelines. They were housed in pairs, in standard laboratory cages, and kept in an air-conditioned animal room at a temperature of $22 \pm 2^\circ\text{C}$ and relative humidity of $57 \pm 2\%$ under a 12-h light/12-h dark cycle with ad libitum access to food and water. The experimental protocol was approved by the Research Ethics Board of Hôpital du Sacré-Cœur de Montréal. AIA was produced with complete Freund's adjuvant (CFA) containing heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) in mineral oil at 10 mg/ml, as described previously [Fernandes et al., 2008]. The rats were injected intradermally with 100 µl of adjuvant at the base of the tail. They developed arthritis 10 days later. The rats were allocated to three groups, each consisting of eight animals. Group 1, serving as normal controls, received 0.1 ml of mineral oil. Group 2 was administered 0.1 ml of CFA and represented the AIA model. Group 3 was given 0.1 ml of CFA with 5 mg/kg/day of TQ by oral gavage. The treatment was initiated on day 0 of the study and continued through day 28. All groups were killed on day 28, when maximum inflammation occurred.

SPECIMEN SELECTION

At the end of the study, the animals were anaesthetized with xylazine/ketamine (10/50 mg/kg, intraperitoneally), and blood was

collected into tubes by cardiac puncture before sacrifice. Blood samples were immediately centrifuged at 3,000 rpm for 10 min, and serum samples were stored at -80°C before being used. Trabecular bone samples were isolated from the medial tibial plateau under sterile conditions.

CELL VIABILITY

TQ cytotoxicity was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described previously [Vaillancourt et al., 2008]. Tests were performed in 96-well plates. Briefly, FLS were incubated for 24 h with increasing concentrations (0–10 μM) of TQ (Sigma–Aldrich). Then, the cells were incubated with 0.5 mg/ml MTT for 15 min at 37°C . Thereafter, 100 μl of solubilization solution (0.04 M HCl–isopropanol) was added. The amount of MTT formazan product was quantified by measuring of optical density at 570 nm with a microplate reader (BioTek Instruments, Winooski, VT).

CELL PROLIFERATION ASSAY

Cell proliferation was assessed by [^3H]-thymidine incorporation as follows: FLS were cultured in 96-well plates at a density of 2×10^4 cells/well in 0.2 ml of 10% FBS–DMEM. Twenty-four hour later, the cells were synchronized by incubation with 1% FBS–DMEM for another 24 h. The medium was removed, and 1% FBS–DMEM containing 1 μM lipopolysaccharides (LPS; Sigma–Aldrich) with or without TQ (0–10 μM) was added to the wells. After incubation for 24 h, the cells were pulsed with 10 $\mu\text{Ci/ml}$ [^3H]-thymidine for an additional 24 h. Finally, they were harvested and counted in a scintillation counter. The data were expressed as cpm/ 2×10^4 cells.

EXPERIMENTAL CULTURE CONDITIONS

FLS (10^5 cells/ cm^2) were pre-treated with increasing concentrations of TQ (0–10 μM) for 1 h, followed by another incubation in 1% FBS–DMEM with 1 μM LPS (Sigma–Aldrich) or 100 μM H_2O_2 for 8 or 24 h for mRNA and protein determination. To explore the involvement of different protein kinases in signalling cascades, FLS were pre-incubated with 10 μM for 1 h, followed by escalating incubation time periods (0–120 min) in the presence of 1 μM LPS.

PROTEIN DETECTION BY WESTERN BLOTTING

Twenty micrograms of total proteins from FLS lysates treated under the indicated conditions were subjected to discontinuous 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Protein transfer and immunodetection as well as semi-quantitative measurements were performed, as described previously [Morquette et al., 2006]. The primary antibodies were rabbit anti-COX-2 (Cayman Chemical Company, Ann Arbor, MI), anti- β -actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphorylated and total p38 mitogen-activated protein kinase (MAPK), anti-extracellular-regulated kinases (ERK) $_{1/2}$ and anti-nuclear factor- κB (NF- κB) p65 (Cell Signaling Technology, Inc., Danvers, MA). After serial washes, primary antibodies were detected by goat anti-rabbit IgG conjugated with horseradish peroxidase (Cell Signaling Technology). Immunoreactive proteins were quantified with Super-

Signal blotting substrate (Pierce Biotechnology, Inc., Rockford, IL) and exposed to clear-blue X-ray film (Pierce Biotechnology, Inc.).

RNA EXTRACTION AND REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION (RT–PCR)

Total RNA was isolated with TRIzol reagent according to the manufacturer's instructions. RNA was evaluated with RiboGreen RNA quantitation kits (Molecular Probes, Eugene, OR), dissolved in diethylpyrocarbonate-treated H_2O , and stored at -80°C until used. One microgram of total RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Fermentas, Burlington, ON, Canada), as detailed in the manufacturer's guidelines. One-fiftieth of the reverse transcriptase reaction product was analyzed by traditional PCR or real-time quantitative PCR [Chen et al., 2010]. The following sense and anti-sense specific primers (Bio-Corp, Inc., Montreal, QC), were tested: human TNF α (forward) 5'-TGG GCA GGT CTA CTT TGG GAT CAT-3', (reverse) 5'-TTT GAG CCA GAA GAG GTT GAG GGT-3'; human IL-1 β (forward) 5'- AAG TAC CTG AGC TCG CCA GTG AAA-3', (reverse) 5'-TTG CTG TAG TGG TGG TCG GAG ATT-3'; human GAPDH (forward) 5'- CAT GTT CGT CAT GGG TGT GAA CCA-3', (reverse) 5'-ATG GCA TGG ACT GTG GTC ATG AGT-3'; human MMP-13 (forward) 5'-AGT TTG CAG AGC GCT ACC TGA GAT-3', (reverse) 5'-TCG TCA AGT TTG CCA GTC ACC TCT-3'; human COX-2 (forward) 5'-CAA ATC CTT GCT GTT CCC ACC CAT-3', (reverse) 5'- GCA CTG TGT TTG GAG TGG GTT TCA-3'.

Quantitative PCR analysis was performed in a total volume of 50 μl containing template DNA, 200 nM sense and anti-sense primers, 25 μl of SYBR Green Master Mix (Qiagen, Mississauga, ON), and 0.5 units of uracil-*N*-glycosylase (UNG; Epicentre Technologies, Madison, WI). After incubation at 50°C for 2 min (UNG reaction) and at 95°C for 10 min (UNG inactivation and activation of AmpliTaq Gold enzyme), the mixtures were subjected to 40 amplification cycles (15 s at 95°C for denaturation and 1 min for annealing and extension at 60°C). SYBR Green dye incorporation into the PCR products was monitored in real time with a Mx3000 real-time PCR system (Stratagene, La Jolla, CA), to determine the threshold cycle (C_t) at which exponential amplification of PCR products begins. After PCR, dissociation curves were generated with 1 peak, indicating amplification specificity. A C_t value was obtained from each amplification curve with the software provided by the manufacturer (Stratagene). The amplicons size for TNF α , IL-1 β , GAPDH, MMP-13 and COX-2 were 128, 169, 156, 145 and 171, respectively.

Relative mRNA expression in FLS was quantified according to the $\Delta\Delta C_t$ method, as detailed in the manufacturer's guidelines (Stratagene). A ΔC_t value was first calculated by subtracting the C_t value for the housekeeping gene GAPDH from the C_t value for each sample. A $\Delta\Delta C_t$ value was then calculated by subtracting the ΔC_t value for the controls (unstimulated cells) from the ΔC_t value for each treatment. Fold changes compared to the controls were then quantified by $2^{-\Delta\Delta C_t}$. Each PCR generated only the expected specific amplicon, as shown by melting temperature profiles of the final product and gel electrophoresis of the test PCRs. Each PCR was performed in triplicate on two separate occasions for each independent experiment.

PGE₂, IL-1 β , AND TNF α IMMUNOASSAY

PGE₂ and cytokine (IL-1 β and TNF α) levels were measured in supernatants of FLS incubated as described above or in serum with enzyme immunoassay (EIA) or enzyme-linked immunosorbent assay (ELISA). The kits were from the Cayman Chemical Company and R&D Systems (Minneapolis, MN), respectively. Detection sensitivity was 9 pg/ml for PGE₂, 0.3 pg/ml for IL-1 β , and 4.4 pg/ml for TNF α . Each assay was performed according to the manufacturer's specifications.

HISTOLOGICAL EXAMINATION

Specimens were obtained from the tibial plateaus of rats at equivalent anatomical sites, fixed in 10% (vol/vol) formalin and decalcified with 10% (vol/vol) formic acid for 24 h. The specimens were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. A Leica DMLS microscope (Leica, Wetzlar, Germany) was connected to a personal computer (using Image J software, V.1.27; National Institutes of Health, Bethesda, MD) for bone morphometry. Bone histomorphometry was performed on three consecutive sections of each specimen. From each section, three representative fields (500 \times 500 μm^2) were identified (original magnification 10 \times). Bone surface (μm^2) and marrow cavity area (μm^2) measurements as

well as all histomorphometric data collected followed the standard convention defined by Parfitt et al. [1987].

ARTHRITIS SCORING

Arthritis severity was evaluated by monitoring the forepaws and hindpaws on day 28 for visual signs of inflammation, such as erythema and swelling, according to a macroscopic scoring system described as follows: 0: no signs of arthritis, 1: swelling and/or redness in one paw; 2: two joints affected; 3: more than two joints affected; and 4: severe arthritis in all paws. Paw diameters also were measured on day 28. All groups were killed on day 28, when maximum inflammation occurred.

DETERMINATION OF SERUM ALKALINE PHOSPHATASE (ALPASE) ACTIVITY AND TRAP LEVELS

ALPase activity in serum was considered as the release of p-nitrophenol hydrolyzed from p-nitrophenyl phosphate (12.5 mmol/L final concentration) at 37°C for 30 min, as described previously [Shi et al., 2006]. Serum TRAP levels were quantified by rat TRAP assay kits from IDS (Boldon Colliery, Tyne and Wear, UK). The detection limit of this assay was 0.1 U/ml.

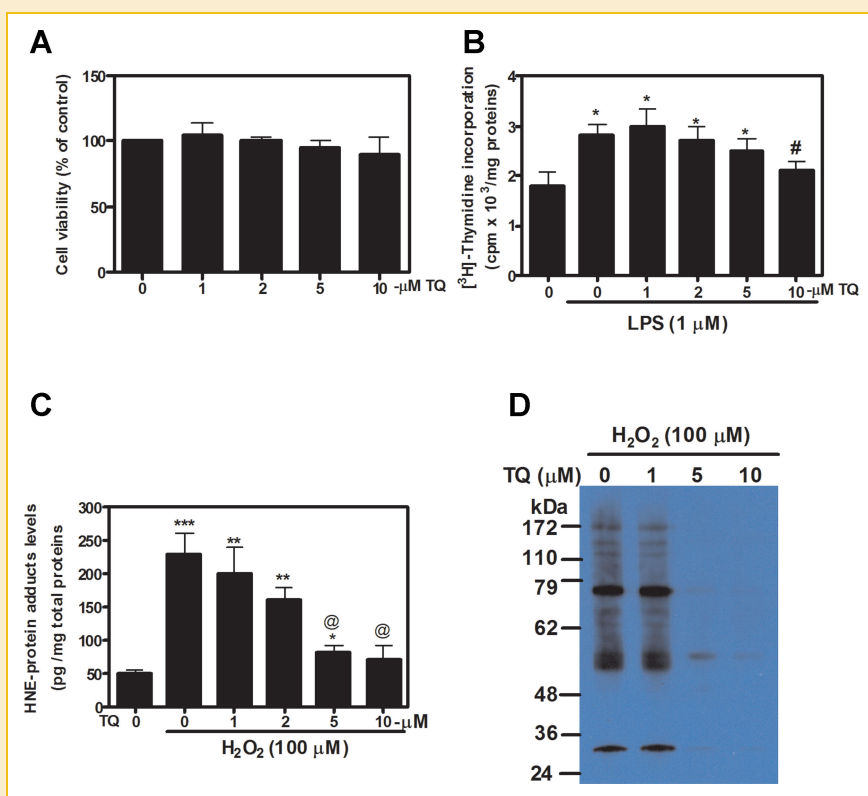


Fig. 1. Thymoquinone (TQ) had no effect on cell viability and reduced cell proliferation and lipid peroxidation (LPO). Human fibroblast-like synoviocytes (FLS) from RA patients were treated with increasing TQ concentrations (0–10 μM) for 24 h (A) or for 1 h before incubation for 24 h in the presence of 1 μM lipopolysaccharides (LPS) (B) or 100 μM H₂O₂ (C,D). Cell viability (A) and proliferation (B) were assessed by MTT and [³H]-thymidine incorporation into DNA, respectively. 4-Hydroxynonenal (HNE)-protein adducts, as a marker of LPO, were quantified by ELISA (C) and analyzed by Western blotting (D) using a specific anti-HNE antibody. Values represent the means \pm SEM of 3–4 separate experiments performed in duplicate. * P < 0.05, ** P < 0.01, *** P < 0.001, compared to untreated cells (control; 1% FBS); @ P < 0.01, & P < 0.001 compared to LPS-treated cells. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TISSUE LEVELS OF HNE-PROTEIN ADDUCTS

Total tissue levels of HNE-protein adducts were assessed in serum from normal, RA and RA + TQ rats by in-house ELISA, as described previously [Morquette et al., 2006]. HNE-modified bovine serum albumin (BSA) served as standard.

STATISTICAL ANALYSIS

The data are expressed as means \pm SEM of eight rats. All statistics were generated by Prism software (GraphPad Software, San Diego, CA). Statistical significance was assessed by two-way ANOVA, and $P < 0.05$ was considered significant.

RESULTS

TQ IS AN ANTI-PROLIFERATIVE AND ANTI-OXIDANT AGENT

First, we tested the cytotoxic effect of TQ on FLS. As illustrated in Figure 1A, TQ at different concentrations did not alter the cell viability. Then, the anti-proliferative and anti-oxidant properties of TQ in LPS-treated human RA FLS. LPS is well known as a potent growth-promoting agent in various cell types [Wang et al., 2009; Xiao-Jun et al., 2010]. Measuring [3 H]-thymidine incorporation, treatment of cells with TQ at 10 μ M inhibited cell growth by 20% ($P < 0.05$) compared to LPS-treated cells (Fig. 1B). Thereafter, to document the anti-oxidant properties of TQ, cells were incubated with H_2O_2 , a free radical donor, in the presence or absence of TQ. Our ELISA and Western blotting data (Fig. 1C,D) revealed that TQ at 5

and 10 μ M prevented HNE generation ($\sim 70\%$, $P < 0.01$), a product of free radical-induced LPO, in cell extracts from H_2O_2 -treated FLS. Collectively, these data suggest that TQ exerts moderate anti-proliferative and strong anti-oxidative effects in isolated FLS. Of note, FLS treatment with TQ up to 10 μ M did not alter the cellular viability.

TQ INHIBITS LPS-INDUCED IL-1 β AND TNF α

Dose-response experiments were first performed to explore the effect of TQ on pro-inflammatory cytokines production. RA FLS were cultured for 1 h in the presence of 1, 2, 5, or 10 μ M TQ, after which they were incubated for another 8 or 24 h for protein and mRNA determination respectively in the presence of 1 μ M LPS. TQ at different concentrations had no effect on basal IL-1 β and TNF α production (data not included). However, combined with 1 μ M LPS, TQ dramatically reduced LPS-induced cytokine production (Fig. 2). At 10 μ M, it significantly decreased LPS-induced IL-1 β (Fig. 2A) and TNF α (Fig. 2C), by 85% and 80% ($P < 0.001$), respectively. In a similar manner, our data showed that TQ inhibited the LPS-induced mRNA expression of IL-1 β (Fig. 2B) and TNF α (Fig. 2D) by $\sim 70\%$ ($P < 0.001$), respectively.

TQ INHIBITS LPS-INDUCED PGE $_2$ RELEASE AND COX-2 EXPRESSION

To establish whether TQ abolishes LPS-induced COX-2 expression, RA FLS were incubated in the previous conditions, and PGE $_2$ release and COX-2 expression were quantified. As shown in Figure 3A, TQ significantly blocked LPS-induced PGE $_2$ release in a dose-dependent

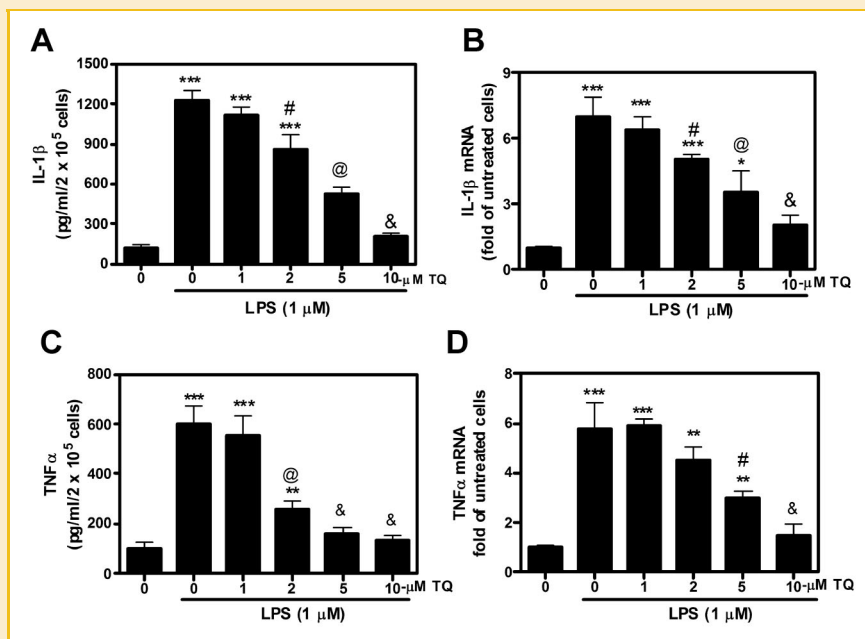


Fig. 2. Thymoquinone (TQ) suppressed pro-inflammatory cytokine production. Confluent human fibroblast-like synoviocytes (FLS) were treated with increasing doses of TQ (0–10 μ M) followed by another incubation for 24 h in the presence or absence of 1 μ M lipopolysaccharides (LPS). The culture medium was collected to quantify interleukin-1beta (IL-1 β , A) and tumour necrosis factor- α (TNF α , C) with commercial ELISA kits. IL-1 β (B) and TNF α (D) mRNAs were quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The protein and mRNA results are expressed as pg/ml and fold changes, considering 1 as the value in unstimulated samples, and represent the means and SEM of four independent experiments performed in duplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, compared to untreated cells (control) (1% FBS); # $P < 0.05$, @ $P < 0.01$, & $P < 0.001$ compared to LPS-treated cells alone.

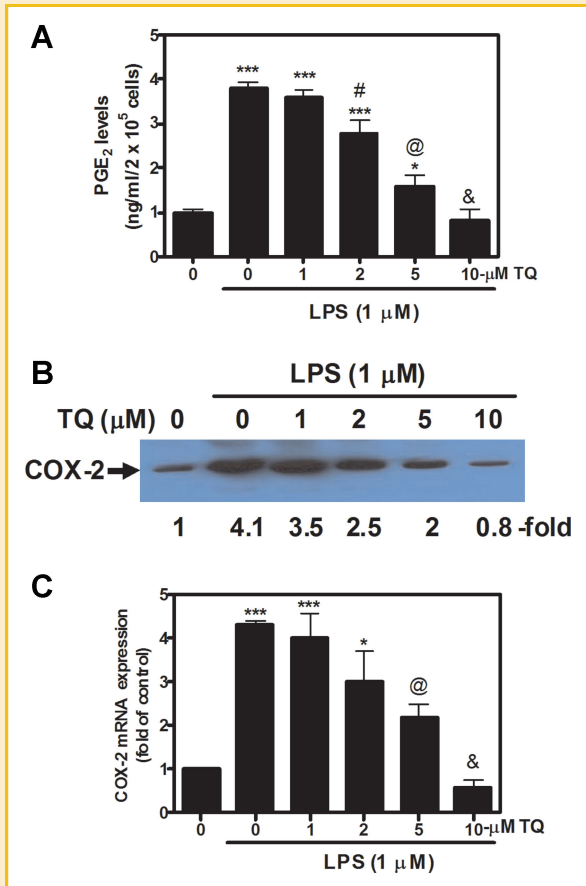


Fig. 3. Inhibition of LPS-induced cyclooxygenase-2 (COX-2) by thymoquinone (TQ). Confluent human fibroblast-like synoviocytes (FLS) were treated with increasing doses of TQ (0–10 μM), followed by another incubation for 24 h in the presence or absence of 1 μM lipopolysaccharides (LPS). The culture medium was collected to quantify PGE₂ with commercial kits (A), and cell lysates were used to quantify (B) COX-2 protein, and (C) mRNA levels by Western blotting and real-time reverse transcriptase-polymerase chain reaction (RT-PCR), respectively. PGE₂ levels are expressed as pg/ml, and protein and mRNA results as fold changes, considering 1 as the value in unstimulated samples, and represent the mean and SEM of four independent experiments. **P* < 0.05, ****P* < 0.001, compared to untreated cells (control) (1% FBS); #*P* < 0.05, @*P* < 0.01, &*P* < 0.001 compared to LPS-treated cells alone. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

manner. To determine if PGE₂ inhibition by TQ treatment is related to COX-2 down-expression, we studied the effects of increasing TQ doses on COX-2 expression at the protein and mRNA levels. As expected, our data revealed that LPS-induced COX-2 expression was gradually reduced by TQ in a dose-dependent manner. Maximum inhibition of both PGE₂ release and COX-2 expression was achieved with 10 μM TQ (*P* < 0.001). Of note, TQ at different concentrations had no effect on basal PGE₂ and COX-2 production (data not included).

MMP-13 IS REDUCED IN THE PRESENCE OF TQ

Since MMPs are involved in cartilage degradation in RA [Jungel et al., 2009], we further characterized the ability of TQ to prevent MMP-13 expression. Figure 4A,B show that TQ prevented LPS-

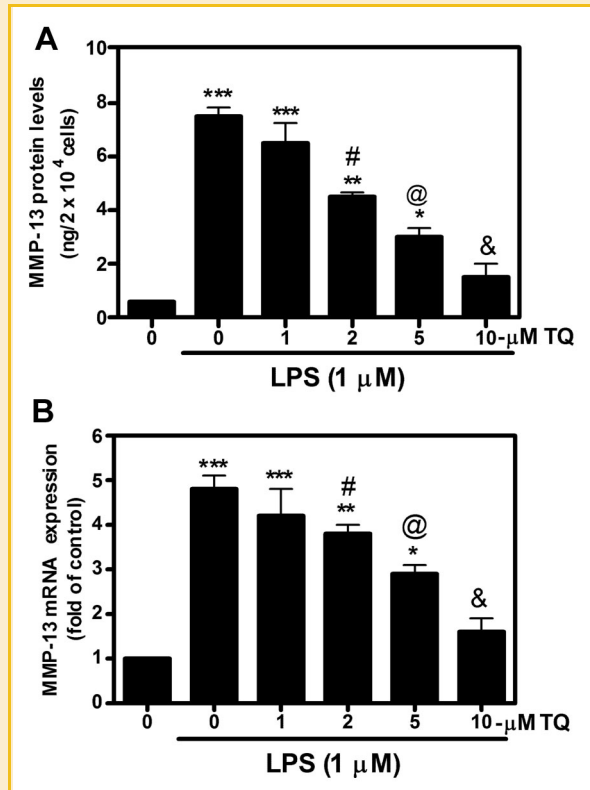


Fig. 4. Thymoquinone (TQ) prevented LPS-induced metalloproteinase-13 (MMP-13). Human fibroblast-like synoviocytes (FLS) were treated with different doses of TQ (1–10 μM) before incubation with 1 μM lipopolysaccharides (LPS) for 24 h. The supernatants were collected, and MMP-13 levels (A) were measured with commercial ELISA kits. MMP-13 mRNA levels (B) were quantified in isolated total mRNA by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The data are expressed as ng/ml for MMP-13 protein and as fold changes of MMP-13 mRNA, considering 1 as the value in unstimulated samples, and represent the means and SEM of four independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared to untreated cells (control; 1% FBS); #*P* < 0.05, @*P* < 0.01, &*P* < 0.001 compared to LPS-treated cells alone.

induced MMP-13 expression at the protein and mRNA levels in a dose-dependent manner. MMP-13 inhibition by TQ at the protein and mRNA levels was more pronounced when the cells were treated with TQ 10 μM, reaching 80% and 70% (*P* < 0.001), respectively. Of note, TQ at different concentrations had no effect on basal MMP-13 production (data not included).

TQ-DEPENDENT INHIBITION OF MAPKS AND NF-κB-SIGNALLING PATHWAYS

To elucidate the signalling pathways that are inhibited by TQ, we analyzed members of the MAPKs and NF-κB pathways by Western blotting with protein samples from RA FLS that had been pre-incubated with 10 μM TQ for 1 h, followed by 15, 30, 60, or 120 min of stimulation with 1 μM LPS. TQ at 10 μM had no effect on basal MAPKs and p65-NF-κB (data not included). However, combined with LPS, TQ at 10 μM blocked LPS-induced p38 MAPK, ERK1/2 and NF-κB-p65 phosphorylation with all incubation time periods (Fig. 5). Taken together, these findings suggest that TQ-inhibited

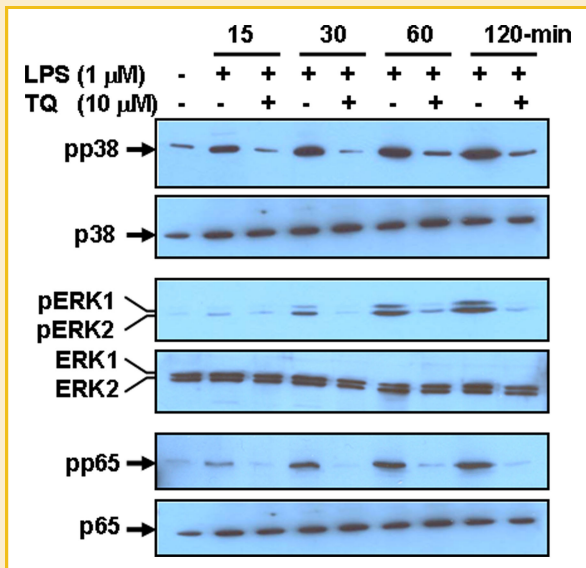


Fig. 5. Thymoquinone (TQ) blocked induction of the signalling pathways. Human fibroblast-like synoviocytes (FLS) were treated with increasing concentrations of TQ (1–10 μ M) for 1 h before incubation for different periods (0–120 min) in the presence or absence of 1 μ M lipopolysaccharides (LPS). Cell lysates were subjected to Western blot analysis for phosphorylated and total levels of p38 MAPK, ERK1/2, and NF- κ B-p65. These blots are representative of similar results obtained in five independent experiments. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

inflammatory and catabolic mediators could be attributed, in part, to abrogation of the MAPKs and NF- κ B-signalling pathways.

TQ SUPPRESSES HNE, IL-1 β , TNF α , AND PGE $_2$ PRODUCTION IN RA RATS

This part of the experiment was designed to verify the ability of TQ to attenuate oxidative stress and inflammatory cytokine production in experimental RA rats. The animals received an injection of adjuvant, and then TQ (5 mg/kg/day) was given orally at time 0. Our findings demonstrated that the levels of HNE-protein adducts (Fig. 6A), IL-1 β (Fig. 6B), TNF α (Fig. 6B) and PGE $_2$ (Fig. 6B) increased significantly by 355% ($P < 0.01$), 190 ($P < 0.05$), 283% ($P < 0.01$) and 215% ($P < 0.01$) respectively in serum from RA rats compared to the controls. Interestingly, daily oral administration of TQ suppressed the over-production of all indicated mediators. RA rats receiving vehicle only (0.9% NaCl + 1% ethanol) presented no effect (data not included). Taken together, our in vivo data confirm the in vitro findings and indicate the biological efficacy of oral TQ in suppressing animal models of arthritis.

TQ NORMALIZES SERUM ALPASE AND TRAP ACTIVITIES IN RA RATS

We next looked at the effect of TQ treatments on bone turnover markers such as ALPase and TRAP. TRAP is exocytosed from osteoclasts along with bone matrix products into the circulation where its activity reflects the bone resorption rate [Janckila et al., 2002]. Figure 6C reveals that serum ALPase activity was

significantly increased in AIA rats (4.6-fold higher, $P < 0.01$) compared to normal controls. Daily oral TQ administration was found to have significant protective effects by decreasing the enzyme's levels in comparison to the RA rats. The data showed a ~50% decrease ($P < 0.05$) in ALPase activity in TQ-treated RA rats compared to RA rats. Furthermore, the serum TRAP profile was similar to that of serum ALPase (Fig. 6C). The augmented serum TRAP activity in AIA rats (3.2-fold increase, $P < 0.05$) was significantly decreased by TQ treatment. The inhibition rate by TQ was 50% ($P < 0.05$). Taken together, we discovered that the levels of LPO and inflammation markers (HNE, IL-1 β , TNF α and PGE $_2$) and bone turnover markers (ALPase and TRAP) in rat serum were elevated in AIA rats (group 2) in comparison to the controls (group 1). Treatment with TQ (group 3) significantly inhibited the increased levels of the indicated bio-markers. Finally, our data suggested that oral TQ administration to adjuvant-immunized rats reduced the progression of arthritis by inhibiting the increase in arthritis score and paw swelling compared to RA rats (Fig. 6D).

TQ SUPPRESSES BONE RESORPTION IN RA RATS

Representative histopathological findings in the rat trabecular bone region are illustrated in Figure 7 with histological hematoxylin and eosin staining. In periarticular, bone from normal rat joints, the marrow cavity was small and bone was intact (Fig. 7A). As opposed to periarticular bone from AIA rat joints, the marrow cavity was greatly expanded (Fig. 7B). These changes included a significant reduction in bone surfaces related to a marked increase in marrow cavity area (Fig. 7D, $P < 0.01$). Interestingly, bone surface and marrow cavity area were the most improved features of TQ administration (Fig. 7C,D, $P < 0.05$).

DISCUSSION

Ours is the first study to investigate the anti-oxidative and anti-proliferative properties of TQ, the main active constituent of *Nigella sativa*, in RA FLS and in an experimental model of RA. We also explored the ability of this drug to attenuate inflammatory and catabolic factors known to be involved in the RA process. In in vitro experiments, human RA FLS were treated with LPS to elicit cell proliferation as well as inflammatory and catabolic responses or with H $_2$ O $_2$ to evoke oxidative stress. In vivo, we tested an adjuvant-induced experimental model of RA. Our results provide a mechanistic explanation for the beneficial properties of TQ in alleviating arthritis as conveyed by traditional medicines in different cultures. In our experimental conditions, TQ up to 10 μ M did not alter the cell viability (data not shown).

First, we obtained data showing that TQ reduced slightly but significantly LPS-induced human FLS proliferation. The moderate reduction in cell proliferation could be attributed to low dose of TQ. Several lines of evidence suggest that TQ exhibits inhibitory effects on cell proliferation, migration, invasion, tube formation, and angiogenesis in vitro and in vivo [Yi et al., 2008; Alhosin et al., 2010; Ravindran et al., 2010]. The anti-proliferative action of TQ would be helpful since RA is characterized by tumour-like expansion of the synovium that is composed of proliferating

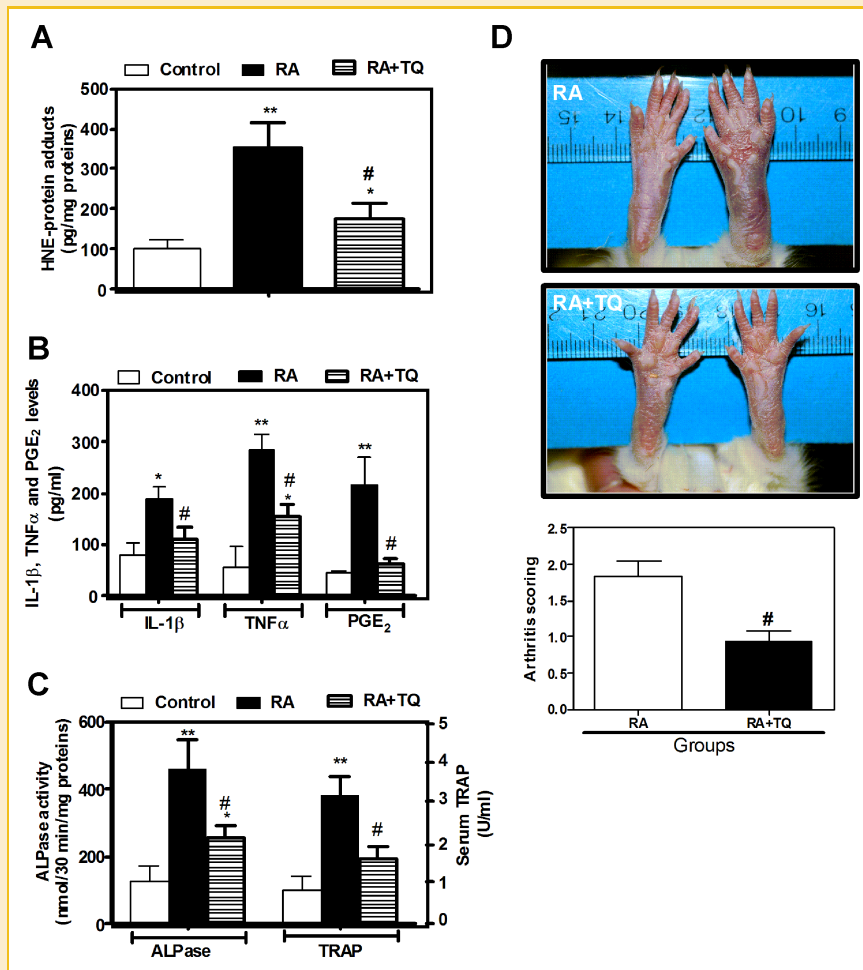


Fig. 6. Effect of thymoquinone (TQ) on serum markers of oxidative stress, inflammation and bone formation and clinical evaluation. The therapeutic effect of TQ on serum levels of (A) 4-hydroxynonenal (HNE), (B) interleukin-1beta (IL-1 β), tumour necrosis factor-alpha (TNF α), and prostaglandin E2 (PGE $_2$), (C) alkaline phosphatase (ALPase) and tartrate-resistant acid phosphatase (TRAP) in rats with adjuvant-induced arthritis (AIA). D: Arthritis scoring in RA rats with or without TQ administration. Values are the means \pm SEM of eight rats. Statistical significance was assessed by unpaired Student's *t*-test, **P* < 0.05, ***P* < 0.01, versus control rats; #*P* < 0.05 versus RA rats.

synoviocytes and infiltrating leucocytes [Firestein, 2003]. Recently, Alhosin et al. [2010] reported that TQ at $>10 \mu\text{M}$ target the anti-apoptotic *UHRF1* gene with subsequent cell cycle arrest and apoptosis. The authors indicated that TQ produces intracellular ROS, promotes a DNA damage-related cell cycle arrest and triggers apoptosis through the activation of a p73-dependent mitochondrial and cell cycle signaling pathway, followed by a down-regulation of UHRF1. In the present study, we did not investigate the apoptotic effect of TQ in RA FLS. To predict the potential impact of such a therapeutic strategy on the development of RA, additional experiments are needed to clarify whether TQ suppresses tissue invasion through FLS death.

Second, our findings showed that TQ reduced the accumulation of HNE-protein adducts, suggesting normalization of redox status by TQ administration. Ample evidence proves that TQ has potent ROS-scavenging abilities in vitro and in vivo [Ragheb et al., 2009; Ismail et al., 2010]. Treatment of rats with TQ caused up-regulation of superoxide dismutase 1, catalase and glutathione peroxidase 2 genes

compared to untreated rats [Ismail et al., 2010]. TQ effectively improved plasma and tissue anti-oxidant capacity and enhanced the expression of liver anti-oxidant genes. In contrast, other studies presented evidence linking the pro-oxidant effects of TQ with its apoptotic actions [Gali-Muhtasib et al., 2008; El-Najjar et al., 2010]. The oxidant-anti-oxidant ability of TQ depended on its concentration and the milieu where it was present. Its concentration needed to induce ROS was higher ($>20 \mu\text{M}$) than in our study.

Third, a set of experiments was performed to investigate the ability of TQ to attenuate inflammatory and catabolic factors known be involved in the RA process. As predicted, TQ administration suppressed LPS-induced IL-1 β , TNF α , COX-2 and MMP-13 production in LPS-treated RA FLS and reduced serum levels of IL-1 β , TNF α and PGE $_2$ in RA rats. TQ has been well documented as an important anti-inflammatory mediator that inhibits IL-1 β , TNF α and COX-2 production in pancreatic cancer cells [El Mezayen et al., 2006; Chehl et al., 2009]. In RA, Tekeoglu et al. [2007] demonstrated a decrease in serum levels of IL-1 β and TNF α in TQ-treated RA rats

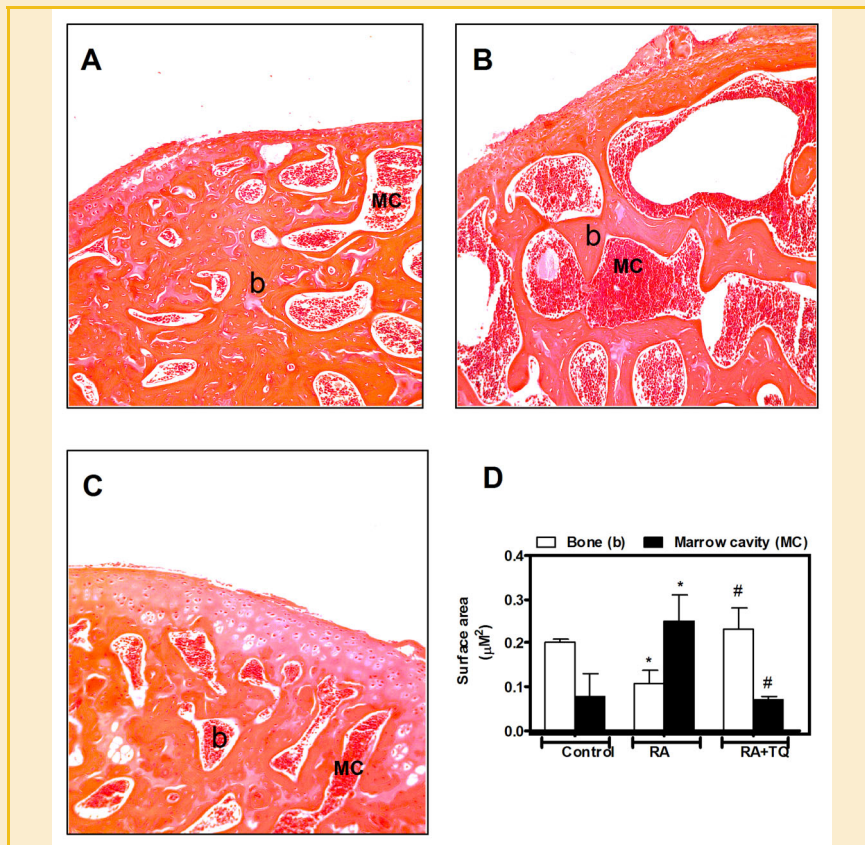


Fig. 7. Histological examination (hematoxylin and eosin staining) of trabecular bone in rats. A: Control rats, (B) adjuvant-induced arthritis (AIA) rats, (C) AIA rats treated with TQ. D: Morphometric analyses of bone surface (b) and marrow cavity (MC) in rat trabecular bone. Values are the means \pm SEM of eight rats. Statistical significance was assessed by unpaired Student's *t*-test, **P* < 0.05 versus control rats; #*P* < 0.05 versus RA rats. Original magnification 10 \times .

compared to RA animals. These authors also reported lower clinical and radiological arthritis scores in the RA rat model compared to the controls. According to data from Houghton et al. [1995], TQ inhibited thromboxane and leukotriene B₄ synthesis from eicosanoids, by suppressing COX-2 and lipooxygenase enzymes in leucocytes. In a recent study, Ravindran et al. [2010] noted that TQ encapsulated in nanoparticles prevented angiogenesis, inflammatory mediators and MMP-9 production in several cell lines. In RA, pro-inflammatory cytokines, such as TNF α and IL-1 β , COX-2 and PGE₂ are abundantly expressed in patients with RA and in the arthritic joints of mice with collagen-induced arthritis, and blockade of these molecules results in a reduction of disease severity [Williams, 2004; Schett et al., 2008].

Fourth, the MAPKs and NF- κ B signalling pathways were explored to investigate the concept that LPS-induced signalling pathways were blocked by TQ addition in cultured RA FLS. MAPKs and NF- κ B expression has previously been described to be elevated in the RA synovium from humans and animals, suggesting their important roles in destructive arthritis [Marok et al., 1996; Schett et al., 2000]. Our Western blot findings clearly indicated that TQ prevents LPS-induced activation of p38 MAPK, ERK1/2 and NF- κ B in RA FLS. These data are in agreement with previous reports indicating that TQ prevented p38 and NF- κ B activation [El-Mahmoudy et al., 2005; Sethi et al., 2008]. In human myeloid KBM-5 cells and human

embryonic kidney A293, it has been reported that TQ suppressed the NF- κ B activation pathway through modulation of the p65 subunit of NF- κ B and inhibition of I κ B kinase [Sethi et al., 2008]. However, our data contrast to other reported showing that TQ-induced p38 MAPK and c-Jun NH(2)-terminal kinase 1/2 in pancreatic cancer cells FG/COLO357 and CD18/HPAF [Torres et al., 2010]. The activation of these protein kinases is essential to induce apoptosis process in these cells.

Finally, given that RA pathogenesis is associated with bone damage, we considered it relevant to test the effect of TQ on bone turnover in RA rats. The present study is the first to demonstrate that TQ administration in RA rats reduced ALPase activity and TRAP levels compared to untreated RA rats. Histology confirmed that TQ prevents bone erosion, as seen on the bone surface and in the marrow cavity. Ample studies have disclosed that significant soft tissue inflammation in most RA patients causes joint damage, and bone erosion occurs within 2 years of disease onset [Fye, 1999]. Regardless, up to 30% of patients first presenting with symptoms of RA show radiographic evidence of bone erosion [O'Dell, 2004]. AIA in rats has been widely adopted as an experimental model for the pre-clinical screening of RA treatments. The model is robust, the incidence rate of the disease is 100%, and AIA in rats shares many features with RA in humans, such as inflammation, marked bone resorption and periosteal bone proliferation [Bendele, 2001].

It has been reported that bone formation is reduced in arthritic disease, whereas bone resorption is increased [Ramprasath et al., 2006]. Markers of bone metabolism, such as ALPase and TRAP, were studied in this investigation. ALPase and TRAP are considered to be biomarkers of bone formation and bone resorption, respectively [Allen, 2003]. Their levels in serum and bone-cultured osteoblasts were altered in the arthritic condition, showing abnormal bone turnover. Our data support previous findings on bone metabolism, where it has been established that serum ALPase and TRAP are significantly elevated [Ramprasath et al., 2006]. Heightened serum ALPase levels could be due to either increases in liver and bone fractions or increments of both isoenzymes.

In summary, our study demonstrated that TQ-inhibited FLS proliferation, oxidative stress as well as catabolic and inflammatory mediators in both FLS and RA rats. Moreover, TQ administration was shown to play a role in preventing abnormal osteoblast metabolism and bone damage in RA rats. These findings suggest that TQ may represent a new therapeutic approach to the treatment of chronic autoimmune arthritis.

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