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Research article

Red Ginseng-containing diet helps to protect mice and ferrets from the lethal infection by highly pathogenic H5N1 influenza virus

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ABSTRACT

The highly pathogenic (HP) H5N1 influenza virus is endemic in many countries and has a great potential for a pandemic in humans. The immune-enhancing prowess of ginseng has been known for millennia. We aimed to study whether mice and ferrets fed with Red Ginseng could be better protected from the lethal infections of HP H5N1 influenza virus than the infected unfed mice and ferrets. We fed mice and ferrets with Red Ginseng prior to when they were infected with HP H5N1 influenza virus. The mice and ferrets fed with a 60-day diet containing Red Ginseng could be protected from lethal infections by HP H5N1 influenza virus (survival rate of up to 45% and 40%, respectively). Interferon-α and -γ antiviral cytokines were significantly induced in the lungs of mice fed Red Ginseng, compared to mice fed an unsupplemented diet. These data suggest that the diet with the immune-enhancing Red Ginseng could help humans to overcome the infections by HP H5N1 influenza virus.

1. Introduction

Influenza viruses belong to the *Orthomyxoviridae* with genomic negative-sense ribonucleic acid. They are classified as A, B, and C by antigenic differences in their nucleocapsid (NP) and matrix (M) proteins [1]. Influenza A viruses are circulating in aquatic birds and have been responsible for human pandemics. Sixteen subtypes of hemagglutinin (HA) and nine subtypes of neuraminidase (NA) of influenza A viruses have thus far been described in aquatic birds [2,3].

Influenza pandemics in humans by influenza A viruses occur three to four times per century. During the 20th century, humans experienced three pandemics including the Spanish pandemic by avian-like H1N1 influenza virus in 1918, the Asian pandemic by the reassorted H2N2 influenza virus in 1957, and the Hong Kong pandemic by the reassorted H3N2 influenza virus in 1968 [4–9]. Among them, the Spanish pandemic was exceptional in terms of its mortality, with over 20 million human deaths [4,5]. In this century, a pandemic involving reassorted H1N1 influenza virus containing the human, avian, and swine-origin genomes of influenza A virus has occurred in 2009 [10].

Highly pathogenic (HP) H5N1 influenza virus has the potential to become a pandemic influenza virus in humans, because this virus continues to infect humans and is global in its occurrence. HP H5N1 influenza virus has successfully negotiated the species barrier from poultry to humans, killing six of 18 infected humans in Hong Kong in 1997 [11]. Since 2003, the virus has spread to many countries including Indonesia, Pakistan, Thailand, and Vietnam [12–14]. As of July 5, 2013, 377 of 633 infected humans have died from infections caused by HP H5N1 influenza virus, a mortality rate of over 59%, despite the intensive care the patients received [15].

The clinical signs of human infection with HP H5N1 influenza virus include high fever, severe diarrhea, seizures, and coma [14,16]. Efforts have been made to develop an effective vaccine to prepare for the anticipated pandemic [17–19].

In seeking other forms of treatment, attention has turned to medicinal plants, which have a history of human disease relief

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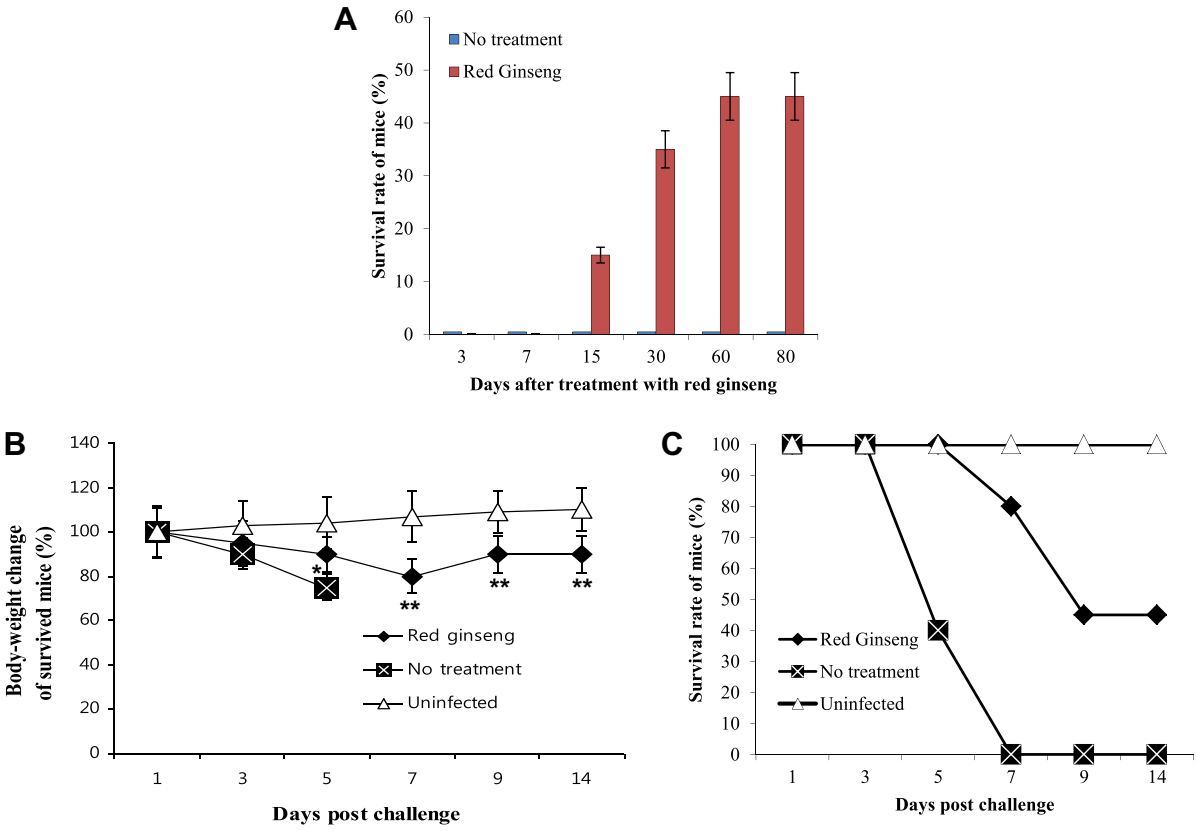

Fig. 1. Survival rate and body changes of mice fed with Red Ginseng against highly pathogenic (HP) H5N1 influenza virus. (A) Mice ($n = 20$) fed with Red Ginseng extract (50 mg/kg) for 80 d were intranasally (i.n.) challenged with HP H5N1 influenza virus [10 mouse lethal dose (10 MLD) 50/mL] and were observed for the survival rate for 14 d. (B, C) Mice ($n = 20$) fed with Red Ginseng extract (50 mg/kg) for 60 d were i.n. challenged with HP H5N1 influenza virus (10 MLD 50/mL) and were observed for (B) body-weight change and (C) survival for 14 d. Groups were compared using repeated measures analysis of variance (ANOVA). $^{*}p < 0.05$. $^{**}p < 0.001$.

dating back to the Neanderthal period [20]. Botanical gardens established to grow medical plants date back to at least the 16th century [21]. Use of herbal medications in the United States began in the early colonial days, when women provided their family with health care. In 1974, the World Health Organization (WHO) recommended the use of herbal medicines in developing countries, whose modern medical infrastructure can be deficient [22].

Panax ginseng has been used as a traditional medicine in China and Korea for over 2,000 years and has been suggested to enhance immune responses, memory, and physical capabilities [23–25]. Ginseng saponins (ginsenosides) are the main substances in the total extracts of ginseng and over 30 ginsenosides have been identified in *Panax ginseng* [26]. The pharmacological effects of ginseng have been reported in the central nervous, cardiovascular, endocrine, and immune systems [27].

The present study was undertaken to investigate whether dietary treatment with Red Ginseng could aid in the survival from lethal infections of HP H5N1 influenza virus and the underlying mechanisms of the protection. For these purposes, mice and ferret models were used.

2. Materials and methods

2.1. Virus and Red Ginseng

The HP H5N1 influenza virus, A/Vietnam/1203/04 (clade 1), was kindly provided by the WHO Collaborating Center for Influenza, the United States Centers for Disease Control and Prevention. HP H5N1 influenza virus was grown in 10-d-old hen eggs (Dukhee farm, Icheon, Republic of Korea) prior to use. All viral experiments were performed in a Biosafety Level-3 (BSL-3) facility approved by the Korean government. Red Ginseng (*Panax ginseng* Meyer) extracts were provided by the Korean Ginseng Co, Daejeon. Korean Red Ginseng (KRG) extract was prepared from the roots of a 6-yr-old fresh *Panax ginseng* Meyer grown in Korea. Red Ginseng was made by steaming fresh ginseng at 90–100°C for 3 h and then drying at 50–80°C. Red Ginseng extract was prepared from the Red Ginseng water extract, which was extracted at 85–90°C for 8 h using three cycles of hot water circulation. The ingredients of the Red Ginseng (*Panax ginseng* Meyer) extracts included 0.71 mg/g of Radical g (Rg) 1, 0.93 mg/g of Radical e (Re), 1.21 mg/g of Radical f (Rf), 0.78 mg/g of Radical h (Rh)1, 1.92 mg/g of Rg2(s), 1.29 mg/g of Rg2(r), 4.62 mg/g of Radical b (Rb)1, 2.41 mg/g of Radical c (Rc), 1.83 mg/g of Rb2, 0.89 mg/g of Rd, 2.14 mg/g of Rg3(s), and 0.91 mg/g of Rg3(r). The total content of the extracts was 19.66 mg/g.

2.2. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory animals of the Korean Veterinary Research and Quarantine Service. The protocol was approved by the Committee on the Ethics of Animal Experiments of Chungnam National University. All surgery was performed under Zoletil anesthesia (Virbac Laboratories, Crros, France), and all efforts were made to minimize suffering. Animals were fed with enough foods and water. The infected animals were monitored twice a day.

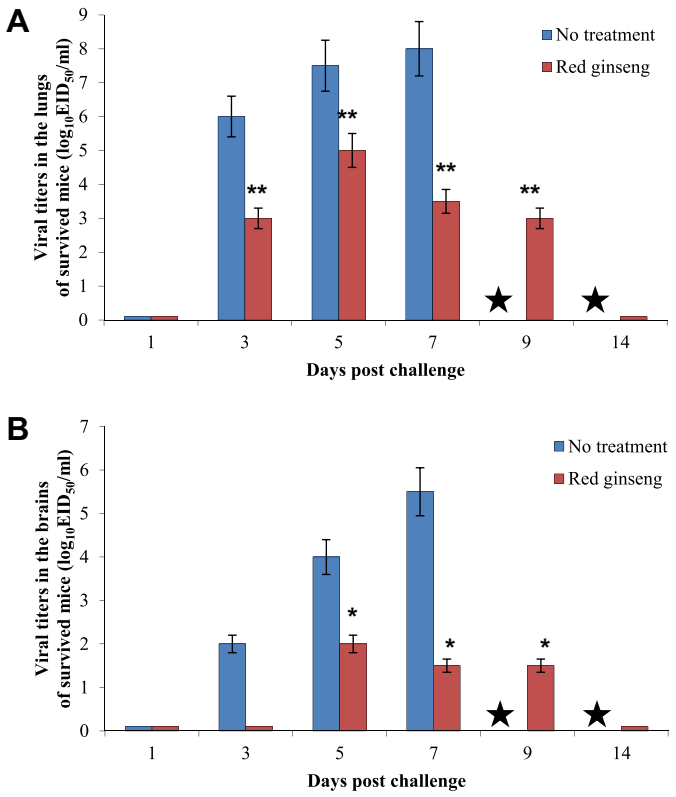

Fig. 2. Viral titers in the lungs and brains of mice fed with Red Ginseng and challenged with highly pathogenic (HP) H5N1 influenza virus. Mice fed with Red Ginseng extract (50 mg/kg) for 60 d were intranasally (i.n.) challenged with HP H5N1 influenza. The surviving mice ($n = 5$) were euthanized prior to when (A) the tissues of lungs and (B) brains were collected, homogenized, and suspended in phosphate buffered saline (PBS). The tissue supernatants were serially diluted and were inoculated into the fertilized eggs prior to when viral titers were determined by log_{10} egg infectious dose (EID) 50/mL. Groups were compared using repeated measures analysis of variance (ANOVA). [1] We could not detect viruses on 9 d and 14 d post challenge (d.p.c.) because all control mice died. $^{*}p < 0.05$. $^{**}p < 0.001$.

2.3. Treatment of mice with Red Ginseng extract and HP H5N1 influenza virus challenge

Three-to-four wk old female mice (NaraBio, Seoul, Republic of Korea) (BALB/c) were fed a daily diet containing Red Ginseng extract (50 mg/kg body weight) for up to 80 d prior to intranasal challenge with 10 mouse lethal dose of 50/mL (10 MLD 50/mL) of virus.

2.4. Time-course survival

Mice fed ($n = 10$ per group) as described above were challenged with HP H5N1 influenza virus as described above 3 d, 7 d, 15 d, 30 d, 60 d, and 80 d after commencement of the diet. Survival rates were observed for 14 d postinfection (d.p.i.).

2.5. Body weight change and survival rate of mice fed Red Ginseng extract for 60 d

Mice ($n = 20$ per group) were fed as described above and challenged with HP H5N1 influenza virus 60 d after commencement of the diet. Body weights of the surviving mice were determined for 14 d.p.i., or until death. Similarly, age-matched mice not fed with Red Ginseng extract were used as comparative controls.

2.6. Determination of tissue viral titers in mice

Mice ($n = 10$ per group) were fed and challenged with the virus as described above. Surviving mice ($n = 5$) were euthanized with a high dose of Zoletil. Lung and brain tissues were immediately collected, homogenized, and suspended in phosphate buffered saline (PBS; pH 7.4; 0.05 g/mL) supplemented with 2× antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO, USA). The tissue supernatants were serially diluted 10-fold in PBS and each diluted sample was inoculated into four 10-d-old hen eggs. The presence of the virus in the allantoic fluids of the inoculated eggs was determined by a HA assay with 0.5% turkey red blood cells (Chungnam National University Animal Resources Center, Daejeon, Republic of Korea). Viral titers were expressed as the log_{10} egg infectious dose 50/mL (log_{10}EID 50/mL) as previously described [28]. The detection limit of viruses was <1 log_{10}EID 50/mL.

2.7. HA assay

The allantoic fluids (50 μL) were individually serially diluted two-fold in PBS in the wells of V-bottom 96-well plates and 50 μL of 0.5% turkey red blood cells in PBS were added. Plates were incubated at room temperature for 30 min prior to when hemagglutination was evaluated.

2.8. Microscopic examination of mouse lung tissue

Mice ($n = 10$ per group) were fed and challenged with the virus as described in the body weight determination experiment. The lungs of the surviving mice ($n = 3$) were immediately collected and the lung tissue was submerged in 10% neutral buffered formalin and embedded in paraffin. Five micrometer-thick sections were cut and stained with hematoxylin and eosin (H&E) stain using a standard protocol. The stained tissue sections were evaluated under a DP70 light microscope (Olympus, Tokyo, Japan).

2.9. Measurement of lung inflammatory cytokines in mice

Mice ($n = 10$ per group) were fed and challenged with the virus as described in the body weight determination experiment. The surviving mice ($n = 3$) were euthanized with a high dose of Zoletil on 3 d.p.i., 5 d.p.i., or 7 d.p.i. and the lungs was collected. The collected lungs were homogenized in PBS and the supernatants were collected. The collected supernatants were used for determining the amount of cytokines such as tumor necrosis factor-alpha (TNF-α), interferon (IFN)-α, IFN-γ, and interleukin (IL)-4 (R&D Systems, Minneapolis, MN, USA). The assays were performed as described by the manufacturer. Fifty μL of sample dilution buffer was added to each well of an enzyme-linked immunosorbent assay (ELISA) plate followed by 50 μL of the particular supernatant. The plate was gently shaken and incubated for 30 min at room temperature. The wells were washed with wash buffer and 100 μL of a dilution of the particular detection antibody was added to each well. After incubation for 1 h at room temperature, each well was washed and 100 μL of horseradish peroxidase-conjugated Avidin was added to each well. Following incubation for 20 min at room temperature, each well was washed and 100 μL of development solution was dispensed. After incubating for 15 min, 100 μL of stop solution was added to each well. The absorbance of the fluid in each well was read at 450 nm using an ELISA plate reader (Tecan, Männedorf, Switzerland). The amount of the individual cytokine was determined based on the standard curve of each cytokine.

2.10. Determination of body weight change and survival rate of ferrets fed with Red Ginseng extract for 60 d

Seven-to-eight wk old ferrets (*Mustela putorius furo*; $n = 10$ per group) obtained from Path Valley Farm (Spring Run, PA, USA) were fed a daily diet containing Korean Red Ginseng extract (50 mg/kg body weight) and were intranasally (i.n.) challenged with a 10 ferret lethal dose 50/mL (10 FLD 50/mL) of HP H5N1 influenza virus 60 d after commencement of the diet. The body weight change of the surviving ferrets and the survival rates of infected ferrets were observed for 14 d.p.i. Similarly, age-matched ferrets that were not fed with Red Ginseng extract were used as controls for comparison.

2.11. Statistical analysis

Groups of data were analyzed by repeated measures analysis of variance (ANOVA) using pairing of samples with IBM SPSS version 20 (International Business Machines Corp, New York, USA). The differences between the Red Ginseng administered and the control were considered along with time and interaction between both. Any analyses showing $p < 0.05$ were considered significant.

3. Results

3.1. Protective efficacy of Red Ginseng in mice against HP H5N1 influenza virus

Given that ginseng is an immune stimulator, it was of interest to determine whether mice fed with Red Ginseng could be protected from the lethal infections of HP H5N1 influenza virus. The effects of time-course feeding of Red Ginseng were evaluated in mice (Fig. 1A). The survival rate of mice increased when the time of ginseng feeding was longer. None of the 20 mice fed for 3 d or 5 d prior to the challenge with the lethal H5N1 influenza virus survived, whereas three (15% survival rate), seven (35% survival rate), nine (45% survival rate), and nine (45% survival rate) out of 20 mice fed for 15 d, 30 d, 60 d, and 80 d prior to the lethal challenge with H5N1 influenza virus survived, respectively. A Red Ginseng feeding period of 60 d was subsequently used, because the efficacy of Red Ginseng for the survival rate of mice against HP H5N1 influenza virus was optimal. After mice fed with Red Ginseng for 60 d were challenged with HP H5N1 influenza virus, the temporal changes in body weight and survival rates were determined (Fig. 1B, C). The surviving mice displayed up to a 20% reduction in body weight, whereas the control mice displayed up to a 25% reduction in body weight until 5 d.p.i., when all control mice had died (Fig. 1B). The survival rate of mice fed with Red Ginseng was initially 80%, but declined to 45% by the final day of observation (14 d.p.i.; Fig. 1C).

Viral titers in the lungs and brains of control mice or mice fed with Red Ginseng were determined following the challenge with HP H5N1 influenza virus. The viral titers in the lungs of Red Ginseng-fed survived mice peaked at 5 d.p.i. with 5.0 EID 50/mL, and were under the detection limit of 1 EID 50/mL on 14 d.p.i. Viral titers in control mice peaked at 7 d.p.i. with 8.0 EID 50/mL (Fig. 2A). The viral titers in the brains of Red Ginseng-fed survived mice peaked at 5 d.p.i. with 2.0 EID 50/mL and were under the detection limit (1 EID/mL) at 14 d.p.i., whereas the titer of unfed mice peaked on 7 d.p.i. with 5.5 EID 50/mL (Fig. 2B).

Lung tissues of mice were stained with H&E 5 d after the challenge with HP H5N1 influenza to evaluate the pathological damage. Lung tissue obtained from Red Ginseng-fed, virus-challenged mice displayed an appearance consistent with mild pneumonia with some lymphocyte infiltration (Fig. 3B), whereas tissue obtained from the control virus-challenged mice displayed severe interstitial pneumonia with heavy lymphocyte infiltration and some mucus in the bronchioles (Fig. 3C).

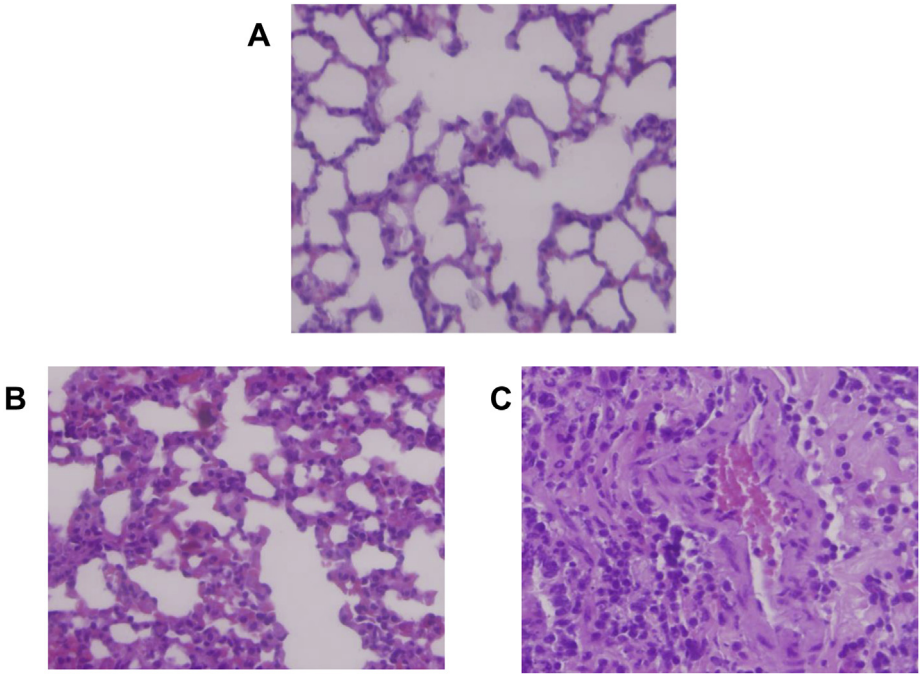

Fig. 3. Evaluation of pathological damage to the lungs of mice fed with Red Ginseng following viral challenge. Mice fed with Red Ginseng extract (50 mg/kg) for 60 d were intranasally (i.n.) challenged with highly pathogenic (HP) H5N1 influenza virus and the surviving mice were euthanized on 5 d postinfection (d.p.i.), and the lungs were collected. Lung tissues were cut and stained with hematoxylin and eosin (H&E) stain. Lung tissues of (A) control uninfected mice, (B) mice fed with Red Ginseng and infected with HP H5N1 influenza virus, and (C), and control mice infected with HP H5N1 influenza virus are shown.

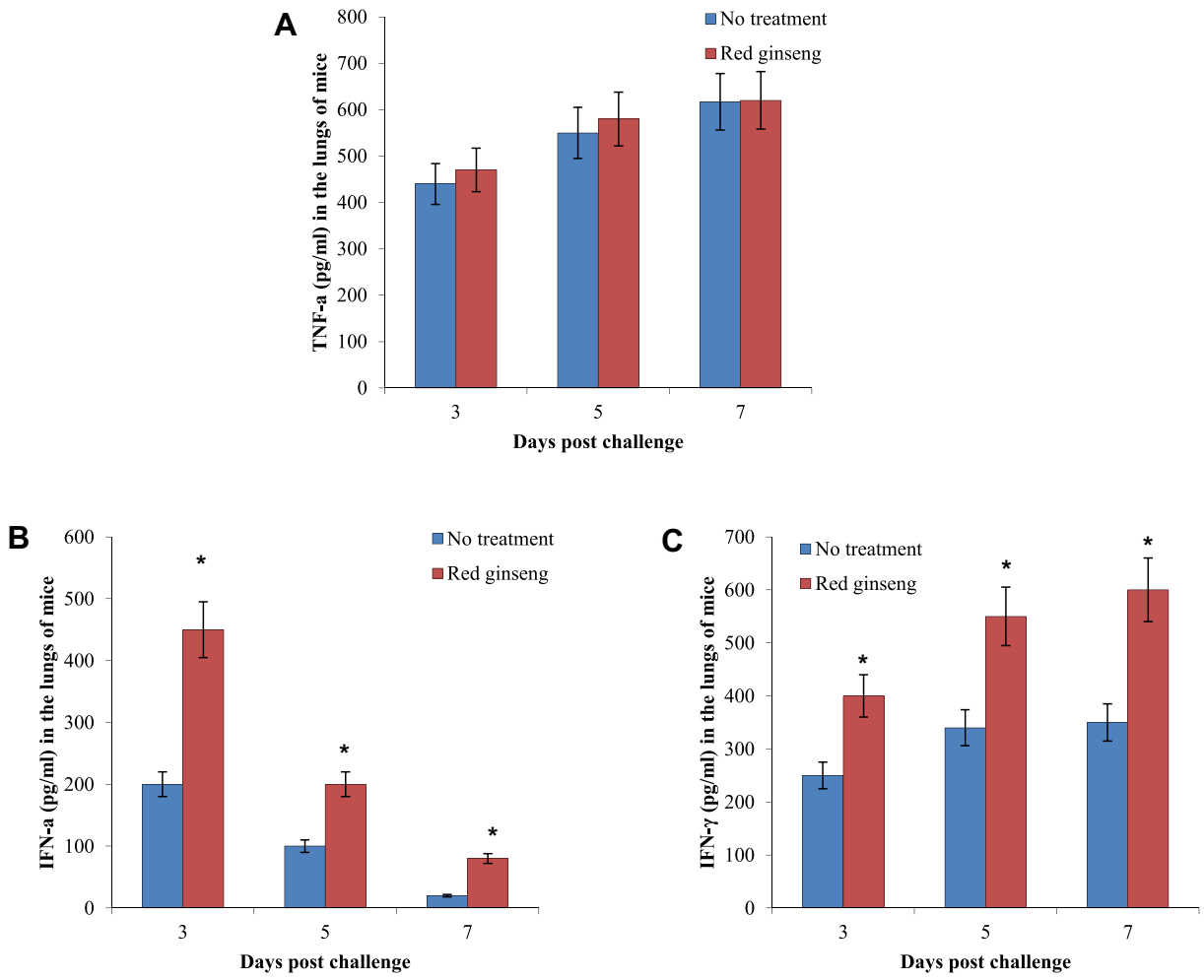

Fig. 4. Evaluation of cytokine induction in the lungs of mice fed with Red Ginseng. Mice fed with Red Ginseng extract (50 mg/kg) for 60 d were intranasally (i.n.) challenged with highly pathogenic (HP) H5N1. The surviving mice ($n = 3$) were euthanized on 3 d, 5 d, or 7 d postinfection (d.p.i.), and the lungs were collected. The collected lungs were homogenized in phosphate buffered saline (PBS) and the induced amount of cytokines of (A) tumor necrosis factor-alpha (TNF-α), (B) interferon-α (IFN-α), and (C) IFN-γ were detected. Groups were compared using repeated measures analysis of variance (ANOVA). $^*p < 0.05$. d.p.c., days post challenge.

3.2. Evaluation of inflammatory cytokines in the lungs of mice

The inflammatory cytokines such as TNF-α, IFN-α, IFN-γ, and IL-4 in the lungs of mice were measured to discern the possible mechanism by which Red Ginseng aided mice in surviving a lethal challenge of the HP H5N1 influenza virus. The induction of the pro-inflammatory cytokine TNF-α was similar between control mice and mice fed with Red Ginseng following the virus challenge (Fig. 4A). However, IFN-α and IFN-γ antiviral cytokines were induced much more in mice fed Red Ginseng than in control mice. IFN-α peaked on 3 d.p.i. (450 pg/mL; Fig. 4B). The IFN-γ level in the lungs of mice fed with Red Ginseng and control mice was 600 pg/mL and 350 pg/mL, respectively, at 7 d.p.i. (Fig. 4C). IL-4 induction was similar between both groups of mice (data not shown).

3.3. Protective efficacy of Red Ginseng in ferrets against HP H5N1 influenza virus

Ferrets are a good animal model for human influenza virus infection [29,30]. Presently, the body weight of surviving ferrets that had been fed with Red Ginseng and lethally challenged with HP H5N1 influenza virus was reduced up to 20% at 7 d.p.i., whereas the body weight of control ferrets was reduced up to 25% at 5 d.p.i. (Fig. 5A). The survival rate of ferrets fed with Red Ginseng approached 40% at 14 d.p.i., the final day of observation, whereas none of the control ferrets lived to 14 d.p.i. (Fig. 5B).

4. Discussion

Human pandemics by new subtypes of influenza viruses are inevitable. HP H5N1 influenza virus is such a candidate. The preparedness for pandemics may include vaccine development, anti-influenza drug development, and immune-enhancing medicine. Ginseng has been regarded as an immune-enhancing compound in humans for a long time. Our study provides evidence for this view. Mice and ferrets fed with Red Ginseng could be protected from lethal challenges of HP H5N1 influenza virus.

When we tested the time-course effects of Red Ginseng in mice against HP H5N1 influenza virus, feeding for at least 15 d was necessary for protection, suggesting that Red Ginseng may act as an immune stimulator rather than a therapeutic agent. This view is entirely consistent with a variety of previous studies [24,31–34]. Repeated oral administration of *Panax ginseng* extract to mice resulted in protection from the infections of Semliki forest virus up to 34–40% [24]. A study with Chinese herbal medicinal ingredients containing ginsenosides from ginseng showed that the inoculation of rabbits with a mixture of rabbit hemorrhagic disease (RHD) vaccine and the herbal ingredients could enhance rabbit

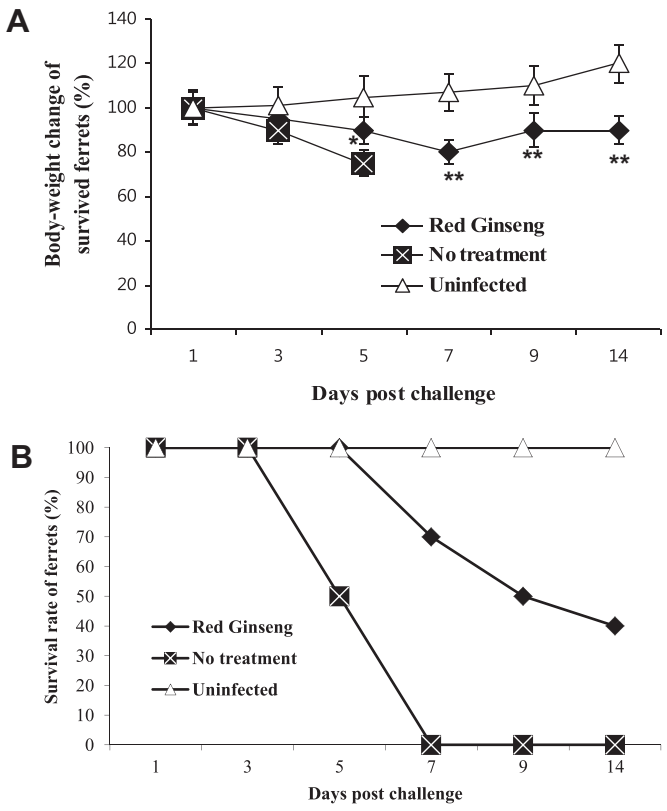

Fig. 5. Body-weight change and survival rate of ferrets fed with Red Ginseng. Ferrets ($n = 10$) fed with Red Ginseng extract (50 mg/kg) for 60 d and intranasally (i.n.) challenged with 10 ferret lethal dose (FLD) 50/mL of highly pathogenic (HP) H5N1 influenza virus were observed for (A) body-weight change and (B) survival rate for 14 d. Groups were compared using repeated measures analysis of variance (ANOVA). $^{*}p < 0.05$. $^{**}p < 0.001$. d.p.c., days post challenge.

lymphocyte proliferation and the inductions of IFN-γ and IL-10 mRNA by T lymphocytes [31]. A study that assessed the immune enhancing prowess of ginsenoside Rg1 from *Panax ginseng* using sheep red cells as an antigen showed that the number of spleen plaque-forming cells, titers of serum hemagglutinin, and the number of antigen-reactive T cells could increase in mice [32]. When ginsenoside Re from the root of ginseng was inoculated into mice with split-inactivated H3N2 influenza virus antigen, serum antibodies, lymphocyte proliferation, and the secretion of IFN-γ and IL-5 were enhanced [33]. Ginseng may also be beneficial for those infected with the human immunodeficiency virus; long-term ginseng use has been linked to slowed depletion of CD4 T lymphocytes in such patients [34].

The present study demonstrates that mice and ferrets fed with Red Ginseng could be protected from the lethal challenges of HP H5N1 influenza virus. The results hold out the potential that Red Ginseng may contribute to protecting humans from pandemic influenza virus prior to when the pandemic vaccine or an effective anti-influenza drug is available. In the event of such a pandemic, an estimated 30% of the global human population would be at risk of infection, because most humans do not have prior immunity to pandemic influenza virus. Considering the vast geographic distribution of HP H5N1 influenza virus and its ability to infect humans, H5N1 influenza virus is a prime candidate as a pandemic cause [35,36]. During such an event, daily consumption of Red Ginseng may increase the likelihood of human survival from exposure to a lethal dose of HP H5N1 influenza virus, at least until an effective vaccine becomes available and prophylactic protection can be established. The pandemic vaccine can be developed only after the pandemic virus is available because HP H5N1 influenza virus continuously evolves [37]. In addition, HP H5N1 influenza virus that is resistant to the most used anti-influenza drug, Oseltamivir, has already emerged [38]. Our results indicate that the underlying mechanism that feeding of mice and ferrets with Red Ginseng help to increase the survival rate of these animals from the lethal infections of HP H5N1 influenza virus may be due to the enhanced inductions of antiviral cytokines of IFN-α and IFN-γ. It is well established that IFN-α and IFN-γ could inhibit the replication of influenza viruses [39,40]. Further studies such as cytokine production, viral titers, and histological pathology in ferrets may be needed to support the immune enhancing effects of Red Ginseng against HP H5N1 influenza virus. At this moment, no commercially available ELISA kits for measuring ferrets' cytokines at the level of proteins exist.

In summary, we studied the effects of Red Ginseng on protective immunity of mice and ferrets against HP H5N1 influenza virus. Our results suggest that taking Red Ginseng daily may contribute to protecting humans from the lethal infections of HP H5N1 influenza virus in the event of a pandemic by HP H5N1 influenza virus.

Conflicts of interest

All authors declare no conflicts of interest.

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