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Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators

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AA, adjuvant-induced arthritis
COX, cyclooxygenase
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IFN, interferon
NOS, nitric oxide synthase
QU, quercetin
RA, rheumatoid arthritis
TNF, tumor necrosis factor

ABSTRACT

Pentahydroxyflavone dihydrate, quercetin (QU) is one of common flavonols biosynthesized by plants and has been suggested to modulate inflammatory responses in various models. In the present study, we investigated *in vivo* effects of oral or intra-cutaneous QU in chronic rat adjuvant-induced arthritis (AA). Growth delay and arthritic scores were evaluated daily after AA induction in Lewis rats. Oral administration of QU (5× 160 mg/kg) to arthritic rats resulted in a clear decrease of clinical signs compared to untreated controls. Intra-cutaneous injections of lower doses (5× 60 mg/kg) of QU gave similar anti-arthritic effects, while 5× 30 mg/kg concentrations were inefficient in this respect. Finally, injection of relatively low QU doses (5× 30 mg/kg) prior to AA induction significantly reduced arthritis signs. As QU was suggested to inhibit macrophage-derived cytokines and nitric oxide (NO), we then analyzed macrophage response *ex vivo*. Anti-arthritic effects of QU correlated with significant decrease of inflammatory mediators produced by peritoneal macrophages, *ex vivo* and *in vitro*. These data indicate that QU is a potential anti-inflammatory therapeutic and preventive agent targeting the inflammatory response of macrophages.

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1. Introduction

Rheumatoid arthritis (RA) is characterized by chronic inflammation of synovial tissue and the destruction of cartilage and bone in the joints [1]. Macrophages play an important role in RA, as the rheumatoid synovium is intensively infiltrated by macrophages and their numbers correlate with clinical scores [2] and articular destruction in RA [3]. Activation of the

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monocyte/macrophages in arthritic patients is characterized by increased expression of interleukin (IL)-1β, TNF-α, cyclooxygenase-2 (COX-2) and other pro-inflammatory mediators [1,4]. Reports have identified NO as another pro-inflammatory mediator of arthritis in human and experimental animal studies [5,6]. Increased concentrations of nitrites, stable metabolites of NO, have been observed in the serum and synovial fluid of patients with rheumatoid arthritis (RA) and osteoarthritis [5,7]. Increased inducible nitric oxide synthase (iNOS) activity and NO production have also been detected in the blood mononuclear cells of RA patients and correlated with the tender and swollen joint counts [5,8].

Recently, we have shown that QU decreases NO and TNF-α production in human inflammatory macrophages in a dose dependant manner [9]. QU is the most common flavones present in plants such as *Ginkgo biloba*, tea, lovage, onion, apple (up to 170 mg/100 g in lovage leaves) and commonly absorbed by humans [10]. In murine models, QU was able to inhibit inflammatory diseases *in vivo* [13–15] and to decrease COX-2 and iNOS-mRNA *in vitro* [11]. First promising *in vivo* results using QU for the treatment of human inflammatory prostatitis [16], chronic pelvic pain [17], or rat colitis and arthritis [13,18] led us to assay, in the present study, *in vivo* effects of this molecule on macrophage activation in rat chronic adjuvant-induced arthritis [19]. Strong inhibition of *in vivo* rat inflammatory responses was observed following therapeutic or preventive QU treatment.

2. Materials and methods

2.1. Reagents

Oral treatment with Quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate, >98% purity powder, Sigma–Aldrich) was performed by gavages with QU suspended in distilled water containing 1% polysorbate 80 and 0.5% carboxymethylcellulose sodium. Later excipient was used for control rats. Intra-cutaneous (I.C.) injections were performed with QU suspended in saline.

2.2. Induction and evaluation of adjuvant arthritis (AA)

Female Lewis rats (Janvier, Le Genest St Isle, France) were housed under standard laboratory conditions with free access to food and water. The temperature was kept at 22 ± 2 °C and a 12-h light/dark schedule was maintained. All animal procedures were performed in strict accordance with the guidelines issued by the European Economic Community ''86/609''. Adjuvant arthritis was induced in 6 weeks old animals by subcutaneous injection at the base of the tail of 300 μl (1.8 mg) of inactivated *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) diluted in emulsion of 8 ml Vaseline, 1 ml polysorbate 80, and 1 ml PBS (phosphate-buffered saline; BioWhittaker, Walkersville, MD). Rats were boosted 1 week later with the same dose of antigen and observed for up to 50 days following immunization for clinical signs of arthritis. Evaluation of AA severity was performed by two independent observers with no knowledge of the treatment protocol. The severity of AA in each paw was quantified daily by a clinical score measurement from 0 to 2 as following: no signs of inflammation (0); swelling alone (greater than two-fold paw diameter) (0.5) or swelling/immobility (1.0) of one paw; swelling (1.5) or swelling/immobility (2.0) of two paws. Weight evolution of the animals was measured daily.

2.3. Macrophages

Animals were anaesthetized with ether and the peritoneal cavity was washed with 10 ml cold phosphate-buffered saline (PBS), pH 7.4. After centrifugation at 1200 × *g* for 10 min at 4 °C, cells were collected, counted and adjusted to 2×10^5 cells/ml with RPMI 1640 culture medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat-inactivated fetal calf serum (FCS) (all from Eurobio, Les Ulis, France). Above culture medium, chemicals, and FCS were endotoxin-free and tested for the absence of direct activation effects on normal macrophages (NO production as activation markers). Following 96 hr incubation in medium alone, cell supernatants from disease-free and AA rats were collected for mediator quantification. To activate the production of various inflammatory mediators from peritoneal macrophages, cells were incubated with lipopolysaccharide (LPS, 5 μg/ml; Sigma). Quercetin was added to the cells simultaneously to their activation. Cells or their supernatants were then tested for the presence of various inflammatory mediators. To detect cell apoptosis, externalization of inner membrane phosphatidylserine and DNA content were analyzed by flow cytometry using fluorescein-conjugated annexin-V and propidium iodide kit (Immunotech, Marseille, France). Results were analyzed and compared using the Student *t*-test for paired data.

2.4. Quantification of inflammatory mediators

Culture supernatants (72 h) were assayed for the stable end product of NO, NO_2^- using the Griess reaction modified as detailed elsewhere [21]. Inhibitory analog of L-arginine, L-NIL (*N*(6)-(1-iminoethyl)-L-lysine/2HCl, Coger SA, Paris, France) [22] was used to inhibit iNOS-mediated NO generation. To detect other rat mediators, we have used appropriate specific ELISA kits as recommended by the manufacturer: PGE2 (R&D, Lille, France), MCP-1 (Tebu, Le Perray-en Yveline, France), TNF-α and IL-1β (Biosource, Monyrouge, France). Total RNA was isolated from peritoneal macrophage with Trizol reagent as recommended (Invitrogen, Cergy Pontoise, France). Subsequently, 2 μg of RNA were reverse transcribed using a set of oligo(dT) primers and 25 U of Superscript II Reverse Transcriptase [23]. The resulting cDNA (1 μl) was then amplified by classical PCR using *Taq*DNA polymerase and primers for iNOS, and β-actin under PCR conditions as previously described [23].

3. Results

3.1. Anti-arthritic effect of oral quercetin

In vivo potential of QU in reducing the severity of AA in Lewis rats was tested. The rat model was chosen since there is a very good homology between human and rat macrophage markers. In contrast to earlier reports, QU formulations and doses used in this work were close to those used as diet supplement for

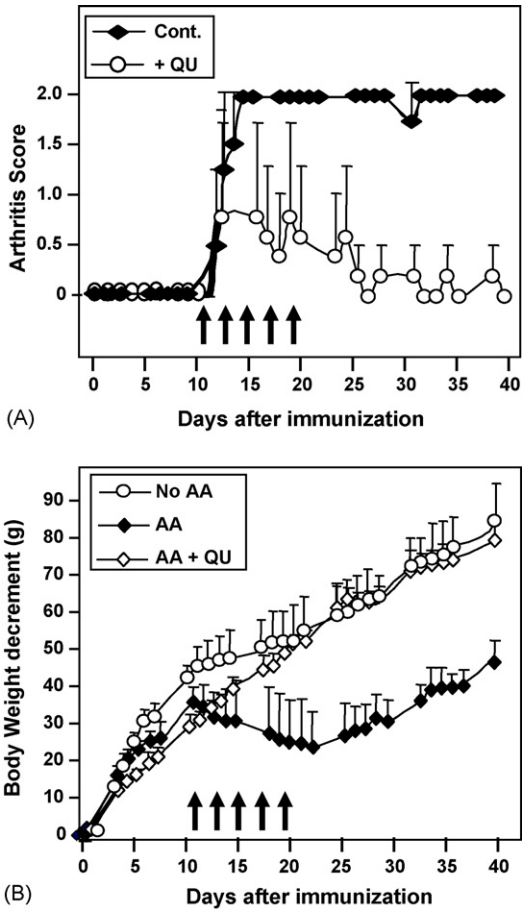

Fig. 1 – The evolution of arthritis severity scores (A) and body weight (B) of AA rats following their treatment with 150 mg/rat oral doses of QU (30 mg every 2 days 5×). Rats were treated following the appearance of clinical signs as illustrated by arrows. Results are means ± S.E.M. from five rats.

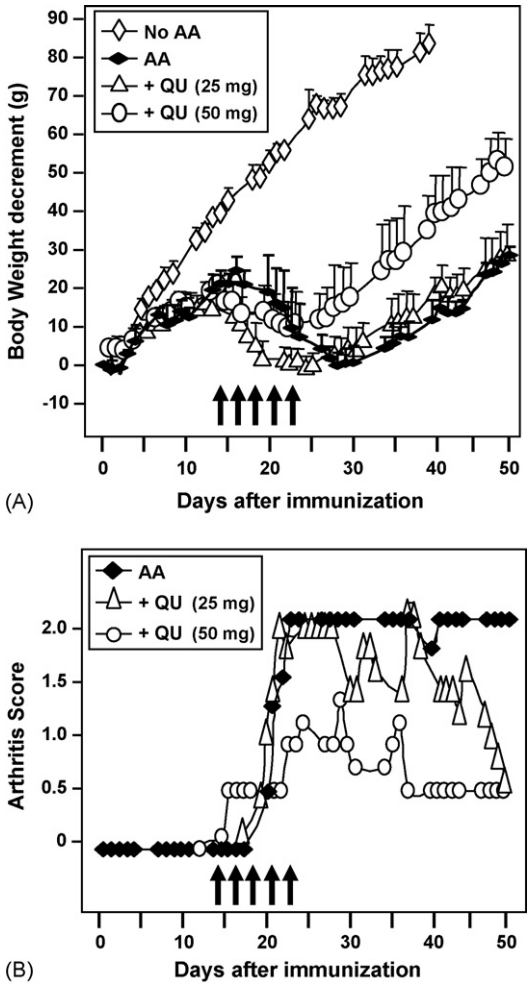

Fig. 2 – Effect of intra-cutaneous QU injections on the severity of AA in rats. The evolution of body weight (A) and arthritis severity scores (B) of AA rats following treatment with five I.C. doses of QU (5 or 10 mg every 2 days 5×) as illustrated by arrows. Results are means ± S.E.M. from five rats.

the treatment of human prostatitis [16]. In later study, patients received 1 g of QU daily during 1 month (250–500 mg/kg total dose). Accordingly, AA Lewis rats were treated with a total of 150 mg/rat of QU (30 mg every 2 days, during 10 days) following the appearance of first AA signs. Results of Fig. 1A indicate that AA severity significantly decreases in animals treated by oral QU, compared to those treated with excipient alone ($p < 0.0004$). Interestingly, no particular behavior, clinical or physiological signs were observed in animals treated with above doses of QU, suggesting that these QU doses are probably not toxic *in vivo*. Furthermore, weight progression in QU-treated rats (Fig. 1B), was very similar to that of healthy, non-AA animals, while untreated AA animals had a significant growth delay.

3.2. *Therapeutic effect of intra-cutaneous quercetin*

It is well known that oral absorption of QU and its intestinal uptake lead to major transformation and chemical changes of this molecule, limiting its bioavailability [14,20]. We thus tested I.C. administration of lower QU doses on AA scores *in vivo*. Arthritic rats were then treated with total doses of 25 or 50 mg/rat of QU (5 or 10 mg every 2 days) following the appearance of first arthritic symptoms. As shown in Fig. 2, I.C. treatment of AA rats with QU resulted in a decrease in the severity of cachexia in animals received 50 mg QU but not in those having 25 mg QU (Fig. 2A). In addition, arthritis scores were reduced in a more significant manner in rats treated with 50 mg, compared to those treated with 25 mg QU (Fig. 2B). Of particular interest, while untreated rats remained affected at day 50 post-immunization, most QU-treated rats have no arthritic signs at that time (Fig. 2B). These data further support the anti-arthritic potential of QU and point to the various QU administration possibilities *in vivo*.

3.3. *Reduced AA clinical signs following preventive injection of quercetin*

Above data on therapeutic effects of QU led us to assay the ability of QU to prevent AA development in rats. QU was thus injected to rats simultaneously with their immunization and

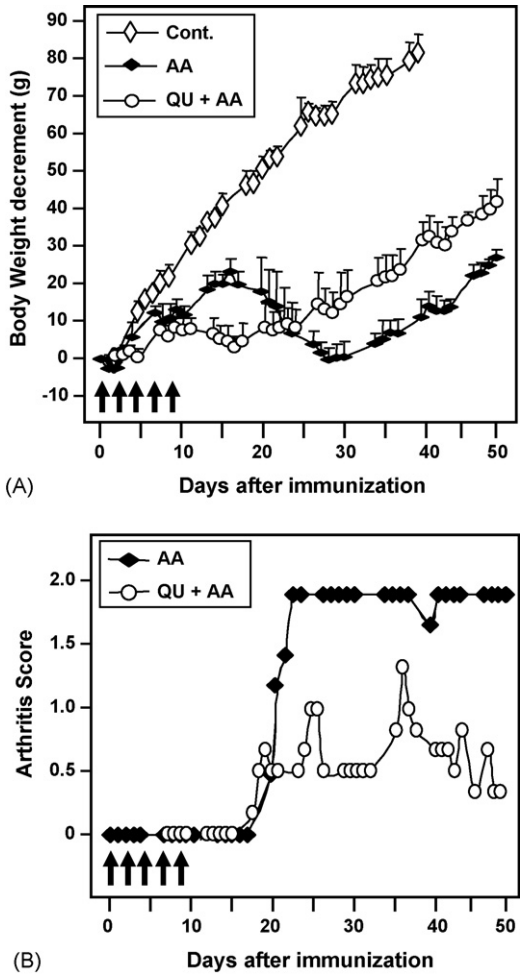

Fig. 3 – Preventive property of intra-cutaneous QU injections on clinical signs of AA in rats. Rats were treated with five I.C. doses of QU (5 mg every 2 days 5×) as illustrated by arrows, simultaneously with their immunization and prior to the appearance of AA. The evolution of body weight (A) and arthritis severity scores (B) were shown as means ± S.E.M. from four rats.

prior to the appearance of any clinical signs. As shown in Fig. 3, I.C. treatment of rats with doses as low as 25 mg/rat of QU (5 mg every 2 days, <180 mg/kg total doses) decreased the intensity of weight loss in AA rats (Fig. 3A). In addition, significant reduction of clinical scores was observed in treated AA rats (Fig. 3B), as only 2/5 rats have developed severe arthritis on both back paws during 2 days, while untreated rats remained affected in both back paws for >30 days. Together, these data reveal the preventive *in vivo* role of QU in AA experimental model.

Finally, analysis of cumulated arthritic scores from all rats (Fig. 4) clearly indicate that high oral doses were the most efficient in reducing arthritic signs, followed by lower I.C. therapeutic or preventive QU doses.

3.4. *Inhibition of macrophage-derived inflammatory mediators by QU*

To explain *in vivo* mechanism(s) of anti-arthritic effects of QU, we tested macrophage inflammatory markers following I.C.

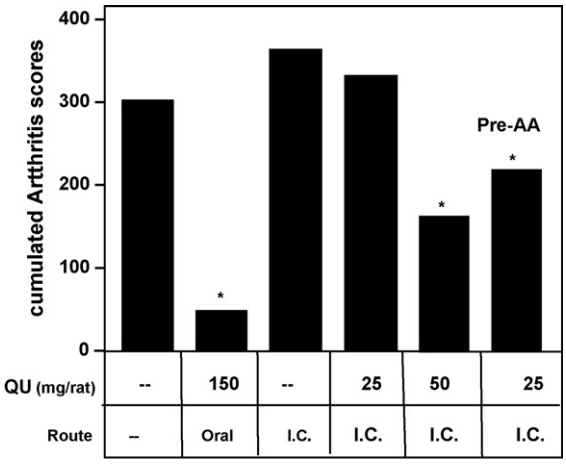

Fig. 4 – Cumulative AA scores in rats treated by oral or intra-cutaneous (I.C.) QU in arthritic rats or prior to AA development (pre-AA). Each column represent total daily AA severity scores over 50 days observation of four rats from each group. $^{*}p < 0.009$ compared to untreated rats.

QU treatment of AA rats. As shown in Fig. 5, freshly isolated peritoneal macrophages from AA rats spontaneously produced more TNF-α and nitrites, the NO metabolites, compared to controls ($p < 0.012$) (Fig. 5). The levels of these mediators were decreased following both preventive (pre-AA) and therapeutic (AA) QU injections ($p < 0.022$). These *ex vivo* data suggest a role for macrophage inflammatory response as a possible target for QU *in vivo*. To further enforce these data, we assess the effects of QU on *in vitro* inflammatory responses of

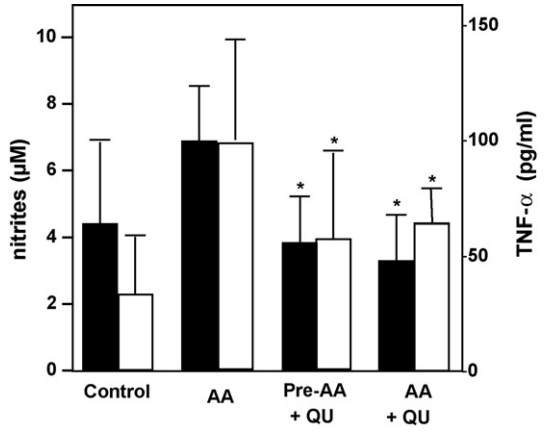

Fig. 5 – Effects of arthritic rat treatment with QU on *ex vivo* generation of nitrites (black bars) and TNF-α (clear bars) from their freshly isolated peritoneal macrophages. Rats were treated with I.C. injections of QU following AA development (10 mg every 2 days 5×) or prior to AA (pre-AA, 5 mg every 2 days 5×). Peritoneal macrophages were collected on day 40 after first adjuvant injection and incubated in medium at 2×10^5 ml^{-1}. Cell supernatants were harvested 72 h later and tested for their nitrites and TNF-α levels. Bars show means ± S.D. ($n = 3$), $^{*}p < 0.023$ compared to untreated AA rats.

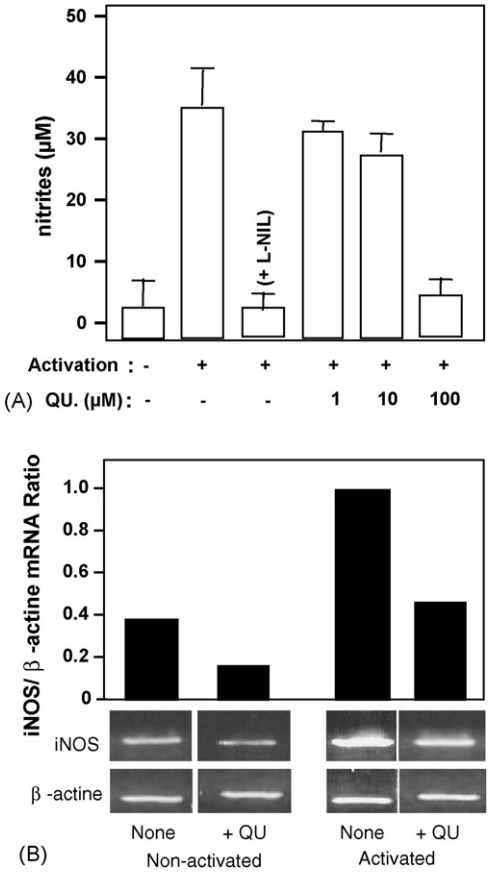

Fig. 6 – Quercetin effects on iNOS pathway in murine peritoneal macrophages. Peritoneal macrophages were incubated in medium alone or activated by LPS. QU dilutions were added simultaneously to cell activation. (A) QU, as specific iNOS inhibitor (L-NIL, 1 mM), decreased NO levels in activated murine macrophages, as measured by the levels of nitrites in 72 h cell supernatants. Bars show means ± S.E.M. (n = 5). (B) QU decreased the levels of iNOS mRNA in murine macrophages as shown by PCR analysis, compared to β-actine.

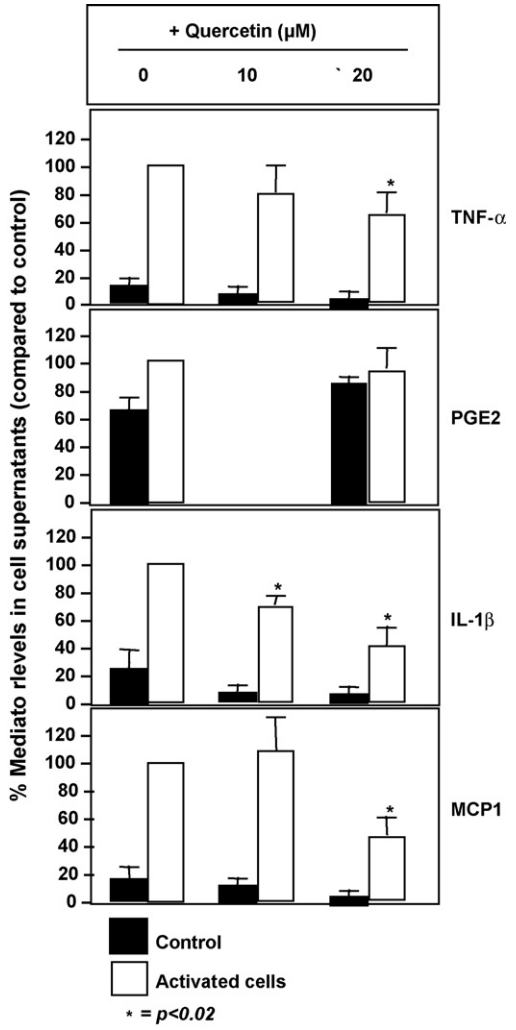

Fig. 7 – Quercetin effects on inflammatory mediator secretion by murine peritoneal macrophages. Freshly isolated peritoneal macrophages were incubated in medium alone or activated by LPS. QU dilutions were added simultaneously to cell activation. Cell supernatants were harvested 72 h post-incubation and tested for their concentrations of TNF-α, PGE-2, IL-1β and MCP-1 using appropriate ELISA kit. Bars show mean ± S.D. (n = 3 rats), $^{*}p < 0.02$ compared to control cells.

freshly isolated murine peritoneal macrophages. Addition of QU during macrophage activation by LPS resulted in a significant inhibition of the levels of nitrites in cell supernatants ($p < 0.006$) (Fig. 6A). This corroborates significant decrease of iNOS-mRNA expression in murine macrophages following QU addition (Fig. 6B). Finally, we tested the effect of QU addition to other macrophage-derived inflammatory mediators, known to be involved during inflammatory arthritis. As shown in Fig. 7, QU decreases the levels of TNF-α ($p < 0.02$), IL-1β ($p < 0.003$) and MCP1 ($p < 0.014$) produced by activation macrophages. In contrast, the production of PGE2, an indicator of COX-2 activity, was not modified following the addition of similar QU dilutions. Above QU doses did not induced direct macrophage toxicity (<12% cell death post-incubation), as quantified by annexin-V/propidium iodide labeling. Thus, QU is a potent inhibitor of most pro-inflammatory factors of macrophages, known to be involved in degenerative arthritis [4].

4. Discussion

The most common flavonol in the human diet is quercetin, present in many different glycosidic forms [10], with quercetin-3-rutinoside, also called quercetin-3-rhamnoglucoside or rutin, being one of the most widespread forms. QU have a wide range of biological activities and a potential therapeutic interest in human. The present study evaluated anti-arthritic potential of QU in both *in vivo* and *ex vivo* rat models, which is very close to its human counterpart. QU significantly reversed growth delay and prevented severe AA development in rats (resumed in Fig. 4). These data corroborates early observations in human and experimental models [9–18,26], and further show that quercetin displays

preventive *in vivo* effects in its purified form. This property correlated with QU-mediated inhibition of murine macrophage activation and inflammatory mediator release *ex vivo* and *in vitro* (Figs. 5–7).

Macrophages, once activated *in vivo* by auto antibody or by antigen-specific T-cell derived lymphokines, are the major source of TNF-α, IL-1, MCP-1 and iNOS-mediated NO generation during immune response [8,24]. Although essential for the elimination of invasive antigens, chronic expression of NO resulted in a variety of inflammatory disorders including rheumatoid arthritis and many other autoimmune diseases [25]. *In vitro*, we have recently showed that QU inhibits the production of TNF-α and NO from activated human macrophages [9]. *In vivo*, present work confirms anti-inflammatory potential of QU as it clearly shows that anti-arthritic property of this molecule corroborated lower NO and TNF-α production capacity of macrophages *ex vivo*. Oral administration of QU reduced clinical scores in AA rats (Fig. 5) at concentrations close to those being used for the treatment of prostatitis/chronic pelvic pain in man [16,17]. Similar to pharmaco-toxicology reports in QU-treated men [16,26,27], we did not observe any apparent toxicity in QU-treated rats. However, due to QU glucoronation during intestinal passage [20], *in vivo* bioavailability of biologically active QU following oral treatment has been shown to decrease (20–50%). We therefore analyzed the effect of intra-cutaneous injection of lower QU doses on AA rats and our data point to the therapeutic effects of QU, obtained with I.C. doses, lower than those used for oral route. Of particular interest, injections of relatively low doses of QU significantly prevented severe AA signs in rats (Fig. 3). Finally, *ex vivo* analysis of freshly isolated rat macrophages pointed to QU-mediated lowering of their activation markers.

The exact mechanism of anti-inflammatory effects of QU in arthritis remains to be clarified. In addition to *ex vivo* data, we clearly show that QU decreases the production of TNF-α, NO, IL-1β and MCP-1, major inflammatory and pro-arthritic mediators of macrophages. We also confirm QU ability to inhibit iNOS transcription in these cells. By contrast to most early data [12], we did not observe PGE-2 decrease following QU addition. This suggests that our therapeutic formulations does not significantly modulate COX-2 pathway. Above QU anti-inflammatory properties corroborate early data indicating that QU decreases the phosphorylation and the activation of Jun N-terminal kinase/stress-activated protein kinase, leading to the suppression of AP-1 activation [28]. QU also inhibits the activation of NF-κB in both human and experimental models [18,29]. Together, decreasing AP-1 and NF-κB levels may explain the anti-inflammatory activities of QU, as both factors are necessary for high generation of most inflammatory mediators analyzed in the present study [30]. While other flavonoids may share some of anti-inflammatory properties of QU, this factor displays particular trypanolytic activities (9) that make it ideal therapeutic tool for African trypanosomiasis: controlling both parasite growth and infection-mediated inflammatory disease. Finally, the absence of apparent toxicity, the preventive effect, and the availability of therapeutic glycosylated forms of this molecule further enforce its interest, compared to most current immunosuppressor agents.

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