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Article *in* Experimental Lung Research · July 2005

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Experimental Lung Research, 31:449–459, 2005

ISSN: 0190-2148 print/1521-0499 online
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EFFECT OF QUERCETIN SUPPLEMENTATION ON LUNG ANTIOXIDANTS AFTER EXPERIMENTAL INFLUENZA VIRUS INFECTION

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□ *In the mice, instillation of influenza virus A/Udorn/317/72(H3N2) intranasally resulted in a significant decrease in the pulmonary concentrations of catalase, reduced glutathione, and superoxide dismutase. There was a decrease in vitamin E level also. These effects were observed on the 5th day after viral instillation. Oral supplementation with quercetin simultaneous with viral instillation produced significant increases in the pulmonary concentrations of catalase, reduced glutathione, and superoxide dismutase. However, quercetin did not reverse the fall in vitamin E level associated with the viral infection. It is concluded that during influenza virus infection, there is 'oxidative stress.' Because quercetin restored the concentrations of many antioxidants, it is proposed that it may be useful as a drug in protecting the lung from the deleterious effects of oxygen derived free radicals released during influenza virus infection.*

Keywords influenza virus, pulmonary antioxidants, quercetin

Several forms of highly reactive oxygen derived free radicals are produced during oxidative processes in living cells. These free radicals participate in many forms of tissue damage, which result in system dysfunction. Under normal conditions, their harmful effects upon the cells are neutralized to a large extent by the antioxidant defense mechanisms of the body. Whenever there is an excessive generation of oxygen-derived free radicals or a diminished production of antioxidants, the system is considered to be in a state of 'oxidative stress' [1, 2].

Received 10 May 2004; accepted 13 July 2004.

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During exposure to a viral infection, the lung is potentially at high risk of injury because it is the primary target for viral replication. Recent studies indicate that acute influenza virus infection is associated with the development of oxidative stress as evidenced by accumulation of lipid peroxidation products in blood and lung [3–8], and it has been proposed that during the infection, the oxygen-derived free radicals generated by the phagocytes may contribute to the lung injury either by oxidizing lipids, proteins, and nucleic acids [8–10] or by activating certain proteases [11]. Oda [3] and Ungheri [7] and their colleagues reported protective role of reactive oxygen intermediate (ROI) in the pathogenesis of influenza virus infection.

There is increasing evidence that a number of products found in fruits and vegetables function as antioxidants. They are being used therapeutically to supplement the systemic antioxidants [12, 13]. Quercetin, a bioflavonoid that occurs naturally in fruits, vegetables, nuts, seeds, red wine, tea, and flowers [14–16], is one such product that possesses antioxidant property. It prevents lipid peroxidation and scavenges superoxide radicals [8, 17, 18]. In a recent study, we had reported the usefulness of quercetin as a protective agent in lung pathology associated with influenza virus infection [8]. However, that study did not deal with the mechanism behind the curative potential of quercetin. The present investigation has been planned to address this issue. It explores whether quercetin restores the changes in concentrations of pulmonary antioxidants associated with influenza virus infection.

MATERIALS AND METHODS

Animal Infection and Treatment

BALB/c male mice (14 to 16 g) were fasted for 24 hours before the study. They were distributed into 4 groups:

Group I ($n = 6$): control group (normal, healthy mice).
Group II ($n = 6$): mice infected with influenza virus A/Udorn/317/72 (H3N2) intranasal instillation.
Group III ($n = 6$): mice infected intranasally with influenza virus A/Udorn/317/72 (H3N2) and supplemented orally with quercetin at a dose of 1 mg/day for 5 consecutive days.
Group IV ($n = 6$): mice treated orally with quercetin at a dose of 1 mg/day for 5 consecutive days.

Virus Dose

Influenza virus A/Udorn/317/72(H3N2) was obtained from the Centers for Disease Control and Prevention (CDC), Atlanta, and was used in all

experiments. Stock preparations of influenza A consisted of infectious chicken embryo allantoic fluid. Viral infectivity was quantified by a conventional in ovo assay. The Reed and Muench method was used to calculate 50% end-points [19], and virus titers are expressed as 50% egg infectious doses (EID_{50}) per milliliter. Stock virus was frozen at −80°C until needed. Virulence of influenza virus is well expressed on the 5th to 6th day after virus instillation [8]. Hence the mice were sacrificed on the 5th day.

Catalase Assay

Catalase activity was measured according to the previously described method [20, 21]. Briefly, lungs were homogenized in phosphate buffer (pH 7.2) and then centrifuged at 1800 ×g for 15 minutes. A 0.1-mL aliquot of clear supernatant was added to substrate solution of 0.050 M hydrogen peroxide in phosphate buffer (pH 7.0). The decrease in absorbance was recorded at 240 nm for 2 to 3 minutes and the decrease per minute was calculated from the initial (1-minute) linear portion of the curve.

Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was measured according to the previously described method [21]. The xanthine–xanthine oxidase system was used to generate superoxide radicals, which in turn reduce cytochrome *c*. One unit of SOD activity was defined as the amount of protein that inhibits the rate of cytochrome *c* reduction by 50% and was measured spectrophotometrically at 550 nm. Enzyme activity was expressed as units per milligram protein.

Assessment of Glutathione

The levels of reduced glutathione (GSH) and glutathione (GSSG) in lung were analyzed spectrofluorimetrically as described earlier [22]. Briefly, lung sections were washed twice in saline and sonicated in 6% trichloroacetic acid (TCA) and then centrifuged at 3600 ×g at 4°C. The determination of GSH was performed by adding aliquots of standard and samples to tubes containing phosphate/EDTA buffer (pH 7.8) and 1 mg/mL opthalaldehyde (200 µL) and then stored in dark for 15 minutes. NaOH, 0.1 N, was used as diluent in place of phosphate-EDTA buffer for GSSG assay. The fluorescence was measured (excitation 350 nm, emission 420 nm) by spectrofluorimetry.

Vitamin E Measurement

Vitamin E was extracted from lung as described previously [23] and was assayed by HPLC using C-18 column (25 × 4.1 mm). The eluent was

methanol-ethanol 1:9 (v:v), 20 mmol/L lithium perchlorate. The flow rate was 1 mL/min, and the injected volume was 20 μL. The eluate was monitored by spectrofluorimetric detector. λex = 292 nm, λem = 325 nm.

Statistical Analysis

The data were analyzed with one way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison tests**.** All the results are expressed as mean ± SD.

RESULTS

Five days after the intranasal instillation of the influenza virus A/Udorn/317/72 (H3N2) in BALB/c mice, there was piloerection. The food intake was reduced and the mice became lethargic. The lung fluid was influenza positive by hemagglutination test (HA) and the bacterium culture was negative.

Concentration of Catalase

The concentration of catalase in the lung homogenate of normal control mice was 26.8 ± 2.4 units/mg protein. In the influenza virus infected mice, it was 16.9 ± 2.9 units/mg protein. There was a 63% decrease in catalase concentration, which was statistically significant ($P < .001$). The results are presented in Figure 1.

Oral administration of quercetin alone did not produce any significant change in catalase concentration of normal mice (Figure 1). However, when given simultaneously with viral instillation, it increased the catalase concentration to 24.9 ± 2.5 units/mg protein ($P < .001$, Figure 1), which was similar to the control value.

Concentration of Superoxide Dismutase

When compared with the SOD concentration in the lung homogenate of normal control mice, the SOD concentration in the influenza-infected mice was found to be decreased by 52%. The respective values were 12.8 ± 1.2 units/mg protein in the control group and 6.7 ± 0.89 units/mg protein in the influenza-infected group. This decrease was statistically significant ($P < .01$, Figure 2). A significant ($P < .001$) increase in SOD concentration was observed when quercetin was supplemented with viral instillation. The SOD concentration became 12.0 ± 1.38 units/mg protein

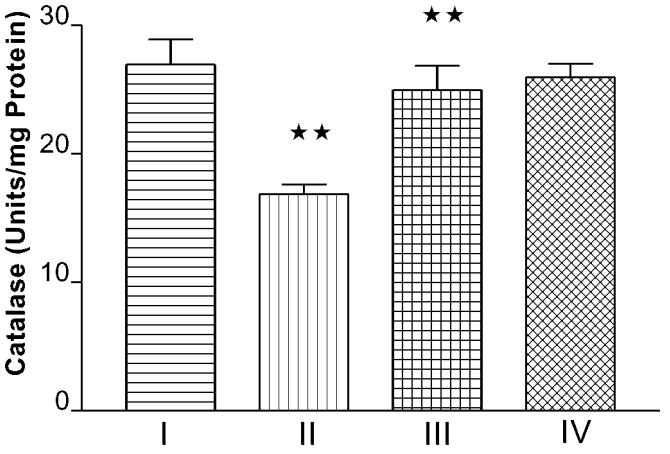

FIGURE 1 Effect of influenza virus infection on the concentration of catalase in lung homogenate before and after supplementation with quercetin. I, Control group; II, influenza virus–infected group; III, influenza virus–infected + quercetin-treated group; IV, quercetin-treated only group. $^{**}P < .001$ versus group I; $^{**}P < .0001$ versus group II.

(Figure 2), which was similar to the control value. Oral intake of quercetin alone did not produce any alteration in the SOD concentration in the normal mice (Figure 2).

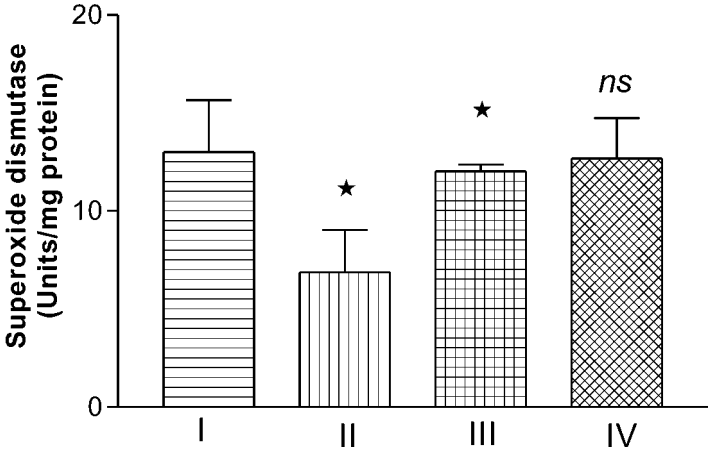

FIGURE 2 Effect of influenza virus infection on the concentration of superoxide dismutase in the lung homogenate before and after supplementation with quercetin. I, Control group; II, influenza virus infected group; III, quercetin-treated + Influenza virus infected group; IV, quercetin-treated group. $^{*}P < .01$ versus group I; $^{*}P < .01$ versus group II.

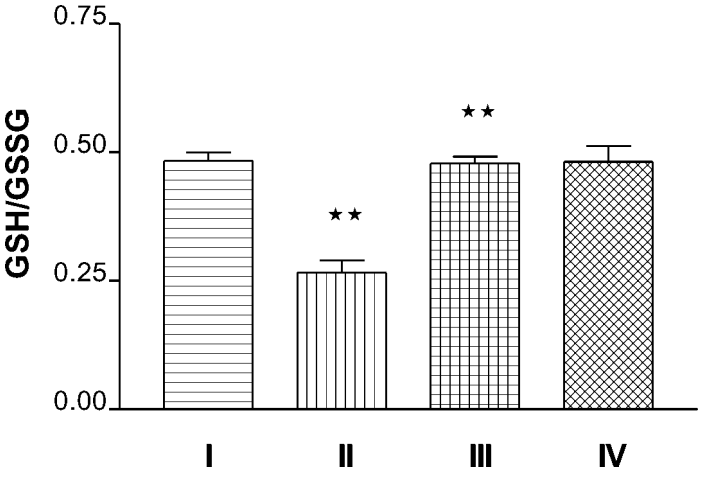

FIGURE 3 Effect of influenza virus infection on the ratio of GSH/GSSG level in the lung homogenate before and after supplementation with quercetin. I, Control group; II, influenza virus infected group; III, quercetin-treated + influenza virus infected group; IV, quercetin-treated group. $^{**}P < .001$ versus group I; $^{**}P < .001$ versus group II.

Ratio of the Concentration of GSH/GSSG

The changes in the ratio of the concentration of GSH/GSSG were investigated. From a control value of 0.484 ± 0.016, the GSH/GSSG ratio decreased to 0.266 ± 0.024 in the influenza-infected mice. There was a decrease of 45.04%, which was statistically significant ($P < .001$). The results are presented in Figure 3. Following simultaneous intake of quercetin along with viral instillation, the GSH/GSSG ratio increased to 0.479 ± 0.013 ($P < .001$, Figure 3). Intake of quercetin alone did not produce any significant alteration in GSH/GSSG ratio in the normal mice (Figure 3).

Vitamin E Level

Vitamin E level in the lung homogenate of control mice was found to be $0.145 \pm 0.006\,\mu$mol/mg protein. After influenza virus instillation, it decreased to $0.056 \pm 0.003\,\mu$mol/mg protein. There was a decrease of 35%, which was statistically significant ($P < .001$). Quercetin supplementation in the virus instilled mice failed to raise the vitamin E level significantly (Figure 4). It did not have any effect in the vitamin E level of normal mice either (Figure 4).

DISCUSSION

In a recent study in mice, we had reported that intranasal instillation of influenza virus A/Udorn/317/72(H3N2) caused airway epithelial damage.

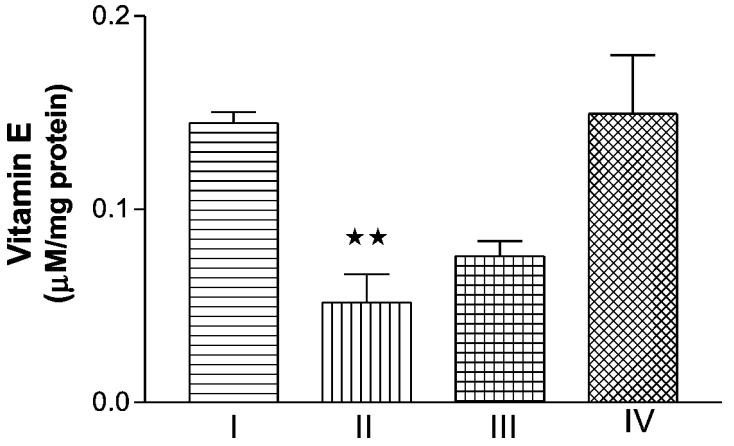

FIGURE 4 Effect of influenza virus infection on the vitamin E level in the lung homogenate before and after supplementation with quercetin. I, Control group; II, influenza virus infected group; III, quercetin-treated + influenza virus infected group; IV, quercetin-treated group. $^{**}P < .001$ versus group I.

There were lesions in alveolar walls with acute inflammatory reaction and development of pneumonitis and bronchitis. There was high recruitment of neutrophils and macrophages also [8].

Several investigators have proposed that the airway inflammation observed during viral infection may be due to damage to various cell components by oxygen-derived free radicals and cytokines from the infiltered cells [8, 24–26]. Even though the antioxidant defense mechanisms detoxify the oxygen-derived free radicals and prevent the tissues from injury, the histopathological changes associated with influenza virus infection reported previously [8] suggest that they may be due to excessive generation of free radicals or diminished production of antioxidants. It has been reported that following influenza virus infection, there is reduction in the pulmonary concentrations of antioxidants such as glutathione, vitamin C, and vitamin E [25, 27, 28]. The first part of the present study is in agreement with these findings.

We observed that 5 days after the instillation of influenza virus, there were marked decreases in pulmonary antioxidant concentrations. There were significant decreases in catalase and SOD concentrations. Catalase is present in almost all mammalian cells. It is localized in the peroxisomes or in the microperoxisomes and is known to be involved in the scavenging of hydrogen peroxide. It catalyses the decomposition of H_2O_2 to H_2O and oxygen and thereby protects the cells from oxidative damage by H_2O_2 and $OH^{\bullet}$ [29]. It was reported earlier that superoxide radicals inhibit catalase activity [28, 30]. It is well known that SOD plays a major role in the destruction of superoxide radicals. It converts superoxide radicals into hydrogen

peroxide via dismutase reaction [31]. Blech and Bordes [32] and Mileva and coworkers [28] have reported that hydroperoxide anions inhibit SOD activity. However, the exact mechanism by which they inhibit the enzyme activities is not known. Thus the decreases in catalase and SOD concentrations observed could be due to the direct attack by free radicals that are generated during respiratory burst by activated macrophages [25, 27, 28]. These findings suggest that there may be oxidative stress during influenza virus infection.

Besides catalase and SOD, we observed that following influenza virus infection, there were decreases in pulmonary GSH and vitamin E levels. Decreases in hepatic GSH and vitamin E levels have been reported in mouse model of endotoxemia [33]. There are also reports that show that the levels of vitamin C and GSH in bronchoalveolar lavage and lung tissue decrease significantly during the early stage of infection [25, 34, 35]. In stress responses, ascorbic acid and GSH serve as cofactors in enzymatic reactions through the formation of noradrenaline or leukotrienes [36]. Vitamin E and glutathione have beneficial effects on the responsiveness of immune system cells such as T lymphocytes, polymorphonuclear leukocytes, and macrophages. Any decrease in endogenous concentration of these antioxidants may result in local immunosuppression [37, 38]. Thus, the general decrease in the antioxidant buffering capacity during influenza virus–induced infection may reduce the ability of tissues to protect themselves from the deleterious effects of oxidative stress.

A bioflavonoid, quercetin has been reported to prevent lipid peroxidation and scavenge superoxide radicals [39–43]. However, its role on pulmonary antioxidant levels during influenza virus infection has not been studied. In our previous study, supplementation of quercetin resulted in significant decreases in superoxide production and lipid peroxidation (LPO) products associated with viral infection [8]. Ferriola and colleagues [44] showed that quercetin inhibited protein kinase C, an enzyme that plays a key role in the activation of NADPH oxidase and respiratory burst. Skaper and colleagues [45] had reported that the addition of quercetin to a variety of cell lines protected the cells from the cytotoxic effects of buthionine sulfoximine (BSO), a compound that decreases the intracellular GSSG, thus resulting in accumulation of oxygen/peroxy radicals, and eventually leading to cell death. The pharmacological effect of quercetin is due to its antioxidant activity, metal-chelating activity, and capacity to inhibit certain enzymes [46, 47]. In the present study, our results show that quercetin supplementation along with influenza virus instillation result in significant increases in antioxidant enzyme levels excepting vitamin E.

Vitamin E is the major lipid-soluble antioxidant in the cell antioxidant defense system and is exclusively obtained from the diet. Plasma vitamin E concentrations vary little over a wide range of dietary intakes and even daily

supplements of the order of 1600 IU/day for 3 weeks increase plasma levels by 2 to 3 times only and on cessation of intake, plasma levels return to pretreatment values in 5 days [48]. Thus, the increase in vitamin E level of plasma is not marked in spite of an excessive dietary intake. This transitory effect may explain why vitamin E homologues have markedly differing antioxidant abilities in biologic systems [49]. However, some recent investigations [28, 50] report that vitamin E supplementation results in significant increases of antioxidant contents in blood and lung. Similarly, the fall in vitamin E level associated with viral instillation was reported to be raised after dietary intake of vitamin E. In our study, such a response was not evident after the administration of quercetin. At present, we do not have an explanation as to why quercetin did not raise the vitamin E level although it restored other antioxidants of lung. One possibility is that such an effect did not occur within the time frame of the study, i.e., 5 days. But, others have reported that in 5 days after viral instillation, simultaneous intake of vitamin E increased the plasma vitamin E level [35]. Another possibility is that quercetin by itself may not be able to raise the vitamin E level, but, when supplemented with vitamin E, it may actually reduce the amount of vitamin E, which has to be taken to raise it to the preinfection level. The latter possibility remains to be verified.

In summary, the present study shows that there is oxidative stress in the lung during influenza virus induced infection. Because quercetin raises the concentrations of antioxidants and brings them to near normal values, it is proposed that it may be used as a drug in alleviating the oxidative stress induced by influenza virus infection.

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