

# PEGylated human catalase elicits potent therapeutic effects on H1N1 influenza-induced pneumonia in mice

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**Abstract** Therapeutic recombinant human catalase (rhCAT) can quench infection-induced reactive oxygen species (ROS), thereby alleviating the associated tissue damage. Although the intranasal route is efficient to deliver native rhCAT to the lung, the therapeutic effect is limited by rapid elimination from the blood. In this study, we modified rhCAT with the active polymer, polyethylene glycol monomethyl ether (PEG)-5000, and analyzed the pharmacokinetics of PEGylated rhCAT in mice. The high tetra-PEGylation ratio was about 60 %, and PEGylation prolonged the half-life of rhCAT in serum (75 vs. 13.5 min for native rhCAT). The protective effects of PEG-rhCAT were investigated in a mouse model of influenza virus A (H1N1)-associated pneumonia. PEG-rhCAT was more effectively delivered than native rhCAT and was associated with higher survival ratio, less extensive lung injuries, reduced ROS levels, and lower viral replication. Collectively, these findings indicate that PEGylation can enhance the therapeutic efficacy of native rhCAT and suggest that PEGylated rhCAT may represent a novel complement therapy for H1N1 influenza-induced pneumonia.

**Keywords** Catalase · Influenza · Pneumonia · Oxidative stress · PEGylation

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## Introduction

Reactive oxygen species (ROS) have been implicated in the pathogenesis of myriad diseases, ranging from cancer (Cerutti 1991) and atherosclerosis (Halliwell and Gutteridge 1990) to Alzheimer's disease (Simonian and Coyle 1996) and human immunodeficiency virus infection (Deng et al. 2010). Although ROS function is a beneficial component of the immune response, unregulated and excessive ROS are toxic, inducing irreparable damage to the cellular membrane, proteins, and nucleic acids. Under normal physiologic conditions, antioxidant enzymes help to maintain the balance of ROS. This functional interaction has led to interest in generating antioxidants as therapeutic agents for ROS-mediated injury and diseases.

Catalase (CAT) is one of the key antioxidant enzymes of the mammalian system. CAT works by catalyzing the decomposition of H<sub>2</sub>O<sub>2</sub> into molecular oxygen and water (Lv et al. 2009; Ogbeyalu et al. 2009; Brian 2009). CAT plays an important role in the development of tolerance to oxidative stress. This process has been defined as protective against several pathological conditions, such as allergies (Yoshimaru et al. 2006), atherosclerosis (Kudchodkar et al. 2007), ischemia (Hanawa et al. 2009), chronic granulomatous (Yu et al. 2008), metastatic tumor progression (Nishikawa et al. 2009; Hyoudou et al. 2004), and aging (Brown-Borg and Rakoczy 2000). The potential of catalase as a therapeutic agent has been explored for many different diseases and in many different forms, including gene therapy (Guy et al. 1998) and recombinant catalase (Yabe et al. 1999), for well over a decade. In a previous study, we found that inhalation of native recombinant human catalase (rhCAT) elicited a protective effect in mice with influenza-associated pneumonia (Shi et al. 2010).

According to global estimates generated by the World Health Organization, 450 million cases of pneumonia are

diagnosed every year. Approximately four million of those cases end in death, accounting for 7 % of the total mortality worldwide. The recent emergence of severe acute respiratory syndrome (SARS)-associated coronavirus, avian influenza A (H5N1) virus, and the 2009 pandemic influenza A (H1N1) virus served to highlight respiratory viruses as important causative agents of severe pneumonia. Nonetheless, the current battery of antivirals available in clinical practice for the treatment of pneumonia is limited (Ruuskanen et al. 2011; Rudan et al. 2008). The continued pandemic threat of these circulating viruses makes identification and development of novel therapeutic strategies, especially for the treatment of influenza A, an urgent matter (Derek 2009).

Pneumonia and severe acute lung injuries often occur after infection with the H1N1 influenza virus. Virus invasion of the bronchi and/or alveoli in the lung destroys these structures, leading to a drastic reduction of the air space that is essential for oxygen/CO<sub>2</sub> exchange (Hurt et al. 2004). The immune response to the virus manifests as increased vasodilation and leakage of leukocytes and neutrophils into the lung tissue, ultimately resulting in life-threatening pulmonary edema and congestion. Studies to identify the mechanisms underlying cell death associated with influenza-induced pneumonia have implicated ROS in promoting the extent of lung injury and respiration compromise (Gangehei et al. 2010; Case et al. 2011; Nakamura et al. 2002). Thus, it has been theorized that quenching ROS levels might be a promising strategy to alleviate the severity of viral pneumonia.

Despite the successes of our recently developed inhalable native rhCAT—both in targeted delivery to the site of infection and in producing a protective effect in mice with influenza viral pneumonia (Shi et al. 2010)—the therapeutic effect was limited by rapid elimination from the circulation. Therefore, in the present study, rhCAT was conjugated to activated polyethylene glycol-5000 (PEG-5000) in an attempt to extend the half-life of the rhCAT and enhance its therapeutic effects on H1N1 influenza-induced pneumonia.

## Materials and methods

### Experimental animals

Imprinting control region (ICR) mice (weighing 16.0–18.0 g) were purchased from the Shanghai SLACCAS Laboratory Animal Co., Ltd. (Shanghai, China). Mice and rats were housed under specific pathogen-free conditions in a federally licensed biosafety level 3 animal facility. The animals were given free access to sterile water and standard rat/mouse chow. All experimental protocols were approved by the Animal Experiment Committee of Fudan University (Shanghai, China).

### Influenza virus

Influenza virus A/FM/1/47 (H1N1) strain was supplied by the Shanghai Center for Disease Control and Prevention (Shanghai, China) and stored in aliquots at  $-70^{\circ}\text{C}$ . For each experiment, an aliquot was thawed to ensure use as a fresh preparation (Michelle et al. 2011).

### PEGylation of rhCAT

MPEG-5000 (Sigma-Aldrich, St. Louis, MO, USA) polymer was activated with cyanuric chloride and coupled to rhCAT by using the previously described protocol (Baysal and Uslan 2001). Briefly, the reaction mixture was diluted with 20 mM Na<sub>2</sub>HPO<sub>4</sub> (pH7.0) and loaded onto a sephacryl S-300 gel filtration column (GE Healthcare, Uppsala, Sweden) that had been equilibrated with buffer A (20 mM phosphate buffer, pH 7.0) using an AKTA purifier protein purification system (GE Healthcare). Fractions corresponding to the protein peaks were collected for analysis by SDS-PAGE. The confirmed PEGylated proteins were dissolved in saline solution, sterilized by filtration (0.22  $\mu\text{m}$  pore size), and diluted to working concentrations for subsequent animal administration.

### Pharmacokinetic analysis of PEG-rhCAT

The Sprague–Dawley rats were randomly divided into three treatment groups ( $n=6$  each): rhCAT; PEG-rhCAT; and saline-treated controls. The treatments consisted of a single intranasal delivery of rhCAT or PEG-rhCAT at 100 kU/kg or an equal volume of saline. Blood samples (0.5 mL) were drawn via the retro-orbital venous plexus into vials containing EDTA-2K at selected post-treatment time points (0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 75, 90, 105, 120, 135, and 150 min). Each sample was allowed to clot for 30 min at 25  $^{\circ}\text{C}$ , after which the serum was collected by centrifugation (2000 $\times$ g for 15 min at 4  $^{\circ}\text{C}$ ). The serum CAT activity was measured by using a catalase activity assay kit (no. A007; Jiancheng Bioengineering Institute, Nanjing, China) and following the manufacturer's instructions.

### Virus infection and CAT treatment

The ICR mice were randomly divided into four treatment groups ( $n=12$  each): virus-infected, native rhCAT; virus-infected, PEG-rhCAT; virus-infected, saline-treated controls; and uninfected, saline-treated controls. The rhCAT (both the native and PEGylated forms) was prepared for administration as previously described (Shi et al. 2007). Mice (except for those in the uninfected, saline-treated control group) were anesthetized with isoflurane and infected with influenza virus A/FM/1/47 (H1N1) via intranasal inoculation (8 TCID<sub>50</sub>).

All mice received their group-appropriate treatments after influenza infection. For treatment, mice from each group were anesthetized twice daily and administered one of the following solutions by the intranasal route: 100 kU/kg native rhCAT solution; 100 kU/kg PEG-rhCAT solution; or 30  $\mu$ L saline (for the uninfected and the virus-infected control groups).

#### Survival studies

Virus-infected mice were randomly divided into three groups ( $n=20$  each); native-rhCAT, PEGylated rhCAT, and untreated control. Treatments were administered as described above for seven days. On the eighth day, mice began to be monitored for survival over the next six days. Survival analysis curves were generated by plotting the number of survivors divided by the total number of mice in a group (expressed as percentage of mice that survived). Data were only plotted out to 14 days after infection since no mortalities occurred beyond this time point.

#### Pathological analysis of rhCAT and PEG-rhCAT therapeutic efficacy

Control (uninfected) mice were treated with saline, and viral pneumonia mice were treated with rhCAT and PEG-rhCAT for four days, after which all mice were sacrificed by exsanguination. Tissues were harvested and suspended in PBS-buffered formalin. Excised lungs were preserved in paraffin blocks, and 10  $\mu$ m tissue sections were cut, placed on glass slides, and stained with hematoxylin and eosin. Microscopic analysis was carried out by three separate pathologists who were blinded to the various experimental treatments.

Tissue inflammation score was assigned to the analyzed sections of each lung, using the mean score obtained from six separate random fields per tissue section. Scores were assigned according to the percentage of lung involvement as follows: none=0,  $\leq 25$  %=1, 26–50 %=2, 51–75 %=3, and  $\geq 76$  %=4.

#### Measurement of influenza virus titers in the mouse lung

Viral load was measured as the hemagglutination assay (HA) titer, as previously described (Wang et al. 2008). Briefly, mouse lung tissue samples from all mice of each group were pooled and homogenized in 1 mL of sterilized normal saline. Aliquots of 100  $\mu$ L of the whole lung homogenate or a twofold serial dilution of the homogenates were placed into the wells of a V-shaped 96-well microtiter plate. Each sample was covered with 100  $\mu$ L of freshly prepared chicken blood corpuscles and incubated at room temperature for 2 h. For the control, 100  $\mu$ L of normal saline was covered with 100  $\mu$ L chicken blood corpuscles. The HA titer was expressed as the reciprocal of the dilution of samples in the last well with complete hemagglutination.

#### Detection of malondialdehyde (MDA) and antioxidant enzymes content and total antioxidant status (TAS)

Mouse lung homogenates were freshly prepared as described above. The TAS and content of MDA, superoxide dismutase (SOD), and CAT were determined using commercially available assay kits (nos. A015, A003–2, A001–1, and A007, respectively; Jiancheng Bioengineering Institute).

#### Statistical analysis

Statistical analyses were performed using SPSS software, version 17.0 (SPSS Inc., Chicago, IL, USA). Data are presented as mean $\pm$ SD, unless otherwise indicated. Intergroup comparisons were carried out with the one-way ANOVA and two-tailed Student's *t* tests. A *p* value of less than 0.05 was considered statistically significant.

## Results

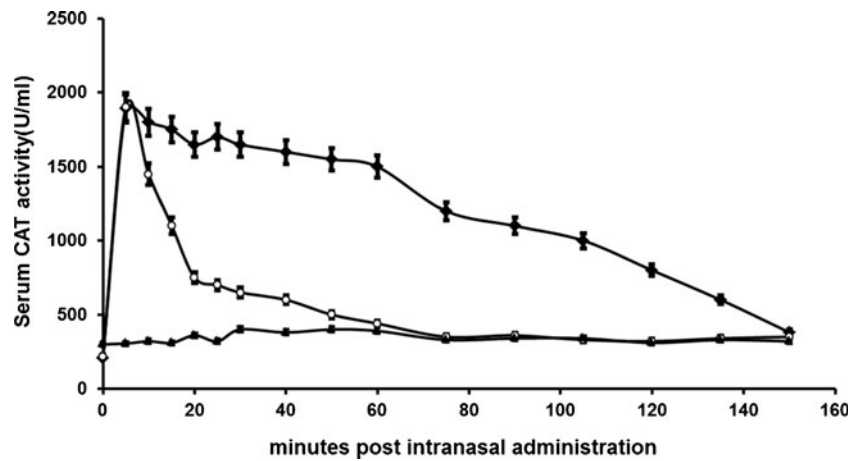
#### Preparation and purification of PEG-rhCAT

PEGylation of rhCAT was carried out as described in material and methods above, and it achieved a high ratio of tetra-PEGylated protein (~60 % of total proteins) and a low percentage of other PEGylated proteins (<10 %), as indicated by high pressure size exclusion chromatograph analysis (Fig. S1–2, Table. S1). The fraction of PEGylated rhCAT (65 kDa in SDS-PAGE, a subunit of tetra-PEGylated protein) was separated from the fraction of unmodified protein (60 kDa in SDS-PAGE) by gel filtration chromatography. In the current reaction and purification condition, the activity of native and PEGylated rhCATs remained very well (Fig. S3). The relative activity of PEGylated rhCAT was similar to native rhCAT (29 vs. 28 kU/mg, respectively), indicating that the PEG modification and the following purification did not affect the protein's function.

#### PEGylation enhanced the pharmacokinetic characteristics of rhCAT

There was no significant difference in CAT activity among the three rat treatment groups prior to intranasal administration of the various treatments (time, 0 min; PEG-rhCAT vs. native rhCAT vs. untreated control). As shown in Fig. 1, CAT activity peaked in both the PEG- and native rhCAT groups (~1900 U/mL) at 5 min post-treatment. In the native rhCAT-treated group, the serum CAT activity rapidly decreased after the 5 min time point, and its half-life was determined to be ~13.5 min. The PEGylated rhCAT also decreased after the 5 min time point, but at a significantly reduced rate, indicating that the PEG modification produced

**Fig. 1** PEGylation markedly prolonged the serum half-life of rhCAT in rats. Rats ( $n=6$  rats/group) were intranasally administered 100 kU/kg PEG-rhCAT (filled diamond) or native rhCAT (open circle), or left untreated (control; filled upright triangle). Blood samples were collected at the indicated time points and CAT activity was measured



serum stability. The half-life of PEG-rhCAT was  $\sim 75$  min, representing a significant improvement over the native form ( $p \leq 0.05$ ).

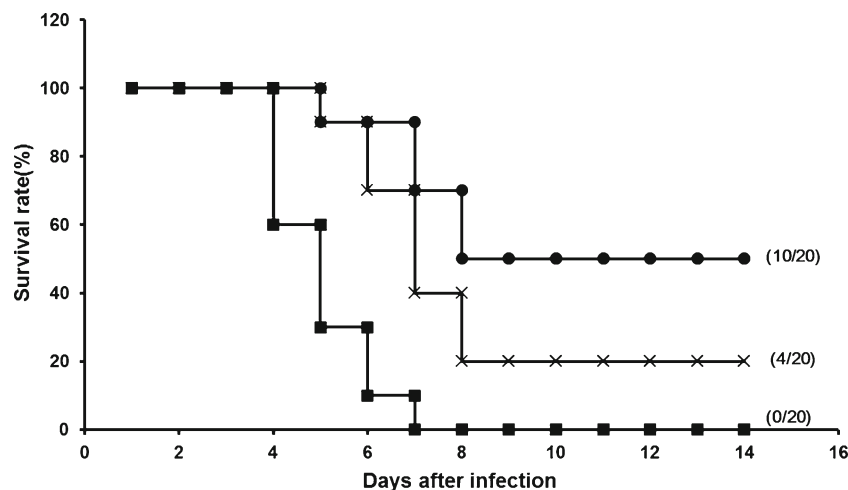
PEGylated rhCAT administration significantly improved survival of mice with influenza pneumonia

Mice infected with H1N1 influenza virus were treated with native rhCAT or PEG-rhCAT for seven days. As shown in Fig. 2, infected mice began to die almost immediately after the fourth day of infection, regardless of treatment. Most deaths occurred during the first week after infection. On the seventh day post-infection, no mice in the virus control group survived; however, 90 % of the PEG-rhCAT-treated mice (18/20) and 40 % of the native rhCAT-treated mice (8/20) were still alive. By the 14th day post-infection, 50 % of the PEG-rhCAT-treated mice (10/20) had survived, but

only 20 % of the native rhCAT-treated mice (4/20) had survived. These findings indicated that both native rhCAT and PEG-rhCAT elicited protective effects, as evidenced by the delay of death and improved overall survival time. Moreover, the effects of PEG-rhCAT were superior to those of the native form (90 vs. 40 % survival rate at day 7; 50 vs. 20 % survival at day 14).

PEGylated rhCAT administration markedly limited pulmonary injury in mice with influenza pneumonia

Since the extent of pulmonary injury has been shown to be associated with morbidity from influenza virus H1N1 (FM1 strain) (Hurt et al. 2004), the ability of PEG-rhCAT to protect lung tissues was investigated. Histopathologic analysis of mice with influenza pneumonia revealed that treatment with either native or PEGylated rhCAT led to reduced



**Fig. 2** PEGylated rhCAT significantly improved the survival rate of mice with viral pneumonia. On day 0, each group of mice ( $n=20$ /group) was infected intranasally with influenza virus at an 8 TCID<sub>50</sub> dose. From day 0–7 post-infection, 100 kU/kg PEG-rhCAT (black circle) or native rhCAT (multiplication sign), or distilled saline

(control; black square), were administered intranasally twice daily. On day 8, the treatment was terminated. The ratio of survivors/total number of mice out to day 14 is denoted at the right-side terminus of each survival curve



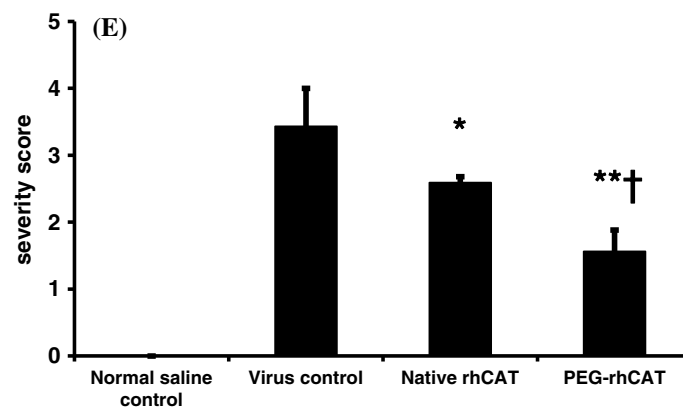
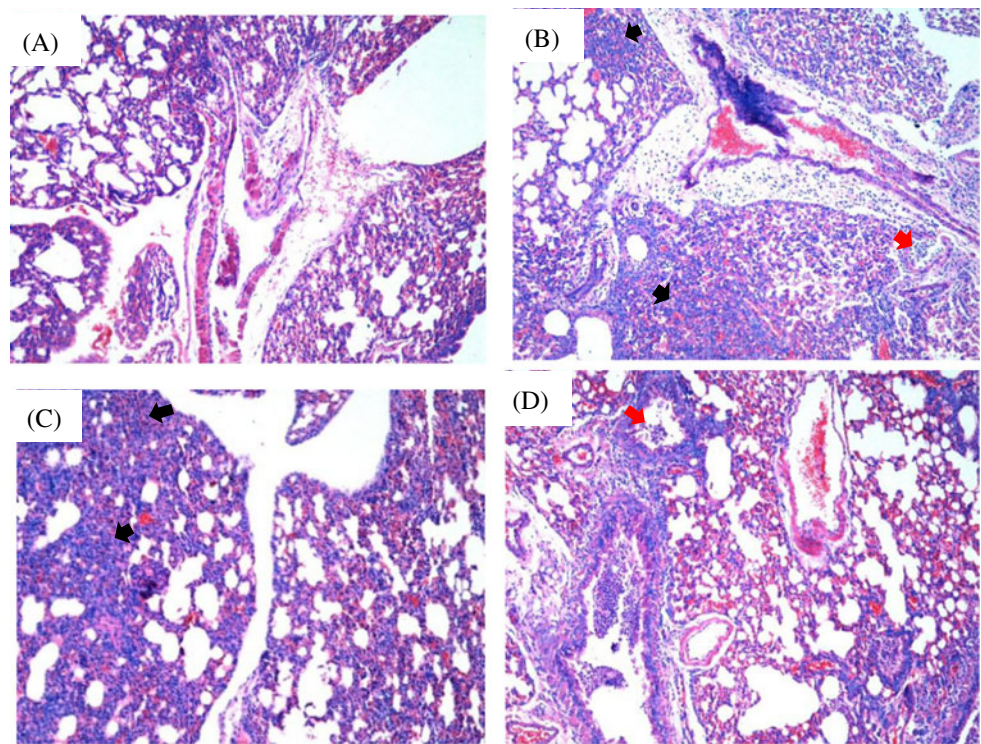
infiltration by inflammatory cells, less extensive interstitial pneumonia and bronchiolitis, and obviously diminished tissue inflammation scores on day 4 post-infection (vs. virus control mice). Pathologic analysis of lungs from the infected mice showed that treatment with either the native or PEGylated rhCAT caused a marked reduction in tissue injury, mononuclear cell infiltration, hemorrhage, and pulmonary edema, as well as significantly reduced tissue inflammation scores (native form,  $2.59 \pm 0.32$ ; PEGylated form,  $1.56 \pm 0.41$ ), as compared with those in the virus control mice ( $3.43 \pm 0.57$ ;  $p < 0.05$ ) on post-infection day 4. In the virus control group, areas of increased tissue consolidation and vascular hemorrhaging were detectable (Fig. 3b). In the rhCAT-treated mice (Fig. 3c, d), areas of tissue consolidation and hemorrhage were less numerous, decreased in

size, and less confluent than in the virus control mice. PEG-rhCAT produced all of the same effects as native rhCAT, but to a greater extent, indicating the PEG-rhCAT had superior protective abilities ( $p \leq 0.05$ ). Moreover, the histopathologic findings of each group corresponded with the mortality rates observed for each treatment type (Fig. 2).

PEGylated rhCAT lowered the viral load in mice with influenza pneumonia

The HA measurement method was employed to determine the effects of the different rhCAT forms on the viral burden of influenza virus in mice. Treatment with either the PEGylated rhCAT or the native form led to decreased amounts of virus in the lung at day 4 post-infection. As

**Fig. 3** PEGylated rhCAT administration markedly limited pulmonary injury in mice with viral pneumonia. Virus-infected mice were administered 100 kU/kg PEG-rhCAT or native rhCAT, or distilled saline (virus control), intranasally twice daily until sacrifice on the 4th day. Influenza virus induced severe interstitial pneumonia (black arrow indicates the mononuclear inflammatory cells infiltrating the wall of the alveoli) and bronchiolitis (red arrow indicates inflammation of the bronchioles with mononuclear inflammatory cells infiltration). Representative hematoxylin and eosin staining of lung tissue sections are shown for healthy, untreated mice (a), virus control mice (b), native rhCAT-treated mice (c), and PEG-rhCAT-treated mice (d). Pooled group ( $n=4$ /group) tissue inflammation severity scores (e) are presented as the mean lung involvement derived from six separate random fields per six tissue sections for each mouse  $\pm$  SEM. The severity scores on the y-axis correspond to percentage of lung involvement: 0, none; 1,  $\leq 25\%$ ; 2, 26–50%; 3, 51–75%; 4,  $\geq 76\%$  lung involvement. \* $p \leq 0.05$ , \*\* $p \leq 0.01$  vs. virus control; † $p \leq 0.05$ , †† $p \leq 0.01$  vs. native rhCAT

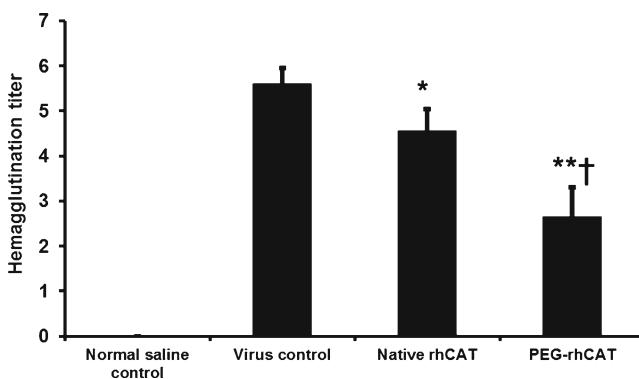


shown in Fig. 4, no virus was detectable in blood samples from the uninfected (saline) control group, indicating that no influenza virus was obtained by extra-experimental means in our study population. All of the experimentally infected mice showed an obvious viral titer. Compared with the virus control mice (infected but untreated; HA titer,  $5.60 \pm 0.35$ ), treatment with either native rhCAT ( $4.56 \pm 0.66$ ) or PEG-rhCAT ( $2.65 \pm 0.40$ ) significantly inhibited the virus, as evidenced by a reduced HA titer. Moreover, the PEGylated form was much more effective than the native form at lowering the viral burden in mice with influenza pneumonia ( $p \leq 0.05$ ).

PEGylated rhCAT administration restored the balance of antioxidant system components in the lungs of mice with influenza pneumonia

Since copious amounts of virus can induce excessive oxidative responses, subsequently exhausting the antioxidant pool and destroying the redox balance, the levels of key antioxidant enzymes were evaluated in mice with influenza pneumonia after rhCAT treatments. Malondialdehyde is a product of lipid peroxidation and is often present in tissues with oxidative injury. As expected, the levels of MDA were significantly increased in mice with influenza pneumonia, as compared to uninfected (saline) control mice. However, treatment with either native rhCAT or PEG-rhCAT led to a significant reduction in MDA levels (vs. the untreated virus control group;  $p \leq 0.05$  and  $p \leq 0.01$ , respectively). Moreover, the PEGylated form was able to reduce the MDA level to a greater extent than the native form ( $p \leq 0.01$ ) bringing it much closer to the level detected in uninfected controls (Fig. 5a).

CAT and SOD are considered the key antioxidant enzymes of the antioxidant defense system. Activities of both CAT and SOD were found to be significantly decreased in untreated mice with influenza pneumonia. This finding indicated that



**Fig. 4** PEGylated rhCAT lowered the viral load in mice with viral pneumonia. Viral load in lung tissue samples ( $n=12$ /group) is presented as mean $\pm$ SD of hemagglutination titer. \* $p \leq 0.05$ , \*\* $p \leq 0.01$  vs. virus control; † $p \leq 0.05$ , ‡ $p \leq 0.01$  vs. native rhCAT

the natural immune response ROS burst induced by an influenza virus invasion had exhausted the enzyme pools of the antioxidant system. Treatment with either PEGylated rhCAT or native rhCAT produced a rapid increase in both CAT (Fig. 5b) and SOD (Fig. 5c) levels in lung tissue. These findings suggested that augmenting the natural CAT pool with rhCAT might help to restore the redox balance under conditions of viral pneumonia. Moreover, the PEGylated form of rhCAT produced a significantly more robust increase in the CAT and SOD levels than in the native form ( $p \leq 0.05$ ).

By measuring the total antioxidant status, it is possible to indirectly estimate the levels of nonenzymatic antioxidant molecules. Untreated mice with influenza pneumonia showed significantly reduced TAS, as compared to uninfected control mice ( $p \leq 0.05$ ; Fig. 5d). This reduction was found to be reversed in response to treatment with either rhCAT or PEGylated rhCAT, and the PEGylated form produced the most robust therapeutic effect (vs. native rhCAT;  $p \leq 0.05$ ).

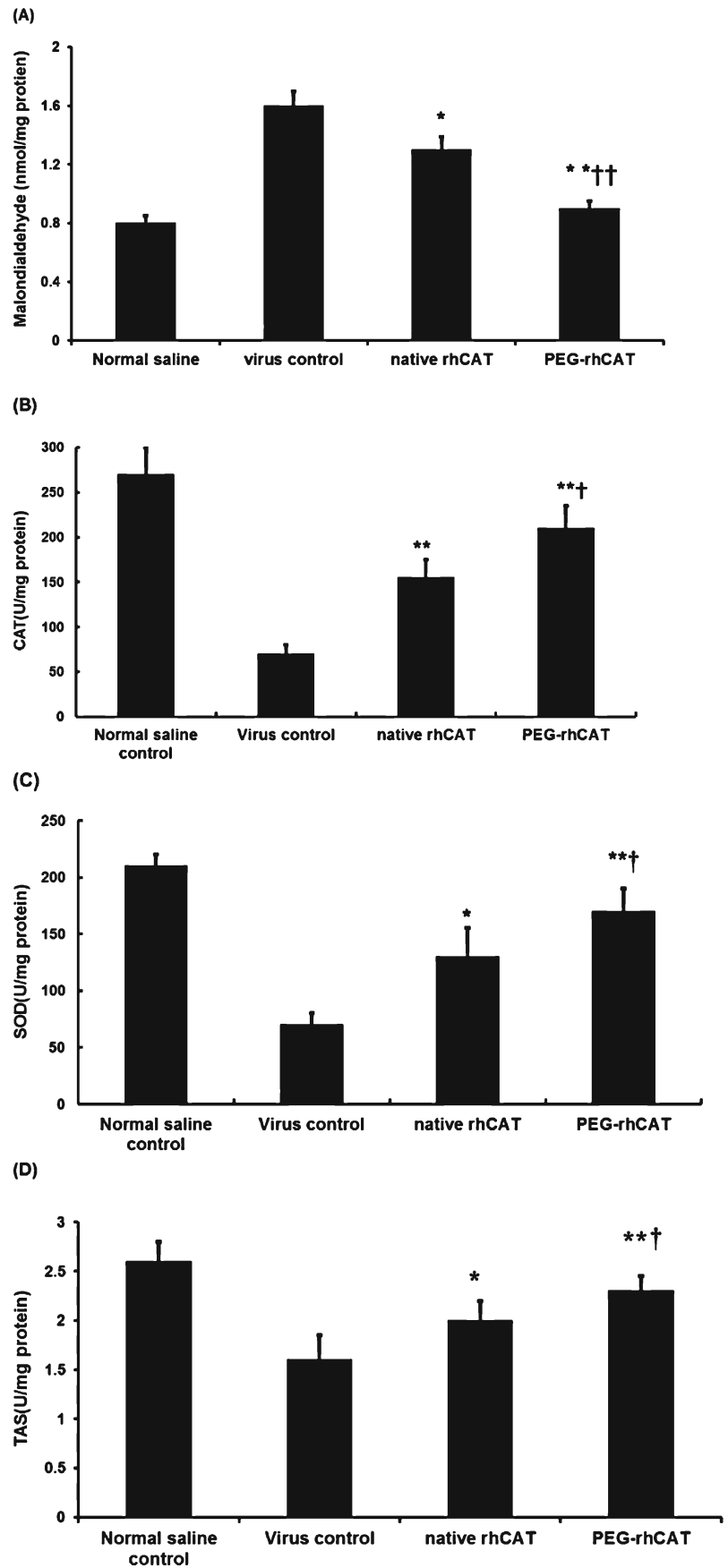
Taken together, these data indicated that PEG-rhCAT administration might represent an effective therapeutic agent to restore the components of the antioxidant system and inhibit the detrimental production of MDA.

## Discussion

Factors implicated in high morbidity and mortality from influenza virus infection includes robust cytokine production (cytokine storm), excessive inflammatory infiltrates, and extensive virus-induced tissue destruction. To date, very few antiviral drugs are approved for the treatment of pneumonia (Wong and Yuen 2008). Despite successful development of several new antiviral agents, such as Oseltamivir and Ribavirin, the pandemic threat of influenza and its high risk of mortality persist due to problems related to drug-induced adverse effects, emergence of resistant viruses, and rapid loss of drug efficacy related to serotype variation. Thus, novel therapeutic strategies based on specific and manipulable virus and host molecular pathways are urgently needed.

The findings from our previous and current studies have described a novel and unique strategy to alleviate viral pneumonia by augmenting the available pool of CAT antioxidant enzyme. Proper administration via the intranasal route was key to achieve targeted delivery to the lung and optimize the potential therapeutic effects. This requirement was based upon the short half-life of our originally designed rhCAT in the native form, which precluded the usual delivery routes of intravenous, subcutaneous, and peritoneal. While intranasal administration of the native rhCAT form was capable of eliciting protective effects against influenza virus pneumonia in mice, it was necessary to extend its half-life of rhCAT to achieve the optimal therapeutic effect.

**Fig. 5** PEGylated rhCAT restored redox balance in mice with viral pneumonia. The levels of malondialdehyde (MDA; **a**), catalase (CAT; **b**), superoxide dismutase (SOD; **c**), and total antioxidant status (TAS; **d**) were measured in lung homogenates from each group ( $n=12/\text{group}$ ). Data are presented as mean $\pm$ SD. \* $p\leq 0.05$ , \*\* $p\leq 0.01$  vs. *virus control*; † $p\leq 0.05$ , †† $p\leq 0.01$ : vs. *native rhCAT*





Catalase is a macromolecular (240 kDa), and the most important factors for serum clearance of catalase were degraded by serum proteases and hepatic uptake. So low-molecular-weight PEG molecules were often chosen to conjugate with catalase, which obviously hindered the clearance rate (Hanawa et al. 2009; Yabe et al. 1999)

Therefore, in the present study we modified rhCAT with active Y-shape PEG-5000, prolonging the half-life by five-fold over that of native rhCAT (to 75 min). Furthermore, the therapeutic effects of rhCAT were also significantly enhanced by the PEG modification. Based on the protective effects observed in mice with influenza pneumonia, we hypothesized that rhCAT may influence multiple pathogenesis-related pathways in this disease condition.

First, the excessive ROS burst induced by virus infection is recognized as a key factor of lung damage. The immune response rapidly exhausts the pool of available antioxidants, as evidenced by low levels of CAT and SOD during the immediate-early stages of severe pathogenic infection. Augmenting the natural antioxidant pool with recombinant CAT can promote the system's ability to eliminate excessive ROS and help to restore redox balance. Indeed, intranasal administration of PEG-rhCAT helped to resolve the influenza-induced perturbations of MDA, CAT, SOD, and TAS in mice with viral pneumonia. In this manner, we presume that PEG-rhCAT therapy may down-regulate ROS, thereby helping to alleviate the destruction of lung tissues and promote survival.

Second, influenza invasion is known to frequently stimulate an excessive immune response, which is accompanied by a robust infiltration of inflammation-related cells, such as monocytes, to the site of infection. The persistent release of inflammatory factors causes significant damage to the underlying tissue and can compromise pulmonary function. In conditions of excessive amounts of ROS, the macrophage-expressed CD200 signaling molecule is activated. Activated CD200 induces expression of several chemokines and proinflammatory cytokines, which in turn recruit peripheral inflammation-related cells to the virus-infected organ (Snelgrove et al. 2006; Rosenblum et al. 2004). By augmenting the antioxidant pool with recombinant CAT, the excessive ROS may be eliminated, thereby helping to quell macrophage-mediated processes, such as apoptosis. The rhCAT-mediated clearance of ROS may also help to halt the massive influx of immunomodulatory cells, as was observed in our histopathological analyses of pulmonary tissue from PEG-rhCAT-treated mice with influenza pneumonia.

Third, virus clearance is initiated by the innate immune system but is dependent upon adaptive immune processes for completion. ROS-mediated tissue injury during severe infections disrupts normal T cell development, thereby impairing adaptive immune responses and inhibiting clearance of an influenza infection (Case et al. 2011; Imai et al.

2008). In our study, untreated mice with influenza pneumonia presented with atrophied thymus and spleen on day 4 post-infection (data not shown). The structural destruction of immune organs had functional consequences on the immune system, impairing maturity of the adaptive immune T cells, and presumably hindering effective virus clearance. Therapeutic administration of rhCAT, however, lowered the viral load, possibly as a result of excessive ROS elimination, which would have decreased destruction of immune-related organs and helped to restore the specific adaptive immune response.

In summary, PEGylation prolonged the circulatory half-life of rhCAT and improved its therapeutic efficacy. PEGylated rhCAT may represent a novel adjuvant therapy to promote the efficacy of the other established therapeutics, such as antiviral drugs, to promote recovery and survival from viral pneumonia.

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**Conflict of Interest Statement** The authors declare that there are no conflicts of interest, financial or otherwise, associated with the publication of this study.

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