PROTECTIVE EFFECT OF POLYPHENOL-RICH EXTRACT ON ACUTE LUNG INJURY IN INFLUENZA VIRUS INFECTED MICE

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ABSTRACT
We have followed the anti-oxidant effects of a plant polyphenol-rich extract (PC) in the lungs of albino mice in the experimental influenza A/Aichi/2/68 (H3N2) (A/Aichi) virus infection. The effect of PC on the superoxide (O₂⁻) and peroxide (H₂O₂) production from alveolar macrophages (aMØ) and on the lung antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) was studied. We also investigated the effect on the lipid peroxidation (LPO) and the total antioxidant activity (TAOA) in the lung tissue. All of the mentioned effects were studied in parallel with the protection on mortality rates and lung virological parameters on days 2, 6 and 9 after the viral challenge. It was shown that the extract significantly restored and stimulated the antioxidant activities in the lungs of influenza virus (IV)-infected mice. The protective effect of PC in the experimental influenza virus infection (EIVI) was related to both the specific antiviral effect of the extract and its antioxidant activity.

Keywords: plant polyphenol extract, influenza virus infection, lung injury, reactive oxygen species, lung antioxidant enzymes, protective effect

Abbreviations: a.i.: after inoculation; aMØ: alveolar macrophage; CAT: catalase; CH: control healthy; CPE: cytopathogenic effect; EIVI: experimental influenza virus infection; H₂O₂: hydrogen peroxide; i.n.: intranasal; IP: index of protection; IV: influenza virus; LD: lethal doses; LPO: lipid peroxidation; M: mortality; MDA: malondialdehyde; MDCK: Madin-Darby canine kidney; MST: mean survival time; NO: nitric oxide; O₂⁻: superoxide; PBS: phosphate buffered saline; PC: polyphenolic complex; p.i.: post infection; RP: ratio of protection; SOD: superoxide dismutase, TAOA: total antioxidant activity; TBARS: thiobarbituric acid reactive products; TCID: tissue culture infectious doses; VC: virus control; VIM: virus-infected mice;

Introduction

Previous investigations proved that a semi-standardized polyphenol-rich extract, obtained from the medicinal plant Geranium sanguineum L. (Geraniaceae), exhibited a pronounced anti-influenza virus effect in vitro and in vivo (18). The virus-inhibitory effect of the preparation in cell cultures was specific and selective (16). However the investigations showed that the in vitro antiviral activity was fairly modest (selectivity indices=6.1-29.1) and this was in contrast with the marked protection in the murine EIVI (indices of protection=64–100%). Thus the therapeutic effect of PC needed explanation. We presumed that it might be attributed to a combination of more than one biological activity, known for natural polyphenols. PC exhibited a stimulating effect on the phagocytic activity of murine blood polymorphonuclear lymphocytes and peritoneal macrophages and showed a beneficial effect on the spontaneous nitric oxide (NO) production by the peritoneal and alveolar macrophages (23). It also possessed antioxidant and radical scavenging capacities (22) and inhibited the proteolytic activity of trypsin (20). Further, in the course of EIVI we demonstrated that in addition to its selective virus-inhibitory activity, PC interfered with the infection alternatively through potentiation and restoration of the host immune response (7), regulation of the host lung protease activities (20), exhibition of antioxidant and radical scavenging properties (9, 12, 24).

The aim of the present study was to examine furthermore the anti-oxidant effects of PC in the lungs of influenza virus infected mice.

Materials and Methods

Plant material
Geranium sanguineum L. (Geraniaceae) has been introduced into the experimental field of the Institute of Botany, Bulgarian Academy of Sciences, Sofia and a voucher specimen was deposited in the Herbarium of the same institute (SOM 5/86). The extract was prepared as described previously (19) and kindly provided by Dr. S. Ivancheva, Institute of Botany, Bulgarian Academy of Sciences.

Mice
Male and female (16-18 g), inbred ICR mice were obtained from the Experimental Animal Station, Bulgarian Academy of Sciences, Slivnitsa. They were quarantined 24 h prior to use and maintained on standard laboratory chow and tap water ad libitum for the duration of the studies.

Chemicals were purchased from Sigma-Aldrich Chemie GmbH, Fluka and Merck, Germany.

Preparation of lung tissue homogenates from mice
On days 2, 6 and 9 p.i. 3 mice of each group were anaesthetized with ether and exsanguinated by section of the subclavian...
arteries. Lungs were perfused in situ with ice-cold 1.15% KCl and homogenized at 4°C in 0.1 M KNa-phosphate buffer, pH 7.4. The homogenates were centrifuged (9000 rpm, 30 min, 4°C) and the supernatants were examined for SOD and CAT activities, TBARS and TAOA.

Alveolar macrophages (aMØ) were collected on days 2, 6, 9 p.i. as described in (23).

O$_2$ generation and H$_2$O$_2$ production were performed as previously described (24).

SOD and CAT activities were evaluated as described before (9).

TAOA and TBARS were determined as in (9).

Protein contents of the samples were estimated according to (10).

Cells, Media and Viruses

Madin-Darby canine kidney (MDCK) cells were passaged and cultivated as described before (20). MDCK cells were provided by Mrs. I. Roeva, Institute of Microbiology, Bulgarian Academy of Sciences.

Viral infection

The infection was induced under light ether anaesthesia by intranasal (i.n.) inoculation of A/Aichi, adapted to mice lungs with infectious titre 10$^7$ TCID$_{50}$/ml (50% tissue culture infectious doses/ml). The virus was maintained by passages in mice lungs; virus stock was kept at -80°C. It was from the collection of the Institute of Microbiology, Bulgarian Academy of Sciences. To induce lethal infection, mice were challenged with 10 LD$_{50}$ (50% lethal doses) in the volume of 0.05 ml phosphate buffered saline (PBS)/mouse.

Experimental design

Mice were separated in 4 experimental groups: mock-infected and PBS-treated – control healthy (CH), mock-infected and PC-treated (PC), IV-infected and PBS-treated - virus control (VC), IV-infected and PC-treated (VC+PC). PC (10 mg/kg in the volume of 0.05 ml PBS) was administered by i.n. instillation 3 h before the infection under light ether anaesthesia. Two additional groups of 12 animals each (VC and VC+PC) were observed for death daily for 14 days post infection (p.i.) (Table 1). After the end of the experiments surviving mice were sacrificed by cervical dislocation under ether anaesthesia.

To determine infectious lung parameters 3 animals from each experimental group were sacrificed on days 2, 6 and 9 p.i., with their lungs being removed aseptically and lung consolidation (score) scored from 0 – normal to 4 - 100% consolidation (Table 1). Lungs were homogenized to 10% suspensions in PBS and ten-fold dilutions (0.2 ml) were assayed for infectivity in MDCK cells. Virus-induced cytopathogenic effect (CPE) was used as a measure of viral replication and scored as in (16). Infectious virus titres were evaluated and expressed in log$_{10}$ TCID$_{50}$/ml. The protective effect was estimated by the reduction of the rate of mortality, the increase of the index of protection (IP) and prolongation of mean survival time (MST) as described before (17). IP was determined from the equation (PR-1)/PRx100, where PR (ratio of protection) was M$_{control}$/M$_{experiment}$, where M was mortality.

Statistical methods

Results from the in vivo experiments are given as arithmetic mean values. Every experimental group was compared with the mean value of CH groups. The results were analyzed statistically by the one-way analysis of variance (ANOVA). Student’s $t$-test was used to evaluate differences in lung scores and lung virus titres. $p<0.05$ was accepted for statistical significance.

Results and Discussion

Influenza is a highly contagious, acute respiratory disease, which remains one of the most serious problems of public health. The pathogenesis of the infection is determined by the development of mechanisms of injury, causing profound changes in tissue metabolism and host response, i.e. rupture of the cell membrane, impairment of the receptor system, overreaction of the immune response etc. An important aspect of the pathogenesis is the so-called “respiratory burst” (3). ROS complicate local inflammation and thus contribute to pulmonary tissue damage, hypoxia and toxicosis (1). To protect themselves against toxic free radicals the organisms

<table>
<thead>
<tr>
<th>Experimental group (PC, 10 mg/kg)</th>
<th>Day p.i.</th>
<th>N survival/total</th>
<th>Lung parameters</th>
<th>Score±SD</th>
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<td>Infectious titre±SD</td>
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<td>(log$<em>{10}$ TCID$</em>{50}$/ml)</td>
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<td>VC$^1$</td>
<td>2</td>
<td>20/20</td>
<td>2.4±0.4</td>
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<td>VC+ PC$^2$</td>
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<td>16/20</td>
<td>5.1±0.3</td>
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<td>VC</td>
<td>9</td>
<td>7/20</td>
<td>4.7±0.5</td>
<td>2.7±0.5</td>
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<td>VC+ PC</td>
<td>8/10</td>
<td>3.1±0.3***</td>
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<td>VC$^1$</td>
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p.i., post infection; 1: virus control; 2: polyphenolic complex; ** $p<0.01$, ***$p<0.001$

| Table 1 |

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have developed defence systems against the damaging effect of ROS, including the enzymes SOD, CAT and glutathione peroxidase, as well as some low molecular substances (5). In addition a significant increase of LPO products in the blood, lungs and livers of infected animals has been observed (4, 11). Thus it seems reasonable that the treatment with antioxidants could be beneficial for the control of influenza virus infection (4, 11). Plant polyphenols have aroused considerable interest because of their broad pharmacological potential. Most of their properties have been ascribed both to the inhibition of enzymes involved in the production of ROS and to their free radical scavenging and iron chelating capacities (6). Plant polyphenols, acting as antioxidants, were shown to protect mice during IV-induced oxidative stress (14).

We have demonstrated previously that the semi-standardized polyphenol-rich extract, obtained from *G. sanguineum* L. inhibited IV replication *in vitro* and exhibited a pronounced protective effect *in vivo* (7, 12, 18, 20). Phytochemical analysis of PC showed that the total polyphenolic content of the extract was 167.8 μg/ml; the extract contained tannins (34%), flavonoids (0.17%), catechins and proanthocyanidines (2 mg/kg). The identification of individual compounds showed that flavonoids - aglycones and glycosides (quercetin, quercetin 3-0-galactoside, morin, myricetin, kaemperol, rhamnasion, retusin, apigenin), phenolic acids (caffeic, ellagic, quinic, chlorogenic), gallotannins and catechins were present (13). Most of the biologically active compounds, identified in the preparation, belong to chemical groups, known both as inhibitors of viral growth (18) and as antioxidants (15). It has been reported that PC possessed antioxidant and radical scavenging capacities in model systems (12, 22). In addition the decrease in the oxidative stress response as a result of the preventive treatment with PC in the EIVI has been observed in different models of oxidative stress: increase of TAOA in the lungs of VIM and reduction of liver SOD and CAT elevated levels to normal (9), restoration of the number and functions of aMØ and decrease of the augmented generation of NO both *in vitro* and *in vivo* (23, 24). All these considerations encouraged more profound investigations on the anti-oxidant effects of PC in the EIVI in mice. Here we present the assessment of the effect of the plant extract on the levels of MDA formation as a measure of LPO in the lungs is presented in Fig. 6 and the experimental data on the effect of PC on the TAOA are shown in Fig. 5.

The obtained results presented a further confirmation of the free-radical character of EIVI (1, 11). Elevated MDA concentrations as markers of the oxidative stress (114-200% of CH, CH being 0.64±0.33 nM/mg protein) correlated proportionally with reduced TAOA levels (60-68% of CH, CH being 1.34±0.15 nM/L). The preventive PC-treatment lead to a significant modulatory effect on both increased LPO- and decreased TAOA levels.

The presented data clearly describe the extract from *G. sanguineum* L. as a preparation of marked antioxidant potential, which significantly restores and stimulates the antioxidant activities in the lungs of influenza A/Aichi VIM. This effect of PC is of special interest since lungs are the target organs of influenza virus infection; from the other side the lungs of adult animals are highly vulnerable to oxidative stress because of their inability to augment antioxidant enzymes activity (21).
Conversely we demonstrated that PC showed a moderate prooxidant character in the intact animals: the extract caused an increase of the $\text{O}_2^-$ generation on day 6 after inoculation (a.i.) (Fig. 1) and of $\text{H}_2\text{O}_2$ production on day 2 a.i. (Fig. 2); a slight reduction of SOD and CAT activities was found on day 2 a.i. (Fig. 2, Fig. 3); the level of MDA was increased on day 6 a.i. (Fig. 5) and a transitory decrease of TAOA was registered on day 2 a.i. (Fig. 6). It is tempting to speculate that the prooxidant capacity of the plant extract, exerted at the early stages of infection, could be part of the non-specific
defensive reaction of the organism before the development of the specific immune response. It has been reported that plant polyphenols are naturally occurring antioxidants but they also exhibit prooxidant properties under certain conditions. Although known for their antioxidant activities, rutin and quercetin exhibit prooxidant effects in healthy animals (14).

Collectively our results suggest that PC may act not only as an antioxidant, but also as a prooxidant. Both the prooxidant as well as the antioxidant effects could be an important mechanism of the host response modulation in the experimental influenza virus infection.

An additional experiment established that the preventive PC-treatment of VIM lead to a significant reduction of mortality rates (IP=77.8%) and marked prolongation of MST (+5.2 days). Lung infectious virus titres (Δlog_{10} TCID_{50}/ml=2.2–3.2), lung weights and lung indices were all reduced; lung lesions as shown by macroscopic and microscopic examination (nor presented) were markedly alleviated.

Conclusions
The obtained results outlined the antioxidant and radical scavenging properties of the plant extract; PC beneficially modulated the oxidative stress response in IV-induced pneumonia. These effects were consistent with a prolongation of MST and reduction of mortality rate, infectious virus titre and lung consolidation. This alternative mechanism of action might contribute to the overall protective effect in the lethal A/ Aichi EIVI in mice. The presented results correspond to our intensive studies on the mode of the protective effect of the plant extract which show positively that the protection may possibly be attributed to the combination of more than one biological activities of the preparation.

Influenza virus, like other viruses, depends on its host cell and thus cellular functions and mechanisms, essential for viral replication might be suitable targets for antiviral therapy. As a result viral growth could be affected independent of the type, strain and antigenic properties of the invading virus.

Taken together, our findings provide evidence that the therapeutic effect of the plant preparation could be attributed to the inhibition of the viral replication as well as to the normalization of the functional-metabolic disorders in the infected cells.

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REFERENCES