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Full Length Research Paper

The antiviral effect of fullerene-liposome complex against influenza virus (H1N1) *in vivo*

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Influenza viruses are important pathogens for humans and the discovery of novel anti-influenza drugs with low toxicity deserves great efforts. Fullerenes have attracted considerable attention in different fields of sciences including antiviral activity. We synthesized a fullerene-liposome incorporated compound and investigated its antiviral activity on influenza virus infection in a mouse model. The results showed that fullerene-liposome could reduce mean pulmonary virus yields, decrease the lung index and eventually significantly prolong mean time to death (MTD) and decrease mortality of H1N1 virus-infected mice. Our data indicated that fullerene-liposome has the anti-influenza activity *in vivo* at much lower concentrations as compared to the Rimantadine, and then reveals that fullerene-liposome is a promising agent in the clinical therapy of influenza infection with favorable water-solubility and low toxicity.

Key words: Fullerene-liposome, influenza virus infection, antiviral activity.

INTRODUCTION

Influenza virus is a negative single-strand RNA enveloped virus and belongs to the Orthomyxoviridae family that include influenza A, B, C and thogotoviruses. Influenza virus can cause highly contagious, febrile, acute respiratory illness in humans and remain a leading cause of morbidity and mortality, especially in the immuno-compromised patients, in very young and elderly populations. Influenza viruses are able to undergo rapid and unpredictable antigenic change, and thus, result in annual seasonal epidemics and recurrence of pandemic influenza at irregular intervals worldwide with varying rates of illness and death (Colacino et al., 1999). Recently, new strain of swine influenza H1N1, reported from Mexico and the United States, shows sustained human-to-human transmission and is responsible for 267,105 reported cases of swine influenza affecting 175 countries, with a total of 2,692 deaths (Khanna et al., 2009).

Significant efforts have been made to explore effective treatment or prevention of influenza virus infections. Vaccination is the primary and efficacious strategy for the prevention and control of influenza. Although, more novel vaccine methodologies are being developed, the protection still varies widely because of its highly strain specificity, variation of influenza virus and the recipient's self-factors(Lambert and Fauci, 2011). Therefore, the availability of effective antiviral drugs would be of obvious value. The adamantanes (amantadine and rimantadine) are effective against influenza A viruses by blocking the proton channel activity of the viral M2 protein, but their clinical application is confined by the lack of activity against influenza B, toxicity and the rapid development of resistance. The neuraminidase inhibitors (zanamivir and oseltamivir) represent the other class of drugs against both influenza A and B with high specificity by interacting with the conserved sialic acid binding site of the viral

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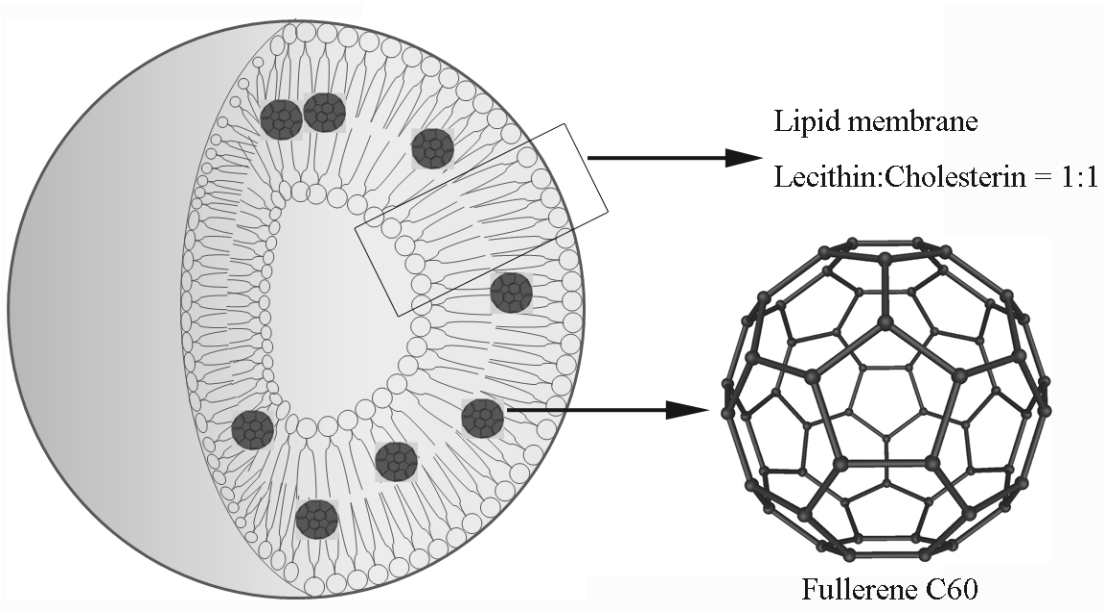

Figure 1. The structure of fullerene liposome.

neuraminidase. Recently, there are some novel antiviral drug developed to target other phases of the life cycle of influenza or immuno-modulatory treatment / antiviral combinations available as alternative treatment, such as antiviral proteins (for example, modified collectins, human immunoglobulin), or oligonucleotides (for example, antisense DNA oligomers, short interfering RNA) (Kandel and Hartshorn, 2001). Due to the rapid development of resistance, toxicity, and other limitations of these antiviral drugs, it remains a high priority to discover antiviral therapy against influenza virus.

As a nanomaterial, fullerene-C60 and its derivatives have shown an excellent potential in controlling oxidative stress, which hint at their use in antioxidant thepeutics in related disease, including ischemia / reperfusion injury, inflammatory apoptosis and neurogenerative diseases (Bakry et al., 2007; Partha and Conyers, 2009). Recently, researchers demonstrated the activities of various C60-based derivative against human pathogens *in vitro*, such as HIV (Durdagi et al., 2008; Marchesan et al., 2005), CMV (Medzhidova et al., 2004), influenza virus (Piotrovskii et al., 2001), VZV, SFV (Bakry et al., 2007) and Gram-negative bacterium (Spesia et al., 2008). Pristine fulleren-C60 is insoluble in water, which is thought to be an obstacle to its biological application. In a previous study, we showed that a nano carbon fullerene liposome directly inactivated the viruses under the light illumination with IC_{50} less than 2μg/ml (Ji et al., 2008). However, no long term studies on their antiviral activity to humans or animals are currently available with respect to possible utilization in the future. Hence, we initiated the present study to evaluate the antiviral effects of fullerene-liposome against influenza virus (H1N1) *in vivo*.

MATERIALS AND METHODS

Viruses and animals

FLU-A (A/Yamagata/120/86 H1N1) was propagated in the allantoic cavities of 10-day-old chicken eggs. After 72 h at 35°C and 12 h at 4°C, the allantoic fluid was harvested and centrifuged at 5000 rpm for 15 min to remove cellular debris, and viral titer was above 1: 640, determined by hemagglutination test. The harvested viruses were then inoculated onto SPF Balb/C mice for species adaption. The lung homogenates were collected and stored in multiple single-use aliquots at -80 for animal inoculation. The viral titer, mouse 50% lethal dose (LD50) was determined as $1 \times 10^{-6.3}$ by Reed-Muench method.

In vivo experiments were carried out with specific-pathogen-free BALB/c mice, 5 to 7 weeks old, obtained from Animal Center of Wuhan University. All the animal research was conducted in compliance with the internationally accepted principles and guidelines for Care and Use of Laboratory Animals of Wuhan University, which is in accordance with the Chinese Animal Protection Act and the National Research Council criteria.

Preparation of fullerene-liposome

Fullerene (C60), provided by Wuhan University 3D Carbon Material Co., Ltd., was dissolved in methylbenzene (12 to 15 mg: 17 to 18 ml). Lecithin/chloroform mixture (1: 1 molar ratio) was added into chloroform / methanol solution (72/24 ml). Two parts were mixed up and condensed by rotary evaporator at 30°C to form a homogeneous lipid membrane. After overnight incubation in vacuum oven, pristine fullerene-liposome was dissolved in ethylether / PBS solution (50/25 ml, pH 7.0) and sonicated for 30 min while purging with nitrogen. The final fullerene-liposome suspension was stored at 4°C after filtration via 0.22 μm filter. This C60-incorporating-liposome was approximately 28.7 to 100 nm in diameter and the average encapsulation efficiency was 80%. The scheme of fullerene-liposome incorporation is shown in Figure 1. Its solution of 10% aqueous phase was employed in the experiment.

Protective efficacy in mice

BALB/c mice were anesthetized by aether inhalation and infected intranasally with 50 µl viral suspension containing 100 LD_{50} of H1N1. The mice infected with virus were randomly divided into 6 groups: fullerene-liposome at a dose of 0.8, 1.6, 3.3 mg/kg/day and Rimantadine at a dose of 90 mg/kg/day were orally administered three times daily to the mice (at 8 h intervals) for 6 days beginning 24 h pre-virus exposure; the virus controls with viral infection and the normal controls without viral infection received no treatment. Considering the low toxicity shown in the cell culture (Ji et al., 2008) *in vivo* toxicity was evaluated under the same dosage in a preliminary and parallel experiment (6 mice per group). Animal weights were determined every day. The mice (20 mice per group) were observed daily for 16 days after infection in the survival study. The protection was estimated by the reduction of the rate of mortality and prolongation of mean time to death (MTD). In the lung virus yield study (8 mice per group), the lung were harvested, weighed, and the lung index was calculated as (lung weights/body weight) x 100%. The harvested lung was homogenized to 10% (w/v) suspensions in test medium on the 6^{th} day after viral exposure. The homogenates were frozen and thawed twice followed by 10 min of centrifugation at 3000rpm. Virus titrations were measured by hemagglutination test.

Statistical analysis

The data were analyzed by SPSS 17.0 software. The data of in vivo experiments was analyzed using Wilcoxon test for survival rates, One-Way ANOVA for MTD and Kaplan-Meier method for survival analysis.

RESULTS

Protective efficacy of fullerene-liposome in H1N1-infected mice

In order to test if fullerene-liposome is active against H1N1 in animal model, mice infected with H1N1 virus developing into fatal viral pneumonia were used as evaluation model. Body weight loss, lethality and median survival time were employed to determine the antiviral efficiency of fullerene-liposome. The signs of murine influenza pneumonia were observed in the mice at Day 3 after inoculation, such as tendencies to huddle, ruffled fur and diminished vitality. As shown in Figure 2, most of mice in virus control group (17/20, survival rate as 15%) died within 16 days because of the disease, with MTD as 9.4 ± 0.71 days. However, survival rate increased and MTD prolonged in all the treatment groups of the infected mice compared to the virus control group ($p < 0.01$). The 0.8 mg/kg/day fullerene-liposome groups showed slightly weaker survival rate (9/20, 55%) and shorter MTD (12.4 ± 0.9days) compared to those in 1.6 mg/kg/day (14/20(70%), 15.05 ± 0.4 days) and 3.3 mg/kg/day (16/20 (80%), 15.5 ± 0.3 days) groups ($p < 0.01$). Furthermore, it is notable that the antiviral effect of two larger dosage groups are equivalent to that of Rimantadine-treated group (13/20 (65%), 14.7 ± 0.5days) regarding to the indexes of survival rate and MTD ($p > 0.05$). However, approximately 30 times more of Rimantadine was needed to achieve the same level of therapeutic effect as was seen with fullerene-liposome.

Changes of lung virus yield in the H1N1 infected mice

To better evaluate the antiviral activity of fullerene-liposome in H1N1-infected mice, infectious viral titers in murine lungs were measured. Oral administration of fullerene-liposome beginning 24 h pre-virus infection significantly decreased the virus titers of mice lung homogenates. As shown in Figure 3B, the viral yields were reduced to 13.1 ± 1.4, 10.8 ± 1.7 and 8.6 ± 1.3 ($p < 0.01$) in the groups treated with fullerene-liposome at 0.8, 1.6, 3.3 mg/kg/day, respectively, whereas the yields in viral control group were 19.3 ± 1.5. Additionally, this inhibitory effect was enhanced in a dose-dependent manner. Furthermore, infection with H1N1 virus led to an increase in mean lung weight, which was due to pulmonary inflammation and edema, and was detectable on day 6 after viral exposure. Our results (Figure 3A) showed that fullerene-liposome treatment dramatically decreased lung index compared to the viral control group during the progress of the disease ($p < 0.01$) and there is no significant differences among drug treatment groups (fullerene-liposome and rimantadine) regarding to reduction of lung index ($p > 0.05$). These results suggested that fullerene-liposome may be effective for prevention of influenza virus infection.

DISCUSSION

Fullerenes have been investigated extensively due to their unique physical and chemical characteristics since their discovery in 1985 (Partha and Conyers, 2009).

However, lack of solubility of fullerenes restricts their utilization in biomedicine and many efforts have been made to address this problem. Liposome has attracted considerable attention as materials for drug-delivery systems. In the previous study, we demonstrated that fullerene-liposome compound presented virucidal effect against influenza virus directly and this virucidal activity was dependent upon both the dosage of fullerene derivatives used in the treatment and the light exposure level. To further investigate the antiviral activity in an integral biological model, we employed an H1N1-infected BALB/c mice mouse model which can be used to screen and evaluate the anti influenza virus agents. The fullerene-liposome treated mice showed better survival rate, prolonged MTD and reduced mean pulmonary virus yields compared to the virus control group.

Previous studies show that C60 incorporated liposome did not exhibit significant phototoxicity and bacterial reverse mutagenicity, which suggested that fullerene-liposome incorporation could be a potent way for

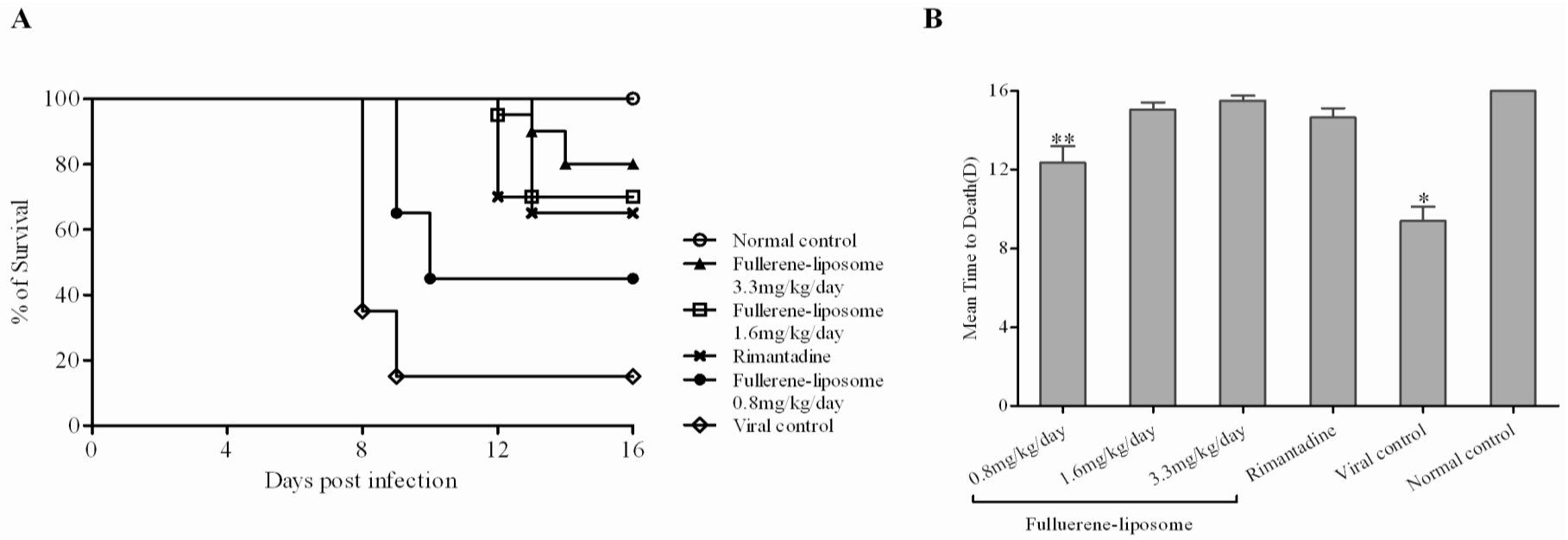

Figure 2. Effects of orally administrated fullerene-liposome on H1N1 challenge model. Fullerene-liposome at 0.8, 1.6, 3.3 mg/kg/day was orally administered 24 h before viral infection with 100 LD_{50} of H1N1. (A) Survival curves for H1N1 infected BALB/c mice with fullerene-liposome. (B) Mean time death (MTD) of BALB/c mice from different experimental group. Mice in normal control group, as well as some mice in other groups, survived during the observation period and the MTD was considered as 16 days. *All the fullerene treated group showed favorable survival rates and longer MTD in H1N1infected mice compared to control group ($p < 0.01$). **The 1.6 mg/kg/day and 3.3 mg/kg/day fullerene-liposome groups and Rimantadine-treated group showed prolonged MTD compared to those in 0.8 mg/kg/day groups ($p < 0.01$).

fullerene application in biomedicine with favorable water-solubility and low toxicity (Kato et al., 2009). Our results demonstrate that fullerene-liposome can exert this anti-influenza activity at much lower concentrations compared to the Rimantadine, which reveals low toxicity of fullerene-liposome and favorable potential application in the field of biomedical sciences.

Previous studies demonstrate that fullerenes and their derivatives can inactivate various enveloped viruses via the photodynamic reactions induced by photoactivation, including SFV, VZV, CMV), (Kasermann and Kempf, 1997) Dengue-2 virus (Lin et al., 2000) influenza virus (Vladimir et al., 2007). On the other hand, different manners of pharmaceutical actions have been demonstrated regarding to the antiviral activity of fullerenes. For example, several biological properties may be responsible for the antiviral activity of fullerene derivatives against HIV *in vitro*, including the antioxidant activity and their unique molecular architecture (Bakry et al., 2007). The antiviral structural activity is mainly reflected in the following: inhibit and make complex with HIV protease, suppress HIV replication, and so on. Besides, Lens et al. (2008) successfully retained biological antioxidative properties of fullerenes with enhanced water solubility by encapsulating fullerenes in biocompatible liposomes. We assume that there could be multiple mechanisms involved in H1N1 inhibition by fullerene-liposome complex.

This is also the first long term study on anti-H1N1 activity of fullerene-liposome to animals. Biodistribution studies of water-soluble fullerene derivatives in BALB/c mice demonstrate that the compounds are not acutely toxic and have a blood pool remnant time of more than 1 h followed by quick and totally clearance thereafter (Cagle et al., 1999). Furthermore, $(Gd@C_{82}(OH)_{22})n$, a fullerene derivative, could regulate ROS production *in vivo* by reducing the activities of enzymes and other

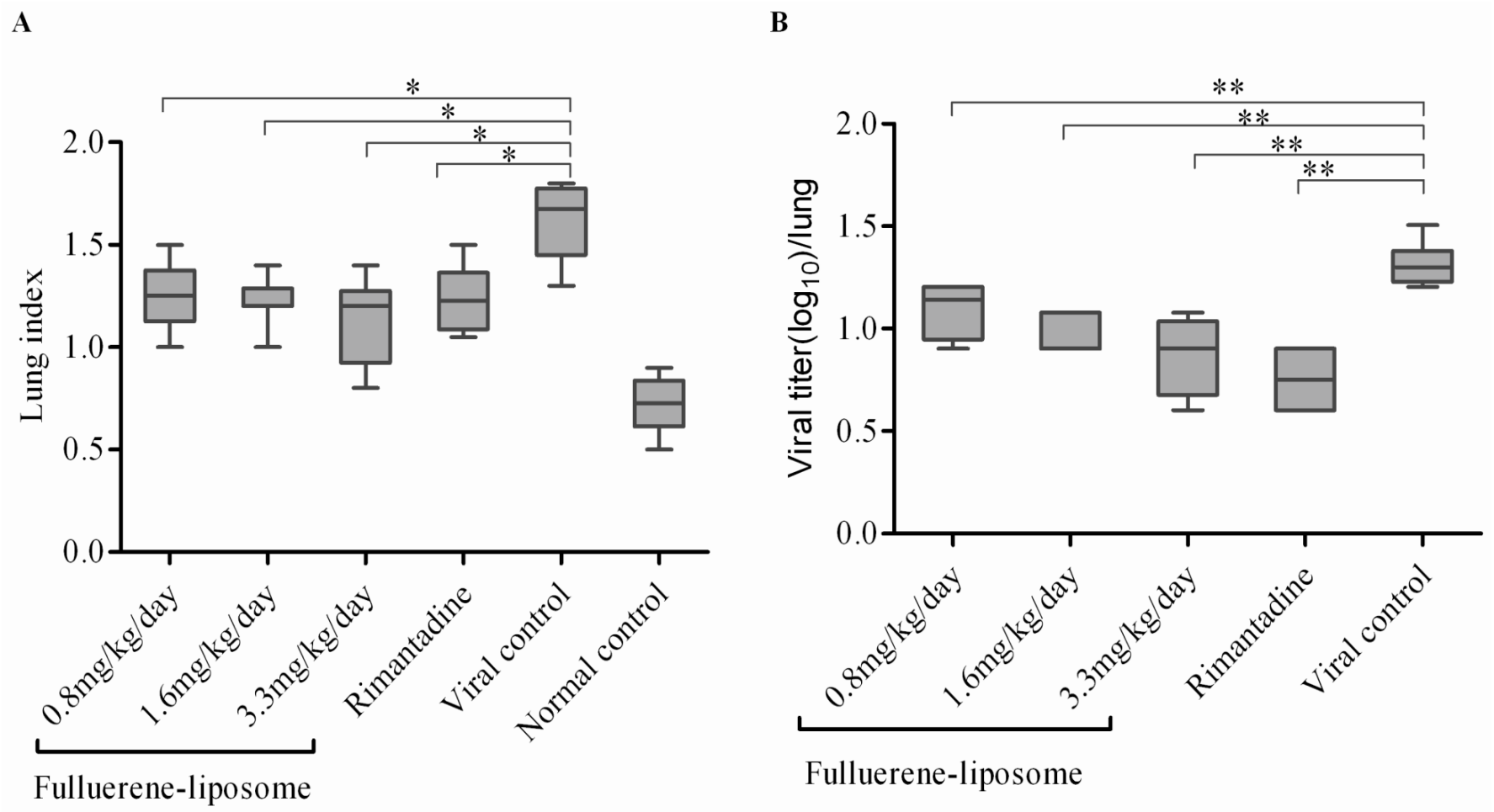

Figure 3. Effect of oral administration with fullerene-liposome on lung virus yield in H1N1 infected mice. Fullerene-liposome at 0.8, 1.6, 3.3 mg/kg/day was orally administered 24 h before viral infection with 100 LD_{50} of H1N1. Mice from each group were sacrificed on day 6 post-infection, and thus lung index was calculated as (lung weight/body weight) x 100%. (A) Viral titer of lungs were determined by Reen-Muench method (B) *All the fullerene-liposome treated group and Rimantadine-treated group showed decreased lung index of H1N1-infected mice compared to viral control group ($p < 0.01$). ** All the fullerene-liposome treated group and Rimantadine-treated group showed lower viral titer of H1N1infected mice compared to viral control group ($p < 0.01$).

parameters related to oxidative stress in tumor-bearing model (Wang et al., 2006). This can explain why fullerene-liposome also exhibits a protective antiviral activity against H1N1 *in vivo* in our study. We assume that fullerene-liposome complex may also be able to regulate the ROS production and further result in a significant protection of viral infected animals. There could be more mechanism involved in, which leaves us

an open question in the future study.

Conclusions

In summary, our results indicated that fullerene-liposome was an effective antiviral agent against influenza virus both *in vitro* and *in vivo*. Other studies have also demonstrated antiviral activities of fullerene against some enveloped viruses, such as HIV, human cytomegalovirus, SFV, VZV. Considering these findings, fullerene-liposome may potentially play a significant role in controlling influenza virus infection with broad spectrum of antiviral activities and low toxicity in the nanomaterial field.

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