A Fungal Cu/Zn-Containing Superoxide Dismutase Enhances the Therapeutic Efficacy of a Plant Polyphenol Extract in Experimental Influenza Virus Infection

Julia Serkedjieva*, Tsvetanka Stefanova, and Ekaterina Krumova

Institute of Microbiology, Bulgarian Academy of Sciences, 26, Acad. G. Bonchev St., 1113 Sofia, Bulgaria. Fax: +359 2 870 01 09. E-mail: jserkedjieva@microbio.bas.bg

* Author for correspondence and reprint requests

Z. Naturforsch. **65 c**, 419–428 (2010); received December 9, 2009/January 13, 2010

The combined protective effect of a polyphenol-rich extract, isolated from *Geranium sanguineum* L. (PC), and a novel naturally glycosylated Cu/Zn-containing superoxide dismutase, produced from the fungal strain *Humicula lutea* 103 (HL-SOD), in the experimental influenza A virus infection (EIVI) in mice, induced with the virus A/Aichi/2/68 (H3N2), was investigated.

The combined application of HL-SOD and PC in doses, which by themselves do not defend significantly mice in EIVI, resulted in a synergistically increased protection, determined on the basis of protective indices and amelioration of lung injury. Lung weights and consolidation as well as infectious lung virus titers were all decreased significantly parallel to the reduction of the mortality rates; lung indices were raised. The excessive production of reactive oxygen species (ROS) by alveolar macrophages (aMØ) as well as the elevated levels of the lung antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT), induced by EIVI, were brought to normal. For comparative reasons the combined protective effect of PC and vitamin C was investigated. The obtained results support the combined use of antioxidants for the treatment of influenza virus infection and in general indicate the beneficial protective role of combinations of viral inhibitors of natural origin with diverse modes of action.

Key words: Plant Polyphenol Extract, Fungal Cu/Zn-Containing SOD, Influenza Virus Infection

Introduction

Influenza is a major public health problem in the industrialized world because of its wide spread, high morbidity rate, and considerable social and economic implications. Influenza remains an important epidemic viral infection with the potential to cause pandemics. Recently, a novel H1N1 influenza virus spread fast in the human population, and the resulting pandemia has already proved to be a significant and very costly cause of mortality and morbidity in the human population. Because recently available vaccines are not completely protective against influenza virus infection and no specific therapy of proven value currently exists for its severe forms, there is large interest in the development of new strategies for its control. The data on the combined inhibitory activity of natural and synthetic antiviral agents, though scarce, suggest that this could be a promising approach to the control of viral infections (Kurokawa *et al*., 1995; Musci *et al*., 1992; Weaver and Arou, 1998) and may be used successfully to increase the antiviral efficacy of the individual compounds. Our group has a substantial experience in this field of research (Gegova *et al*., 1993; Serkedjieva, 2000; Serkedjieva and Ivanova, 1997; Serkedjieva and Zgorniak-Nowosielska, 1993; Serkedjieva *et al*., 2003).

We have studied intensively the mode of the anti-influenza virus activity of the semi-standardized polyphenol-rich extract isolated from *Geranium sanguineum* L. (PC). It was shown that its *in vitro* virus-inhibitory effect was specific and selective. PC affected the synthetic stages of A/Rostock viral replication; virus-specific RNA and protein synthesis were selectively inhibited (Serkedjieva and Hay, 1998). We have demonstrated that the plant preparation markedly protected mice from mortality in the murine experimental influenza virus infection (EIVI) (Ivanova *et al*., 2005; Serkedjieva *et al*., 2007; Murzakhmetova *et al*., 2008). PC interfered with the infection alternatively through enhancement and restoration of the host im-

0939–5075/2010/0500–0419 $ 06.00

mune response (Ivanova *et al*., 2005), regulation of the host lung protease activities (Serkedjieva *et al*., 2007), and exhibition of antioxidant and radical scavenging properties (Murzakhmetova *et al*., 2008). The variety of biological activities of the plant extract was related to the presence of large quantities of potential bioactive compounds, mainly polyphenols (Pantev *et al*., 2006).

The findings that reactive oxygen species (ROS) play an important role in influenza infection pathogenesis (Akaike *et al*., 1996) suggest that the use of exogenic superoxide dismutase (SOD) could be a new approach for the treatment of the disease (Oda *et al*., 1989). We have established that Cu/Zn-containing SOD from *Humicula lutea* 103 (HL-SOD) increased the survival rate and prolonged the survival time in EIVI (Angelova *et al*., 2001). It should be noted that HL-SOD is a naturally glycosylated enzyme, which could be isolated in few cases only. This characteristic is very important with regard to its *in vivo* pharmacological activity; in all probability its half life in plasma and blood is prolonged. The combined application of HL-SOD with rimantadine hydrochloride resulted in a synergistic amplification of the protective effect (Serkedjieva *et al*., 2003).

The aim of the present study was to investigate the combined protective effect of PC and HL-SOD on the lung injury of mice, induced by EIVI, as well as the combined effect on the production of ROS by alveolar macrophages (aMØ) and on the lung antioxidant enzymes SOD and catalase (CAT).

Material and Methods

Compounds

The medicinal plant *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 (N SOM 5/86). The preparation of the extract has been described in detail before (Serkedjieva and Hay, 1998). The polyphenol content of PC was controlled by thin layer chromatography and by quantitative determination of tannins, flavonoids, and catechins (Pantev *et al*., 2006).

Ascorbic acid (vitamin C, vit C) and trypsin were purchased from Sigma-Aldrich Chemie GmbH (Diesenhofen, Germany). Rimantadine hydrochloride (Rim) was obtained from Hoffman-La Roche Inc. (Nutley, NJ, USA).

Microorganism, cultivation and equipment

The fungal strain *Humicula lutea* 103 was used throughout and maintained at 4 ºC on beer agar, pH 6.3. Cultivation was performed in a 3-l bioreactor ABR-09, developed by CLBA, Bulgarian Academy of Sciences, Sofia. The composition of the culture media was as described earlier (Angelova *et al*., 2001). The preparation of the cell-free extract, analysis, purification, and characterization of HL-SOD were as described in Angelova *et al*. (2001).

Cell cultures and virus

Madin-Darby canine kidney (MDCK) cells were provided by Dr. I. Roeva, Institute of Microbiology, Bulgarian Academy of Sciences, Sofia and were passaged as described in Serkedjieva *et al*. (2007). The human influenza virus A/Aichi/2/68 (H3N2), adapted to mice lungs and maintained by passages in mice lungs and fertile hen's eggs was used (A/Aichi). The viral infectious titer was 10^5 $TCID_{50}$/ml (50% tissue culture infectious doses/ml), the hemagglutination titer was 1024. The viral stocks were stored at –80 ºC.

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. Experiments with animals were indispensable in investigations, concerning the treatment of influenza infection and the evaluation of their oxidative status. The number of experimental animals was reduced as much as possible, depending on statistical significance. Refinement of the tests with animals was achieved by careful planning of multifactor experiments. The animals were bred under standard conditions, accepted by the Bulgarian Veterinary Health Service. Specialized personnel took care of their welfare.

Mouse lung extracts

On days 6 and 9 post infection (p.i.) 3 mice of each group were anaesthetized with diethyl ether and, exsanguinated by section of the subclavian

arteries. Lungs were removed aseptically, washed in cold phosphate buffered physiological saline (PBS) and blotted dry. Tissue pieces of about 1 g were disintegrated mechanically in ice-cold PBS and subsequently by an ultrasound disintegrator (MSE, Sheffield, England) for 3 min (interruption of sonication every 15 s). The homogenates were centrifuged (9,000 × *g*, 30 min, 4 ºC) and the supernatants were examined for SOD and CAT activities.

The SOD activity was assessed by the nitro blue tetrazolium (NBT) reduction method (Beauchamp and Fridovich, 1971). The reaction mixture contained 56 mM NBT, 0.01 M methionine, 1.17 mM riboflavin, 20 mM NaCN, and 0.05 M phosphate buffer, pH 7.8. Superoxide was measured through the increasing absorbance at 560 nm at 30 ºC after 6 min of incubation from the beginning of illumination. One unit of specific SOD activity was defined as the amount of enzyme protein required for inhibition of the reduction of NBT by 50% (A_{560}) and was expressed as units/mg of protein (U/mg protein).

The CAT activity was determined by monitoring the decomposition of 18 mM H_2O_2 at 240 nm (Beers and Sizer, 1952). One unit of activity was that, which decomposed 1 mM of H_2O_2/min/mg protein at 25 ºC and pH 7.0. The specific activity was given as U/mg protein.

Protein contents of the samples were estimated according to Lowry *et al.* (1951).

aMØ were collected on days 6 and 9 p.i. by 5 washings of the broncho-alveolar cavity of 6 mice with 1 ml ice-cold HBSS. The phenotype of the cells was determined by light microscopy of cell populations immediately after being obtained.

The superoxide anion (O_2^-) release was measured using the SOD-inhibitable reduction of ferricytochrome *c* as previously described (Serkedjieva *et al.*, 2007). Cytochrome *c* reduction was measured at 550 nm in an ELISA reader (Organon Teknika, Salzburg, Austria). The amount of O_2^- produced per well was determined as the difference in cytochrome *c* reduction between wells without and with SOD.

The H_2O_2 assay was based on horseradish peroxidase-mediated oxidation of phenol sulfonphthalein (phenol red) and was performed as previously described (Toshkova *et al.*, 2006). The reaction was read in an ELISA reader at 610 nm. H_2O_2 concentrations were calculated using a standard curve (Werner, 2003).

Viral infection and experimental design

The infection was induced under light diethyl ether anesthesia by intranasal (i.n.) inoculation of influenza virus A/Aichi. This virus causes hemorrhagic pneumonia in mice. To cause lethal infection, mice were infected with ten 50% lethal doses (LD_{50}) of the virus in a volume of 0.05 ml PBS per mouse. PC was applied nasally 3 h before viral infection in the dose 2.5–10 mg/kg. HL-SOD was inoculated in the dose 125–500 U/mouse/d either intravenously (i.v.) or intraperitoneally (i.p.) from the 4th to 7th day or from the 3rd to 6th day after viral challenge. Vit C was applied i.p. 24 and 2 h before and 24, 48, and 72 h after viral inoculation in the dose 100 mg/kg. Rim was used as a positive control (Dolin *et al.*, 1982) and was applied orally (p.o.) 24 and 2 h before and 24, 48, and 72 h after viral challenge in the dose 40 mg/kg.

Mice were separated in 5 experimental groups: Group 1, mock-infected and PBS-treated – control healthy (CH); group 2, influenza A virus (IAV)-infected and PBS-treated – virus control (VC); group 3, IAV-infected and PC-treated (PC); group 4, IAV-infected and HL-SOD-treated (HL-SOD); group 5, IAV-infected and PC + HL-SOD-treated (PC + HL-SOD). In the experiments with vit C, group 4 consisted of IAV-infected and vit C-treated (vit C) mice and group 5 of IAV-infected and PC + vit C-treated (PC + vit C) mice. Following the challenge, mice were observed daily to monitor changes in body weight and to record death for 14 d.

Toxicity control for the combinations was run in parallel. After the end of the experiments surviving mice were sacrificed by cervical dislocation under diethyl ether anesthesia.

For the virological experiments the experimental groups consisted of 10 animals each, virus control groups (VC) consisted of 12 animals. Additional groups of 3 animals from each experimental group were sacrificed on day 7, their lungs were weighed, and lung consolidation was scored 0–normal, 1–25% consolidation, 2–50% consolidation, 3–75% consolidation, 4–100% consolidation. Mice lungs were homogenized to a 10% suspension in PBS, and ten-fold dilutions were assayed for infectious virus in MDCK cells. Virus titers were expressed as log 50% tissue culture infectious doses/0.2 ml (log $TCID_{50}$/0.2 ml) (Reed and Muench, 1938). Virus controls were as described above.

The protective effects of PC, HL-SOD, Rim, and the combinations PC + HL-SOD and PC + vit C were estimated by the reduction of lung virus infectious titers, lung consolidation, lung weights, lung indices, and rates of mortality, the increase of indices of protection and prolongation of mean survival times (MST) as described in Serkedjieva and Ivanova (1997). The index of protection (PI) was determined according to the equation PI = (PR – 1)/PR · 100, where PR (ratio of protection) is $M_{control}/M_{experiment}$ and M is the mortality. The combined effect was evaluated according to Webb (1966). The effect of the combination ($E_{1,2} = PI_{1,2}/100$) and the effects of the individual substances ($E_1 = PI_1/100$ and $E_2 = PI_2/100$) are related in the equation $E_{1,2} = E_1 + E_2 - E_1 \cdot E_2$; the combined effect is synergistic if $E_{1,2} >$, additive if $E_{1,2} =$, and antagonistic if $E_{1,2} < E_1 + E_2 - E_1 \cdot E_2$.

Statistical methods

Results from *in vivo* experiments are given either as arithmetic mean values or their ratios from 2–4 experiments. For the biochemical parameters, 5–7 measurements were made for every sample. Every experimental group was compared with the mean value of the group of healthy animals on the respective day of investigation. The results were analyzed statistically by the one-way analysis of variance (ANOVA). The two-tailed Student's *t*-test was used to evaluate differences in lung weights, lung virus titers, and lung scores. Fisher's exact test was used for comparison of the mortality rates. $p < 0.05$ was accepted for statistical significance.

Results

Intranasal inoculation of the A/Aichi virus to mice produced a damaging infection of the lungs which, depending on the dose of the viral inoculum, was highly lethal to the animals. The experiments were carried out in conditions of 70–80% mortality of virus control (5 LD_{50}). The pattern of the development of viral infectivity in the lungs of virus-infected mice (VIM) clearly showed that infectious titers were markedly reduced during the whole period of observation by the combined application of PC and HL-SOD (Fig. 1).

The combined use of HL-SOD and PC in doses, which by themselves were ineffective or with low potency (1/4–1/8 of the effective doses), led to a substantial growth of survival and resulted in increased protective effects, determined on the basis of the indices of protection. The calculated enhancement was of the synergistic type. On day 6 p.i., at the peak of infection, all infectious parameters – lung consolidation, lung virus titers, lung weights, mortality rates of infected animals – were reduced significantly; lung indices were raised, and survival times markedly increased by drug combinations (Table I). This tendency was observed also on day 9 p.i., the convalescent stage of infection. The combinations were well tolerated by the experimental animals and the improved protection was not associated with increased toxicity.

For comparative reasons the combined protective effect of PC and vit C, possessing antioxidant

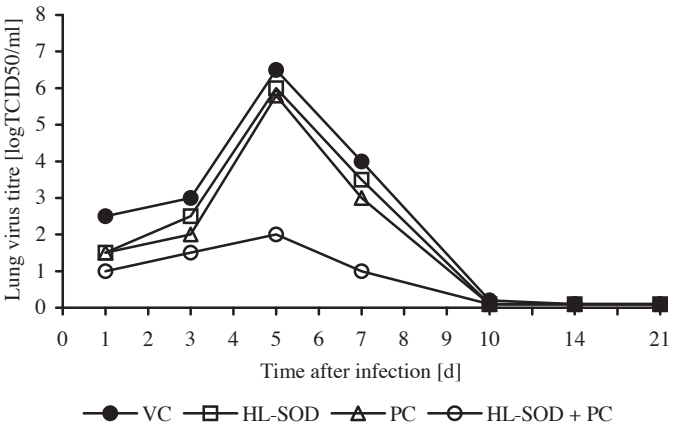

Fig. 1. Infectious virus titers in the lungs of mice, infected with A/Aichi and treated with PC, HL-SOD, and their combination. PC, 2.5 mg/kg; HL-SOD, 125 U/mouse/d, applied i.p. as described in Materials and Methods; VC, virus control.

Table I. Combined protective effect of PC and HL-SOD in EIVI in mice.

Group	Dosage	Protection index[a] (%)	Combined effect[b]	Lung parameters				
				Day p.i.	Weight [g]	Lung index[c] (%)	Score[d]	Virus titer [log $TCID_{50}$/ml]
Virus control				6	0.25	1.18	3.5	6.7
				9	0.2	1.08	3.5	2.5
PC[e]	10.0	82.4		6	0.22	1.0	2.0	2.1
				9	0.21	0.98	1.0	1.3
PC1[e]	2.5	14.6[i]		6	0.24	1.22	3.0	6.5[i]
				9	0.22	1.17	2.5	2.5[i]
PC2[e]	1.25	0		6	0.21	1.2	4.0	6.7[i]
				9	0.25	1.2	3.5	3.5[i]
HL-SOD[f]	500.0	87.5		6	0.3	1.0	2.3	6.1[i]
				9	0.21	1.1	0.5	2.3[i]
HL-SOD1[f]	125.0	21.7[i]		6	0.23	1.17	4.0	6.2[i]
				9	0.22	1.2	3.5	3.0[i]
HL-SOD2[g]	500.0	23.1[i]		6	0.26	1.2	3.5	6.3[i]
				9	0.23	1.12	3.0	2.3[i]
HL-SOD3[g]	250.0	16.5[i]		6	0.25	1.15[i]	3.0	6.5[i]
				9	0.23	1.12	2.5	2.5[i]
PC1 + HL-SOD1	2.5 + 125	76.4	synergistic	6	0.21	0.98	1.0	2.0
				9	0.22	1.0	1.0	1.2
PC1 + HL-SOD3	2.5 + 250	64.5	synergistic	6	0.24	1.0	0.5	1.0
				9	0.22	1.02	0.25	1.3
PC2 + HL-SOD3	1.25 + 250	52.6	synergistic	6	0.26	1.1	1.0	3.7
				9	0.22	0.9	1.0	2.8
Rimantadine[h]	40.0	87.9		6	0.24	1.02	0.5	1.0
				9	0.21	0.98	0.5	0.5
Healthy control					0.21	1.0		

[a] (PR-1)/PR · 100, where PR (protective ratio) is $M_{control}/M_{experiment}$ and M is the mortality. Mortality in the VC group was 84.2%.
[b] The combined effect is synergistic if $E_{1,2}$ >, additive if $E_{1,2}$ = , and antagonistic if $E_{1,2} < E_1 + E_2 - E_1 \cdot E_2$. $E_{1,2} = E_1 + E_2 - E_1 \cdot E_2$, where $E_{1,2} = PI_{1,2}/100$, $E_1 = PI_1/100$, and $E_2 = PI_2/100$.
[c] Lung weight/body weight · 100.
[d] Scores 0–4, assigned to % visible consolidation.
[e] mg/kg, administered i.n. 3 h before viral infection.
[f] U/mouse/d, administered i.v. 4–7 d after viral infection.
[g] U/mouse/d, administered i.p. 3–6 d after viral infection.
[h] mg/kg, administered p.o. 24, 2 h before and 24, 48, 72 h after viral infection.
[i] The difference to virus control is not significant ($p < 0.05$).

properties, was investigated. The results are presented in Table II. An enhancement of protection of the synergistic type was observed when vit C and PC were applied in the doses 2.5 and 100 mg/kg, respectively. The MST was prolonged to 3.9 days. The combination of 100 mg/kg vit C and 1.25 mg/kg PC was indifferent and in any case the achieved survival rate was not significantly different from that of VC. All lung parameters were ameliorated (Table II).

Furthermore we investigated the effect of a selected PC + HL-SOD combination (2.5 mg/kg and 125 U/mouse/d) on oxidative stress response. As a first approach we studied the effect of the individual components and their combination on ROS generation from aMØ of VIM. EIVI induced an about 1.6-fold increase of O_2^- production on day 6 p.i. and an 1.8-fold increase on day 9 p.i., CH being (3.04 ± 0.09) nM $O_2^-/10^6$ cells for 1 h (Fig. 2A). While PC treatment did not affect the generation of O_2^-, HL-SOD suppressed their accumulation. The superoxide release in VIM after PC + HL-SOD treatment was reduced and was brought to normal levels on both days of obser-

Table II. Combined protective effect of PC and vitamin C in EIVI in mice.

Group	Dosage	Protection index[a] (%)	Combined effect[b]	Lung parameters				
				Day p.i.	Weight [g]	Lung index[c] (%)	Score[d]	Virus titer [log $TCID_{50}$/ml]
Virus control				6	0.24	1.2	4.0	6.0
				9	0.24	1.18[i]	4.0	2.7
PC1	2.5[e]	12.8[i]		6	0.25	1.15[i]	2.0	4.5
				9	0.23	1.12	2.0	3.0[i]
PC2	1.25[e]	0.0		6	0.24	1.22[i]	3.5[i]	6.5[i]
				9	0.25	1.15	3.0	2.5[i]
Vitamin C	100.0[f]	26.5[i]		6	0.21	1.05	2.5	4.5
				9	0.21	1.05	2.0	3.1[i]
PC1 + vitamin C	2.5 + 100	53.5	synergistic	6	0.28	1.02	1.5	3.0
				9	0.21	1.05	1.0	1.8
PC2 + vitamin C	1.25 + 100	26.7[i]	indifferent	6	0.22	1.1	2.5	4.5
				9	0.21	1.05	2.0	3.2[i]

[a–e, i] As in Table I. Mortality in the VC group was 75.5%.
[f] mg/kg, administered i.p. 24, 2 h before and 24, 48, 72 h after viral infection.

vation. EIVI triggered also a marked enhancement of H_2O_2 production (Fig. 2B), peaking on day 6 p.i. [224% of CH, CH being (4.4 ± 0.12) nM $H_2O_2/10^6$ cells]. While PC did not change significantly the level of H_2O_2 production at both time points, HL-SOD was more effective on day 6 p.i. PC + HL-SOD treatment noticeably reduced the excessive discharge, induced by EIVI, and led to normalization of the H_2O_2 levels.

Furthermore we inspected the effect of PC, HL-SOD, and their combination on the lung antioxidant enzymes SOD and CAT. EIVI induced a slight increase of SOD activity on day 6 p.i., and its maximum was reached on day 9 p.i. [150% of CH, CH being (6.8 ± 1.06) U/mg protein] (Fig. 3A). The maximum of CAT activity induced by EIVI was measured on day 6 p.i. [148% of CH, CH being (4.4 ± 0.55) U/mg protein]; on day 9 p.i. no raise of CAT levels was registered (Fig. 3B). PC + HL-SOD treatment led to normalization of the enzyme levels.

Discussion

It has been shown that EIVI is accompanied by profound changes in cell/tissue metabolism, which lead to intensive generation of ROS (Akaike *et al.*, 1996). ROS are known to play a dual role

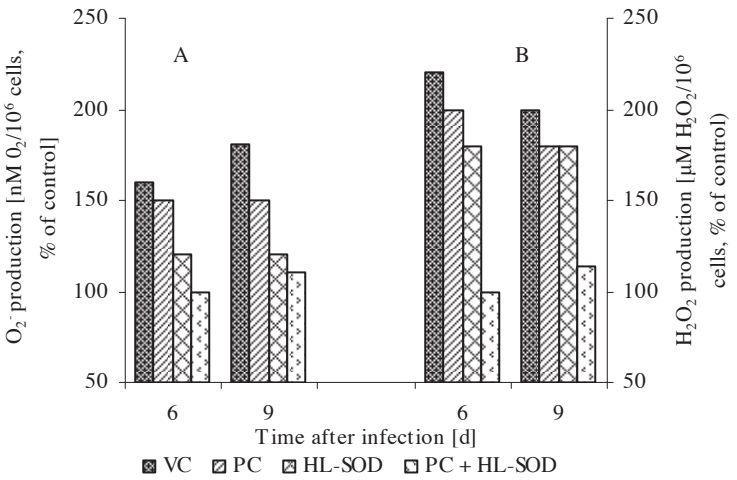

Fig. 2. (A) O_2^- and (B) H_2O_2 production in aMØ of influenza VIM, treated with PC, HL-SOD, and their combination. PC, HL-SOD, VC as in Fig. 1.

during influenza infection. They have a part in pulmonary tissue damage, hypoxia, and toxicosis due to their toxic properties; on the other hand, they can induce apoptosis in virus-infected cells, thus contributing to the decrease in the total viral load. In this way ROS offer the first line of defense against infection, preceding the alternative defense mechanisms of the organism (Arora and Houde, 1992). The presented results together with our previous data (Murzakhmetova *et al.*, 2008) show that influenza A/Aichi virus infection is accompanied with deficits in antioxidant enzyme activities and an increase in ROS and lipid peroxidation (LPO) levels in the lungs, livers, and sera, which can be overcome through supplementation with antioxidants. Plant polyphenols acting as antioxidants also demonstrate protective effects during IAV-triggered oxidative stress (Kumar *et al.*, 2005; Murzakhmetova *et al.*, 2008). It has been also demonstrated that treatment with SOD improves lung pathology and reduces lethality in IAV-induced pneumonia (Angelova *et al.*, 2001; Oda *et al.*, 1989).

Because of their location at the alveolar air tissue interface, the aMØ are the first line of cellular defense against infectious microorganisms that enter the lungs. Otherwise macrophages can contribute directly to IAV-induced lung pathology by releasing excessive amounts of reactive nitrogen intermediates and ROS. Recently O_2^- has been suggested to be related to the occurrence and exacerbation of interstitial pneumonia. H_2O_2 plays an important role in host defense and oxidative biosynthetic reactions. To protect themselves against toxic radicals, cells have developed a variety of antioxidant defenses. These include enzymes such as SOD, which dismutates superoxide, CAT, which converts hydrogen peroxide into water and oxygen, and glutathione peroxidase, which destroys toxic peroxides (Akaike *et al.*, 1996).

In the present paper we wanted to test the hypothesis of the advantageous combined treatment of the murine A/Aichi EIVI with a plant polyphenol-rich preparation and a novel glycosylated fungal SOD.

As a first approach we examined the effects of PC, HL-SOD, and their combination on ROS generation in aMØ. Though PC did not affect markedly the production of ROS during infection, applied in the dose of 2.5 mg/kg (Figs. 2A, B), previously we have shown that the preventive treatment with 10 mg/kg PC modulated the excessive generation of O_2^- and H_2O_2 (Toshkova *et al.*, 2006). In addition, a beneficial effect has been observed on the enhanced NO production. HL-SOD suppressed the generation of O_2^-. The combined PC + HL-SOD treatment further reduced the excessive discharge of O_2^- and even more dramatically the accumulation of H_2O_2 and lead to normalization of ROS levels (Figs. 2A, B).

We have found previously that the preventive treatment of VIM with PC (10 mg/kg), in addition to the decrease of ROS and NO generation from aMØ (Toshkova *et al.*, 2006), brought to normal the levels of malondialdehyde (MDA), used as a

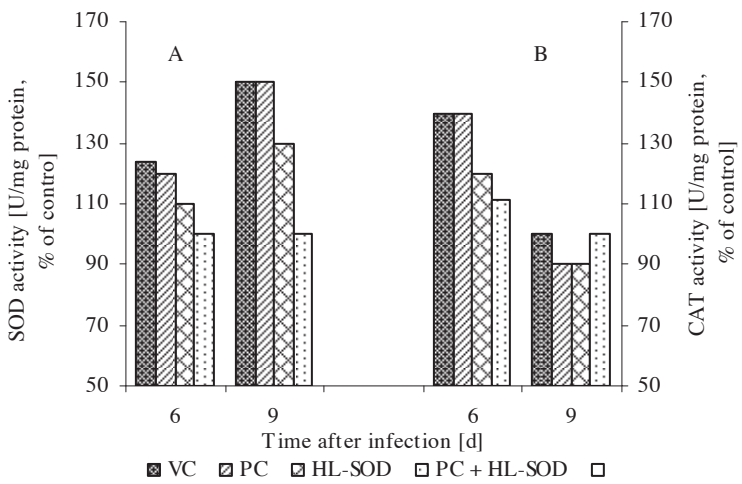

Fig. 3. (A) SOD and (B) CAT activities in the lungs of influenza VIM, treated with PC, HL-SOD, and their combination. PC, HL-SOD, VC as in Fig. 1.

biomarker of LPO) in the lungs, livers and sera of VIM at each time point of observation (Murzakhmetova *et al.*, 2008). Of special interest is the antioxidant effect of PC in the lungs of VIM since lungs are the target organs of IAV infection.

Furthermore, we inspected the effect of PC, HL-SOD, and their combination on the antioxidant enzymes SOD and CAT in the lungs of VIM. In alternative models of oxidative stress it has been observed that the failure of the host to induce enzyme expression according to its demand resulted in degeneration and cell death (Semrau *et al.*, 1998). EIVI induced a slight increase of SOD activity on day 6 p.i.; its maximum was reached on day 9 p.i. [150% of CH, CH being (6.8 ± 1.06) U/mg protein] (Fig. 3A). The maximum of CAT activity induced by EIVI was measured on day 6 p.i. [148% of CH, CH being (4.4 ± 0.55) U/mg protein] (Fig. 3B). The observed rise of SOD and CAT activities in VIM corresponded to the elevation of O_2^- and H_2O_2 levels. However, the produced antioxidant enzymes obviously were not in sufficient amounts to cope with the excessive generation of ROS (Fig. 2). PC, applied in the dose of 2.5 mg/kg, did not affect the production of SOD and CAT during infection. Nonetheless we have observed that the preventive treatment with 10 mg/kg PC modulated the excessive generation of the two enzymes both in the lungs and livers of IAV-infected animals (unpublished). Similar results were obtained by Kumar *et al.* (2005); they detected a rise of lung antioxidant enzymes during EIVI and reported that oral supplementation with quercetin increased their pulmonary concentrations. The application of HL-SOD caused a reduction of lung SOD and CAT levels at each time point. PC + HL-SOD treatment reduced the activated enzyme levels to normal and thus evidenced the modulatory effect of the combination on the lung antioxidant enzymes (Fig. 3).

The presented results confirmed the free-radical character of EIVI and provided evidence for the beneficial effect of the combined treatment with PC and HL-SOD. Thus, it could be speculated that the modulation of oxidative stress is an alternative mode of action of the combination in addition to its specific virus-inhibitory activity.

It has been found that PC showed also prooxidant characteristics (Murzakhmetova *et al.*, 2008). In the intact animals the level of MDA was increased by 30% in the lungs on days 6 and 9 p.i. and in the livers on day 6 p.i. Similar results have been observed in alternative assays: PC (10 mg/kg) caused an increase of the generation of H_2O_2 on day 2 and of O_2 on day 6 p.i. (Toshkova *et al.*, 2006). It is tempting to speculate that the prooxidant capacity of the plant extract at the early stages of infection could be part of the nonspecific defensive reaction of the organism before the development of the specific immune response. Both the prooxidant as well as the antioxidant effects could be important mechanisms of the host response modulation in EIVI. Although known for their antioxidant activities, rutin and quercetin, for instance, exhibit prooxidant effects in healthy animals (Kumar *et al.*, 2005). Hence, therapeutic strategies to modulate, but not totally obliterate, the generation of ROS, and so reduce the impact of the cellular injury in the lung, would appear to have considerable benefit.

Our results are in agreement with the accumulating evidence that a great number of aromatic, medicinal, and other plants contain chemical compounds exhibiting antioxidant properties, and some of their physiological effects are actually due to their antioxidative activities. In this respect a particular interest has been given to plant polyphenols. The natural polyphenols have an ideal structure for capturing free radicals, and their antioxidant activity surpasses the effect of known antioxidants such as the vitamins A and E (Murzakhmetova *et al.*, 2008 and references cited therein).

The effect of the combination of the plant polyphenol-rich extract and the fungal SOD on the inspected biochemical parameters was studied in parallel with the virological factors of the infection, *e.g.* rate of mortality, mean survival time, infectious lung virus titer and consolidation of the lungs, lung weights, and lung indices (Table I). In additional experiments clear evidence of protection was observed for animals receiving PC + HL-SOD treatment; a significant reduction of mortality rates (IP = 52.6–76.4) and marked prolongation of MST (up to +5.2 days) was achieved. Lung infectious virus titers (Δlog $TCID_{50}$/ml = 3.9–5.7), lung weights, and lung indices were reduced; lack of body weight increase was reversed, lung lesions as shown by macroscopic and microscopic examination were markedly alleviated. Light microscopy examination of mice lungs revealed reduction of the hemorrhages and alveolar edema; no cells of the lymphoid type were observed. The protective activity of the

combinations in the mouse model was associated with reduction in lung virus titers and pneumonitis, as well as with improved body weight during the infection. Thus alleviation of major influenza symptoms was attained.

It should be noted that while the 4-fold intraperitoneal treatment with as much as 1000 U/mouse/d HL-SOD was not efficient (not shown), a 4- to 8-fold reduced dose enhanced the protective efficacy of PC, applied in otherwise inefficient doses (2.5 and 1.25 mg/kg).

For comparative reasons the combined protective effect of PC and vitamin C was investigated (Table II).

An enhancement of protection of the synergistic type was observed when vit C and PC were applied in the doses 2.5 and 100 mg/kg, respectively. All lung parameters were ameliorated (Table II). It has been suggested that in addition to its antioxidant properties vitamin C may affect the incidence and severity of the common cold and other respiratory infections (Hemila, 2004). Li *et al*. (2006) showed that vitamin C was required for an adequate immune response in limiting lung pathology after IV infection.

The 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.

In conclusion, the obtained results clearly demonstrated that the fungal Cu/Zn-containing SOD enhanced the therapeutic efficacy of the plant polyphenol-rich extract and outlined the antioxidant and radical scavenging properties of their combinations; the combinations beneficially modulated the oxidative stress response in IAV-induced pneumonia. This alternative mechanism of action by all means contributed to the overall protective effect in lethal murine EIVI in addition to their specific virus-inhibitory activity.

The present results indicate that tissue damage during influenza virus infection may greatly be reduced by combined antiviral therapy and in general by the beneficial role of the combined use of viral inhibitors with diverse mechanisms of action. This could be explained with the effective integration of different functions in the control of the infection. Thus, the appropriate combined use of antiviral agents, among them antioxidants, is a promising approach for the control of the disease.

Acknowledgements

The authors acknowledge the skilful technical assistance of Mrs. K. Todorova, Institute of Microbiology, Bulgarian Academy of Sciences. This study was supported by research grants K-1007 and L-1518 from the National Council for Science, Bulgaria.

Akaike T., Noguchi Y., Ijiri S., Setoguchi K., Suga M., Zeng Y., Dietzschold B., and Maeda H. (1996), Pathogenesis of influenza virus-induced pneumonia: Involvement of both nitric oxide and oxygen radicals. Proc. Natl. Acad. Sci. USA **93**, 2448–2453.

Angelova M., Dolashka-Angelova P., Ivanova E., Serkedjieva J., Slokoska L., Pashova S., Toshkova R., Vassilev S., Simeonov I., Hartman H.-J., Stoeva S., Wesser U., and Voelter W. (2001), A novel glycosylated Cu/Zn-containing superoxide dismutase: Production and potential therapeutic effect. Microbiol. UK **147**, 1641–1650.

Arora D. J. and Houde M. (1992), Modulation of murine macrophage responses stimulated with influenza glycoproteins. Can. J. Microbiol. **38**, 188–192.

Beauchamp C. and Fridovich I. (1971), Superoxide dismutase: Improved assay applicable to polyacrylamide gels. Anal. Biochem. **44**, 276–287.

Beers R. F. and Sizer I. W. (1952), A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. **195**, 133–140.

Dolin R., Reichman R. C., Madora H. P., Maynard R., and Linton P. N. (1982), A controlled trial of amantadine and rimantadine in the prophylaxis of influenza A infection. N. Engl. J. Med. **307**, 580–584.

Gegova G., Manolova N., Serkedjieva J., Maximova V., Uzunov S., Dzeguze D., and Indulen M. (1993), Combined effect of selected antiviral substances of natural and synthetic origin. II. Antiinfluenza activity of a combination of a polyphenolic complex isolated from *Geranium sanguineum* L. and rimantadine *in vivo*. Acta Microbiol. Bulg. **30**, 37–40.

Hemila H. (2004), Vitamin C, supplementation and respiratory infections, a systematic review. Military Med. **169**, 920–925.

Ivanova E., Toshkova R., and Serkedjieva J. (2005), A plant polyphenol-rich extract restores the suppressed functions of phagocytes in influenza virus-infected mice. Microb. Infect. **7**, 391–398.

Kumar P., Khanna M., Srivastava V., Tyagi Y. K., Raj H. G., and Ravi K. (2005), Effect of quercetin supplementation on lung antioxidants after experimental influenza virus infection. Exp. Lung Res. **31**, 449–459.

Kurokawa M., Nagasaka K., Hirabayashi T., Uyama S., Sato H., Kageyama T., Kadota S., Ohyama H., Hozumi T., Namba T., and Shiraki K. (1995), Efficacy of traditional herbal medicines in combination with acyclovir against HSV type 1 infection *in vitro* and *in vivo.* Antiviral Res. **27**, 19–37.

Li W., Maeda N., and Beck M. A. (2006), Vitamin C deficiency increases the lung pathology of influenza virus-infected gulo-/-mice. J. Nutr. **136**, 2611–2616.

Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951), Protein measurement with the Folin-phenol reagents. J. Biol. Chem. **193**, 265–275.

Murzakhmetova M. K., Moldakarimov S. B., Tancheva L., Abarova S., and Serkedjieva J. (2008), Antioxidant and prooxidant properties of a polyphenol-rich extract from *Geranium sanguineum* L. *in vitro* and *in vivo.* Phytother. Res. **22**, 746–751.

Musci I., Gyulai Z., and Beladi I. (1992), Combined effects of flavonoids and acyclovir against HSV in cell cultures. Acta Microbiol. Hung. **39**, 137–149.

Oda T., Akaike T., Hamamoto T., Suzuki F., Hirano T., and Maeda H. (1989), Oxygen radicals in influenza-induced pathogenesis and treatment with pyran polymer-conjugated SOD. Science **244**, 974–976.

Pantev A., Ivancheva S., Staneva L., and Serkedjieva J. (2006), Biologically active constituents of a polyphenol extract from *Geranium sanguineum* L. with antiviral activity. Z. Naturforsch. **61c**, 508–516.

Reed L. J. and Muench H. (1938), A simple method of estimating fifty per cent endpoints. Am. J. Hyg. **27**, 493–497.

Semrau F., Kühl R. J., Ritter S., and Ritter K. (1998), Manganese superoxide dismutase MnSOD and autoantibodies against MnSOD in acute viral infections. J. Med. Virol. **55**, 161–167.

Serkedjieva J. (2000), Combined antiinfluenza virus activity of *Flos verbascum* infusion and amantadine derivatives. Phytother. Res. **14**, 571–574.

Serkedjieva J. and Ivanova E. (1997), Combined protective effect of an immunostimulatory bacterial preparation and rimantadine in experimental influenza A virus infection. Acta Virol. **41**, 65–70.

Serkedjieva J. and Zgorniak-Nowosielska I. (1993), Combined antiinfluenza activity of a plant preparation SHS-174 and amantadine derivatives. Acta Virol. **37**, 258–264.

Serkedjieva J. and Hay A. J. (1998), *In vitro* antiinfluenza virus activity of a plant preparation from *Geranium sanguineum* L. Antiviral Res. **37**, 121–130.

Serkedjieva J., Roeva I., Angelova M., Dolashka P., and Voelter W. (2003), Combined protective effect of a novel fungal Cu/Zn-containing superoxide dismutase and rimantadine hydrochloride in the murine experimental influenza A virus infection. Acta Virol. **47**, 53–56.

Serkedjieva J., Toshkova R., Stefanova T., Antonova-Nikolova S., Teodosieva A., and Ivanova I. (2007), Effect of a plant polyphenol-rich extract on the protease and protease-inhibitory activities in the lungs of influenza virus-infected mice. Antiviral Chem. Chemother. **18**, 75–82.

Toshkova R., Stefanova T., Nikolova N., and Serkedjieva J. (2006), A plant extract ameliorates the dysfunctions of alveolar macrophages in influenza virus-infected mice. PharmacologyOnLine **3**, 778–784.

Weaver E. and Arou G. M. (1998), Synergistic anti-NDV activity of a pokeweed antiviral protein, ribavirin and guanidine. Can. J. Microbiol. **44**, 702–705.

Webb L. (1966), In: Inhibitors of Enzymes and Metabolism General Principles of Inhibition. Mir Publishing House, Moscow, p. 484.

Werner E. (2003), Determination of cellular H_2O_2 production. Sci. STKE **4**, PL3.