Original Article

Protective effects of quercetin during influenza virus-induced oxidative stress

Telugu Akula Narasa Raju[1] MSc, Ayyagari Naga Vijaya Lakshmi[1] MSc, Tamatam Anand[1] MSc, Linga Venkateshwar Rao[1] MSc, PhD and Gita Sharma[2] MSc, PhD

[1]*Department of Microbiology, Osmania University, Hyderabad, India*
[2]*Biotechnology Division, Zydus Cadila Healthcare Research Centre, Ahmedabad, India.*

Oxidative stress was found to have a role in many viral diseases including AIDS, hepatitis and influenza. In the present study the pathology of influenza viral infection in the lungs, which may lead to oxidative stress, was investigated and an attempt was made to study the efficacy of anti-oxidants as therapeutic agents. Adult male mice of Swiss albino type were infected with influenza virus (A/Hong Kong/8/68) and studied for the anti-oxidant status in the lungs by evaluating the lung enzymatic anti-oxidant system including superoxide dismutase and catalase. Superoxide radical generation, which might increase by the activated alveolar macrophages, was estimated by nitroblue-tetrazolium reduction assay. We have also estimated lipid peroxidation levels in lung through thiobarbutiric acid reactive substances assay. We also examined the ability of flavonoid quercetin in protecting from influenza virus-induced oxidative stress. The influenza-infected group showed decreased levels of superoxide dismutase and catalase; however, anti-oxidant supplemented groups showed these activities to be the same as in the control group. The lipid peroxide levels were increased in virus-infected mice. Administration of quercetin lowered the lipid peroxide levels significantly. Formazan positive cells were increased by 80% in the virus-infected group and supplementation with quercetin reduced their number to 44%.

Key words: anti-oxidants, influenza virus, oxidative stress, quercetin.

Introduction

Influenza viruses are known to cause worldwide epidemics and pandemics. Despite much research that has been carried out on these viruses, the actual mechanism involved in the pathogenesis is not clear. The major symptoms include extensive haemorrhage, necrosis of ciliated epithelial cells, infiltration of lymphoid cells and oedema in the alveolar space.[1] Several studies have suggested that an overreaction of the host immune system may be responsible for these pathological effects. Accumulation of neutrophils and macrophages in the lungs could participate in the development of the disease, as they produce reactive oxygen species such as superoxide ions (O_2^-) and hydrogen peroxide (H_2O_2), which in turn causes tissue damage by either directly oxidizing cellular biomolecules including lipids, proteins and nucleic acids[2] or by activating certain nucleases or proteases.[3]

Earlier studies have reported that influenza viral infection causes a decrease in the levels of endogenous concentration of anti-oxidants such as vitamin E and glutathione[4] and an increase in the levels of xanthine oxidase, an enzyme which generates superoxide radicals.[5] Evidence for the presence of oxidative stress has suggested that anti-oxidants could be used as therapeutic agents. There is a single report available on the efficacy of anti-oxidants as therapeutic agents during influenza virus infection. Oda *et al*.[5] documented that administration of pyran polymer conjugated superoxide dismutase protected mice against potentially lethal doses of influenza virus.

Quercetin is a member of a group of polyphenolic compounds known as flavonoids. It occurs naturally in fruits, vegetables, seeds and flowers.[6,7] Quercetin has been shown to scavenge superoxide radicals[8] and protect from lipid peroxidation[9] and chelate metal ions[10] to form inert complexes. Warren *et al*.[11] reported that quercetin inhibits degranulation and superoxide production by neutrophils activated by phorbol ester (PMA). The present work was conducted in order to determine the protective role of quercetin against oxidative stress caused during influenza virus infection.

In this study, influenza virus-induced oxidative stress was assessed by evaluating the lung enzymatic anti-oxidant system, which includes superoxide dismutase and catalase. Superoxide production was assessed by nitroblue-tetrazolium (NBT) reduction assay and lipid peroxidation was estimated to assess the oxidative damage to lipids. Furthermore, the protective abilities of quercetin were assessed on the above parameters.

Materials and methods

Chemicals

Cytochrome c, xanthine oxidase, xanthine, NBT and tetraethoxypropane (TEP) were purchased from Sigma Chemical Company (Sigma, MO, USA). All other chemicals were of analytical grade and obtained from local firms.

Correspondence address: Dr Gita Sharma, Department of Microbiology, Osmania University, Hyderabad 500 007, India.
Tel: 91 040 7018 951 ext 246; Fax: 91 040 7019020
Email: tanraju@hotmail.com
Accepted 15 May 2000

Virus

Influenza virus type A/Hong Kong/8/68, obtained from the National Institute of Virology, Pune, was propagated in the allantoic fluid of 10-day-old white leghorn chick embryos (supplied by Venkateshwara Hatcheries, Hyderabad). Quantification of the virus was done by haemagglutination assay.

Animals and experimental design

Healthy adult Swiss albino male mice (25–30 g) purchased from the National Institution of Nutrition (NIN), Hyderabad, were fed on a pellet diet and water ad libitum. The animals were divided into four groups each of six animals, designated as CON – control, INF – infected, QT – quercetin treated and IQT – infected and quercetin treated. Mice were infected intranasally with 30 µl of 75 HA units of virus under anaesthetic. The control group received uninfected allantoic fluid of the same quantity. The QT and IQT groups received 1 mg of quercetin orally, daily, from the day of inoculation till the day they were killed. Mice were killed on the eighth day, as the maximum pathological effects are seen at 8-day post infection.

Tissue collection

Mice were killed by cervical dislocation and an incision was made which opened the ribcage to expose the lungs. The inferior venacava was transected and lungs were perfused with 5 ml of cold phosphate buffer saline (PBS), pH 7.2, through the right ventricle. The lungs were then immediately excised and stored at –20°C till further use.

Collection of alveolar macrophages

Alveolar macrophages were collected as described earlier by using a special device, which contains a long polyethylene tubing connected with two 5 ml sterile syringes through two-holed rubber stoppers.[12] Mice were sacrificed by cervical dislocation and an incision was made to expose the trachea. A small lateral incision was made between two of the cartilage rings and a tube inserted in the trachea. 0.5 ml of 0.05 M PBS (pH 7.2) was injected into the lungs and then collected with another syringe. This process was repeated until the lungs were lavaged with 5 ml of PBS. The collected fluid was centrifuged at 650 *g* for 10 min and the supernatant was discarded. The cell pellet was resuspended in PBS at the concentration of 1 mg/ml protein.

Assessment of superoxide radical production by nitroblue-tetrazolium (NBT) reduction assay

Park *et al.*[13] described the procedure to assess the superoxide radical production. Nitroblue-tetrazolium in the presence of superoxide radicals, is reduced to an insoluble cytoplasmic blue-black formazan precipitate. 50 µl of cell suspension was mixed with 50 µl of 0.2% NBT in PBS and incubated for 15 min at 37°C in a humid chamber and for 15 min at room temperature. After incubation, slides were washed and cells were counterstained with Leishman's stain. Formazan positive cells (F+) were scored as those cells containing blue-black formazan granules in the cytoplasm. All results were expressed as the number of F+ cells per 200 cells.

Preparation of sample for biochemical studies

Lungs were weighed and homogenized in phosphate buffer pH 7.2. The homogenates were centrifuged at 1800 *g* for 15 min and the clear supernatants were separated and used for further biochemical studies.

Lipid peroxidation

Lipid peroxidation was determined with the lung homogenate by the method of Burge and Aust based on the formation of thiobarbituric acid reactive species (TBARS).[14] Tetraethoxypropane was used as an external standard.

Superoxide dismutase (SOD) enzyme assay

Superoxide dismutase (SOD) activity was measured by the method of James *et al.*[15] 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% at 550 nm. Enzyme activity was expressed as units per mg protein.

Catalase assay

Catalase activity was determined according to the method of Machly and Chance.[16] One unit of enzyme activity was defined as the amount of protein required that decomposes 1 µmol/L of H_2O_2 per min at 25°C, estimated in terms of decreased absorbance at 240 nm.

Protein estimation

Protein was determined according to the method described by Lowry *et al.*[17]

Statistical analysis

The data were analysed with one-way analysis of variance (ANOVA) followed by Dunnett *t*-test. All the results are expressed as mean ± SD.

Results

Mice were infected with Influenza virus type A/Hong Kong/8/68 intranasally and the lung antioxidant status was studied. Supplementation with quercetin, an antioxidant, was also studied to determine the protective effects. Only traces of viral activity in the lung homogenate were observed with no significant difference in the infected group, as well as in the supplemented group. No significant changes in bodyweight were observed.

The levels of lipid peroxidation were reported based on the content of TBARS in the lung extract. Lipid peroxidation levels were increased by 56% during viral infection when compared with the control group (Table 1). However, in the supplemented group the elevated level was lowered from 56% to 39%. The assessment of superoxide radical production was done by NBT reduction method (Table 2). Formazan positive cells were increased by 80% during viral infection, but it decreased to 44% after supplementation with quercetin. Superoxide dismutase and catalase activities decreased in the infected group by 18% and 16%, respectively. However, supplemented groups showed activities similar to those of the control group.

Discussion

There is increasing evidence to support the involvement of oxidative stress in many human pathological conditions, such as rheumatoid arthritis, ischaemic heart disease, cancer, age-

Table 1. Lipid peroxidation (TBARS assay)

	CON	INF	QT	IQT
Lipid peroxidation nmol MDA per 100 mg protein	160 ± 7.6	251 ± 2.8	168 ± 3.0	207 ± 4.8

Levels of lipid peroxidation expressed as nmol malondialdehyde (MDA) formed per 100 mg protein. Values are the mean ± SD of six animals. $P < 0.05$ vs corresponding control. CON, control; INF, infected; QT, quercetin treated; IQT, infected and quercetin treated; TBARS, thiobarbutric acid reactive substances.

Table 2. Assessment of superoxide production (NBT reduction assay)

	CON	INF	QT	IQT
Formazan positive cells per 200 cells	63.6 ± 7.5	114.5 ± 4.1*	74.6 ± 9.7	91.3 ± 5.7

Each value is expressed as the mean ± SD value of three slides (200 cells per each slide); *$P < 0.05$ *vs* corresponding control; NBT, nitroblue-tetrazolium; CON, control; INF, infected; QT, quercetin treated; IQT, infected and quercetin treated.

Table 3. Superoxide dismutase and catalase activities during influenza viral infection and after supplementation with quercetin

	CON	INF	QT	IQT
SOD Units/mg protein	13.02 ± 1.2	10.4 ± 0.89*	12.93 ± 1.6	14.5 ± 1.38
Catalase Units/mg protein	30.4 ± 2.4	26.5 ± 2.9*	30.38 ± 1.15	29.4 ± 2.5

Each value is the mean ± SD of six animals. Activities of the enzymes were calculated per min per mg of protein as described in Materials and methods.
* $P < 0.05$ *vs* corresponding control. SOD, superoxide dismutase; CON, control; INF, infected; QT, quercetin treated; IQT, infected and quercetin treated.

ing and several infectious diseases. Reactive oxygen species that are produced during oxidative stress participate in radical chain reactions, causing lipid peroxidation and protein oxidation, which lead to membrane structure alteration and enzyme inactivation.

Hennet *et al.*[4] reported that influenza virus infections results in a decrease in the endogenous concentration of anti-oxidants such as vitamin E, vitamin C and glutathione. Previous reports in our lab reported that oxidative stress plays a major role in the pathology of influenza viral infection.[18] Anti-oxidant enzymes, such as catalase and peroxidase, showed increased activities in the virus infection until 48 h and thereafter a decrease in their activity was observed, with a maximum loss in activity at 8-day post infection. However, their levels had recovered to normal after 15 days post infection. Depletion of anti-oxidants such as vitamin E, glutathione and vitamin C and elevated levels of reactive oxygen species and pro-oxidant cytokines[19] are the biomarkers for the presence of oxidative stress during influenza virus infection. Evidence for the presence of oxidative stress in influenza viral infection suggested that anti-oxidants could be used as therapeutic agents in these conditions. Flavonoids such as quercetin (3,3,4,5 and 7 pentahydroxy flavone) and rutin have been demonstrated to possess anti-oxidant activity. Quercetin scavenges oxygen radicals, such as superoxide radical and hydroxyl radical and also chelate metal ions.[9,10]

In the present investigation, we have studied the anti-oxidant status of the lung during influenza virus infection and after dietary supplementation with an anti-oxidant, quercetin. We have measured the activities of anti-oxidant enzymes, such as superoxide dismutase and catalase. Catalase and SOD activities were decreased during influenza virus infection. Superoxide dismutase plays a major role in the destruction of superoxide radicals. It converts superoxides into H_2O_2, which is further metabolized by catalase and peroxidase. However, in the supplemented groups, the activity of both enzymes was similar to that of the control group. Hassan and Fridovich have reported that superoxide radicals inhibit catalase enzyme and H_2O_2 suppresses the SOD activities in the cell.[20] However, the exact mechanism by which they inhibit the enzyme activities is not known. Lipid peroxidation, which can cause alteration in the membrane structure, was increased during viral infection. Supplementation with quercetin alone did not show any significant effect on lipid peroxide level. However, supplementation after viral infection showed a significant decrease in the lipid peroxide level. This substantially supports the earlier report that quercetin prevents capillary fragility from free radical damage. Formazan positive cells (F+) were increased during viral infection, which indicates the increased production of superoxide radicals by activated macrophages. The hyperactivated phagocytic cells, which produce free radicals,[21] might be a prime cause for epithelial clearance in the trachea and haemorrhage in the lungs, which are the major symptoms during influenza viral infection. The number of F+ cells in the viral infected group was almost double than in the control group. Administration of quercetin decreased their number, which suggests that quercetin scavenges the superoxide radicals and thereby protects cells from oxidative damage. Several studies are now in progress to understand the metabolism and mechanism of action of quercetin in several pathological conditions.[22–25] From the present data we conclude that quercetin is effective in alleviating the oxidative stress induced during influenza virus infection.

Acknowledgements. Mr TA Narasa Raju is grateful to the University Grants Commission, India for providing financial support to carry out this work.

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