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ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · MARCH 2011

Impact Factor: 2.91 · DOI: 10.1021/jf104982y · Source: PubMed

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Protective Effect of Apple Polyphenols against Stress-Provoked Influenza Viral Infection in Restraint Mice

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ABSTRACT: This study was conducted to investigate the effects of apple polyphenol extract (APE) against influenza virus in mice loaded with restraint stress. The high-performance liquid chromatography (HPLC) fingerprint of APE was recorded, and the percentage composition of polyphenols was determined as 81.7%. Our results showed that restraint stress significantly promoted the mortality and duration of complications of mice infected with the H1N1 virus. However, oral administration of APE (100 and 200 mg/kg) improved the survival rates and prolonged living time of stressed mice infected with influenza virus in a dose-dependent manner. APE was further found to significantly improve the number of immunocytes, ratio of CD4 helper cells, secretion of IL-2, and capabilities of natural killer (NK) cytotoxicity (LU10/spleen) in spleens of restraint-stressed mice. In addition, APE also significantly decreased the level of lipid peroxidation and increased oxygen radical absorbance capacity (ORAC) in splenocytes. These results indicated that the protective effects of APE on mice infected with influenza virus were related to the alleviation of stress-induced impairment of immune functions and its antioxidant property might contribute to the immune recovery.

KEYWORDS: Apple polyphenol, restraint stress, NK cytotoxicity, influenza virus

INTRODUCTION

Influenza is an acute infectious disease caused by RNA viruses of the family Orthomyxoviridae (the influenza viruses).[1] Most people will recover completely in about 1−2 weeks after virus infection, but others will develop life-threatening complications, such as pneumonia. Influenza viruses killed millions of people in the 20th century, and over 90% of deaths occurred in the elderly population and individuals presenting fatigue or stress, who have weak immunity.[1,2] Stress has been shown to induce significant changes in immune response of host toward viral or bacterial pathogens.[3] Our previous studies have indicated that restraint stress might cause immunocompromisation by affecting the number and cytotoxicity of natural killer (NK) cells.[4,5] NK cells are a type of cytotoxic lymphocytes, which are major components of the innate immune system. They are important in the early clearance of the influenza virus because this virus is able to undergo continuous genetic variation, allowing it to evade prior adaptive immune responses.[6] Therefore, stress-induced immunocompromisation of innate immunity might provoke influenza viral infection.

It was reported that dietary factors can modulate the development of influenza.[7] Apples (*Malus* spp., Rosaceae) are one of the most commonly consumed fruits around the world. The old European proverb "an apple a day keeps the doctor away" addresses the health effects of the fruit.[8] Recent years, studies revealed that apple polyphenols are the most important bioactive components in apples. The major polyphenol compounds are flavonoids, procyanidins, chlorogenic acids, catechins, and phenolic acids.[9] They were suggested to possess various pharmacological functions, such as anti-inflammation,[10] anitoxidantion,[11] cholesterol-lowering activities,[12] immunoregulation,[13] etc. Some of these pure polyphenol compounds, such as resveratrol, quercetin, catechin, and epigallocatechin gallate, were reported to inhibit the replication of various viruses, including the influenza virus.[14−16] However, most of these studies had considered the *in vitro* antiviral properties of polyphenols. Few animal results have been reported because of the use of a nonsuitable animal model as evaluation methodology. Moreover, there has been no previous study on the effect of these compounds on viral infection provoked by stress. Accordingly, we investigated the effects of apple polyphenols on stress-provoked influenza infection with a restraint-stressed mice model. Furthermore, contribution of the immunomodulatory properties of apple polyphenols to their effect on stress-provoked influenza infection was also investigated.

MATERIALS AND METHODS

Isolation and Characterization of Apple Polyphenol Extract (APE). APE, Appijfnol (batch number 1004007-16), which contains 81.7% polyphenols, was purchased from the Tianjin Jianfeng Natural Product R&D Co., Ltd. (Tianjin, China). APE was isolated and purified from the pomace of red Fuji apple as described previously.[12] In brief, the pomace was extracted twice with 70% ethanol at a ratio of 1:4 (kilogram of pomace per liter of ethanol) for 1.5 h. The ethanol phases were pooled, filtered, and concentrated. The extract was purified through an AB-8 macroporous resin column after being defatted with *n*-hexane. The final extract was reddish brown and stored at −20 °C. The

Received: December 28, 2010
Revised: March 1, 2011
Accepted: March 1, 2011

dx.doi.org/10.1021/jf104982y | *J. Agric. Food Chem.* XXXX, XXX, 000–000

yield was about 0.4 kg of APE per 100 kg of apple pomace. Contents of individual polyphenols in APE were determined using high-performance liquid chromatography−mass spectrometry (HPLC−MS). HPLC−MS analysis was carried out on Agilent 6210 LC/MSD TOF mass spectrometers (Agilent, Santa Clara CA).

Materials and Chemicals. Authentic standards of chlorogenic acid, proanthocyanidin B2, phloridzin, epicatechin, catechin, phloretin, rutin, and quercetin were purchased from Sigma (St. Louis, MO). Ribavirin was purchased from Star Lake Bioscience Co., Inc. (Zhaoqing, China). Mouse IgG1-fluorescein isothiocyanate (FITC) or -phycoerythrin (PE), anti-CD3 (FITC), anti-CD4 (PE), and anti-CD8 (PE) were all purchased from Beckman (Brea, CA). Commercial enzyme-linked immunosorbent assay (ELISA) kit for mouse IL-2 was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). The malondialdehyde (MDA) kit, total superoxide dismutase (SOD) kit, and glutathione peroxidase (GPx) kit were purchased from Jiancheng Bioengineering Institute (Nanjing, China). 2,2-Azobis(2-amidinopropane)dihydrochloride (AAPH), sodium fluorescein (FL), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, a water-soluble vitamin E analogue) used in the oxygen radical absorbance capacity (ORAC) assay were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Viruses and Cells. The influenza A/PR/8/34 (H1N1) virus was provided by the Chinese Center for Disease Control and Prevention (Beijing, China). The virus strain was propagated in specific pathogen-free embryonated eggs and adapted for lethality in mice after three passages in the animals. YAC-1 tumor cell line, a Moloney-virus-induced mouse T-cell lymphoma of A/SN origin, was obtained from Institute of Health Care Science (Suntory Ltd., Japan). The YAC-1 cell was used to test NK cytotoxic activity for its noted sensitivity to NK cells. The cell line was cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 humidified atmosphere before tests.

Animals. Male-specific pathogen-free BALB/c mice (13−15 g) were purchased from the Guangdong Medical Laboratory Animal Center (Guangzhou, China). All mice were kept in a pathogen-free animal room under controlled conditions at 23 ± 1 °C. A 12 h light−dark cycle was maintained, with lights on from the time 06:00 to 18:00. The mice were provided with the standard laboratory diet and water. All studies were conducted in accordance with the guidelines (publication number 85-23, revised 1996) set by the National Institutes of Health and the U.S. Department of Agriculture.

Evaluation of APE Antiviral Effects in Restraint-Stressed Mice. The experiments were designed to test the effects of APE against influenza in restraint-stressed mice. The experimental mice were divided into six groups, normal control, virus control (virus only), model control (stress + virus), positive control (stress + virus + 100 mg/kg ribavirin), and two APE groups (stress + virus + 100 or 200 mg/kg APE). APE groups received APE suspended in drinking water at dosages of 100 and 200 mg/kg by oral gavage for 10 consecutive days. The positive control group received ribavirin at dosages of 100 mg/kg, while the other three control groups received water only. On the 2nd day of administration, mice were physically restrained in a 50 mL polypropylene centrifuge tube with holes for 18 h. After recovery for a day, the animals were anesthetized by inhalation of ether vapor and then an approximate 2× LD_{50} amount of virus (50 μL) was instilled onto the nares. The experiment recorded the mortality and mean day to death (MDD) of mice dying prior to day 21.[17] The experiment was repeated to determine lung index on the 4th day after infection.

Evaluation of APE Influence on Immune Functions in Restraint-Stressed Mice. Another experiment was designed to test the effects of APE on immune functions in restraint-stressed mice without virus infection. The preparation of splenocytes was modified according to the previously described method.[4] The spleens of all mice in this experiment were collected, and splenocytes were prepared by disrupting the spleen with a grinder in phosphate-buffered saline (PBS, pH 7.4). After a 10 min centrifugation at 370 relative centrifugal force (rcf) to separate debris, erythrocytes were lysed using ammonium chloride reagent. The cells were washed twice with PBS and suspended in 1 mL of cold RPMI-1640 medium with 10% FBS. The total splenocyte number was determined with a Beckman-Coulter Z2 cell counter (Brea, CA). The obtained splenocytes were used for the following tests.

Determination of T Lymphocyte Subsets. Samples containing 1×10^6 splenocytes in RPMI-1640 medium were treated with selected monoclonal antibodies conjugated with FITC or PE (Beckman, Brea, CA). We used the following double-staining combinations: anti-CD3 (FITC)/anti-CD4 (PE) and anti-CD3 (FITC)/anti-CD8 (PE). Mouse IgG1-FITC and -PE were used as control staining. After 15 min of incubation at room temperature in the dark, the cells were washed with PBS, resuspended in 0.5 mL of cold PBS, and analyzed using a FACS Epics XL (Beckman, Brea, CA). Usually, 10 000 cells were scanned for each sample, and the results were expressed as the percentage of cells yielding a specific fluorescence in a gated lymphocyte region.

Cytokine Secretion of Splenocytes. The effects of APE on IL-2 secretion in the splenocytes of restrain-stressed mice were investigated. The prepared splenocytes (5×10^6 cells/mL) were cultured with ConA (10 μg/mL) at 37 °C for 48 h on 96-well culture plates in a 5% CO_2 humidified atmosphere. The supernatant was harvested and kept frozen at −20 °C. Levels of IL-2 in the supernatant were determined using commercial ELISA kits.

NK Cell Cytotoxicity Assay. The experiments employed two fluorescent stains as previously reported.[4] Results were plotted, and the number of cells required to produce 10% specific cytotoxicity (one lytic unit, 1 LU10) was established from the best fit of the curve. The number of LU10 per spleen (LU10/spleen) was thus calculated and used to express final results.

Measurement of MDA Contents, ORAC Level, and SOD and GPx Activities in Splenocytes. Splenocytes were homogenated in PBS at a concentration of about 5×10^7 cells/mL. The splenocyte lipid peroxide content was determined by measuring thiobarbituric-acid-reactive substances (TBARS) with a commercial MDA kit. The effects of antioxidant capacity in splenocytes was evaluated by the ORAC assay. ORAC is a method used to measure the oxidative degradation of the fluorescent molecule after being mixed with free-radical generators, such as azo-initiator compounds. Azo-initiators are considered to produce peroxyl free radical by heating, which damages the fluorescent molecule, resulting in the loss of fluorescence. The presence of an antioxidant is able to protect the fluorescent molecule from the oxidative degradation. The procedures for the ORAC assay on spleen cells were modified from the previously described method by Kurihara et al.[18] The automated ORAC assay was carried out on a decay of the fluorescein signal and determined with an excitation/emission filter pair of 485/527 nm in a GENios Lueifcrase microplate reader (TECAN) at 37 °C. Fluorescein was used as a target for the free radical to attack, and the reaction was initiated with AAPH. Trolox was used as a control standard. Final results were calculated on the basis of the difference in the area under the fluorescein decay curve between the blank and each sample. The activities of total SOD and GPx were measured according the guide of commercial kits.

Measurement of the Expression of Cu/ZnSOD, MnSOD, and GPx mRNA Levels in Splenocytes. Antioxidant enzyme gene expression was semi-quantitatively assessed using reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from samples of splenocytes using TRIzol Reagent according to the protocol of the manufacturer (Invitrogen, Carlsbad, CA). A 3 μg amount of total RNA was reverse-transcribed into cDNA at 42 °C for 1 h in 20 μL of reaction mixture containing mouse Moloney leukemia virus reverse transcriptase (Tiangen, CA) with oligo$(dT)_{15}$ primer (Tiangen, CA) followed by PCR amplification. PCR was carried out with 1 μL of cDNA,

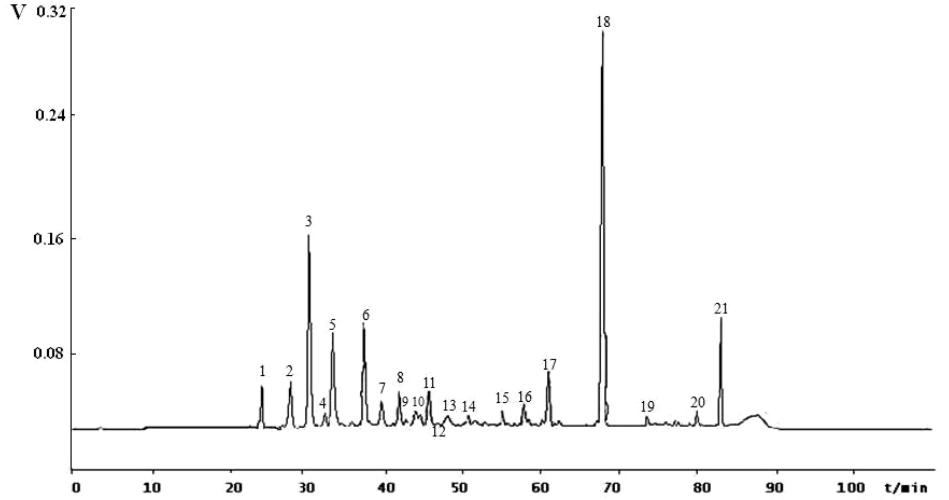

Figure 1. Chemical profile of APE analyzed by HPLC. Several batches of APE were analyzed by HPLC−ultraviolet (UV) with a HPLC column (Luna C18 100A, 250 × 64.6 mm^2 inner diameter) at 208 nm, and similar profiles were observed. The elution profile was programmed at a flow rate of 1 mL/min, while the gradient mobile was phase-composed of 2% acetic acid (solvent A) and 0.4% acetic acid with 80% acetonitrile (solvent B). The ratio of A/B was programmed from 90:10 to 20:80 in 80 min. Electrospray ionization−mass spectrometry (ESI−MS) was in positive-ion mode. Identification of peaks are as follows: 2, catechin; 3, chlorogenic acid; 5, proanthocyanidin B2; 6, epicatechin; 17, rutin; 18, phloridzin; 20, quercetin; 21, phloretin; 1, 4, 7−16, and 19, proanthocyanidins (dimers and trimers).

2.5 μL of 10× Taq reaction buffer (Tiangen, CA), 2 μL of dNTP mixture, 1 μM forward primer, 1 μM reverse primer, and 1 μL of Taq polymerase (Tiangen, CA) in a total volume of 25 μL. The cDNA was amplified using specific primers with 30 cycles at 94 °C for 30 s, an annealing temperature of 58 °C for 40 s, and then 72 °C for 50 s, with final incubation at 72 °C for 7 min. The PCR primers for mouse Cu/ZnSOD mRNA were (forward) 5′-ATGGCGATGAAAGCGGTGTG-3′ and (reverse) 5′-TTACTGCGCAATCCCAATCAC-3′, and the product size was 456 base pairs (bp). The PCR primers for mouse MnSOD mRNA were (forward) 5′-AAGCACAGCCTCCCAGACCT-3′ and (reverse) 5′-TCACTTCTTGCAAGCTGTGTATCTT-3′, and the product size was 597 bp. The PCR primers for mouse GPx mRNA were (forward) 5′-GAAGTGCGAAGTGAATGG-3′ and (reverse) 5′-TGGGACAGCAGGGTTT-3′, and the product size was 255 bp. The primers for the mouse housekeeping gene β-actin mRNA were (forward) 5′-GAGGGAAATCGTGCGTGAC-3′ and (reverse) 5′-GCTGGAAGGTGGACAGTGAG-3′, and the product size was 446 bp. The PCR products were fractionated on a 1% agarose gel and visualized by ethidium bromide staining. The band intensity of ethidium bromide fluorescence was measured using an image analysis system (Bio-Rad, Hercules, CA), then quantified with Quantity One analysis software (Bio-Rad, Hercules, CA), and expressed as the ratio to β-actin.

Statistical Analysis. The data were presented as the mean ± standard error (SE). Statistical analysis of the data was performed using the SPSS 13.0 statistical package. One-way analysis of variance (ANOVA) was applied to analyze differences in data of biochemical parameters among the different groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Chemical Profile of APE. The typical HPLC chromatogram of APE is shown in Figure 1. APE contains 81.7% polyphenols, and contents of individual polyphenols in APE were determined using HPLC. APE contained 17.4% chlorogenic acid, 6.1% proanthocyanidin B2, 5.2% phloridzin, 2.6% epicatechin, 1.2% catechin, 0.3% phloretin, 0.2% rutin, and 0.1% quercetin. Under the present experimental conditions, other phenolic compounds and proanthocyanidins (dimers and trimers) were not quantified.

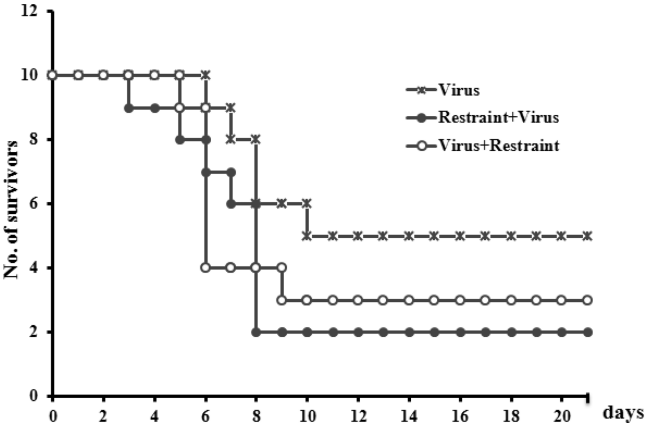

Figure 2. Effect of restraint stress on survival rates of virus-infected mice. At 1 day before (restraint + virus) or after (virus + restraint) H1N1 infection, BALB/c mice were fixed in a restraint cage for 18 h. The survival days of each mouse were recorded until the 21st day after viral infection. Data were obtained from 10 animals in each group.

Effects of APE against Influenza Virus in Restraint-Stressed Mice. At 1 day before or after H1N1 infection, BALB/c mice were fixed in a restraint cage for 18 h. As shown in Figure 2, mice in the virus-infected group began to die on the 6th day after viral infection and 50% mice survived at the end of this experiment. However, when mice were loaded with restraint stress before infected with virus, they began to die on the 4th day after infection and only 20% of mice survived. Similarly, when mice were loaded with restraint stress after viral infection, mice began to die on the 5th day and 30% of mice survived in the end. Results indicated that restraint stress before or after viral infection significantly decreased the survival rates of virus-infected mice.

dx.doi.org/10.1021/jf104982y |J. Agric. Food Chem. XXXX, XXX, 000–000

Table 1. Effects of APE on Virus Infection in Restraint-Stressed Mice

group		survive/total	MDD (day)[a]	lung index (mg/g)[b]
normal control		10/10	>21.0	7.1 ± 0.3
virus control		5/10	8.9 ± 2.1[c]	15.8 ± 2.3[c]
restraint + virus	model control	2/10	7.3 ± 1.8[d]	17.5 ± 3.1[d]
	50 mg/kg ribavirin	9/10	12.6 ± 0.0[e]	8.5 ± 1.6[e]
	100 mg/kg APE	6/10	13.8 ± 2.6[e]	11.3 ± 3.5[e]
	200 mg/kg APE	9/10	16.3 ± 0.0[e]	9.8 ± 1.9[e]

[a] MDD of mice dying prior to day 21. [b] The lung index was calculated according to the following formula: lung index = lung weight (mg)/body weight (g). The results represented the mean ± SE of values obtained from 10 BALB/c mice in each group. [c] Significantly different from the normal control mice at $p < 0.01$. [d] Significantly different from the virus control mice at $p < 0.01$. [e] Significantly different from the model control mice at $p < 0.01$.

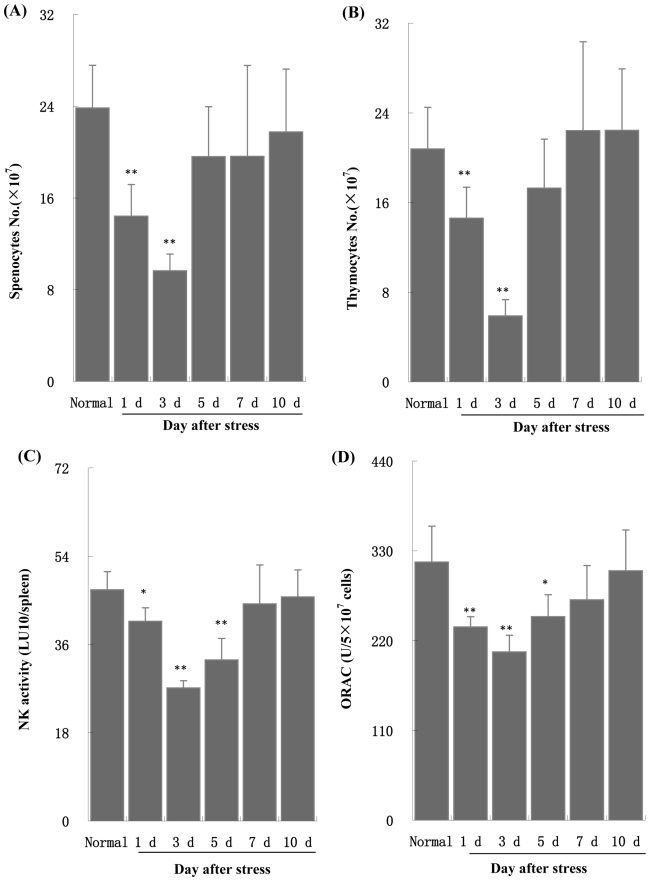

Figure 3. Temporal changes of (A) splenocytes, (B) thymocytes, (C) NK cell activity, and (D) ORAC level after restraint stress. BALB/c mice were fixed in a restraint cage for 18 h and recovered for different days. The results represented the mean ± SE of values obtained from seven mice in each group. The significance of differences from the normal control group was at (*) $p < 0.05$ and (**) $p < 0.01$.

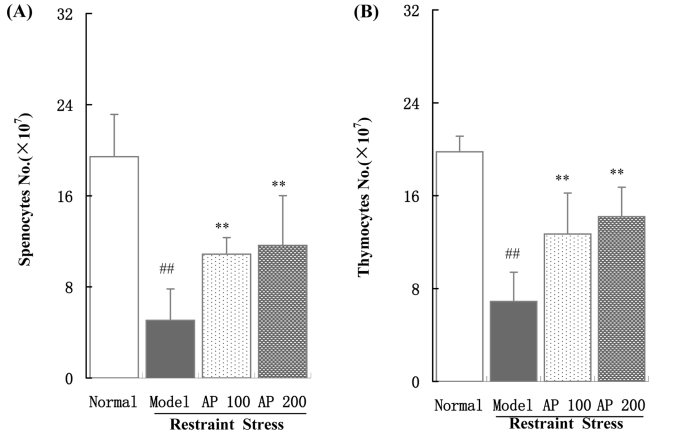

Figure 4. Effects of APE on (A) splenocytes and (B) thymocytes in restraint-stressed mice. BALB/c mice were administered with APE (100 and 200 mg/kg) 2 days before restraint stress for 5 consecutive days. Mice were fixed in a restraint cage for 18 h and sacrificed 3 days later. The results represented the mean ± SE of values obtained from seven mice in each group. The significance of differences was from the normal control group at (*) $p < 0.05$ and (**) $p < 0.01$ and from the model group at (#) $p < 0.05$ and (##) $p < 0.01$.

The effect of APE against H1N1 influenza was evaluated with mice treated with restraint stress before viral infection. At 3 days after virus infection, behavioral changes, such as a tendency to huddle, ruffled fur, diminished vitality, and reduced food intake, as well as weight loss were observed in mice. However, APE treatment significantly alleviated these symptoms and decreased weight loss. As shown in Table 1, in the model control group, 20% of mice survived and the MDD was decreased from 8.9 ± 1.2 to 7.3 ± 1.8 days ($p < 0.01$). Positive drug ribavirin, at a dosage of 100 mg/kg, significantly improved the survival rate to 90% and MDD to 12.6 ± 0.0 days ($p < 0.01$). Similarly, APE considerably demonstrated inhibitory effects against the virus-induced death, with 60 and 90% of mice surviving in the APE treatment groups at 100 and 200 mg kg^{-1} day^{-1}, respectively. The MDD was also prolonged to 13.8 ± 2.6 and 16.3 ± 0.0 days ($p < 0.01$), respectively. Results showed that APE improved survival rates and prolonged living time of stressed mice in a dose-dependent manner.

Effects of APE against Influenza-Caused Pneumonia in Restraint-Stressed Mice. The lung index was calculated as a parameter of pneumonia caused by the influenza virus. As shown in Table 1, the baseline of the lung index in the normal group was 7.1 ± 0.3 mg/kg, while it increased to 15.8 ± 2.3 mg/kg ($p < 0.01$) in the virus control group and further increased to 17.5 ± 3.1 mg/kg ($p < 0.01$) in the stress model group. In comparison to the model group, ribavirin significantly recovered the lung index to 8.5 ± 1.6 mg/kg ($p < 0.01$). Similarly, lung indexes were also recovered to 11.3 ± 3.5 and 9.8 ± 1.9 mg/kg ($p < 0.01$), respectively, by low and high dosages of APE administrations. Results demonstrated that APE had protective effects against virus-caused pneumonia.

Effects of APE on Immune Functions of Restraint-Stressed Mice. The effects of restraint stress on immune functions of mice were determined in this study. Results demonstrated that restraint for 18 h significantly reduced the number of splenocytes

dx.doi.org/10.1021/jf104982y |J. Agric. Food Chem. XXXX, XXX, 000–000

Table 2. Effects of APE on T-Lymphocyte Subsets, IL-2 Secretion, and NK Cytotoxicity in Restraint-Stressed Mice[a]

group		Th ($CD3^+CD4^+$) (%)	Ts ($CD3^+CD8^+$) (%)	IL-2/spleen (IU)	NK cytotoxicity (LU10/spleen)
normal control		20.8 ± 1.5	9.8 ± 1.0	946.2 ± 89.9	49.3 ± 6.0
restraint	model control	16.4 ± 1.1[b]	10.7 ± 1.5	397.6 ± 65.7[b]	25.3 ± 5.5[b]
	200 mg/kg APE	19.6 ± 1.3[c]	9.8 ± 1.2	750.2 ± 92.3[c]	47.4 ± 10.2[c]
	400 mg/kg APE	20.9 ± 1.3[c]	10.1 ± 1.6	820.2 ± 100.2[c]	48.4 ± 12.3[c]

[a] The results represented the mean ± SE of values obtained from 10 BALB/c mice in each group. [b] Significantly different from the normal control mice at $p < 0.01$. [c] Significantly different from the model control mice at $p < 0.01$.

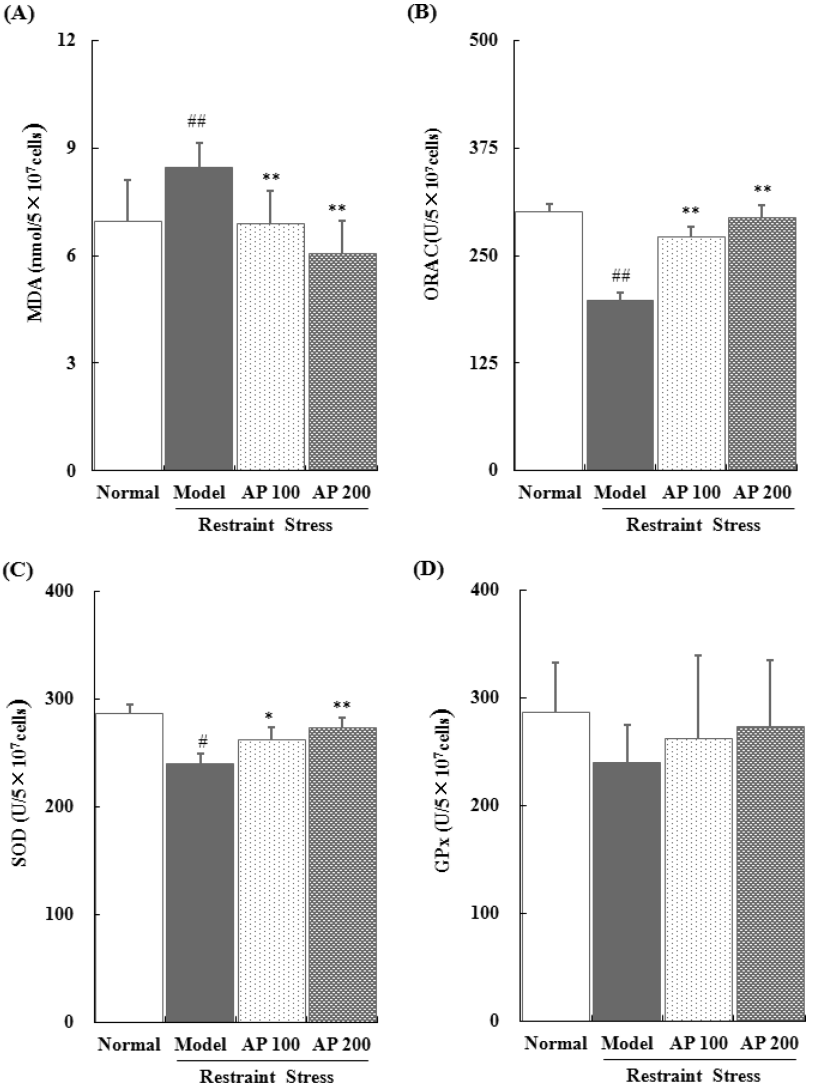

Figure 5. Effects of APE on (A) MDA, (B) ORAC, (C) SOD and (D) GPx of splenocytes in restraint-stressed mice. BALB/c mice were administered with APE (100 and 200 mg/kg) 2 days before restraint stress for 5 consecutive days. Mice were fixed in a restraint cage for 18 h and sacrificed 3 days later. The results represented the mean ± SE of values obtained from seven mice in each group. The significance of differences was from the normal control group at (*) $p < 0.05$ and (**) $p < 0.01$ and from the model group at (#) $p < 0.05$ and (##) $p < 0.01$.

and thymocytes (panels A and B of Figure 3). As shown in Figure 3C, restraint stress also markedly suppressed NK cell cytotoxicity per spleen (LU10/spleen). Furthermore, the antioxidant capacity of immunocytes was also significantly decreased. All parameters above decreased from the 1st day and to the minimum on the 3rd day after restraint stress. It indicated that restraint stress suppressed immune responses with a maximum on the 3rd day after restrain loading, similar to the previous report.[5] Therefore, we further investigated the effects of APE using the above model with 3 days recovery from restraint.

As shown in panels A and B of Figure 4, restraint stress reduced the number of splenocytes and thymocytes significantly ($p < 0.01$), while oral administration of APE (100 and 200 mg/kg) obviously improved the number of immunocytes ($p < 0.01$). The percentage of each type of T lymphocyte was obtained by double fluorescent staining. As shown in Table 2, the percentage

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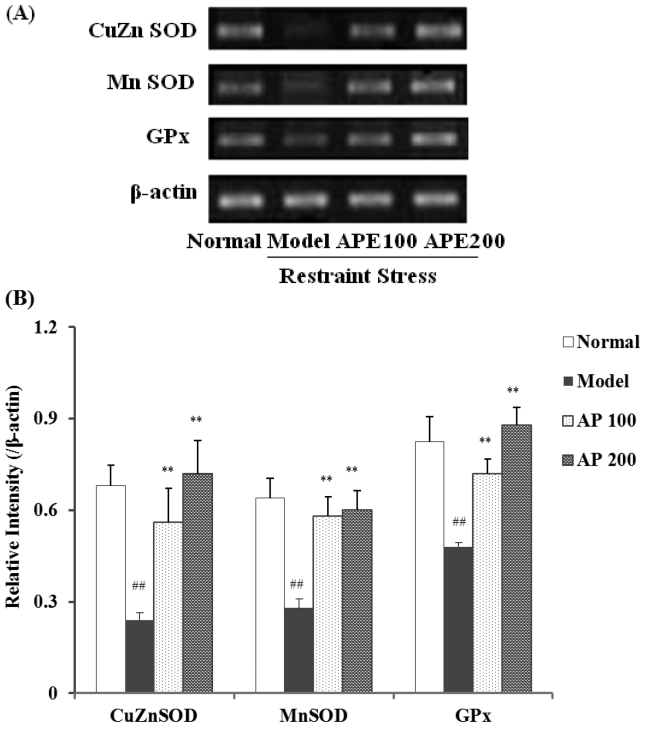

Figure 6. Effects of APE on Cu/ZnSOD, MnSOD, and GPx mRNA expressions of splenocytes in restraint-stressed mice. (A) Agarose gel electrophoresis of RT-PCR amplication of Cu/ZnSOD, MnSOD, GPx, and β-actin mRNAs. (B) Densiometric analysis of PCR products of Cu/ZnSOD, MnSOD, and GPx mRNAs. Results were generated as relative intensity units by densitometry and expressed as the ratio to β-actin. Data represented the mean ± SE of values obtained from seven mice in each group. The significance of differences was from the normal control group at (**) $p < 0.01$ and from the model group at (##) $p < 0.01$.

of Th ($CD3^+CD4^+$) cells in the spleen of restraint-stressed mice was decreased significantly ($p < 0.01$) but not for the percentage of Ts ($CD3^+CD8^+$) cells. IL-2 secretion in mice splenocytes was also decreased after restraint stress loading. However, oral administration of APE (100 and 200 mg/kg) significantly recovered the percentage of Th ($CD3^+CD4^+$) cells and IL-2 secretion in restraint-stressed mice ($p < 0.01$). Moreover, the effects of APE on NK cytotoxicity in spleen was assessed by flow cytometry using NK-cell-sensitive YAC-1 target cells. Results showed that NK cell activity per spleen was suppressed by restraint stress significantly ($p < 0.01$), and oral administration of APE (100 and 200 mg/kg) improved the suppressed NK cell activity per spleen significantly.

Effects of APE on MDA Contents and ORAC Level of Splenocytes in Restraint-Stressed Mice. The extent of lipid peroxidation and antioxidant capability of splenocytes was evaluated by the TBARS and ORAC method, respectively. The basal value of lipid peroxide was 7.0 ± 1.1 nmol in splenocytes at a concentration of about 5×10^7 cells. It was markedly increased to 8.5 ± 0.7 nmol in restraint-stressed mice for the same amount of cells. Administration of APE (100 and 200 mg/kg) reduced the stress-induced increases of lipid peroxide to 6.9 ± 0.9 and 6.1 ± 0.9 nmol, respectively (Figure 5A). The ORAC value was calculated as the ratio of the area under the fluorescence decay curve for 10 umol/L Trolox as a standard. As shown in Figure 5B, the ORAC level of splenocytes in normal mice was 301.5 ± 8.3 units in splenocytes at a concentration of about 5×10^7 cells while remarkably decreasing to 197.5 ± 9.2 units ($p < 0.05$) in restraint-stressed mice. Administration of APE (100 and 200 mg/kg) significantly increased ORAC to 273.0 ± 10.8 and 294.5 ± 13.8 units ($p < 0.05$), respectively. Results above indicated that APE has protective effects on the antioxidant defense of immunocytes in stressed mice.

Effects of APE on SOD and GPx Activities and mRNA Expressions of Splenocyte in Restraint-Stressed Mice. The activities of important antioxidases were evaluated by determining the SOD and GPx activities. As shown in Figure 5, the basal activity of total SOD activity was 286.7.0 ± 8.2 units in splenocytes at a concentration of about 5×10^7 cells. It was markedly decreased to 240.1 ± 9.1 units in restraint-stressed mice for the same amount of cells. Administration of APE (100 and 200 mg/kg) increased the stress-induced decreases of SOD activity to 263.0 ± 10.7 and 273.6 ± 9.1 units, respectively (Figure 5C). The GPx activity in splenocytes of restraint-stressed mice was not changed significantly in the model group or APE administration groups (Figure 5D). The mRNA expressions of antioxidases were further determined by the RT-PCR method. The expression of Cu/ZnSOD, MnSOD, and GPx mRNA levels in splenocytes of stressed mice decreased when compared to the control group. Administration of APE (100 and 200 mg/kg) significantly upregulated the mRNA levels of Cu/ZnSOD, MnSOD, and GPx (Figure 6).

DISCUSSION

During influenza infection, NK cells play an important role as the innate defense.[19,20] Researches demonstrated NK cell number and cytotoxicity in mice spleen were significantly elevated to a maximum on the 3rd day after influenza infection and decreased thereafter.[20] It is due to the fact that NK cells are required for early clearance of virus and subsequent promotion of adaptive immune response. However, our results found that restraint stress significantly reduced the number of splenocytes and thymocytes and also markedly suppressed NK cytotoxocity (LU10/spleen) in mice spleen. Accordingly, when stressed mice were infected with H1N1 influenza, the NK cell number and cytotoxicity were insufficient to respond to virus infection and, consequently, lead to increased mortality, duration of sickness, and secondary complications. These results suggested that stress can facilitate the infection of the influenza virus via a suppressed immune response.

Previous studies reported that apple polyphenols may offer protection against a wide variety of viral infections *in vitro*.[21,22] Meanwhile, there were also other studies reporting the inhibitory effects of plant-derived polyphenols on influenza virus infection in recent years.[23−25] In this study, APE contains 81.7% polyphenols, including chlorogenic acid, proanthocyanidin B2, phloridzin, epicatechin, phloretin, rutin, quercetin, and other polymeric polyphenols. Some of these pure compounds were reported to possess antiviral activities.[14−16] However, those studies were conducted *in vitro*, and few results *in vivo* were reported. In the present study, we used the restraint-stressed mice model to evaluate the effects of APE against influenza, and results showed that oral administration of APE (100 and 200 mg/kg) could significantly elevate survival rates and prolong living time of mice subjected to restraint stress before viral infection. The results reflected that the old saying of "an apple a day keeps the doctor away" proved to be true. Moreover, the mechanism study showed that APE significantly improved immunocyte

number and NK cytotoxicity (LU10/spleen) in restraint-stressed mice. APE also altered the balance of CD4/CD8 T cells by increasing the ratio of CD4 helper cells, which plays an important role in establishing and maximizing the capabilities of NK cells. Furthermore, APE recovered the secretion of IL-2 in splenocytes of restraint-stressed mice. IL-2 stimulates NK cells to produce both type 1 (α and/or β) and type 2 (γ) interferon (IFN),[26] which are important for virus clearance. Results above suggested the protective effect of apple polyphenols against stress-provoked influenza viral infection by improving immune functions of mice loaded with restraint stress.

In recent years, there is growing interest in the fact that immune functions can be modulated by plant polyphenols.[27] Different studies have endorsed the challenge to elucidate the role of polyphenols on the immune system. Bayer and Kim indicated the immunosuppressive effects of polyphenols.[28,29] However, numerous studies reported that polyphenols could enhance immunity and play a key role in immune defense because of its antioxidant properties.[27] Our previous results suggested a correlation between immunologic response and antioxidant capacity in immunocytes.[4] In this study, the oxidative and antioxidative situations in splenocytes were investigated by determining the MDA content and ORAC level in the prepared splenocytes. The promotion of the MDA content and the decrease of the ORAC level in splenocytes of restrained mice indicated that immunocytes were under oxidative stress. Immunocytes are susceptible to lose their number and activities because of the effects of reactive oxygen species (ROS).[30] Our results showed that the treatment with APE could reduce the MDA content and restore the ORAC level in splenocytes of restrained mice. Lu and Yeap Foo[31] reported that the DPPH-scavenging activities of apple polyphenols and superoxide anion radical-scavenging activities were 2−3- and 10−30-fold, respectively, better than those of the antioxidant vitamins C and E. Our results showed that APE administration plays an important role in the activation of antioxidant enzymes, by increasing the activities and mRNA expressions of SOD and GPx in splenocytes of restraint-stressed mice. Therefore, APE was a potent antioxidant *in vitro* and *in vivo*. Apart from the ROS scavenger property, apple polyphenols also inhibited the activation of ROS production pathways. Studies demonstrated that apple polyphenols downregulate nuclear factor-κB (NF-κB) signaling.[32] NF-κB, a redox-sensitive transcriptional factor, is involved in cellular responses to stimuli, such as stress. Apple polyphenols were further reported to inhibit the expression of tumor necrosis factor-α (TNF-α),[10] which is an important cytokine to activate ROS production in cells. These results indicated that APE exerts antioxidant effects by not only increasing the capacity of free radicals absorbance but also indirectly inhibiting stress-induced ROS production in immunocytes. Accordingly, we concluded that APE modulated the development of influenza by elevating immune functions of stressed animals, which may be due to its antioxidant activities directly or indirectly in immunocytes.

Although people may consume polyphenols from other vegetables or fruits in their diet, most of them are not necessarily leading to the highest concentrations of active metabolites in target tissues. The fact is that the chemical profile of the polyphenols determines the rate and extent of absorption and the nature of the metabolites in the plasma.[33] In this study, APE contains 81.7% polyphenols, including 17.4% chlorogenic acid, 6.09% proanthocyanidin B2, 5.2% of phloridzin, 2.6% epicatechin, 1.2% catechin, 0.3% phloretin, 0.2% rutin, and other polymeric polyphenols. The polyphenol profile of APE differs from that of renowned polyphenol extracts, such as green tea and grape seed extracts. Furthermore, APE is originated from apple pomace, which is a byproduct of the apple cider processing industry and represents about 15−30% the original fruit. An acute oral toxicity test and a 90 day subchronic toxicity test showed no toxicity at a dosage of 2000 mg/kg of APE.[34] For these reasons, exploration of potential health care usages for apple pomace is possible and valuable.

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Funding Sources

This work was partially supported by grants from the National Natural Science Foundation of China (30972217).

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; ORAC, oxygen radical absorbance capacity; NK, natural killer; APE, apple polyphenol extract; FITC, fluorescein isothiocyanate; PE, phycoerythrin; MDA, malondialdehyde; SOD, superoxide dismutase; GPx, glutathione peroxidase; AAPH, 2,2-azobis(2-amidinopropane)dihydrochloride; FL, sodium fluorescein; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; FBS, fetal bovine serum; MDD, mean day to death; PBS, phosphate-buffered saline; TBARS, thiobarbituric-acid-reactive substances; RT-PCR, reverse transcription-polymerase chain reaction; ANOVA, analysis of variance; IFN, interferon; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor-α

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