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. 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$_2$O$_2$), which in turn causes tissue damage by either directly oxidizing cellular biomolecules including lipids, proteins and nucleic acids or by activating certain nucleases or proteases.

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 and an increase in the levels of xanthine oxidase, an enzyme which generates superoxide radicals. 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. 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. Quercetin has been shown to scavenge superoxide radicals and protect from lipid peroxidation and chelate metal ions to form inert complexes. Warren et al. 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.

Materials and methods

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

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

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 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.

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 H₂O₂ 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, in the supplemented group the activities were restored to 83% and 81%, respectively.

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-
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. reported that influenza virus infections result in a decrease in the endogenous concentration of antioxidants 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. 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 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.

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 \( \text{H}_2\text{O}_2 \), 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 \( \text{H}_2\text{O}_2 \) suppresses the SOD activities in the cell. 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, 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. From the present data we conclude that quercetin is effective in alleviating the oxidative stress induced during influenza virus infection.

**Table 1. Lipid peroxidation (TBARS assay)**

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<th>CON</th>
<th>INF</th>
<th>QT</th>
<th>IQT</th>
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<tr>
<td>Lipid peroxidation</td>
<td>160 ± 7.6</td>
<td>251 ± 2.8</td>
<td>168 ± 3.0</td>
<td>207 ± 4.8</td>
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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.

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

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<tr>
<td>Formazan positive cells per 200 cells</td>
<td>63.6 ± 7.5</td>
<td>114.5 ± 4.1*</td>
<td>74.6 ± 9.7</td>
<td>91.3 ± 5.7</td>
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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**

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<tr>
<td>SOD Units/mg protein</td>
<td>13.02 ± 1.2</td>
<td>10.4 ± 0.89*</td>
<td>12.93 ± 1.6</td>
<td>14.5 ± 1.38</td>
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<tr>
<td>Catalase Units/mg protein</td>
<td>30.4 ± 2.4</td>
<td>26.5 ± 2.9*</td>
<td>30.38 ± 1.15</td>
<td>29.4 ± 2.5</td>
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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.
References


