Quercetin reduces markers of oxidative stress and inflammation in sarcoidosis

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SUMMARY

Background & aims: Oxidative stress and low antioxidant levels are implicated in the aetiology of sarcoidosis, an inflammatory disease. Quercetin is a potent dietary antioxidant that also displays anti-inflammatory activities. Consequently, the aim is to examine the effect of quercetin supplementation on markers of oxidative stress and inflammation in sarcoidosis.

Methods: A double-blind intervention study has been conducted with two groups of non-smoking, untreated sarcoidosis patients, matched for age and gender. One group was given 4x500 mg quercetin (n = 12) orally within 24 h, the other one placebo (n = 6). Plasma malondialdehyde levels were used as marker of oxidative damage, plasma ratios of TNFα/IL-10 and IL-8/IL-10 as pro-inflammatory markers.

Results: Quercetin supplementation improved the antioxidant defence, indicated by the increased total plasma antioxidant capacity, plasma ratios of TNFα/IL-10 and IL-8/IL-10 as pro-inflammatory markers.

Conclusion: Quercetin supplementation reduced markers of oxidative stress and inflammation in the blood of sarcoidosis patients. The effects of quercetin supplementation appeared to be more pronounced when the levels of the oxidative stress and inflammation markers were higher at baseline.

1. Introduction

Sarcoidosis is a chronic inflammatory disease of which the exact cause still needs to be elucidated. Besides the presence of a chronic inflammatory process, sarcoidosis is also associated with the occurrence of oxidative stress, i.e. an imbalance between the production of and the protection against reactive oxygen species (ROS). This is deduced from increased levels of biomarkers of oxidative damage such as exhaled ethane and both 8-isoprostane and oxidized proteins in the bronchoalveolar lavage fluid (BALF) of sarcoidosis patients. Recently, we have found that the total antioxidant capacity of sarcoidosis patients is approximately 75% of that of matched controls.

To increase the total antioxidant capacity in chronic diseases associated with enhanced oxidative stress, such as sarcoidosis, antioxidant supplementation has gained a lot of interest the past few years. A good candidate for such supplementation could be the dietary antioxidant quercetin. Indeed, it has recently been shown that quercetin supplementation effectively increases both the plasma quercetin concentration and the total plasma antioxidant capacity in healthy volunteers. Moreover, it is known that, within the flavonoid family, quercetin is the most active scavenger of reactive oxygen species (ROS) and reactive nitrogen species (RNS) both in vitro and in vivo. For example, the antioxidant capacity of quercetin is several times that of various endogenous antioxidants including glutathione and vitamin E. This can be explained by the presence of two antioxidant pharmacophores within the molecule that both have the optimal configuration for free radical scavenging.
Interestingly, quercetin displays more characteristics that make it an excellent candidate for antioxidant supplementation in sarcoidosis. Foremost, several studies have indicated that quercetin, both added in vitro and supplemented in vivo, also displays anti-inflammatory effects. Indeed, it is known that this flavonoid is capable of reducing LPS-induced levels of various pro-inflammatory cytokines including TNF-α and IL-8, two cytokines known to be elevated in sarcoidosis. Secondly, de Boer et al have demonstrated that quercetin accumulates in the lungs of rats. This finding suggests that the flavonoid is expected to exert its positive effects especially in this organ, which is also primarily involved in sarcoidosis. Moreover, this specific tissue distribution of quercetin correlates well with the observations of Kumar et al that oral quercetin supplementation offers protection against pulmonary damage induced by influenza virus infection in mice.

The combination of its tissue specific distribution and potent anti-oxidative as well as anti-inflammatory capacities prompted us to study the effect of quercetin supplementation on in vivo markers of oxidative stress and inflammation in sarcoidosis patients. Moreover, to mimic a severe inflammatory burden that might occur by incidental exposure to e.g. dust particles, cigarette smoke or other triggers, an additional in vivo LPS challenge was performed in the blood to study the protecting potential of this quercetin supplementation.

2. Materials and methods

2.1. Materials

Quercetin and lipopolysaccharide (LPS, E. coli 026:B6) were purchased from Sigma Chemical Co. (St. Louis, USA). RPMI 1640 medium containing l-glutamine was obtained from Gibco (UK). Human TNF-α (7300 pg/ml), human IL-10 (4000 pg/ml) and human IL-8 (10 ng/ml) were acquired from CLB/Sanquin (Amsterdam, the Netherlands). All other chemicals were of analytical grade.

3. Methods

3.1. General information

All participating patients were recruited via their own physician. All participants were fully informed, both written and orally, about the aim and details of the study and have given their written informed consent.

The study was carried out at the University Hospital Maastricht after approval of the protocol by the Medical Ethics Committee of Maastricht University and the University Hospital Maastricht and registration of the study at www.clinicaltrials.gov (NCT-00402623). Based on foodstuff questionnaires, it was concluded that all participants had comparable dietary habits with an average daily intake of quercetin of approximately 15 mg. None of the participants took any medication or vitamin or food supplements either prior to or during the study. Randomisation occurred by dividing all participants into trios based on their age and gender and then by randomly giving placebo treatment to one individual out of each trio.

3.2. Participants

Eighteen Caucasian non-smoking patients with symptomatic sarcoidosis (age 45 ± 12, 10 male and 8 female) were enrolled. Sarcoidosis had been diagnosed based on both clinical features and bronchoalveolar lavage (BAL) fluid analysis results (data not shown). Moreover, a biopsy confirmation of the disease had been performed in 11 out of the 18 sarcoidosis patients. The clinical symptoms of all patients included respiratory symptoms, i.e. dyspnea, coughing and chest pain. None of the participants suffered from extra-pulmonary involvement of sarcoidosis during the study. The characteristics of the study population are summarized in Table 1.

3.3. Lung function measurement

Lung function measurements included forced expiratory volume in 1 s (FEV₁), forced vital capacity (FVC) and diffusing capacity of the lung for carbon monoxide (DLCO). FeV₁ and FVC were measured with a pneumotachograph, DLCO by the single-breath method (Masterlab, Jaeger, Würzburg, Germany). Values were expressed as a percentage of those predicted based on age and gender.

3.4. Supplementation study

Prior to the actual supplementation period, participants were subjected to a two-day wash-out period. During this period, they were not allowed to consume food rich in flavonoids in general or quercetin in particular. This food included non-organic onions, apples, red wine, tea, organic and freshly pressed fruit juices, berries (e.g. blueberries and elderberries), grapes, cherries, raisins, parsley, broccoli, cabbage, green beans and tomatoes. Participants also had to minimise the use of herbs and spices during this period.

The wash-out period was followed by a 24-h supplementation period during which all participants had to take 4 capsules containing either 500 mg quercetin or a placebo. The capsules were taken throughout the day, i.e. during lunch, during dinner, just before bedtime and the last during breakfast the following morning, 3 h before the second blood withdrawal. Before and after this supplementation period, venous blood samples were drawn into EDTA-containing vacutainer tubes (Vacutainer, Becton–Dickinson, Belgium) and kept on ice prior to processing which occurred within 1 h after blood collection. During supplementation, the same dietary restrictions as during the wash-out period were applied.

3.5. Preparation of the blood samples

Blood was aliquoted into eppendorfs for both the ascorbic acid and the glutathione analysis: for the former 10% trichloric acid (TCA) was used to preserve the samples for the latter. Another aliquot of blood was used for the ascorbic acid determination required for the blood-based cytokine production.

Table 1: Characteristics of the participants.

<table>
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<th>Quercetin-receiving group</th>
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Controls are matched on age and gender and do therefore not significantly differ from the patients regarding these parameters. Age is expressed in year, length in cm, weight in kg, time since diagnosis in years and DLCO (diffuse capacity of the lung for carbon monoxide), FEV₁ (forced expiratory volume in 1 s) and FVC (forced vital capacity) in % of the predicted value based on age and gender. Data are expressed as range (mean ± SEM).
assay as described in that section below. The remaining blood was centrifuged (3000 rpm, 5’ at 4 °C) to obtain plasma. Deproteinization of an aliquot of this plasma, using 10% TCA 1:1 followed by centrifugation (13,000 rpm, 5’ at 4 °C), was carried out for the trolox equivalent antioxidant capacity (TEAC) and uric acid (UA) measurement. All samples were stored at −80 °C prior to analysis.

3.6. Determination of total plasma quercetin concentration

Total quercetin (sum of quercetin aglycone and quercetin glucuronides/sulfates) concentrations in plasma were analysed by means of HPLC with coulometric array-detection after enzymatic hydrolysis as described previously.18 3’-O-methoxy quercetin (isorhamnetin) and 4’-O-methoxy quercetin (tamarixetin) aglycone and glucuronides/sulfates were not detected in the plasma.

3.7. Markers of oxidative stress: antioxidant status and malondialdehyde (MDA) levels

The antioxidant status was evaluated by measuring the total plasma antioxidant status as well as the plasma levels of the individual antioxidants ascorbic acid, uric acid and glutathione as described previously.17

In short, the trolox equivalent antioxidant capacity (TEAC) was determined as a measure for the total antioxidant status. A solution of 20 mM ABTS (2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) and 2.5 mM ABAP (2,2’-Azobis-(2-aminopropane)HCl) was incubated at 60 °C to generate ABTS radicals (ABTS*) until the absorbance at 734 nm was 0.070 ± 0.02. During the experiments, the ABTS* stock solution was protected from light and kept on ice. The samples were incubated with the ABTS radical solution for 5 min and subsequently the absorbance at 734 nm was measured. The decrease in absorbance, caused by the antioxidant capacity in the sample, was measured and related to that of trolox standards. The TEAC is expressed as μM trolox equivalents. The relative contribution of uric acid, vitamin C and quercetin to the total TEAC value is calculated using the TEAC value described for each individual antioxidant, i.e. 1, 1 and 6.24 respectively.10

Vitamin C was determined by HPLC upon its oxidation by ascorbate oxidase to dehydroascorbate. The latter reacted with o-dithiobis 2-nitrobenzoic acid (0.3 mM) in 0.1% tri-fluoroacetic acid (0.4 mM) and 5,5’-dithiobis 2-nitrobenzoic acid (0.3 mM) in phosphate buffer (pH 7.8) and methanol with fluorimetric detection (355–425 nm).

Uric acid was determined by HPLC using a Hypersil BDS C-18 end-capped column (125 × 4 mm, particle size 5 μm from Agilent, Palo Alto, CA, USA) with a mobile phase of 0.08 M phosphate buffer (pH 7.8) and methanol with fluorimetric detection (355–425 nm).

Total glutathione was determined using a recycling method. To 50 μl of each sample, 100 μl of a solution containing NADPH (0.4 mM) and 5,5’-dithiobis 2-nitrobenzoic acid (0.3 mM) in phosphate buffer (143 mM, pH 7.4) was added in a 96- well plate. The reaction was started by adding 50 μl of a solution containing glutathione reductase (4 U/ml). The increase in absorption at 405 nm was followed in time. This increase was related to that of calibrators containing GSH.

The determination of MDA levels is based on the formation of a coloured adduct of MDA with 2-thiobarbituric acid (TBA).20 In short, 100 μl plasma was added to 900 μl of a reagent (containing 0.12 mol/l TBA, 0.32 mol/l o-phosphoric acid and 0.01% (m/v) EDTA) and incubated for 1 h at 100 °C. After cooling, the MDA-product were extracted with 500 μl butanol. Thirty μl of the butanol layer was injected on an HPLC system (Agilent, Palo Alto, CA, USA) equipped with a fluorescence detector, set on excitation wavelength of 532 nm and emission wavelength of 553 nm with a Nucleosil C18 column (150 × 3.2 mm, particle size 5 μm from Supelco Inc. PA, USA). Samples were eluted with 65% (v/v) 25 mmol/l phosphate buffer pH 4.8, 35% (v/v) methanol. A calibration curve was constructed using malonaldehyde bis(diethylacetal) as standard.

3.8. Markers of inflammation: basal and LPS-induced levels of TNFα, IL-8 and IL-10

Both basal and LPS-induced levels of all cytokines were quantified before and after the supplementation using PeliKine Compact human ELISA kits (CLB/Sanquin, the Netherlands) as described previously.21 Consequently, the LPS-induced cytokine production assay was performed previously.

3.9. Ex vivo LPS-induced cytokine production assay

Blood was collected both before and after quercetin supplementation. Within 1 h after this collection, the blood-based cytokine production assay was performed as described previously.21 To this extent, blood was aliquoted into 24-well sterile plates and diluted 1:4 with RPMI 1640 (supplemented with L-glutamine). Subsequently, 0.1 ng/ml LPS was added to induce cytokine production and the plates were incubated for 24 h at 37 °C in 5% CO2. Cell-free supernatant fluids were then collected by centrifugation (6000 rpm, 10’ at 4 °C) and stored at −20 °C prior to analysis. All incubations were performed in triplicate.

3.10. Statistics

In general, the data of both the quercetin and placebo group have been reduced to the delta (after-before) for each patient after which the average delta values for each parameter were compared using a Mann Whitney U test. Only exception to this approach was the relative contribution of the various antioxidants to the TEAC value. For this analysis, the absolute individual values within the quercetin group were evaluated by a Mann Whitney U test.

The relation between either the MDA levels or the basal cytokine production and the effect of the quercetin supplementation on them has been appraised using Spearman’s rank correlation coefficient.

All statistical analyses were performed using SPSS 15.0. A one-tailed probability value (P-value) equal to or less than 0.05 was considered to be statistically significant.

4. Results

Baseline plasma quercetin levels were below the lower limit of detection of 0.054 μM in all sarcoidosis patients prior to supplementation. After supplementation, the quercetin plasma concentration, reaching an average of 0.27 ± 0.04 μM, could be quantified in all patients (Fig. 1).

Endogenous blood glutathione (GSH) levels were unaffected by supplementation in all patients (Fig. 2A). The total plasma antioxidant capacity, i.e. the total sum of all plasma antioxidants that is expressed as trolox equivalent, was significantly enhanced (3%, P < 0.01) after supplementation in the quercetin-receiving patients (Fig. 2B).

For the quercetin-receiving group, the relative contribution of not only the exogenous quercetin but also of the endogenous antioxidants uric acid and vitamin C to the total plasma antioxidant status is depicted in Fig. 3. These data reveal that total antioxidant capacity increased only 0.3% due to the presence of quercetin in the plasma. Combined with the observation that the total increase due to quercetin administration was 3%, this finding suggests that
Quercetin might increase the concentration of endogenous antioxidants in plasma, although this indirect effect is relatively small. Supplementation did not affect the plasma levels of uric acid and vitamin C (data not shown), indicating that these endogenous antioxidants are not involved.

Malondialdehyde (MDA), a marker of oxidative damage to lipids, was significantly reduced after supplementation with quercetin (Fig. 4A). Interestingly, the effect of quercetin supplementation on the plasma MDA levels was dependent on the levels of this marker prior to supplementation. This indicates that the higher the MDA level at baseline, the more pronounced the reduction of this marker of oxidative stress by quercetin supplementation (Fig. 4B).

The ratios of pro-versus anti-inflammatory cytokines TNFα/IL-10 and IL-8/IL-10 were used as inflammatory markers. Basal cytokine levels were used to evaluate the direct anti-inflammatory effects of the quercetin supplementation, while the ex vivo LPS-induced cytokine levels were analysed to mimic the possible protective effects of such a supplementation against an additional inflammatory burden. Both the basal and ex vivo LPS-induced TNFα/IL-10 and IL-8/IL-10 ratios were significantly decreased upon quercetin supplementation (Figs. 5 and 6). Although less pronounced than for the plasma MDA levels, the effect of quercetin supplementation on basal TNFα and IL-8 levels also showed some dependency on these cytokine levels prior to supplementation (Fig. 7A and B). This indicates that the higher the basal cytokine production, the more prominent the inhibiting effect of quercetin on this cytokine level. No such effect of the supplementation could be observed on either the basal plasma level of the anti-inflammatory cytokine IL-10 (Fig. 7C) or on any of the LPS-induced cytokine levels (data not shown).

No correlations were found between the increase of the total quercetin plasma concentration or the increase in total plasma antioxidant capacity and the effect of supplementation on the markers of oxidative stress and inflammation measured.

In the placebo-receiving group, no significant differences were found between the values before and after supplementation regarding any of the parameters measured.

5. Discussion

Sarcoidosis is an inflammatory disorder in which oxidative stress appears to be involved, as indicated by several biomarkers including a reduced antioxidant capacity. To the best of our knowledge, this is the first study demonstrating a positive effect of a one day antioxidant supplementation on this reduced antioxidant capacity in sarcoidosis.
After ex vivo LPS-stimulation of blood, TNFα/IL-10 and IL-8/IL-10 levels, two markers of inflammation, are significantly reduced by the in vivo quercetin supplementation. These ex vivo results are in agreement with our previous in vitro findings showing that quercetin decreased LPS-induced TNFα- and IL-8 production when added to the blood of sarcoidosis patients in the test tube. Interestingly, the applied one day quercetin supplementation also resulted in a significant decrease of the ratios of pro- versus anti-inflammatory cytokines TNFα/IL-10 and IL-8/IL-10 as well as of the plasma levels of the lipid peroxidation product MDA, a marker of oxidative stress. These results confirm that quercetin is capable of empowering the compromised antioxidant defence system of sarcoidosis patients as well as of mitigating the inflammation present in sarcoidosis.

A possible explanation for the anti-inflammatory effects of quercetin can be found in the interplay between oxidative stress and inflammation. ROS are directly involved in the occurrence of oxidative stress. Moreover, ROS are also capable of promoting inflammation by activating the transcription factors nuclear factor kappa B (NF-κB) and activator protein-1. These transcription factors induce pro-inflammatory cytokines such as TNFα. By scavenging ROS, quercetin might not only offer protection against oxidative stress, the flavonoid may simultaneously mitigate inflammation. It has been reported that quercetin is capable of inhibiting TNFα production as well as TNFα gene expression via modulation of NF-κB in human peripheral blood mononuclear cells. At the same time, it is known that NF-κB activation will induce radical producing enzymes and thus promote radical formation. Consequently, an anti-inflammatory effect of quercetin that decreases NF-κB activation will prevent this route of radical formation and will thus also reduce oxidative stress. This shows that the anti-inflammatory and antioxidant effects of quercetin are intertwined.

During the scavenging of ROS, quercetin is converted into oxidation products. These products might react with critical sulfhydril groups, thereby impairing vital cellular functions. However, the level of glutathione (GSH), the most abundant endogenous thiol, is not affected by supplementation of 4x500 mg quercetin orally within 24 h. This suggests that GSH levels are easily sufficient to absorb the reactive oxidation products of quercetin. This outcome could have been anticipated based on the relatively low plasma quercetin concentration achieved by the current supplementation (0.27 ± 0.04 μM) compared to the high cellular concentrations of GSH (1–10 mM).

Interestingly, the effects of quercetin supplementation appear to be more pronounced when the baseline levels of MDA, TNFα and IL-8 are increased. Only when MDA levels are high, quercetin supplementation reduces this marker of oxidative stress. Similarly, although to a lesser extent, quercetin supplementation only reduces the TNFα/IL-10 ratio or the IL-8/IL-10 ratio when the basal levels of these inflammatory markers are enhanced. The extent of the effect of the applied quercetin supplementation appears to be greatly dependent on the individual baseline levels of oxidative stress and inflammation in the patient. It was expected that the extent of the effect also correlated with the quercetin plasma level.

Fig. 4. The effect of quercetin supplementation on plasma malondialdehyde (MDA) levels (panel A) and the relation between these levels at baseline and the net effect of the quercetin supplementation hereon (panel B) in sarcoidosis patients. This net effect, i.e. the difference between MDA at baseline versus that after quercetin supplementation, is plotted (y-axis) against the MDA levels (x-axis). Data in panel A are individually expressed for all 12 sarcoidosis patients receiving quercetin and all 6 sarcoidosis patients receiving placebo (light grey bars represent the mean). Data in panel B are individually expressed for only the 12 sarcoidosis patients receiving quercetin.

Fig. 5. The effect of quercetin supplementation on basal levels of the ratios TNFα/IL-10 (panel A) and IL-8/IL-10 (panel B) in sarcoidosis patients. Data are individually expressed for all 12 sarcoidosis patients receiving quercetin and all 6 sarcoidosis patients receiving placebo; light grey bars represent the mean. *P < 0.05 compared to the “before” measurement prior to the quercetin supplementation.
achieved in the patient. However, such a correlation could not be demonstrated. The variation in baseline value between the patients, as well as the strong dependency of the effect on those baseline values, might have concealed the anticipated correlation between quercetin plasma level and observed effect.

Our results indicate that beneficial effects of antioxidant supplementation can only be expected in people with enhanced oxidative stress or inflammation. Although this seems trivial, it is often not realised and might explain the negative outcome of many clinical studies where antioxidants were supplied to healthy subjects.25,26 The present study shows that the baseline value of oxidative damage and inflammation is a major discriminator in the beneficial effect of antioxidant supplementation.

In several diseases associated with enhanced oxidative stress, the use of antioxidants, i.e. N-acetyl cystein and lipoic acid, is proven to be clinically beneficial.27 In these antioxidant supplementation studies, besides having an antioxidant effect, the reduction of the occurring inflammation has also been implicated.27

Recently, it has been demonstrated that Infliximab, a TNFα antibody, improved lung function in stable pulmonary sarcoidosis.28 This finding is in line with studies regarding the use of Infliximab in the treatment of other chronic inflammatory diseases such as Bechterew,29 Crohn’s disease30 and rheumatoid arthritis.31 However, the latter studies report an inverse correlation between the effects of this anti-TNFα therapy and the severity of the pathologies, i.e. the more severe the disease, the less effective this anti-TNFα antibody therapy is. This might be due to the limited capacity of anti-TNFα therapy that was restricted by the use of a fixed dosing regime independent of the baseline TNFα level. In the present study, no limitation of the quercetin effect was seen. In

Fig. 6. The effect of quercetin supplementation on ex vivo LPS-induced cytokine production. After LPS stimulation, both the relative ratios of TNFα/IL-10 (panel A) and of L-8/IL-10 (panel B) show a significant decrease in the quercetin-receiving group. Data are individually expressed for 11 sarcoidosis patients receiving quercetin and 5 sarcoidosis patients receiving placebo; light grey bars represent the mean. Results are expressed relative to the cytokine release under stimulation of LPS prior to the supplementation. *P < 0.05 compared to the LPS-induced cytokine production in the control without quercetin.

Fig. 7. The correlation between basal cytokine levels and the net effect of the quercetin supplementation in sarcoidosis patients on the cytokine levels. The net quercetin effect (x-axis) is the difference between the non-stimulated cytokine level at baseline versus that after supplementation and is plotted against the cytokine level at baseline (x-axis). The cytokines are TNFα (panel A), IL-8 (panel B) and IL-10 (panel C). Data are individually expressed for all 12 sarcoidosis patients receiving quercetin.
contrast to regular anti-TNFα therapy, it was found that the more outspoken the markers of oxidative stress and inflammation, the more effectively quercetin reduces these markers, including the TNFα level.

In conclusion, the present study is the first to demonstrate that supplementing a high dose of the antioxidant quercetin over a 24-h period reduces markers of oxidative stress and inflammation in sarcoidosis, provided these markers are elevated. However, in a chronic disorder such as sarcoidosis, supplementation has to proceed over a much longer time period. The safety, tolerability and an improvement of lung function associated with long-term use of quercetin in sarcoidosis remain to be investigated. The current findings suggest that strengthening the endogenous antioxidant shield with the dietary quercetin results not only in more protection against oxidative damage, but also in an increased defence against inflammation. Interestingly, both the anti-oxidative and anti-inflammatory effects of quercetin appeared to be more pronounced when the level of respectively the occurring oxidative stress and inflammation is higher at baseline. This observed correlation indicates that beneficial effects of antioxidant supplementation can especially be expected in people with elevated oxidative stress and inflammation, i.e. patients suffering from a disease of which the pathology is associated with both processes such as sarcoidosis.

Conflict of interest

The authors declare that they have no conflict of interest.

Statement of authorship

AWB conceived and carried out the study, analyzed the data and drafted the manuscript. MD recruited and guided the patients. VdB determined the total plasma quercetin concentrations. AB and GH conceived the study, participated in its design and helped to analyze the data and to draft the manuscript. All authors read and approved the final manuscript.

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