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Immunomodulaton and attenuation of lethal influenza A virus infection by oral administration with KIOM-C

Eun-Ha Kim [a], Philippe Noriel Q. Pascua [a], Min-Suk Song [a,c], Yun Hee Baek [a], Hyeok-Il Kwon [a], Su-Jin Park [a], Gyo-Jin Lim [a], Se mi Kim [a], Arun Decano [a], Kwang Jin Lee [b], Won-Kyung Cho [b], Jin Yeul Ma [b,*,1], Young Ki Choi [a,c,*,1]

[a] College of Medicine and Medical Research Institute, Chungbuk National University, 12 Gaeshin-Dong Henugduk-Ku, Cheongju 361-763, Republic of Korea
[b] Korean Medicine (KM) Based Herbal Drug Research Group, Korea Institute of Oriental Medicine, 1672 Yuseongdea-ro, Yuseong-gu, Deajeon 305-811, Republic of Korea
[c] ID.bio Co., 151 Chungbuk National University, 12 Gaeshin-Dong Henugduk-Ku, Cheongju 361-763, Republic of Korea

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ABSTRACT

Herbal medicine is used to treat many conditions such as asthma, eczema, premenstrual syndrome, rheumatoid arthritis, migraine, headaches, menopausal symptoms, chronic fatigue, irritable bowel syndrome, cancer, and viral infections such as influenza. In this study, we investigated the antiviral effect of KIOM-C for the treatment of influenza A virus infection. Our results show that oral administration of KIOM-C conferred a survival benefit to mice infected with the 2009 pandemic H1N1 [A(H1N1)pdm09] virus, and resulted in a 10- to 100-fold attenuation of viral replication in ferrets in a dose-dependent manner. Additionally, oral administration of KIOM-C increased the production of antiviral cytokines, including IFN-γ and TNF-α, and decreased levels of pro-inflammatory cytokines (IL-6) and chemokines (KC, MCP-1) in the Bronchoalveolar lavage fluid (BALF) of A(H1N1)pdm-infected mice. These results indicate that KIOM-C can promote clearance of influenza virus in the respiratory tracts of mice and ferrets by modulating cytokine production in hosts. Taken together, our results suggest that KIOM-C is a potential therapeutic compound mixture for the treatment of influenza virus infection in humans.

1. Introduction

Influenza viruses, one of the major pandemic diseases worldwide, represent a grave threat to human health. The 1918 Spanish pandemic was caused by an influenza A (H1N1) virus that later contributed 5 of its 8 genes to generate the 1957 Asian (H2N2) virus. This reassortment event was followed by a replacement of the H2 HA and the PB1 gene with avian H3 HA and PB1 genes to create the 1968 Hong Kong influenza (H3N2) virus (Guan et al., 2010; Morens et al., 2009). Multiple reassortment events, including human, avian, and swine influenza A virus strains, then occurred, from which the 2009 pandemic H1N1 [A(H1N1)pdm09] virus emerged (Brockwell-Staats et al., 2009; Michaelis et al., 2009).

At present, there are two major classes of anti-influenza drugs currently available: amantadine and rimantadine are adamantane derivatives that belong to the matrix protein 2 ion-channel class of inhibitors (Brockwell-Staats et al., 2009; Englund, 2002; Wang et al., 1993) whereas oseltamivir and zanamivir are two of the currently prescribed neuraminidase inhibitors (Englund, 2002). However, resistant viruses against these classes of prophylactic agents have arisen in recent years (Hurt et al., 2009; Yang et al., 2010) requiring the need for additional anti-influenza therapy options. Through the years, the use of herbal extracts and compounds with anti-influenza virus activities and adjuvant-like or immunomodulatory effects started to gain interest (Droebner et al., 2007; Pleschka et al., 2009; Palamara et al., 2005; Ehrhardt et al., 2007; Wang et al., 2006). Herbal extracts and compounds, naturally produced from oriental medicines, are believed to demonstrate multiple bioactivities (Hudson and Towers, 1999) and are thought to be safe for both oral and nasal applications (Chung et al., 2012). If proven effective, they would provide more affordable and broad-spectrum alternative means for mitigating influenza A virus infections.

Over 100 herbal medicine formulas are being tested by the Center for Herbal Medicine Improvement Research at the Korea Institute of Oriental Medicine from 2010 to screen for novel antiviral compounds. These traditional herbal medicines from various herbal plants have been commonly used to treat various clinical symptoms such as fever, abdominal pains, myalgia, headache, malaise, nonproductive cough, sore throat, and rhinitis for centuries

* Corresponding authors. Address: College of Medicine and Medical Research Institute, Chungbuk National University, 12 Gaeshin-Dong Heungduk-Ku, Cheongju City 361-763, Republic of Korea. Tel.: +82 43 261 3384; fax: +82 43 272 1603 (Y.K. Choi), tel.: +82 42 868 9466; fax: +82 42 868 9573 (J.Y. Ma).
E-mail addresses: jyma@kiom.re.kr (J.Y. Ma), choiki55@chungbuk.ac.kr (Y.K. Choi).
[1] These corresponding authors contributed equally to this work.

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(Jun, 1999). Their recent efforts led to the discovery of KIOM-C, a compound mixture that can attenuate a clinical disease in pigs suffering from porcine circovirus-associated disease (PCVAD) by promoting increase in body weight and stimulating immune responses (Chung et al., 2012). Chung et al. (2012) reported that KIOM-C has been proposed as an efficient remedy for post-weaning stress among piglets and provide protection against viral infections.

In this study, we evaluated the efficacy of KIOM-C against infection with a mouse-adapted A(H1N1)pdm09 A/Korea/CJ01/2009 (A/Korea/maCJ01/2009) virus. Following A/Korea/ma- CJ01/2009 infection, groups of BALB/c mice were orally administered KIOM-C, then survival rates and immune responses in Bronchoalveolar lavage fluid (BALF) were determined. Furthermore, we tested the potential antiviral effects of KIOM-C in a ferret model by measuring viral replication rate in their infected lungs.

2. Materials and methods

2.1. Preparation of KIOM-C

KIOM-C was prepared by the Herbal Drug Research Group at the Korea Institute of Oriental Medicine. The compound mixture consists of *Scutellariae Radix*, *Glycyrrhizae Radix*, *Paeoniae Radix Alba*, *Angelicae Gigantis Radix*, *Platycodon grandiflorum*, *Zingiber officinale*, *Lonicera japonica Thunberg*, and *Saposhnikovia divaricate schiskin* which were purchased from Yeongcheon Oriental Herbal Market (Yeongcheon, Korea). Each voucher specimen was deposited in the herbal bank and 2.4565 mg of KIOM-C formula were placed in 15 L of distilled water and extracted by heating for 3 h at 115 °C (Gyeongseo Extractor Cosmos-600, Inchon, Korea). After extraction, KIOM-C was filtered out using testing sieves (150 μm) (Retsch, Haan, Germany) and stored at 4 °C prior to use.

2.2. HPLC analysis

The standard chemicals for Chlorogenic acid, Paeoniflorin, Cimifugin, Nodakein, Vanillylacetone, Baicalin, Platycodin D, Glycyrrhizin, 6-gingerol and Decursin were obtained from Sigma. Co. (USA), other Chemfaces Co. (China). The dried sample for each oriental medicine herb materials were purchased from traditional markets (South Korea, and China) in 2012. HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (USA). And Trifluroacetic acid (TFA) was purchased from Sigma. Co. (USA). The twice distilled water was filtered by a pump (Division of Mixllipore, Waters, Milford, MA, USA) and filter (FH-0.2 μm, Waters, Milford, MA, USA). Samples were prepared by dissolving 2 mg of the standard chemicals Chlorogenic acid, Paeoniflorin, Cimifugin, Nodakein, Vanillylacetone, Baicalin, Platycodin D, Glycyrrhizin, 6-gingerol and Decursin in 10 ml of methanol and adjusting the concentration to 200 ppm.

The KIOM-C sample extract was injected into a Dionex Ultimate 3000 HPLC system (Thromo scientific). The chromatographic columns used in this experiment are commercially available; one was obtained from RS-tech (0.46 × 25 cm, 5 μm, C18, Daejeon, Korea). The injection volume was 10 μl, and the flow rate of the mobile phase was 1.0 ml/min delivered by a Dionex Ultimate 3000 Pump. The wavelength of the UV detector was fixed at 210, 254 280 and 320 nm. Data were analyzed using Chromeleon 7 software. The composition of the mobile phases was reservoir A (Water/Trifluroacetic acid = 99.9/0.1, vol.%) and reservoir B (Acetonitrile 100%). The run time was 70 min and the solvent program was the linear gradient method (90:10-30:70, A: B vol.%).

2.3. Cells and virus

Madin-Darby Canine Kidney (MDCK) cells obtained from ATCC were maintained in Eagle's minimal essential medium (EMEM) (LONZA, Inc., Allendale, NJ) supplemented with 10% fetal bovine serum (LONZA, Inc.), 1% penicillin/streptomycin (Gibco-Invitrogen, Inc., Carlsbad, CA), and 1% non-essential amino acids (Gibco-Invitrogen, Inc.).

Virus strain A/Korea/CJ01/2009 is a human A(H1N1)pdm09 isolate from human Korea (Song et al., 2010). It was serially passaged in mice until it generated a mouse-adapted, highly virulent strain termed A/Korea/maCJ01/2009. Virus was propagated for 48 h at 37 °C in the allantoic cavities of specific-pathogen-free 10-day-old chicken eggs. Clarified allantoic fluids were aliquoted and then stored at −70 °C. One day later, virus was calculated the 50% tissue culture infectious dose ($TCID_{50}$) in MDCK cells by the method of Reed and Muench (1938).

2.4. Cell viability assay

MDCK cells were left untreated or treated with the indicated amounts of KIOM-C ranging from 0.001 to 20 mg/ml, and oseltamivir ranging from 001 to 100 mM/ml for 48 h; all compounds were multi proportionally diluted in serum-free medium. Cell-proliferation and metabolism were measured using the CCK8-assay. Briefly, the cells were treated with CCK-8 solution (10 μl/well, dojindo) and incubated for 4 h at 37 °C. The absorbance was measured using a Microplates Synergy HT plate reader at 450 nm. The untreated control was set at 100%, and the treated samples were normalized to this value according to the following equation: cell viability rate (%) = optical density (OD) of the treated cells – OD of blank control/OD of negative control – OD of blank control × 100. The concentration of KIOM-C that caused the death of the 50% of the cells was defined as the 50% cytotoxic concentration (CC_{50}).

2.5. Plaque reduction assay

For plaque reduction assays, confluent monolayer MDCK cells were seeded in 6 well tissue culture plate (5×10^5 cells/well) and incubated at 37 °C for 24 h under 5% CO_2 incubator. The medium was removed and the cells washed twice with Phosphate buffered saline (PBS, LONZA, Inc.). The cells were then infected with 0.001 MOI of A/Korea/maCJ01/2009 for 1 h, the inoculum was removed and the test medium containing the variable concentration of KIOM-C (0.001–20 mg/ml) or oseltamivir (001–100 mM/ml) were added. After 1 h incubation at 37 °C, the supernatant was removed and the cells were washed twice with PBS, and replaced with overlay medium (EMEM containing 1 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin, 0.7% agarose, without serum). Cells incubated during 48 h at 37 °C in 5% CO_2 incubator. Then, the test plates were fixed with 10% formaldehyde solution for 30 min. Agarose was removed and stained with 1% (w/v) crystal violet solution. The plaques were counted by visual examination and percentage of plaque reduction was calculated as relative to the control without KIOM-C. A required concentration to reduce the 50% plaque number (EC_{50}) was calculated by regression analysis of the dose–response curves generated from these data.

2.6. Experimental inoculation of animals and KIOM-C treatment

Groups of 24 five-week-old female BALB/c ($H\text{-}2D^d$) mice (Samtaco, Pyungteack, Korea) were lightly anaesthetized with Avertin (Sigma–Aldrich, St. Louis, MO) and inoculated intranasally (i.n.) with 5 times the 50% mouse lethal dose (MLD_{50}) of A/Korea/maCJ01/2009 ($10^{4.5}$ $TCID_{50}$/ml) in a volume of 30 μl. Following

infection, mice of each group were orally administered with 4 mg/ml (80 μg/kg/day), 8 mg/ml (160 μg/kg/day), or 20 mg/ml (400 μg/kg/day) of KIOM-C in a total volume 300 μl to BALB/c mice at 1, 3, 4, and 5 days post-infection (dpi) and then monitored daily for morbidity and assessed by measuring body weight losses and survival rates for up to 14 dpi. The mice showing more than 25% of body weight loss were considered dead and euthanized. The MLD_{50} was expressed as $\log_{10}$ $TCID_{50}$. The research protocol for the use of mice in this study were conducted in strict accordance and adherence to relevant policies regarding animal handling as mandated under the Guidelines for Animal Use and Care of the Korea Center for Disease Control (K-CDC) and was approved by the Medical Research Institute (approval number CBNU-IRB-2012-GM01). Animal care and the use of an enhanced biosafety level 3 containment laboratory were approved by the Animal Experiment Committee of Bioleaders Corp. (permit number BLS-ABSL-12-010).

Young female ferrets 15–18 weeks-of-age, seronegative for circulating influenza A H1N1, H3N2, and influenza B viruses, were obtained from China (Wuxi Sangosho Pet Park Ltd., Jiangsu). Groups of ferrets (9 animals per group) were anaesthetized and challenged intranasally (i.n) with $10^{5.0}$ $TCID_{50}$/ml of A/Korea/maCJ01/2009 (H1N1) and 3 ml of 8 mg/ml (30 mg/kg/day) or 20 mg/ml (75 mg/kg/day) of KIOM-C was administered orally for 5 days. Ferrets were assessed by measuring body weight loss, body temperature, and clinical sign for up to 7 dpi. Control-treated mice and ferrets were administered with same volume of PBS. All dilutions were conducted with endotoxin-free PBS (Sigma, St. Louis, MO).

Following challenge, observations were recorded according to the schedule defined in Fig. 4. Observations included the use of a scoring system based on work previously described (Reuman et al., 1989; Zitzow et al., 2002). 0 = alert and playful; 1 = alert but playful only when stimulated; 2 = alert but not playful when stimulated; 3 = neither alert nor playful when stimulated. Observations were recorded daily for all groups after 24 h post-challenge until the end of the study. Blinded evaluation was done by monitoring ferret activity without reference to the KIOM-C treatment group.

2.7. BALF samples and cytokine detection by multiplex microbead immunoassay

Bronchoalveolar lavage fluid (BALF) samples were collected from mice lungs at 1, 3, 4 and 5 days after viral infection or uninfected control group. The solution thus obtained was centrifuged at 12,000 rpm for 5 min at 4 °C, aliquoted, and stored at −70 °C until the analysis. A multiplex biometric immunoassay containing fluorescently-dyed microspheres conjugated with a monoclonal antibody specific for a target protein, was used for cytokine measurement according to the manufacturer's instructions (Bio-Plex Mouse Cytokine Assay; Bio-Rad Inc., Hercules, CA). Cytokines measured were: Interleukin 1 beta (IL-1β), IL-6, IL-10, Tumor necrosis factor-alpha (TNF-α), Interferon-gamma (IFN-γ), keratinocyte-derived chemokine (KC/CXCL1) and monocyte chemoattractive protein (MCP-1/CCL2). Briefly, 20 μl BALF samples were diluted 1:4 and incubated with antibody-coupled beads. Complexes were washed, then incubated with biotinylated detection antibody and, finally, applied with streptavidin–phycoerythrin prior to assessing cytokine concentration titers. Concentrated mouse recombinant cytokine was provided by the vendor (Bio-Rad Laboratories). A range of 1.95–32,000 pg/ml recombinant cytokines was used to establish standard curves and to maximize the sensitivity and the assay dynamic range. Cytokine levels were determined using a multiplex array reader from Luminex™ Instrumentation System (Bio-Plex Workstation from Bio-Rad Laboratories). The cytokines concentrations were calculated using software provided by the manufacturer (Bio-Plex Manager Software).

2.8. Tissue sample and nasal wash collection

To determine titers of infectious virus in lungs of infected mice, lung samples from three mice per group were aseptically collected at 3, 5, 7 and 9 dpi. Lung tissues from euthanized mice were extracted and homogenized in EMEM containing antibiotics (0.1% penicillin–streptomycin; Gibco). Ten-fold serial dilutions of supernatants were added in quadruplicate to a monolayer of MDCK cells seeded in 96-well cell culture plates 18 h before infection, allowed to absorb for 1 h at 37 °C in 5% CO_2 incubator and removed supernatant. Fresh EMEM (without serum, with 1 μg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-trypsin) was then added to the cells and the cells continued to incubate for 48 h at 37 °C in 5% CO_2 incubator. Virus cytopathic effect (CPE) was observed daily and the viral titer was determined by the hemagglutination (HA) test using 0.5% turkey red blood cells.

Ferret nasal washes were collected in 1 ml 1× PBS with antibiotics at 1, 3, 5, 7, and 9 days after virus challenge. Lung samples of ferrets at 3, 5, or 7 dpi were harvested and homogenized in the same volume (1 g/1 ml) of 1× PBS containing antibiotics. Tissue homogenates were clarified by centrifugation at 12,000*g* and supernatants were transferred to new tubes. Collected samples were immediately serially diluted 10-fold and then inoculated into 10-day-old embryonated chicken eggs for virus titration as computed with results expressed as $\log_{10}$ 50% egg infective doses (EID_{50}) per gram of tissue collected. All EID_{50}, and MLD_{50} calculations were done by the method of Reed and Muench (1938).

2.9. Statistical analysis

The data were analyzed using GraphPad Prism version 5.00 for Windows (GraphPad Software, La Jolla, CA). *P* values of less than 0.05 ($p < 0.05$) were considered to be statistically significant.

3. Results

3.1. HPLC analysis

Optimized HPLC analysis for identification of main components in KIOM-C was carried out using a C18 column and the flow rate of the mobile phase was fixed at 1.0 ml/min. The gradient elution

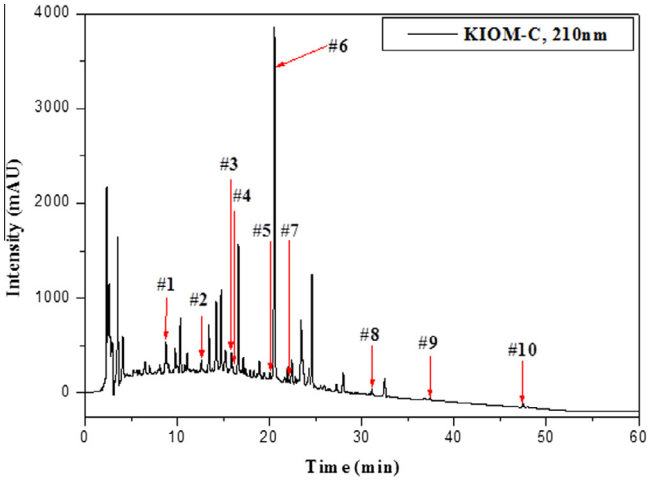

Fig. 1. Identification analysis of Chlorogenic acid (1), Paeoniflorin (2), Cimifugin (3), Nodakein (4), Vanillylacetone (5), Baicalin (6), Platycodin D (7), Glycyrrhizin (8), 6-gingerol (9) and Decursin (10) in KIOM-C by RP-HPLC-DAD.

proportions of water and acetonitrile were tested to achieve the desired separation and in order to improve the peak shape and inhibit the peak tailing, trifluoroacetic (TFA) acid was added to the water. The UV wavelengths of ten components were set based on the maximum UV spectra absorption of each component. After three independent trials, HPLC analyses identified Paeoniflorin, Cimifugin, Nodakein, Canillylacetone, Platycodin D, 6-gingerol and Decursin at 210 nm whereas Glycyrrhizin, Baicalin, Chlorogenic acid were detected at 254, 280, and 320 nm, respectively. After further processing and analysis of the independent profile runs, results revealed that some of the components of KIOM-C include: Chlorogenic acid (1, tR: 8.779 min), Paeoniflorin (2, tR: 12,613 min), Cimifugin (3, tR: 15.836 min), Nodakein (4, tR: 15.846 min), Canillylacetone (5, tR: 20.388 min), Baicalin (6, tR: 20.542 min), Platycodin D (7, tR: 22.228 min), Glycyrrhizin (8, tR: 31.050 min), 6-gingerol (9, tR: 37.425 min) and Decursin (10, tR: 47.486 min) (Fig. 1). Among these components, the extraction efficiency of Baicalin, a plant flavonoid, was the highest (15.03–15.12%) suggesting that it could be one of the active ingredients of KIOM-C.

Table 1
Effects of KIOM-C on A/Korea/maCJ01/2009 (H1N1).

Compound	CC_{50}[a]	Viral strain A/Korea/maCJ01/2009	
		EC_{50}[b]	SI[c]
KIOM-C	10 (±2.747)	2.5 (±0.5)	4
Oseltamivir	62.5 (±3.69)	10 (±3.42)	6.25

[a] Determined as the concentration of the compounds that reduced the cell viability to 50% of control (cells without addition of compound). Each represents the mean ± SD from three independent experiments. The unit for CC_{50} values shown in the table is mM/ml except KIOM-C, which is mg/ml.

[b] Determined as the concentration of the compounds needed to reduct plaque to 50% of control value (cells with only viral infection). Each represents the mean value from three independent experiments. The unit for EC_{50} values shown in the table is mM/ml except KIOM-C, which is mg/ml.

[c] Selectivity index (CC_{50}/EC_{50}).

3.2. Antiviral effect of KIOM-C in MDCK cells

The initial goal of our study was to determine whether KIOM-C has anti-influenza virus activity in cell culture. Cytotoxocity test was performed based on cell viability following treatment of the two compounds and when performed with Oselatmivir and KIOM-C, both have CC_{50} value of 62.5 ± 3.69 mM/ml and 10 ± 2.74 mg/ml (Oselatmivir and KIOM-C, respectively). Plaque reduction assay was also used to test the antiviral capacity of KIOM-C. Results show that at EC_{50} of 10 ± 3.4, Oseltamivir reduced plaque titers by 50% while KIOM-C exhibited inhibitory activity at EC_{50} values 2.5 ± 05 mg/ml and a selective index (CC_{50}/EC_{50}) of about 4 (Table 1). This suggests that KIOM-C can inhibit influenza virus infection.

3.3. Orally-administered KIOM-C promotes survival in influenza A virus-infected mice

The therapeutic potential of a novel herbal medicine against influenza virus infection was evaluated in a murine model of influenza A infection (Fig. 2). Briefly, 24 h after infection with 5 MLD_{50} of virus, each group of mice were orally administered 4, 8, or 20 mg/ml KIOM-C four times. Survival rates and body weight loss were measured every other day following infection. In contrast, all mice orally administered with 20 mg/ml KIOM-C survived up to 14 days after virus infection but displayed about 20% weight loss (Fig. 2A). Interestingly, we observed that survival was different between groups that received 8 mg/ml and 20 mg/ml KIOM-C. In this regard, the survival rate of mice administered 20 mg/ml KIOM-C (100% survival) was higher than that of virus-infected mice that received 4 mg/ml or 8 mg/ml KIOM-C (16.6% and 50% survival, respectively) (Fig. 2B).

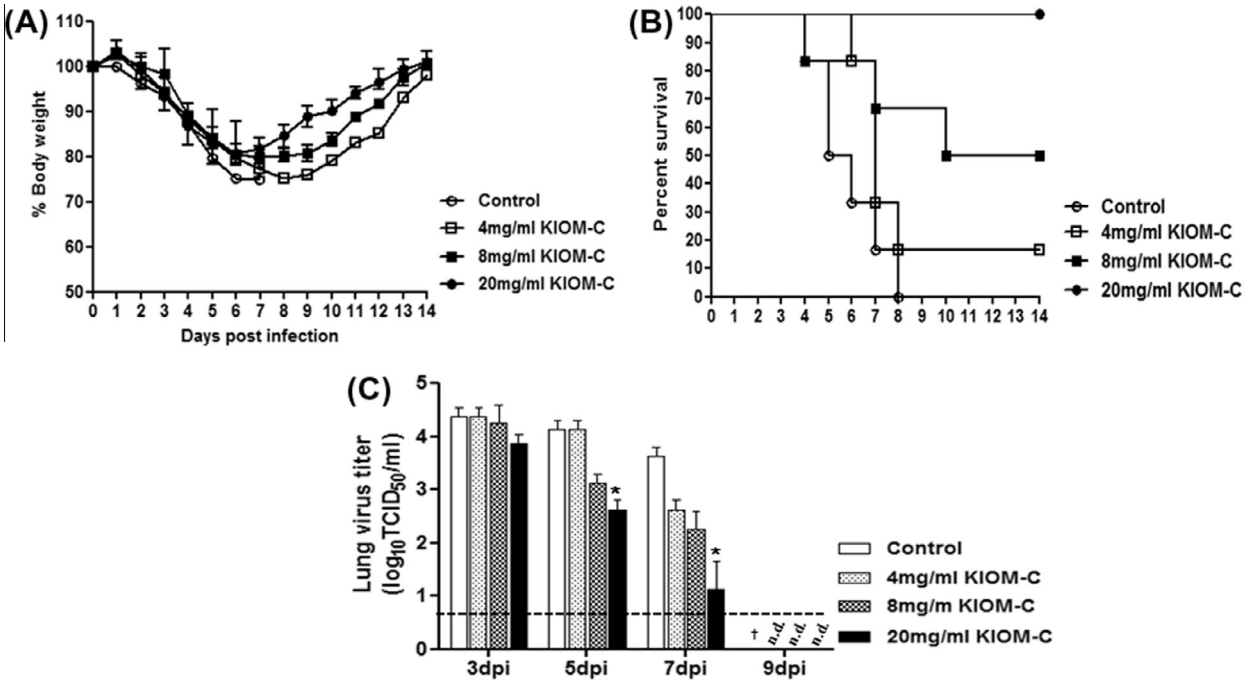

Fig. 2. Oral administration with novel herbal medicine (KIOM-C) provided protection against lethal infection with H1N1 influenza virus in mice. BALB/c mice intranasally infected with 5 MLD_{50} of A/Korea/maCJ01/2009 (H1N1) virus. Twenty-four hours after post-infection, mice were oral administrated 300 μl of KIOM-C (4, 8, and 20 mg/ml) orally. Mice were monitored for changes in (A) body weight and (B) survival rate daily for 14 days. (C) Influenza virus titers were detected at 3, 5, 7 and 9 days post-challenge. The data are presented as GMT ± SD of 3 mice per group. * Indicates $p < 0.05$ compared to the control group. The lower limit of detection (0.75 $log_{10}TCID_{50}$/g) is indicated by a dotted line. † indicate dead control group. n.d. indicates not detected.

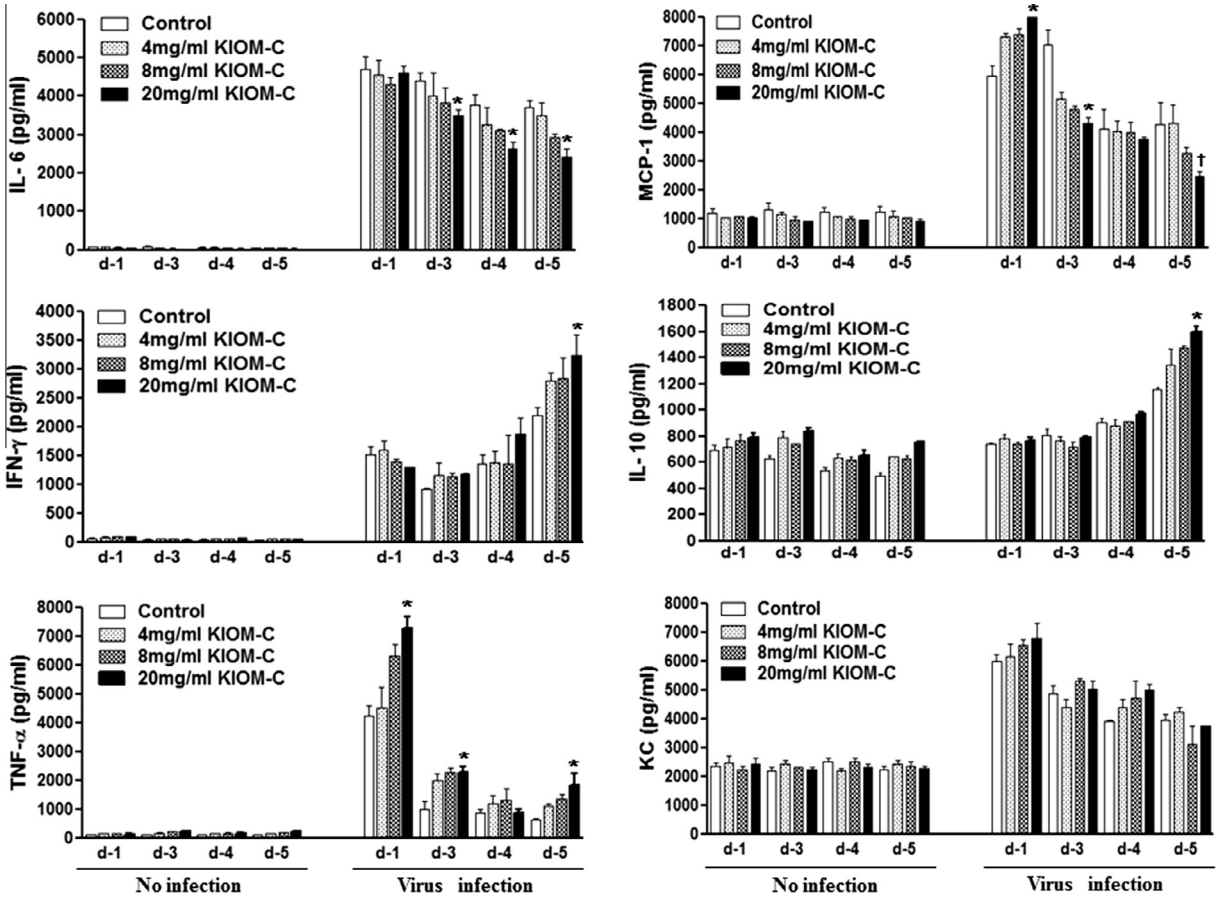

Fig. 3. Cytokine and chemokine production by influenza virus-infected mice treated with KIOM-C. BALB/c mice ($n = 3$) were infected with A/Korea/maCJ01/2009 (H1N1) influenza virus at 5 MLD_{50} per mouse. Twenty-four hours post-infection, mice were orally administrered with 4, 8, or 20 mg/ml of KIOM-C. At 1, 3, 4, and 5 days after treatment, mice were sacrificed and lung BALF was harvested. Cytokine production in lung BALF was analyzed including: IL-6, IFN-γ, TNF-α, IL-10, KC, and MCP-1 by BioPlex analysis. ∗ Statistical significance as compared to mice infected with virus and treated only with PBS was determined by *t-test* ($p < 0.05$).

After evaluating the ability of KIOM-C to increase the survival rate of mice from lethal challenge with A/Korea/maCJ01/2009, we additionally assessed the ability of KIOM-C to inhibit viral growth in lungs of infected mice. A day after infection, mice in each group were orally administered four times with 4 mg/ml, 8 mg/ml, or 20 mg/ml of KIOM-C. At 3, 5, 7 and 9 dpi, lungs were collected from infected mice (3 mice per day) and MDCK cells were inoculated with supernatants from tissue homogenates for virus detection by $TCID_{50}$ titration. Mice that received PBS (control) showed the highest lung viral titers (4.25–3.75 $\log_{10}$ $TCID_{50}$/g mean titer) until 7 dpi and all mice succumbed to infection by 9 dpi (Fig. 2C). In contrast, mice groups that received KIOM-C treatment demonstrated lower virus lung titers starting at 5 dpi. The group that received 20 mg/ml of KIOM-C, among the treatment groups, showed the lowest virus titers in infected lungs, particularly at 5 and 7 dpi compared to control group (4.25 and 3.75 versus 2.75 and 1.25 $\log_{10}$ $TCID_{50}$/g) ($p < 0.05$). Although the 4 mg/ml and 8 mg/ml treated groups also presented reduced lung viral titers, values did not appear to be significantly different from those of the control group ($p = 0.3426$ and $P = 0.1533$, respectably) (Fig. 2C). These results indicate that KIOM-C have potential antiviral activity against influenza A viruses in a dose-dependent manner.

3.4. KIOM-C administration induces cytokine production in mice with infected influenza A virus

To determine whether KIOM-C administration could modulate cytokine production during lethal influenza virus infection, we examined the level of cytokines in BALF from the infected mice at 1, 3, 4, or 5 dpi. As shown in Fig. 3, pro-inflammatory cytokines (IL-6) and chemokines (MCP-1) were produced in lower amounts in BALF of KIOM-C-treated mice relative to control mice starting from 3 dpi through 5 dpi. In contrast, the levels of IFN-γ and IL-10 increased in all groups of mice at 5 dpi in a dose-dependent manner, but the highest expression levels ($P < 0.05$) were observed in mice that received 20 mg/ml (Fig. 3). In addition, the levels of TNF-α were significantly higher in KIOM-C-administered mice in a dose-dependent manner in 3 of 4 time points measured. The expression of KC was not significantly different among the groups. These results suggest that KIOM-C administration may modulate the secretion levels of pro-inflammatory cytokines and chemokines in infected lungs.

3.5. Protection against influenza A infection by KIOM-C in ferrets

To confirm the therapeutic capacity of KIOM-C against influenza virus, groups of ferrets, which are widely believed to be the ideal small animal model for influenza research, were infected with $10^{5.0}$ $TCID_{50}$/ml of A/Korea/maCJ01/2009 virus and then administered 3 ml of 8 mg/ml or 20 mg/ml KIOM-C, or PBS, once a day for 5 days. Administration of 4 mg/ml KIOM-C in mice did not inhibit of influenza virus. Body weights, temperatures, and clinical sign of individual ferrets were monitored for 7 dpi. A/Korea/maCJ01/2009 infection resulted in mean maximum weight loss of approximately 8% at 4 dpi among infected ferrets treated with PBS (Fig. 4A). In addition, the recovery of the control group was delayed as compared to the 20 mg/ml KIOM-C-treated group (Fig. 4A). The KIOM-C-treated ferrets showed fever peaking at 2 dpi with a grad-

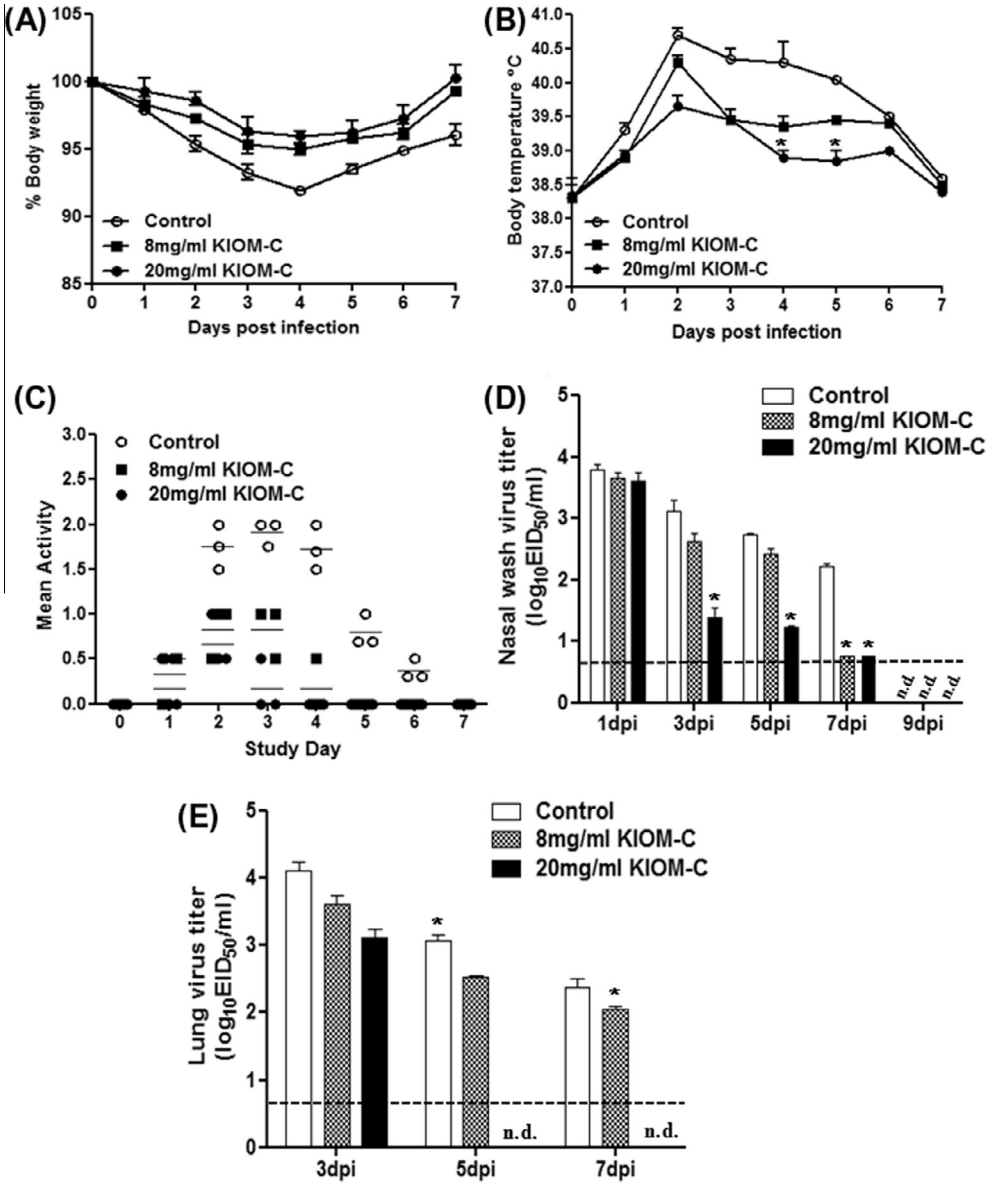

Fig. 4. Oral administration of the novel herbal medicine KIOM-C resulted in reduced viral loads in lung tissue during influenza A virus infection in ferrets. Ferrets ($n = 24$) were intranasally infected with A/Korea/maCJ01/2009 (H1N1) virus. Twenty-four hours post-infection, ferrets were orally administered KIOM-C (8 or 20 mg/ml) once a day for 5 days. Ferrets were monitored for changes in (A) body weight and (B) in rectal temperature for 7 days. (C) Ferrets were monitored for clinical observations and at 24 h intervals until 7 dpi, using the following scoring system. (D) Ferret nasal washes were collected at 1, 3, 5, 7, and 9 dpi and viral titers were measured in 10-day-old embryonated chicken eggs. (E) Ferrets were sacrificed day 3, 5, and 7 post-challenge and lung viral titer determined ($\log_{10}$ EID_{50}/g). $*$ Statistical significance as compared to ferrets infected with virus and only treated with PBS was determined by *t-test* ($p < 0.05$). The limit of virus detection was set at < 0.75 $\log_{10}$ EID_{50}/g (ml) and the virus titers were compared by a standard *t*-test. n.d. indicates not detected.

ual decrease in body temperatures beginning at 3 dpi, while relatively high temperatures were observed until 5 dpi in the PBS-treated control group ($p < 0.05$) (Fig. 4B). Eight mg/ml treated-group scored 0–1 of activity level at 2 dpi (1 out of 3 ferrets) and 3 dpi (2 out of 3 ferrets) at all time points. Similarly, the 20 mg/ml-treated group at 2 dpi and 3 dpi had low activity scores; 2 ferrets with 0.5–1 at 2 dpi and 3 ferrets with 0–0.7 at 3 dpi. In contrast, the control group showed high activity scores, ranging from 1.5 to 2 from 48 h post-challenging to 4 dpi (Fig. 4C). These results indicate that oral administration of KIOM-C also effectively improved the clinical signs of virus infection in ferrets including sneezing, nasal discharge, and decrease in physical activity.

To confirm whether KIOM-C administration also attenuated viral replication in the respiratory tracts of ferrets, viral growth was monitored from nasal wash specimens at 1, 3, 5, 7, or 9 dpi via culture in 10-day-old embryonated chicken eggs (Fig. 4D). The control group exhibited a peak titer of 3.75 $\log_{10}$ EID_{50}/ml at 1 dpi and the virus persisted until 7 dpi (2.5 $\log_{10}$ EID_{50}/ml). The animals administered with 8 mg/ml KIOM-C showed a decreasing trend of viral titers from 3 (2.7 $\log_{10}$ EID_{50}/ml) to 7 dpi compared to those of the control group ($p < 0.05$). In contrast, the 20 mg/ml KIOM-C-treated group showed significantly lower nasal viral titers at 3, 5 and 7 dpi compared to the other groups (control and 8 mg/ml KIOM-C) ($p < 0.05$) (Fig. 4D). To assess whether KIOM-C also affected virus replication in the lungs, ferrets infected with A/Korea/maCJ01/2009 of each group were humanely euthanized at 3, 5, and 7 dpi and virus titers were measured in lung tissue homogenates. Viral titers in the control group peaked at 3 dpi (4.0 $\log_{10}$ EID_{50}/g) and were detectable through 7 dpi (2.5 $\log_{10}$ EID_{50}/g). Notably, animals treated with 20 mg/ml KIOM-C displayed 10 times lower viral ti-

ters (3.0 $\log_{10}$ EID_{50}/g) at 3 dpi and virus was already undetectable at 5 dpi ($p < 0.05$) (Fig. 4E). Collectively, these results demonstrate that KIOM-C can attenuate virus replication in both the upper respiratory tract and the lungs of ferrets in a dose-dependent manner.

4. Discussion

With the persistent burden of global economic crisis, the discovery and screening of herbal medicine or their compound mixtures as alternative means to treat many inflammatory diseases, cancers, and even combat viral infections are gaining steam (Jia et al., 2013; Lu et al., 2012; Ge et al., 2010). With respect to influenza virus infections, the availability of these alternative medicines in the absence of appropriate vaccines or chemical antiviral agents would provide helpful options to prevent looming outbreaks, epidemics or even pandemics. Here, we demonstrated that oral administration of a compound mixture (KIOM-C) impaired the replication of the A(H1N1)pdm09 in mice and ferret models. HPLC analysis revealed several components of KIOM-C which may be of medical importance. For example, Glycyrrhizin, an active component found in *Licorice roots* (Park et al., 2010), has been found as a broad-spectrum antiviral agent reported to suppress many enveloped viruses, such as vaccinia virus, and vesicular stomatitis virus (Harada, 2005; Pompei et al., 1979). It was also shown to protect mice from influenza virus infection by inducing IFN-γ production in T-cells (Abe et al., 1982; Utsunomiya et al., 1997). Interestingly Baicalin, the most dominant compound (at 15.03–15.12%) of KIOM-C, have been shown to rescue mice from lethal infection with the A/FM/1/1947 (H1N1) virus by eliminating the virus and reducing of lung infectivity and pneumonia (Chu et al., 2007). Furthermore, Baicalin was also demonstrated to block Chlamydia trachomatis infection *in vitro* (Hao et al., 2010), inhibit human immunodeficiency virus-1 entry to host cells (Li et al., 2000), and suppress Sendai virus growth in mice (Dou et al., 2011). Thus, it is tempting to speculate that Baicalin could be the main active component of KIOM-C. Although we could not demonstrate the antiviral effect of its individual compound in the present study, we clearly demonstrated that oral administration of KIOM-C led to reduction of lung influenza virus titers and protected mice from lethal challenge with A/Korea/maCJ01/2009 virus.

Inflammatory cytokines and chemokines are produced during influenza virus infection (Herter et al., 2005; Vacheron et al., 1990). However, the multiple functions of cytokines could either be beneficial or detrimental to virus-infected hosts. Cytokines promote lymphocyte activation and infiltration of sites of infection and exert direct antiviral effects but dysregulation of such functions, such as during a cytokine storm, their overall effects (e.g., excessive induction of apoptosis) may become harmful and deleterious. Upon virus challenge, notable pattern of cytokine regulation was observed in KIOM-C treated mice relative to control groups, particularly at high concentrations (20 mg/ml). We found that while the pro-inflammation TNF-α was robustly induced early (at 1 dpi) and waned thereafter, anti-inflammatory IL-10 and immunoregulatory IFN-γ production were significantly increased later in infection (at 5 dpi). Similarly, we have previously reported that oral administration of KIOM-C into porcine circovirus-associated disease (PCVAD)-afflicted pigs demonstrated high levels of serum TNF-α and INF-γ compared to non-treated pigs (Chung et al., 2012). On the other hand, induction of IL-6 and MCP-1 expressions was consistently lower compared to non-treated mice indicating promotion of modulated immune responses throughout the infection. It is of note that Baicalin could suppress inflammatory responses, impede expression of TLR2/4 and NF-κB, and inhibit macrophage activation in murine models (Hao et al., 2012; Liu et al., 2008; Zhu et al., 2012). Thus, we hypothesize that administration of KIOM-C post-infection could help ameliorate clinical disease by enhancing induction of antiviral cytokines and correspondingly promote efficient modulation of the host immune responses that overall promotes protection of mice from lethal infection with a mouse-adapted A(H1N1)pdm09 virus. Notably, dysregulated induction of inflammatory (both pro and anti) and immunoregulatory cytokines contribute to aberrant innate immune responses against the 1918 pandemic and highly pathogenic H5N1 viruses resulting in a high incidence of mortality (Perrone et al., 2008; de Jong et al., 2006; Kobasa et al., 2007).

Although mice have been used for influenza vaccine research for decades, ferrets are distinctively susceptible to influenza virus infection and develop clinical manifestations typically seen among humans. Therefore, ferrets are considered an excellent animal model for the study of influenza virus infection (Matsuoka et al., 2009). Consistent with studies in mice, ferrets treated with 20 mg/ml KIOM-C showed attenuated clinical disease (body weight loss and increased temperatures) and significant reductions in pulmonary viral titers (Fig. 4). Moreover, oral administration of at least 8 mg/ml of KIOM-C was sufficient to decrease viral shedding from nasal washes of infected ferret and impede viral growth in lungs.

In conclusion, we demonstrate that the novel herbal compound mixture, KIOM-C attenuates production of pro-inflammatory cytokines and simultaneously induce a strong IFN-γ response following viral infection in mice. High doses of KIOM-C promoted the clearance of infectious virus in the respiratory tract of both mice and ferrets. These findings suggest therapeutic utility for the oriental medicine KIOM-C when used as an oral anti-influenza virus treatment. Since the herbal remedy was administered, in a daily basis for 4–5 days, 24 h after experimental inoculation, data suggest the efficacy of KIOM-C in early phase of virus exposure/infection. To elucidate whether timing of treatment is significant for immune modulation along with the precise mechanism by which KIOM-C stimulates the host's antiviral functions, further studies will be needed. Regardless, our study shows that KIOM-C could be a novel, orally active antiviral agent with the potential to be effective herbal remedy for prophylaxis and treatment of influenza virus infections.

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