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Quercetin reduced inflammation and increased antioxidant defense in rat adjuvant arthritis

C. Gardi[1,2*§], K. Bauerova[3*], B. Stringa[2], V. Kuncirova[3], L. Slovak[3], S. Ponist[3], F. Drafi[3], L. Bezakova[3], I. Tedesco[1], A. Acquaviva[2], S. Bilotto[1], G. L. Russo[1§]

[1]Institute of Food Sciences, National Research Council, 83100, Avellino, Italy; [2]Department of Molecular and Developmental Medicine, University of Siena, via A. Moro, I-53100, Siena, Italy; [3]Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Dubravska cesta 9, SK-841 04, Bratislava, Slovak Republic.

* Equal contribution

[§]Corresponding author:
Concetta Gardi
Department of Molecular and Developmental Medicine
University of Siena,
via A. Moro 2, I-53100, Siena, Italy
E-mail: concetta.gardi@unisi.it;
Tel: +39 0577234002

Gian Luigi Russo
Istituto Scienze dell'Alimentazione
Consiglio Nazionale delle Ricerche
via Roma 64
83100, Avellino, Italy
E-mail: glrusso@isa.cnr.it;
Fax: +39 0825 781585; Tel: +39 0825 299331

Running Title: Quercetin in adjuvant arthritis

Key words:

Quercetin; adjuvant arthritis; NF-κB; ERK pathway; inflammation; oxidative stress.

Highlights:

- Administration of quercetin is investigated in a rat model of adjuvant arthritis

- Quercetin ameliorates several markers of inflammation and oxidative stress

- Quercetin inhibits inflammation mainly by suppressing NF-κB and ERK pathways

Abstract

Novel therapies for rheumatoid arthritis also include the use of naturally occurring compounds possessing antioxidant properties. In the present work, the effects of oral administration of quercetin were investigated in a rat model of adjuvant arthritis. Arthritis was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* in incomplete Freund's adjuvant. The experimental groups were treated with an oral daily dose of 150 mg/kg b.w. of quercetin for 28 days. Results indicated that quercetin was able to ameliorate all markers of inflammation and oxidative stress measured. Quercetin lowered levels of interleukin-1β, C-reactive protein, and monocyte chemotactic protein-1 and restored plasma antioxidant capacity. In addition, quercetin inhibited the enzymatic activity of pro-inflammatory 12/15-lipoxygenase in lung and liver and increased the expression of heme oxygenase-1 in joint and lung of arthritic rats. Finally, quercetin inhibited the 2-fold increase of NF-κB activity observed in lung, liver and joint after induction of arthritis.

1. Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune disease affecting approximately 1% of the whole world population. Patients with RA have a reduced life quality (joints and bones degeneration, muscle weakness, persistent pain) and require long-life therapy. A common effect of long-term therapy is the development of resistance to treatment and also an increased occurrence of adverse effects. Due to these reasons, a continuous need for new agents in the therapy of RA is envisaged. Primary and dominant processes in the etiopathogenesis of RA are immunological mechanisms, closely related to redox imbalance in the organism, which may potentiate chronic inflammatory processes [1]. Our studies [2-4] are in agreement with findings of other authors who referred to the important role of oxidative stress in the pathogenesis of RA [5-7].

In the last decade, the potential involvement of flavonoids with antioxidant properties in RA has been evaluated [8-10]. In this context, the limited side effects of quercetin (QUE) and its well-known pharmacological activities suggested a potential application as an adjuvant natural drug for the treatment of RA [10]. QUE (3,30,40,5,7-pentahydroxyflavone) is the major dietary flavonol found in fruits, vegetables and beverages, such as tea and red wine [11]. Several epidemiological and experimental studies support the antioxidant, anti-inflammatory, anti-angiogenic, anti-proliferative and pro-apoptotic effects of this molecule [12-14]. In Western populations, the estimated daily intake of total flavonols is in the range of 20–50 mg/day, of which about 15-20 mg correspond to QUE glycosides [15].

The existence of a functional link between the intake of QUE and other flavonoids and RA is supported by circumstantial evidence deriving from pre-clinical studies on primary cells and animal models, as well as clinical studies. Early in the 1997, it was reported that QUE suppressed the increase in the mRNA for interleukin 8 (IL-8) and monocyte chemotactic protein-1 (MCP-1) in cultured human synovial cells stimulated by tumor necrosis factor-alpha (TNF-α) in a dose dependent manner. TNF-α is present in synovial fluid and induces the expression of pro-

inflammatory cytokines in synovial cells of patients with RA. The suppression was dose dependent and probably induced by the inhibition of TNF-α mediated nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation [16]. A decade later, the anti-RA capacity of QUE was confirmed in synoviocytes isolated from rabbit where the molecule inhibited proliferation of 30-40 % at very low micromolar concentrations (< 10 μM). It must be considered that proliferation of synoviocytes in RA contributes to the establishment of the so-called "pannus formation", a lesion accompanied by restriction of joint movement and the generation of pro-inflammatory cytokines [17]. In human rheumatoid synovial fibroblasts activated by interleukin 1 beta (IL-1β), QUE inhibited proliferation and induced apoptosis starting from 20 μM concentration. The mode of action was double: i. inhibition of both the expression of IL-1β-induced mRNA and protein of matrix metalloproteases MMP-1, MMP-3, and COX-2 and PGE2 production; ii. inhibition of extracellular signal-regulated kinases (ERK) signal pathways and NF-κB activation both mediated by IL-1β [18].

The anti-RA effect of QUE was confirmed in several animal models of experimentally induced arthritis. In a rat model of gouty arthritis, QUE treatment (100-400 mg/kg) ameliorated edema by decreasing histological signs of acute inflammation and attenuating several markers of inflammation [19]. QUE was more effective than hesperidin, but less than rutin (all tested at a dose of 80 mg/kg and administered intraperitoneally) in inhibiting acute and chronic inflammation in rats where experimental arthritis was induced following the method of adjuvant-carrageenan-induced inflammation [20]. Considering that rutin differs from QUE for the presence of rutinose in position 3, it is possible to hypothesize that the 2-fold higher efficacy of rutin than QUE in arthrogram scores can be attributed to pharmacokinetic factors [20]. In a subsequent work, the same group, comparing the effects of different flavonoids on different rats and mice models, confirmed that rutin was the only effective against chronic-like arthritis, principally in adjuvant arthritis (AA), but QUE resulted the most active in reducing the paw edema induced by carrageenan [21]. In AA induced in

female Lewis rats by subcutaneous injection of inactivated *Mycobacterium butyricum*, oral administration of QUE (5×160 mg/kg) clearly decreased clinical signs of arthritis. Importantly, the dosage was selected to be comparable to that administered to patients affected by prostatitis who received QUE as dietary supplement 1 g/day [22]. When the molecule was given by intracutaneous injection in AA-induced rats at lower doses (5×60 mg/kg), the anti-arthritic effects were similar, while injection of relatively low doses (5×30 mg/kg) prior to AA induction significantly reduced arthritis signs, suggesting multiple approaches (different doses and modes of administrations) to exploit the clinical potentiality of QUE as an anti-arthritic agent. Finally, analysis of cumulated arthritic scores clearly indicated that high oral doses were most efficient in reducing arthritic signs, followed by lower intracutaneous therapeutic or preventive QUE doses [22].

More recently, the study of the effects of QUE in RA was extended to human subjects with contradictory results compared to pre-clinical studies. In a randomized controlled trial aimed to investigate the efficacy of antioxidant supplementation in RA patients, QUE was administered together with vitamin C (166 mg + 133 mg/capsule, respectively) for 4 weeks in 26 subjects. No changes in the levels of serum pro-inflammatory cytokines and C-reactive protein (CRP) in RA patients after supplementation were observed [23]. In a more recent work, 51 women affected by RA were supplemented with 500 mg/day of QUE for 8 weeks. As in the previous study, measurements of several markers of inflammation, such as plasmatic total antioxidant capacity, malondialdehyde, oxidized low density lipoprotein (Ox-LDL) and high sensitivity CRP did not show any significant difference between QUE and placebo groups [24]. On the opposite, in an *ex vivo* study, where neutrophils were isolated from RA patients versus healthy subjects and stimulated by *in vitro* prepared immune complex before treatment with 4 different flavonols (galangin, kaempferol, QUE, and myricetin), QUE was the most effective in reducing superoxide anion production with an IC_{50} of 1.71 μM [25]. It is worthwhile to note that the applied concentrations were in the physiological range of those measured *in vivo* after supplementation with QUE or other flavonols [15, 26]. Matsuno et al. (2009) performed a study with osteoarthritic patients and RA

patients, in which QUE was administered in form of glucosamine-chondrotin-QUE glucoside combination. The patients were treated for 3 months with oral doses of QUE glucoside (45 mg/day). Significant improvement in pain symptoms, daily activities (walking and climbing up and down stairs) and changes in the synovial fluid properties were observed in osteoarthritic patients. No beneficial effects were observed in RA subjects [27].

Therefore, in our study we re-investigated the effect of QUE orally administered in a dose of 150 mg/kg in AA with the aim to prove its anti-arthritic potential, as well as to study its mechanisms of action. We focused on the key two processes in arthritis: inflammation and oxidative stress. Both processes were evaluated in plasma and in selected tissues as joint, liver and lung homogenates.

2. Materials and methods

2.1 Animals, experimental design and treatments

Male Lewis rats weighing 160-180 g were obtained from the Breeding Farm Dobra Voda (Slovakia). The rats had free access to standard pelleted diet and tap water. The experimental protocol was approved by the Ethics Committee of the Institute of the Experimental Pharmacology and Toxicology, by the Slovak State Veterinary and Food Administration in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and by Slovak legislation. AA was induced by a single intradermal injection of heat-inactivated *Mycobacterium butyricum* in incomplete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). The injection was performed near the tail base. The experiment included healthy animals (CO), healthy animals treated with QUE (CO-Q) in an oral daily dose of 150 mg/kg b.w. (body weight) during 28 days, arthritic animals not treated (AA), arthritic animals treated with QUE (AA-Q) in an oral daily dose of 150 mg/kg b.w. during 28 days. In each group 10 animals were used. After the animals have been sacrificed under deep ketamin/xylasine anesthesia, blood for plasma preparation and tissues for homogenate preparation (joint, lung and liver) were taken at the end of the experiment (day 28). Tissue were immediately frozen and stored at -80°C until analysis. Blood samples were centrifuged at 2400×g for 15 min at 4°C and stored at -80°C until analyses.

2. 2 Change of body weight

Change of body weight (CBW; g) was measured on days 1, 14 and 28. CBW was calculated as the difference of the body mass measured on days 14 and 28 and the body weight measured at the beginning of the experiment (day 1).

2.3 Arthritic score

The arthritic score was measured as the total score of hind paw volume (ml, max. points 8) plus paw diameter of forelimb (mm, max. points 5) plus diameter of scab in the site of *Mycobacterium butyricum* application, measured parallel to the spinal column (mm, max. points 5) for each animal [28].

2.4 Measurement of C-reactive protein (CRP) in plasma

For the determination of rat CRP concentration in plasma (µg/ml), the ELISA kit from Immunology (Consultant Laboratories, Inc.) was used. The reaction of secondary biotin-conjugated anti-rat CRP antibody was evaluated by streptavidin-horseradish peroxidase (HRP). The tetramethyl-benzidine reaction with HRP bound to immune complex was measured at 450 nm (Microplate reader Labsystems Multiskan RC). The results were calculated using the standard calibration curve on internal standards.

2.5 Measurement of monocyte chemotactic protein-1 (MCP-1) in plasma

For determination of MCP-1 concentration in plasma (pg/ml), Instant ELISA kit from eBioscience® was used. Assay procedures were applied as described in the product manual. Rat chemokine present in the samples binds to anti-rat chemokine antibodies adsorbed to the microwells. The reaction of secondary biotin-conjugated anti-rat chemokine antibody was evaluated by streptavidin-HRP. Tetramethyl-benzidine reaction with HRP bound to immune complex was measured at 490 nm in comparison with reference wavelength 620 nm (microplate reader MRX II, Dynex, USA). The results were calculated from standard calibration curve on internal standards.

2.6 Measurement of interleukin 1β (IL-1β) in plasma

For the determination of IL-1β concentration in plasma, the ELISA kit from R&D Systems Quantikine® was used. Assay procedures were used as described in the product manual. Rat cytokine present in the samples binds to anti-rat cytokine antibodies absorbed in the microwells. The reaction of secondary biotin-conjugated anti-rat cytokine antibody is evaluated by HRP. The tetramethylbenzidine reaction with HRP bound to immune complex was measured at 490 nm in comparison with the reference wavelength 620 nm (microplate reader MRX II). The results were calculated using the standard calibration curve on internal standards.

2.7 Plasma antioxidant power

The total antioxidant capacity was measured in the plasma of rats treated as indicated above employing the ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)) radical cation decolorization. Briefly, a mixture containing ABTS solution (10 mM) and hydrogen peroxide (2 mM) was stored in the dark at 4°C overnight before use. The produced ABTS•$^+$ solution was diluted (1:10) to obtain an absorbance of approximately 0.31 at 660 nm. In a cuvette 1000 μL of a buffer pH 3.6, 10 μL of plasma and finally 25 μL of ABTS• were added before measurement of the absorbance (660 nm) after 5 minutes. A blank was run in each assay and determinations were carried out in triplicate. Results were expressed in micromolar equivalent of ascorbic acid, an antioxidant present in the plasma [29].

2.8 Tissue activity of 12/15-lipoxygenase (LOX) in liver and lung

Concentration of proteins in lung and liver homogenates was determined by using the Bradford method [30] and expressed in mg/ml of enzyme preparation (cytosolic fraction from rat lung and

liver tissues). Linoleic acid (99%, Sigma-Aldrich, USA) was used as a substrate prepared in solubilized state in the concentration of 0.2143×10^{-5} - 0.7143×10^{-5} M as previously described [4]. The assay of LOX was monitored as an increase in the absorbance at 234 nm which reflects the formation of the hydroperoxylinoleic acid. For the LOX activity assay, an UV/VIS Spectrometer Perkin-Elmer Lambda 35 (USA) was used. The reaction medium contained a 50 mM Tris-HCl buffer (pH 7.0), 2.5 µL of the enzyme protein and solubilized linoleic acid.

2.9 Immunoblot analysis of heme oxygenase-1 (HO-1)

Protein samples from joint and lung homogenates were resolved on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membrane (Whatman GmbH, Dassel, Germany). Immunoblots were performed using primary antibodies for HO-1 (1:1000; Abcam, Cambridge, UK) and β-actin was used as loading control. Immunodetected proteins were visualized using ECL kit (BioRad, Hercules, CA, USA).

2.10 Preparation of whole cell extract for NF-κB determination

Whole cell extracts from joint, left lung and liver were prepared by using Active Motif nuclear extract kit (Carlsbad, CA, USA) as described by the manufacturer. Protein concentration in whole cell extracts was determined by the Bradford protein assay (BioRad) using bovine serum albumin as standard. NF-κB activation was monitored by TransAM NF-κB p65 Transcription Factor Assay Kit (Active Motif). Absorbance was measured at 450 nm using microplate reader (Perkin Elmer Applied Biosystems). Results were expressed as absorbance per milligram of total protein.

2.11 Western blot analysis of extracellular signal-regulated protein kinase (ERK)

Phosphorylation of ERK was analyzed by Western blotting. Samples were homogenized in lysis buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Triton X-100) containing protease inhibitor cocktail, 1 mM Na_3VO_4, 5 mM β-glycerophosphate, and protein concentration was determined by the method of Bradford [30]. Sixty μg of total protein of each extract was separated by 12% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Whatman). Membranes were incubated overnight at 4°C with a primary antibody anti-phospho-44/42 ERK (1:1000, Cell Signalling, Celbio, Milan, Italy) and then with the secondary antibody HRP-conjugated (BioRad) (1:5000). Immunodetected proteins were visualized using ECL assay kit (BioRad) following the manufacturer's recommended protocol. β-actin was used as the loading control.

2.12 Statistical analysis

Mean and S.E.M. values were calculated for each parameter in each group (8 – 10 animals in each experimental group). Statistically significant differences among treated group, untreated group and control groups were tested using parametric Analysis of Variance (ANOVA). Post hoc tests (Tukey-Kramer (ANOVA)) were applied in situation where differences among groups were significant at level of significance α=0.05. After post hoc testing the following significance designations were specified as follows: extremely significant ($P < 0.001$), highly significant ($P < 0.01$), significant ($P < 0.05$), not significant ($P > 0.05$). Since, for measurement of ABTS, HO-1 and ERK kinase samples from 4-5 animals were used, unpaired Student`s t-test was applied.

3. Results

3.1 Effect of QUE on clinical parameters and on selected inflammatory parameters in plasma

The ability of QUE to ameliorate the clinical parameters following induction of AA in rats was evaluated. As reported in Table 1, a significant decrease in body weight and an increase in arthritic score were observed at both days monitored in AA group. The administration of QUE in healthy and in arthritis animals did not change clinical parameters observed in this study. At day 28, the arthritic score almost doubled in the AA group compared to control and QUE treated groups and this increase persisted after administration of QUE to AA animals. When selected markers of inflammation (such as IL-1β, CRP and MCP-1) were measured, their level was significantly increased as a result of arthritis induction, as expected (Table 2). QUE ameliorated all parameters with significant difference achieved for IL-1β and MCP-1. The QUE treated group did not show any significant change in inflammatory markers compared to healthy control animals. A slight decrease in IL-1β and CRP and a small increase in MCP-1 levels were assessed in plasma following QUE mono-treatment (Table 2).

3.2 Quercetin increased plasma antioxidant power

We measured the antioxidant capacity in plasma of AA rats following QUE treatment using ABTS assay. As reported in Fig. 1, the total antioxidant capacity decreased of about 25-30% in AA group. However, QUE treatment (AA-Q group) almost re-established the basal levels, confirming the capacity of this molecule to counteract the oxidative stress associated to AA induction. Moreover, healthy animals treated with QUE (CO-Q group) showed a higher antioxidant capacity compared to controls (CO) (Fig. 1).

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3.3 Effect of QUE on activity of 12/15-LOX in liver and lung homogenates.

The enzyme 5-LOX catalyzes the conversion of arachidonic acid into the leukotrienes, whose production has been associated to inflammation in arthritis. Suppression of 5-LOX expression ameliorates clinical parameters in RA and AA [31, 32]. A similar role can be attributed to 15-LOX [33]. Therefore, the level of expression of 12/15-LOX were measured in lung and liver of rats treated with QUE. In our previous paper evaluating effect of pinosylvin in AA, we found that inflammatory pathological changes were present also in organs primarily not affected by AA as lung and liver [4]. For this reason, also in this study these two organs were included in experimental evaluation. The effect of QUE on activity of 12/15-LOX was comparable in both homogenates. Activity increased in arthritic animals in comparison with healthy animals. After administration of QUE a significant decrease to the control levels was assessed in the AA group. A minor although significant decrease of 12/15-LOX activity was also observed in healthy rats administered with QUE (CO-QUE group) in comparison with the control group (Fig. 2).

3.4 Effect of QUE on HO-1 expression

HO-1 expression was evaluated as tissue marker of antioxidant defense in joint and lung. The rationale for the selection of these two tissues resides in the observation that this enzyme plays a cytoprotective role not only in the lung [34], but also in the synovial tissue of RA patients, where it regulates joint inflammation [35]. AA rats showed a different trend in HO-1 expression from the two organs analyzed. HO-1 protein level increased in joint (Fig. 3A), but decreased in the lung (Fig. 3B), whereas no significant changes were detected in the liver (data not shown). In healthy animals, QUE did not affect HO-1. A protective effect was observed in both joint and lung from AA animals receiving QUE. In fact, in lung from AA-Q rats QUE restored HO-1 to control levels (Fig. 3B),

while in joint further increased HO-1 expression (about 1.5 times) compared to AA animals (Fig. 3A).

3.5 Effect of QUE on the levels of NF-κB activation

Since NF-κB activation plays a central role in inflammatory and oxidative processes and its role in arthritis has been largely investigated [36, 37], levels of p65 DNA binding were measured in joint, lung and liver. A marked increase of NF-κB activation was observed in tissues from AA animals (ranging from 1.87 to 2.21 fold increase). In AA-Q rats QUE strongly inhibited NF-κB activation in joint (-55.2%), lung (-57.8%) and liver (-55.8%) while QUE did not affect this parameter in healthy animals (CO-Q) (Fig. 4).

3.6 QUE reduced ERK activation in AA animals

To better investigate the protective effect of QUE in AA, we measured the level of activation of ERK pathway. In fact, it has been reported that ERK activation is enhanced in patients meeting RA criteria compared to other diagnoses [38]. In addition, ERK inhibitors have been suggested as a treatment for RA patients, including those who are nonresponsive to the anti-cytokine therapies [39]. As reported in Fig. 5, in AA rats a significant increase in p44/42 ERK was observed in joint and lung (panels A and B), while not significant changes were detected in liver (data not shown). QUE treatment strongly decreased ERK phosphorylation in AA animals, while in healthy animals QUE did not show any significant effect in both joint and lung. These results indicate that the activation of ERK may be involved, at least in part, in the QUE-mediated protective effects observed in AA-animals.

4. Discussion

The present manuscript highlights the capacity of QUE to improve the anti-inflammatory status and antioxidant defenses in AA rats. At systemic level, QUE increases the plasma antioxidant capacity (Fig. 1) at a dosage (150 mg/kg) corresponding to the approximate daily intake of QUE glycosides (15-20 mg/die) within a regular diet in the human population [15]. Considering the complex series of biotransformation reactions which QUE undergoes following its uptake and metabolism at intestinal and hepatic levels [14, 15], it is unlikely that the effects reported in Fig. 1 are attributable to QUE aglycone; probably, a mixture of QUE metabolites differently conjugated (e.g., addition of methyl, sulfate groups and glucuronic acid) are responsible for the results of ABTS assay shown in Fig. 1. Although we are aware that the use of this method is controversial, it has been previously applied to demonstrate the plasma antioxidant capacity of QUE in rats [40]. In addition, data exist in the literature on the possibility that QUE can maintain its antioxidant properties *in vivo*, in different cellular models, evidencing that the *in vivo* scavenging activity of QUE generates different oxidation products [41-43]. Based on this observation, we expected a rationale improvement of the antioxidant defenses and anti-inflammatory status in those tissues more directly involved in RA pathogenesis, such as joint, lung and liver. Data reported in the present work go in this direction and the beneficial effects of QUE resemble the pleiotropic and multifunctional nature of the molecules discussed elsewhere [14, 15, 44]. In fact, we hypothesized that the protection offered by QUE against AA in the different assays here reported (Fig. 2-5) can be explained evoking the ability of the molecule to trigger different molecular targets.

The ability of QUE to potentiate an anti-inflammatory response in AA was supported by the ability of the molecule to inhibit 12/15-LOX activity (Fig. 2). Our results are confirmed by recent observations demonstrating that several flavonoids may act as LOX inhibitors by inhibiting the formation of LTB4. In the case of QUE, the molecule showed an IC_{50} of 4.0 ± 1.2 μM on the production of LTB4 by human neutrophils [45]. In addition, *in vitro* studies demonstrated the

ability of QUE to directly bind and inhibit soybean LOX-1 with a Lineweaver-Burk double reciprocal plot attesting an uncompetitive inhibition type [45]. However, it must be mentioned that the positive role of 5-LOX in sustaining RA has been partially questioned by a recent work where it was reported that products of the 5-LOX activity were not required for the development of disease in Lyme model of arthritis [46]. For these reasons, the Authors suggest caution when targeting 5-LOX as therapy for inflammatory diseases.

More complex, but also fascinating is the potential mechanism explaining the increased expression of HO-1 following QUE treatment in AA-rats (Fig. 3). HO-1 is an oxidative stress-responsive protein that shows anti-inflammatory and antioxidant activities [47] not only in the lung [34], but also in the synovial tissue of RA patients [35]. In our model, AA rats showed an increase in the expression of HO-1 in the joint, which can be interpreted as a stimulus of antioxidant defense. Kitamura [48] showed that the levels of this protein are significantly elevated in human synovial fluid and correlated with the plasma levels of CRP, a cytokine of the acute phase of inflammation. This is in according with our data, which show increased levels of CRP in AA rats. These studies could support the idea that the local concentration of HO-1 in synovial fluid may reflect the severity of inflammation in the joint [48]. In addition, as suggested in the literature [49], QUE and other polyphenols can exert a protective effect against inflammation and oxidative damage upregulating HO-1. Conversely, levels of HO-1 decreased in the lung of AA rats, suggesting that in this organ oxidative stress overwhelmed the antioxidant defense. The reason for the different response in joint and lung is not clear, but it may be related to the cell type-specific effects of HO-1 [50]. Anyway, QUE is able to restore the expression of lung HO-1 to control levels. A similar protective effect of QUE on HO-1 was also observed in rats in a model of lung injury by paraquat [51]. These results are of particular importance since pharmacological up-regulation of HO-1 has been demonstrated to provide a protective response in inflammatory diseases [50], including arthritis [52].

Regarding the possible mechanism of action of QUE, various hypotheses can be put forward. It is known that HO-1 is one of the antioxidant enzymes whose expression is under the control of Nrf2-

Keap1 system [53, 54]. Recent data suggest that one possible mechanism explaining the antioxidant effects of phytochemicals via Nrf2 activation may derive from their electrophilic nature [55]; in fact, dietary phytochemicals can be oxidized to electrophilic hydroquinones and quinones which, in turns, can react with and oxidize specific cysteine residues in Keap1, allowing the transcription factor Nrf2 to leave its docking and inactive position into the cytoplasm and translocate to the nucleus. Examples of this mechanism are already present in the literature [55, 56] and can be extended to QUE also [57, 58]. In other words, we are hypothesizing that the increased oxidative stress in AA rats triggers a partial oxidation of QUE generating its electrophilic forms which are able to interact and oxidize specific (and still unknown) cysteine residues among the 27 present on Keap1 causing its inactivation and, consequentially, allowing Nrf2 activation. Studies are in progress to verify experimentally this hypothesis.

The possibility that QUE can regulate NF-κB activity and inhibit inflammatory cytokine expression reducing inflammatory reactions in different systems has been largely explored in the literature [59-61]. Involvement of NF-κB in RA has been demonstrated in several papers [4, 62, 63] and its inhibition by QUE has also been associated to protection against RA [10]. We confirmed this effect measuring a reduction of NF-κB activation in all tissues examined in AA rats following QUE treatment. Similar findings have been observed *in vivo* in other animal models of inflammation [64, 65] and in agreement with previous data obtained *in vitro* [66, 67]. Although the molecular mechanisms involved in the suppressive effects of flavonoids on NF-κB are still not clear, several hypotheses can be suggested. One of the proposed mechanisms is the direct inhibition of the intracellular signaling pathways leading to the activation of NF-κB. QUE can significantly reduce phosphorylation and degradation of IκBα (inhibitor of κBα) and nuclear level of NF-κB [58, 64]. Based on these observations, one might assume that QUE inhibits inflammatory responses, including the production of inflammatory cytokines and activity of LOX, mainly through the suppression of NF-κB activation.

Together with NF-κB, also ERK are central regulators in inflammatory processes, including the development of RA [68, 69]. Activation of NF-κB and MAPK induces the production of pro-inflammatory cytokines and MMPs in RA [18, 62]. In a murine model of collagen-induced RA, the selective MEK inhibitor, PD184352, inhibited paw edema and clinical arthritis scores in a dose-dependent manner [70]. In agreement with this observation, a different ERK inhibitor, FR180204, has been shown to be effective against RA enforcing the search for potent ERK inhibitors in the therapy of RA [39]. In this view, we observed an activation of ERK 44/42 in AA rats which was reduced to basal level following QUE treatment. The easiest explanation of this result is the well-known capacity of QUE to act as a direct inhibitor of MEK, with a K_i of about 1-2 μM [44]. Structural studies indicate that QUE directly binds with MEK-1 *ex vivo* and *in vitro* in a pocket separate from but adjacent to the ATP-binding site of MEK-1. It is interesting that QUE exerted stronger inhibitory effects than PD098059, a well-known pharmacologic inhibitor of MEK-1 [44, 71]. The capacity of QUE to behave as a not-specific kinase inhibitor (reviewed in [15, 44]) can be also evoked to explain the inactivation of NF-κB presented in Fig. 4. In fact, it has been shown that QUE inhibits both IKKα and IKKβ with apparent IC_{50} values of 11 and 4 μM, respectively, reducing the Vmax and increased the Km, indicating a mixed-type of inhibitory mechanism [72]. On the other hand, it is also known that QUE may attenuate *in vitro* TNF-α-stimulated inflammatory mediator production by suppressing the activation of the ERK-mediated NF-κB pathway that is mediated by cellular peroxides [73].

Data discussed above suggest that the effect of QUE on the production of pro-inflammatory cytokines in AA rats could occur through the blocking of the ERK signaling pathway and regulation of NF-kB activation, indicating a protective role of QUE against inflammation generated by oxidative stress in AA. However, our results do not support an effect of this molecule in decreasing the arthritic score becoming clinically relevant nor in preventing weight loss in AA rats (Table 1). The pathophysiology of altered body weight in RA is complex and probably multifactorial. It has been reported that high levels of proinflammatory cytokines, particularly TNF-α and IL-1β, lead to

an increased hepatic protein synthesis [74]and to an increase in muscle proteolysis via the ubiquitin-proteasome pathway, attributed in part to NF-κB activation [75, 76]. Nevertheless, Roubenoff et al. [77] correlated the weight loss with TNF-α production by spleen mononuclear cells (r = 0.68, P < 0.007), while a weaker correlation was seen with IL-1 production (r = 0.45, P < 0.04). In our experiment we have similarly shown that although the IL-1β plasmatic levels were lowered in a significant way after 28-day of QUE treatment, a correction of body weight loss was not observed. Apart from inflammation, observed weight loss is the result of a complex network of interconnected factors, like animal mobility and food intake and may participate in vicious circle that results in poor disease outcomes. In adjuvant arthritis generated hind paw swelling caused pain and limited mobility of animals, which are unable to feed ad libitum. Moreover together with hind paw joints also the temporomandibular joints are swollen and damaged.

The lacking effect of QUE on clinical parameters raised conflictual data in the literature with one study suggesting that oral administration of QUE (5×160 mg/kg) clearly decreases clinical signs of arthritis in AA Lewis rats [22], while a different report indicated that QUE (200 mg/kg *per os*) did not prevent arthritis [78]. Our results support the latter conclusion, with important and novel information. We hypothesize that the described protective effects of QUE in ameliorating inflammatory and oxidative status of rat tissues are not sufficient to overcome the imaginary threshold that separates pro-inflammatory processes induced by RA from the anti-inflammatory cellular responses triggered by QUE. This may be a consequence of the relatively low dose employed in the present work designed to mimic dietary supplementation of QUE. However, the effect of QUE on healthy rats observed in the present study suggests a potential preventive use of the molecule. We hypothesize that the chronic administration of QUE at a dose superimposable to that applied in the present study "before" the induction of AA may result in a better response to inflammation. In fact, target tissues may react more efficiently to the pro-inflammatory insult since their anti-inflammatory and antioxidant defenses have been already potentiated by QUE. In such scenario, the threshold can be bypassed resulting in absence of disease or a mild form of arthritis.

An alternative possibility is to associate QUE with methotrexate (MTX) in combination therapy to follow disease progression and inflammation in arthritic rats. MTX in small doses became the most frequently used disease-modifying anti-rheumatic drug in the therapy of RA. In AA rats, the combined administration of MTX with natural compounds (e.g., N-feruloylserotonin, pynosilvin, carnosine) resulted in a potentiation of the therapeutic effect of MTX at low dose with a significant improvement of all inflammatory markers measured [4, 28, 79]. Both possibilities will be investigated in the near future.

5. Conclusion

This study demonstrated that QUE, orally administrated in a rat model of AA, ameliorated all markers of inflammation and oxidative stress measured. The molecule achieved this goal modulating key processes involved in cellular antioxidant defenses, including down-regulation of NF-κB pathway and inhibiting ERK phosphorylation. The ability of QUE to trigger multiple cellular pathways is in agreement with its functional pleiotropy and results in an improvement of inflammatory response and reduction of oxidative stress in arthritis.

Conflict of interest

The authors of this paper declare no conflict of interest.

Acknowledgments

Figure legends

Fig. 1 - Effect of quercetin on plasma antioxidant power. The total antioxidant capacity was measured in the plasma of rats treated as described in Methods section employing the ABTS assay. Data are means ± SEM. *$P < 0.01$ and **$P < 0.001$ *vs* CO; $^{+}P < 0.01$ and $^{++}P < 0.001$ *vs* AA. CO, control group; AA, adjuvant arthritis group; CO-Q, quercetin treated control group; and AA-Q, quercetin treated adjuvant arthritis group.

Fig. 2 - Effect of quercetin on 12/15-LOX activity. Activity of LOX was assessed lung and liver. Data are means ± SEM. ***$P < 0.001$*vs* CO, +++$P < 0.001$ *vs* AA. CO, control group; AA, adjuvant arthritis group; CO-Q, quercetin treated control group; and AA-Q, quercetin treated adjuvant arthritis group.

Fig. 3 - Effect of quercetin on HO-1 expression in rat joint (A) and lung (B). Levels of protein were measured by immunoblot and densities of the bands were quantified with an imaging densitometer. Protein quantification was expressed as a ratio to β-actin. Data are means ± SEM. **$P < 0.01$ *vs* CO, ***$P < 0.001$ *vs* CO, ++$P < 0.01$ *vs* AA, +++$P < 0.001$ *vs* AA. CO, control group; AA, adjuvant arthritis group; CO-Q, quercetin treated control group; and AA-Q, quercetin treated adjuvant arthritis group.

Fig. 4 - Effect of quercetin on NF-κB activation. Levels of NF-κB were assessed by measuring p65 DNA binding in joint, lung and liver. Data are reported as percentage of control values. CO joint 1.27 ± 0.02, CO lung 0.46 ± 0.04, CO liver 0.5 ± 0.06 Abs/mg of total protein. Data are means ± SEM. **$P < 0.01$ *vs* CO, ***$P < 0.001$ *vs* CO, ++$P < 0.01$ *vs* AA, +++$P < 0.001$ *vs* AA. CO, control group; AA, adjuvant arthritis group; CO-Q, quercetin treated control group; and AA-Q, quercetin treated adjuvant arthritis group.

Fig. 5 - Effect of quercetin on ERK activation. Levels of p44/42 MAPK were measured by immunoblot in rat joint (A) and lung (B). Densities of the bands corresponding to p44/42 were quantified with an imaging densitometer. Quantification of proteins was expressed as a ratio to β-actin. Data are means ± SEM. ***$P < 0.001$ *vs* CO, +++$P < 0.001$ *vs* AA. CO, control group; AA, adjuvant arthritis group; CO-Q, quercetin treated control group; and AA-Q, quercetin treated adjuvant arthritis group.

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