EFFECT OF QUERCETIN ON OXIDATIVE/NITROSATIVE STRESS IN THE THORACIC AORTA OF ADJUVANT-INDUCED ARTHRITIS RATS

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Rheumatoid arthritis (RA) is a systemic inflammatory disease and its cardiovascular complications (CVC) are the leading cause of morbidity. An early manifestation of CVC is endothelial dysfunction, which can generate functional and morphological vascular abnormalities. Both oxidative/nitrosative stress and inflammation are involved in causing endothelial dysfunction in patients with RA. Excessive production of reactive oxygen and nitrogen species (RONS) accounts for cartilage and bone destruction as well as for endothelial dysfunction associated with RA. The purpose of this study was to evaluate the oxidative/nitrosative stress in the aortic tissue as well as the antioxidant effects of Quercetin (Que) in a rat model of adjuvant-induced arthritis (AIA). Arthritis was induced in Wistar rats by injection of Freund's adjuvant (FA) into the left hind footpad and AIA was confirmed 7 days later by clinical examination. Five groups of rats were compared: a non-arthritic control group and four AIA groups treated for 21 days, starting the first day after AIA induction with physiological NaCl solution, indomethacin (2 mg/kg/day), Arcoxia (10 mg/kg/day) or Que (20 mg/kg/day), respectively. The clinical severity of arthritis was evaluated using a macroscopic scoring system and by quantifying the change in the paw volume (as an indicator of edema) with a plethysmometer. At the end of the experiment, several variables were measured in the aortic tissue homogenate: levels of free radicals [(malondialdehyde (MDA) and carbonylated proteins (CP)]; reduced glutathione (GSH) activity; nitrite plus nitrate (NOx) production; and inducible nitric oxide synthase (iNOS) protein expression. In the AIA rat aortic tissue homogenate, MDA and CP levels,

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NOx production and iNOS expression were increased, while GSH activities were decreased compared with those of the non-AIA control rats. The levels of MDA, CP, iNOS and NOx were lower in Que-treated AIA-rats than in non-AIA controls. However, Que administration raised GSH activities in the aortic tissue of AIA rats. This study suggests that Que treatment can be beneficial in attenuating the articular inflammation and oxidative/nitrosative stress associated with RA.

Keywords: oxidative/nitrosative stress, adjuvant-induced arthritis, Quercetin, rats.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune systemic inflammatory disease of unknown etiology and pathogenesis, characterized by chronic inflammatory arthropathy with a progressive, deforming and destructive course, and multiple systemic manifestations (1). Various mechanisms are involved in the pathogenesis of RA, and one of them relates to increased production of reactive oxygen and nitrogen species (RONS). The occurrence of RA is associated with macrophage, lymphocyte and neutrophil accumulation in the synovial fluid, accompanied by the production of enzymes involved in free radicals generation. The augmented level of RONS in the synovial cavity increases inflammation and joint damage (1-3). Endothelial dysfunction, which is the first stage of atherosclerosis, is observed in RA patients with high levels of inflammatory activity and is a predictor of increased cardiovascular mortality among them. RONS contributes to the pathogenesis of the endothelial dysfunction caused by systemic inflammation characteristic of RA (4-7).

Pain and inflammation are the earliest symptoms in RA, followed by various degrees of joint destruction. Inflammation is initiated and propagated by the production of cytokines, chemokines and cell adhesion molecules (8). Prostaglandin (PG) synthesis by cyclooxygenase (COX) from the arachidonic acid metabolism is one of the main pathways in the pathogenesis of acute inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) have been widely used for the therapy of inflammatory diseases, but they have many side effects due to their COX inhibition (9). Therefore, the NSAIDs and glucocorticoids available nowadays are not a very good treatment option because of their systemic, gastric and renal toxicities (10).

Adjuvant-induced arthritis (AIA) is characterized by inflammation and aggressive pannus formation, which leads to cartilage and bone degradation. In rats, AIA is a model of inflammatory joint disease associated with RA (11).

Quercetin is an essential flavonoid with antioxidant properties, which exerts lipoxygenase inhibition as well as anti-inflammatory, anti-tumor, immunomodulatory, antiproliferative and vasodilatory effects. It is also considered to be a scavenger of peroxyl radicals and to attenuate the molecular expression of tumor necrosis factor-alpha (TNF-α)-induced adhesion in endothelial cells (12). Recent studies show that Que is a powerful antioxidant that exerts endothelium-independent vasodilatory

effects, protective effects on nitric oxide (NO) and endothelial functions, and anti-atherogenic effects in inflammatory lesions and those triggered by oxidative stress (13).

For years, researchers have been trying to discover new pharmacological and non-pharmacological antioxidants to reduce the effects of oxidative/nitrosative stress on cardiovascular risk that accompanies RA. One of the research directions needing more attention is the testing of natural products with antioxidant potential, which may prove to be useful in the treatment of RA.

In the present study, we evaluated whether Quercetin administration decreased the clinical arthritis severity and attenuated oxidative/nitrosative stress.

MATERIALS AND METHODS

DRUGS AND CHEMICALS

Indomethacin (IND), Arcoxia (ARC) and Freund's adjuvant (FA) used in this experiment were purchased from Sigma Chemical Company Inc., UK. The chemicals were of analytical grade. Quercetin was extracted, dosed and encapsulated at the "PROPLANTA" Applied Vegetal Biotechnologies Center in Cluj-Napoca, Romania.

Indomethacin, Arcoxia and Quercetin were dissolved in physiological NaCl solution (PS) and were administered *via* an intra-gastric tube (0.6 ml/rat) daily for 21 days, starting on the first day after AIA confirmation.

EXPERIMENTAL DESIGN AND SAMPLING

The study involved 35 albino Wistar adult male rats (weight 250±27 g at 12 weeks old).

All animals were provided by the Biobase of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania, and were cared for in the Biobase of the Physiology Department of the same University. They were isolated for 10 days prior to their introduction in the study for acclimatization. The animals received a standard diet and their access to water was not restricted. The experimental protocol, approved by the Ethics Committee of the University of Medicine and Pharmacy Cluj-Napoca, agrees to the Guide for the care and use of laboratory animals NIH publication No. 85-23, revised 1996.

Adjuvant-induced arthritis in rats

Adjuvant-induced arthritis (AIA) was induced in rats as a typical inflammatory arthritic pain model (14, 15). The 28 Wistar rats used in the present investigations underwent chronic, unilateral inflammation: the remaining seven

served as untreated (non-inflammated) control animals. Arthritis was induced by injecting 0.1 ml of Freund's complete adjuvant (FA) into the plantar region of the left hind paw. AIA was confirmed 7 days after the injection by clinical examination.

Experimental design

The 35 Wistar rats divided into five groups (n=7) were used to investigate the effects of Quercetin on clinical arthritis severity and oxidative/nitrosative stress in the thoracic aorta. Two control groups – Group I (AR-) (healthy, non-arthritic control rats) and Group II (AR+, arthritic control rats) – were given PS daily (0.6 ml/rat/day) for 21 consecutive days *via* an intragastric tube (0.6 ml/rat/day). The three arthritic groups (Groups III, IV and V) were administered IND (AR+IND) (2 mg/kg/day), ARC (AR+ARC) (10 mg/kg/day) or Que (AR+Que) (20 mg/kg/day) daily, for the same period, also using an intragastric tube (0.6 ml/rat/day).

Evaluation of the clinical arthritis severity

At the onset of arthritis (day 7) and on days 14, 21 and 28, disease severity was assessed visually by two independent observers who were unaware of the treatment received by rats. A modified macroscopic scoring system was used to monitor the severity of arthritis, as previously mentioned (16, 17). Briefly, the severity of inflammation in each paw was graded on a scale from 0 to 4, where zero=no signs of arthritis, 1=detectable swelling and/or redness of the paw or one digit, two=two joints involved with moderate swelling and redness, three=more than two joints involved, four=severe arthritis of the entire paw and all digits. The total score was the cumulative value in each of the four paws, with a maximum of 12 for each rat. Rats with a total score >2 were considered to have arthritis.

Paw volume measurement

The clinical severity of arthritis was also determined by quantifying the change in the paw volume (as an indicator of edema) with a plethysmometer (model 7140; Ugo Basile, Varese, Italy), a micro controlled device specifically designed to accurately measure the volume of inflammatory edema in rats. It consists of a plastic device for water with different diameters, which serves to dip in the paw of the studied animal. A transducer measures the small differences in water level caused by the edema volume. The digital device of the plethysmometer then calculates the exact volume of the paw. The volume of a hind paw is reported as mean±standard error of the mean in milliliters. All measurements were made at the same time every day. The hind paw volumes were measured in both control and test groups on days 0, 7, 14 and 21 of treatment, symmetrically in the two paws (17, 18).

Once all the measurements and evaluations were performed, 24 h of the last treatment (day 28) rats were anaesthetized with pentobarbitone sodium (ip: 60 mg/kg rat) and sacrificed by cervical decapitation. The thoracic aorta of all animals was quickly dissected, washed in ice-cold saline and dried on filter paper. A portion of each thoracic aorta was taken from all the groups, and homogenate was prepared in 0.3 M phosphate buffer (pH 7.4) for the biochemical analysis.

BIOCHEMICAL ASSAYS AND ANALYSIS

The aortic tissue homogenate levels of oxidative and nitrosative stress parameters were determined.

Levels of oxidative stress in the aortic tissue homogenate were estimated by measuring free radical production: lipid peroxides and protein carbonylation.

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) levels (by fluorescein dosage, Conti method) (19). The results were expressed in [nmol/mg protein].

Protein carbonylation was estimated by measuring carbonylated protein (CP) levels. The CPs as products of the reaction between reactive oxygen species and proteins were determined, using the hydrochloric guanidine method (20). The results were expressed in [nmol/mg protein].

The aortic antioxidant status was estimated by measuring the levels of reduced GSH in the aortic tissue homogenate. The aortic activity of GSH was assayed as described by Hu (21), and expressed as specific activity in [nmol/mg protein].

The nitrosative stress in the aortic tissue homogenate was indirectly determined by measuring the nitrite plus nitrate (NOx) production (the results were expressed in [nmol/mg protein]) and by evaluating the expression of inducible nitric oxide synthase (iNOS) (the results were expressed in [ng/mg protein]). The level of iNOS was measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN), and the level of NOx was measured using the Griess method (22).

STATISTICAL ANALYSIS

Results were expressed as the mean values standard deviation (SD) of means. Statistical significance was tested at a confidence interval of 95% ($P<0.05$). Data were analyzed using the Statistical Package for the Social Sciences version 17.0 (SPSS-17). All experimental parameters measured were analyzed by one-way analysis of variance (ANOVA) followed by the Scheffe multiple comparisons post-test.

RESULTS

Quercetin effects on the clinical signs in the AIA rats

After 7 days, animals began to show evidence of clinical inflammation in one or both left hind paws. The first manifestation of disease was erythema of one or more ankle joints, followed by involvement of the metatarsal and interphalangeal joints. The typical time course for the development and progression of disease, as assessed by the mean arthritis severity score and the paw volume, is shown in Fig. 1 and Table 1. The disease was progressive, with joint recruitment involving tarsal, metatarsophalangeal and then interphalangeal joints. In the non-treated arthritic group (AR+, Group II), the disease incidence was 100% (all animals in the group were affected) in day 7, and remained as such throughout the experiment. In contrast, treatment with indomethacin, Arcoxia or Quercetin exerted a significant attenuation in the incidence of AIA: 60% with indomethacin treatment ($P<0.05$), 60% with Arcoxia treatment ($P<0.05$) and 40% with Quercetin treatment ($P<0.05$).

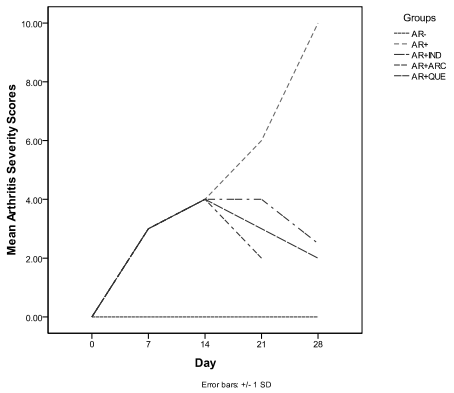


Fig. 1 – Arthritis severity scores in rats during the development of adjuvant induced arthritis. Effect of indomethacin, Arcoxia and Quercetin during the development and progression of arthritis, shown by the arthritis severity scores measured over a period of 28 days. Arthrogram is expressed in points (PTS). Results are the means±SD for seven animals in each group. AR-: non-arthritic control rats; AR+: non-treated control arthritic rats; AR+IND: arthritic rats treated with indomethacin; AR+ARC: arthritic rats treated with Arcoxia; AR+Que: arthritic rats treated with Quercetin. Significant differences ($P< 0.05$) *vs*. AR+

Our results showed a large increase in the affected left hind paw volume of non-treated arthritic rats (AR+, Group II) compared to non-arthritic control rats (AR-, Group I). This increase in the paw volume was statistically significant compared to the non-arthritic rats on day 7 ($P<0.0001$). It was observed that 7 days after the injection with FA on non-treated arthritic rats (AR+, Group II), the paw volume of the affected left hind paw was very significantly increased ($P<0.0001$) compared to the left hind paw of the non-arthritic control rats (AR-, Group I). The arthritic rats treated with indomethacin (AR+IND, Group III) or Arcoxia

(AR+ARC, Group III) had a less significant increase in the paw volume compared to non-treated arthritic rats (AR-, Group II); the paw volume significantly decreased on day 21 ($P<0.001$) and remained as such until the end of the experiment. In the arthritic rats treated with Quercetin (AR+Que, Group IV), the left hind paw volume was significantly decreased on day 21 ($P<0.05$) and very significantly decreased on day 28 ($P<0.001$), compared to non-treated arthritic rats (AR+, Group II) (Table 1).

Table 1

Differences between the affected left hind paw volumes in normal rats versus arthritic rats. The changes in hind paw volume after the induction of arthritis over time. Rats in all arthritic groups showed an increase in the affected hind paw volume until day 7. The arthritic rats treated with vehicle showed a very significant increase ($P<0.0001$) in their hind paw volume over the period of 21 days. The arthritic rats treated with indomethacin, Arcoxia or Quercetin showed a very significant decrease ($P<0.0001$) in the affected hind paw volume over the period of 21 days. Not significant differences between indomethacin, Arcoxia and Quercetin were observed over the period of 28 days. Hind paw volume is expressed in percentage (%). Results are the means±SD for seven animals in each group. AR-: non-arthritic control rats; AR+: non-treated control arthritic rats; AR+IND: arthritic rats treated with indomethacin; AR+ARC: arthritic rats treated with Arcoxia; AR+Que: arthritic rats treated with Quercetin. Significant differences *P< 0.05, **P< 0.001, ***P< 0.0001 *vs* AR-; aP< 0.05, ^{aa}P< 0.001, ^{aaa}P< 0.0001 *vs* AR+

	Day	1st Group (AR-)	2nd Group (AR+)	3rd Group (AR+IND)	4th Group (AR+ARC)	5th Group (AR+Que)
Left hind paw volume	**0**	2.22± 0.15	2.22± 0.15	2.38± 0.2	1.89± 0.11	2.42± 0.1
	7	2.22± 0.15	4.47± 0.57***	4.32± 0.97***	4.31±0.72***	4.43± 0.76***
	14	2.22± 0.15	4.43± 0.76***	3.84± 0.49***^{a}	4.13±0.47***^{a}	4.15±0.7***^{a}
	21	2.22± 0.15	4.66± 0.44***	3.59± 0.31***^{aa}	3.57± 0.64***^{aa}	3.91±0.55***^{a}
	28	2.22± 0.15	5.13± 0.6***	3.39± 0.56***^{aaa}	3.46± 0.35***^{aaa}	3.64±0.53***^{aa}
Right hind paw volume	**0**	2.19± 0.17	2.19± 0.17	2.32±0.17	1.86±0.11	2.39±0.07
	7	2.19± 0.17	2.89± 0.19*	2.58±0.27*^{a}	2.68±0.29*^{a}	2.61±0.33*^{a}
	14	2.19± 0.17	2.61± 0.33*	2.24±0.16^{aa}	2.15±0.34^{aa}	2.85±0.24*^{a}
	21	2.19± 0.17	2.87± 0.26*	2.46±0.35*^{a}	2.09±0.12*^{a}	2.72±0.23*
	28	2.19± 0.17	2.58± 0.26*	2.68±0.4*	2.16±0.1^{aa}	2.28±0.19^{a}

Effect of Quercetin treatment on oxidative stress in thoracic aortic tissue homogenate of AIA rats

Lipid peroxidation. The results summarized in Fig. 2a show the levels of MDA in thoracic aortic tissue homogenate measured on day 28 [nmol/mg protein]. The levels of MDA increased very significantly ($P<0.0001$) after the induction of AIA (AR+, Group II) compared to the non-arthritic rats (AR-, Group I).

Administration of indomethacin (AR+IND, Group III) or Arcoxia (AR+ARC, Group IV) in AIA rats for 21 days significantly reduced (P<0.05) the level of MDA compared with the non-treated arthritic controls (AR+, Group II). The AIA rats treated with Quercetin (AR+Que, Group V) for 21 days exhibited a very significant decline (P<0.0001) in their MDA level when compared to non-treated arthritic controls (AR+, Group II). The results show that Quercetin has more efficiently decreased the MDA levels in aortic tissue homogenate than indomethacin or Arcoxia (Fig. 2a).

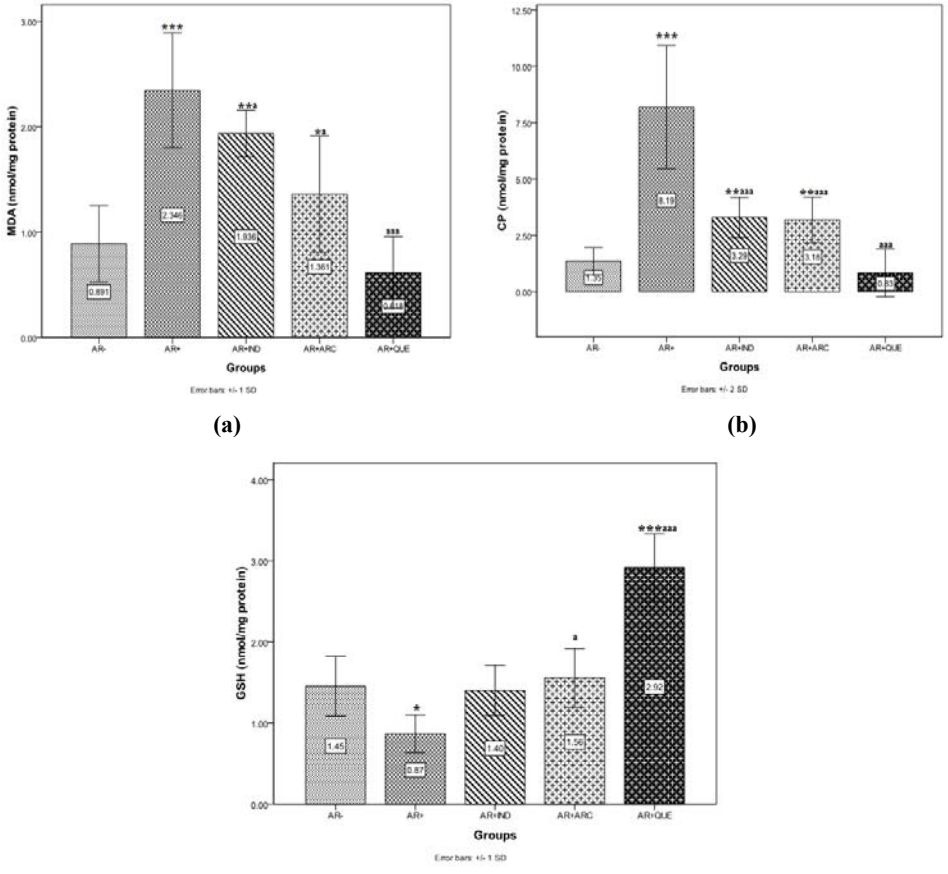


Fig. 2. – The effects of indomethacin, Arcoxia and Quercetin treatment on: (a) lipid peroxidation (MDA) (nmol/mg protein), (b) protein carbonylation (CP) (nmol/mg protein) and (c) reduced glutathione (GSH) (nmol/mg protein) levels in aortic tissue homogenate in control and adjuvant-induced arthritis rats, measured over a period of 28 days. Results are the means±SD for seven animals each group. In AR-: non-arthritic control rats; AR+: non-treated control arthritic rats; AR+IND: arthritic rats treated with indomethacin; AR+ARC: arthritic rats treated with Arcoxia; AR+Que: arthritic rats treated with Quercetin. Significant differences *P< 0.05, **P< 0.001, ***P< 0.0001 *vs*. AR-; ^{a}P< 0.05, ^{aa}P< 0.001, ^{aaa}P< 0.0001 *vs*. AR+

Protein peroxidation. Similar results were obtained for CP determination (Fig. 2b). After AIA induction, the CP levels in the aortic tissue homogenate were very significantly increased ($P<0.0001$) in Group II rats (AR+) as compared to the non-arthritic controls in Group I (AR-). The AIA rats treated with indomethacin (AR+IND, Group III) or Arcoxia (AR+ARC, Group IV) for 21 days exhibited a very significant decline ($P<0.0001$) in the CP levels comparatively to those of the non-treated arthritic controls (AR+, Group II). Treatment with Quercetin of AIA rats (AR+Que, Group V) for 21 days decreased very significantly ($P<0.0001$) the CP levels in aortic tissue homogenate in comparison with non-treated control arthritic rats (AR+, Group II). The results show that Quercetin was more efficient than indomethacin or Arcoxia in reducing the CP levels in aortic tissue homogenate (Fig. 2b).

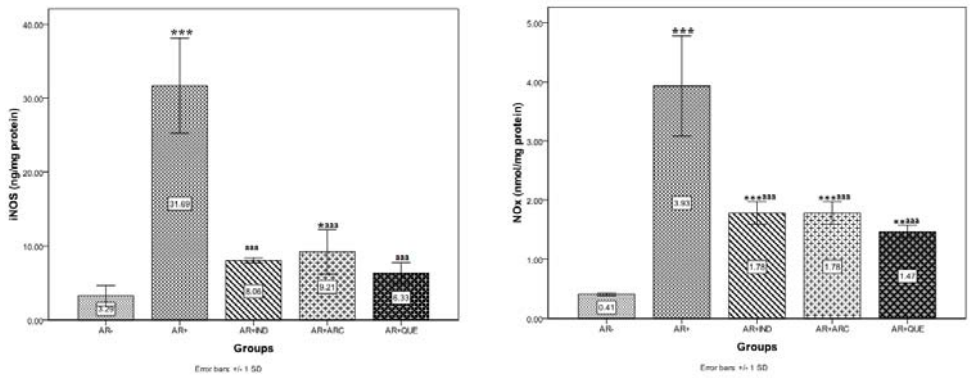
Non-enzymatic activity. The results in Fig. 2c show the levels of reduced glutathione (GSH) in aortic tissue homogenate measured on day 28 in AIA rats [nmol/mg protein]. The aortic GSH activity was significantly lower ($P<0.05$) on day 28 in AIA rats (AR+, Group II) as compared to non-arthritic controls (AR-, Group I). Comparatively to non-treated arthritic controls (AR+, Group II), the levels of GSH were non-significantly ($P>0.05$) and significantly ($P<0.05$) increased respectively, in AIA rats treated with indomethacin (AR+IND, Group III) or Arcoxia (AR+ARC, Group IV) for 21 days, but very significantly increased ($P<0.0001$) in AIA rats treated with Quercetin (AR+Que, Group V) for 21 days (Fig. 2c).

Effect of Quercetin treatment on nitrosative stress in thoracic aortic tissue homogenate of AIA rats

Inducible nitric oxide synthase (iNOS) protein expression. The results in Fig. 3a show the levels of iNOS in aortic tissue homogenate measured on day 28 [ng/mg protein]. The iNOS level in aortic tissue homogenate on day 28 was very significantly higher ($P<0.0001$) in AIA rats (AR+, Group II) compared to the non-arthritic animals (AR-, Group I). Comparatively to non-treated arthritic controls (AR+, Group II), the iNOS levels were significantly decreased ($P<0.0001$) in the AIA rats treated with indomethacin (AR+IND, Group III) or Arcoxia (AR+ARC, Group IV) for 21 days, but presented a very significant decline ($P<0.0001$) in AIA rats treated with Quercetin (AR+Que, Group V) for 21 days (Fig. 3a).

Nitrite plus nitrate production. The results in Fig. 3b show the levels of nitrate plus nitrate (NOx) in aortic tissue homogenate measured on day 28 [nmol/mg protein]. The NOx level in aortic tissue homogenate was significantly higher ($P<0.0001$) on day 28 in AIA rats compared to the non-arthritic animals (AR-, Group I). After treatment with indomethacin (AR+IND, Group III) or Arcoxia (AR+ARC, Group IV) for 21 days, the NOx levels were very significantly lower ($P<0.0001$) than those of the non-treated arthritic controls (AR+, Group II),

but presented a very significant decline (P<0.0001) in AIA rats treated with Quercetin (AR+Que, Group V) for 21 days. The results show that Que improved the effect of IND or ARC on the NOx levels in aortic tissue homogenate (Fig. 3b).



(a) **(b)**

Fig. 3 – The effects of indomethacin, Arcoxia and Quercetin treatment on: (a) inducible nitric oxide synthase (iNOS) protein expression (ng/mg protein) and (b) nitrite plus nitrate (NOx) production (nmol/mg protein) levels in aortic tissue homogenate in control and adjuvant-induced arthritis rats, measured over a period of 28 days. Results are the means ±SD for seven animals each group. In AR-: non-arthritic control rats; AR+: non-treated control arthritic rats; AR+IND: arthritic rats treated with indomethacin; AR+ARC: arthritic rats treated with Arcoxia; AR+Que: arthritic rats treated with Quercetin. Significant differences *P< 0.05, **P< 0.001, ***P< 0.0001 *vs*. AR-; ^{a}P< 0.05, ^{aa}P< 0.001, ^{aaa}P< 0.0001 *vs* AR+

DISCUSSION

This study was performed on an AIA rat model to determine the possible favorable effects that the antioxidative substance tested by us (Quercetin) may exert on the severity of clinically active arthritis and the oxidative/nitrosative status in the aortic tissue of experimental animals. The effects of Que were evaluated in comparison with two NSAIDs: indhometacin and Arcoxia.

Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology (1). Patients suffering from RA have an increased number of inflammatory monocytes circulating in peripheral blood and an elevated number of macrophages in the joints. Inflammatory mediators derived from these cells, particularly interleukin (IL)-1β and TNF-α, have a critical role during synovial inflammation and bone destruction in patients with RA (1, 23-25).

Recent studies indicate that Quercetin, an essential flavonoid, attenuates various inflammatory functions of macrophages in human or animal models. It inhibits inflammatory reactions by regulating the generation of inflammatory cytokines such as IL-6, TNF-α and interferon-gamma and associated activation

protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) signaling pathway in immune cells *in vitro* and *in vivo* (23, 26, 27).

The AIA rat model is robust, the disease incidence rate is 100%, and AIA in rats shares many features with RA in humans – *e.g*., in inflammation, marked bone resorption and periostal bone proliferation (28). AIA in rats has been adopted as an experimental paradigm for the pre-clinical screening of RA treatments. In this model, we measured RA markers. Our data showed increased arthritis scores and edema in AIA rats (Fig. 1 and Table 1) and amelioration in the clinical severity of arthritis due to the oral treatment with NSAIDs (indomethacin or Arcoxia). The analgesic and anti-inflammatory effects of IND and ARC, NSAIDs often used for the treatment of RA, partly produced by COX inhibition, are well known. The anti-inflammatory effect of IND is accompanied by a suppression of the oxidative burst of the stimulated blood phagocytes in animals treated with IND (29). In our study, the oral administration of Que in AIA rats reduced the progression of arthritis by diminishing the arthritis score and edema. We observed a significant reduction in clinical arthritis severity and edema after twenty-one days of Que treatment, since all our AIA rats had a high initial mean clinical arthritis severity. Our results demonstrate that the significant reduction in clinical arthritis severity and edema (Fig. 1 and Table 1) after 21 days of Que treatment could be attributed to a marked decrease in the tender joint count. In accordance with recent studies (6, 7, 10, 18, 29), our results show that clinical arthritis severity correlates better with tender joint count than with other measures of RA disease assessment. These results were interpreted as a consequence of this antioxidant's capacity to inhibit the activation of macrophages and the secretion of proinflammatory mediators from these cells (1, 2, 12).

Proteolytic activity is not the only source of cartilage and bone damage associated with RA, RONS exerting direct effects through oxidation and degradation of cartilage and bone major components, including collagen and hyaluronic acid (26). Numerous recent studies have detected an increased production of RONS in the serum and synovial fluid of patients with RA (30, 31). Excessive production of RONS disrupts the redox balance and amplifies the inflammatory response *via* NF-κB, which is the central regulator of the cellular inflammatory response that controls many genes involved in inflammation, while inflammation affects the vascular endothelium and causes cardiovascular complications that increase mortality in patients with RA. NO is produced by the action of iNOS and, in high concentrations, it acts as a free radical with a toxic impact, exerting pathological effects through rapid interaction with the superoxide anion and the formation of peroxynitrite (ONOO-), which leads to endothelial dysfunction. iNOS is synthesized in a variety of cell physiological and pathological conditions, including monocytes/macrophages, mast cells, microglia, Kupffer cells, neutrophils, fibroblasts, keratinocytes, hepatocytes, astrocytes, smooth muscle cells and vascular endothelial cells (4). Its expression and activity in inflammatory

processes increases explosively. The expression of iNOS gene in the macrophage is controlled by transcription factors, including NF-κB (32, 33).

MDA is a frequently used marker of lipid peroxidation and CP is the marker of protein peroxidation. Clinical studies have shown elevated plasmatic levels of MDA in patients with RA (29, 34). In our previous studies with AIA rats, we observed an increase in oxidative stress that was made evident through the plasmatic levels of MDA and PC, as well as a significant reduction of the total antioxidant status in plasma (17). In the current research, we found an increased concentration of both MDA and CP and a decreased level of GSH in the aortic tissue of AIA rats. Similarly, in our present study we observed that nitrosative stress had a higher level in AIA rat aortic tissue, which has been indirectly proven by the rise of NOx and iNOS levels. These results confirm that AIA induce a growth of oxidative/nitrosative stress in the aortic tissue. Administration of Que was more effective than NSAIDs in reducing oxidative/nitrosative damage in the aortic tissue (Fig. 2 and Fig. 3).

The results of our investigation confirmed the previously reported effect of NSAID treatment in rats with AIA. In our study, IND 2 mg/kg/day and ARC 10 mg/kg/day suppressed, but did not prevent, arthritis development. The therapeutic effect of IND and ARC has been also confirmed by significant edema reduction in the affected joints. Such an effect could also be attributed to the oxidative/nitrosative stress-reducing properties of NSAIDs, as already reported in patients with RA (9, 10, 29, 36). The anti-inflammatory effect of NSAIDs is accompanied by suppression of oxidative/nitrosative stress. Quercetin is known to be a multifunctional agent due to its anti-inflammatory and anticancer activities, as well as a powerful antioxidant through its ability to be a scavenger of RONS and free radicals (27, 35, 37). Recent *in vitro* and *in vivo* studies have demonstrated the ability of Que to inhibit NO production and iNOS expression, iNOS inhibition representing one of the mechanisms by which Que exerts anti-inflammatory effects (38-40). Quercetin is an antioxidant that is efficient through direct scavenging of ROS, including superoxide anion (27), and RNS, including NO (27, 39) and peroxynitrite, and through enhancing endogenous antioxidant activity (27). In our present study, we found that Que suppressed arthritis development and decreased oxidative/nitrosative stress in the aortic tissue when given in a dose of 20 mg/kg/day.

CONCLUSION

Our results indicate that IND, ARC and Que administrations ameliorate the clinical severity of arthritis. What is more, our results show that oxidative/ nitrosative stress plays an important role in AIA pathogenesis and could be controlled through a suitable combination therapy of an antioxidant substance, as demonstrated for Que.

We suggest the possibility of therapeutic co-administration of Quercetin along with conventional drugs (NSAIDs) for the treatment of active RA.

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