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Research Article

Wanglaoji Herbal Tea Protects Against Influenza-induced Pneumonia in Restraint-Stressed Mice via its Anti-inflammatory Effects

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Abstract

Background and Objective: Wanglaoji Herbal Tea (WHT) is considered a traditional remedy for "Shanghuo" in Southern China. Studies have proven that WHT has the potential to modulate immunity against viral epidemics. However, experimental data supporting the pharmacological activities of WHT are limited. The current study aimed to investigate the protective effects and related mechanisms of WHT against influenza-induced pneumonia in mice exposed to restraint stress. **Materials and Methods:** In this study, restraint-stressed mice infected with A/FM/1/47 (H1N1) virus were employed as the model to investigate the anti-inflammatory effects of WHT. On the second day of oral administration of WHT, all mice except the normal and virus control groups were restrained for 18 h. Mice were infected with influenza virus after recovering from restraint stress for 3 days. Body weight, morbidity and mortality were recorded daily for 21 days. In addition, the oxidative activity (MDA, SOD and iNOS), inflammatory markers (TNF-α and IL-1β), histopathologic changes, influenza-related mRNA expression (NP) and influenza-related protein expressions (MAVS, p-IRF3, IFN-β and NF-κB) in the lung tissues were determined on the fourth day of post-viral infection. The data was statistically analyzed by one-way ANOVA using the SPSS software (Version 19). Multiple Tukey's *post hoc* test was used to determine the statistical significance. **Results:** The results showed that WHT reduced morbidity and mortality. Meanwhile, WHT ameliorated oxidative damage by decreasing the level of MDA and increasing SOD activity. Further experimental results showed that WHT not only improved mitochondrial antiviral signaling protein (MAVS) and p-IRF3 protein expression to promote the IFN-β responses but also down-regulated NF-κB protein expression to dampen excessive inflammatory cytokine responses in the restraint-stressed mice infected with the influenza virus. These results demonstrated that WHT elevated the hosts' immune system and ameliorated influenza-induced pneumonia. **Conclusion:** The WHT reduces pneumonia caused by influenza virus infection in restraint-stressed mice by regulating the MAVS antiviral signaling pathway.

Key words: Wanglaoji Herbal Tea (WHT), restraint stress, influenza virus, mitochondrial antiviral signaling protein (MAVS), susceptibility

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

In Southern China, herbal teas are usually prepared in Traditional Chinese Medicine (TCM) to improve health. Wanglaoji Herbal Tea (WHT), which originated in the Qing Dynasty, has been used as a traditional remedy for "Shanghuo" for more than 200 years. "Shanghuo", a traditional concept in Chinese medicine, is an uncoordinated response to stress and a disruption of homeostasis with physical and mental fatigue symptoms[1]. Recently, WHT is widely consumed as a functional herbal beverage to prevent poor health and stress[2,3]. The WHT contains 7 kinds of raw plant materials, namely, *Prunella vulgaris*, *Chrysanthemum morifolium*, *Folium microcotis*, *Glycyrrhiza uralensis Fisch*, *Lonicera japonica Thunb*, *Mesona chinensis Benth* and *Plumeria rubra acutifolia,* most of which have heat-reducing and detoxifying effects[4-6]. Studies have shown that chemicals in these raw plant materials have anti-inflammatory and antiviral activities. Studies also have proven that herbal teas and natural products have the potential to restore strength and modulate immunity against viral epidemics[7-10]. Therefore, WHT has the potential value to reduce the effects influenza-induced pneumonia.

Influenza virus infection is a major public health concern and influenza pandemic causes a large number of deaths worldwide[11]. Lung tissue destruction induced by a cytokine storm and excessive inflammation are the major factors resulting in high mortality from influenza virus infection[12-14]. The influenza pandemic of 1918-1919 resulted in a catastrophic health risk, principally due to pulmonary injury caused by an excessive primary immune response of the host[15]. These facts underscore the role of the inflammatory response in the pathogenesis of a wide assortment of influenza viruses. Thus, it is necessary to inhibit the inflammatory response mediated by nuclear factor κB (NF-κB) and the immunopathological damage induced by an influenza virus.

Epidemiological surveys have shown that people who have weakened immunity, such as newborns, the elderly, the sick and individuals presenting fatigue or stress are more inclined to be infected with an influenza virus[15,16]. It has long been known that stress can alter the behavior and homeostatic state of the host and result in an augmented susceptibility to bacterial pathogens or viruses[8,17]. Previous studies have indicated that restraint stress enhanced the susceptibility to influenza virus and aggravated the complications of influenza virus, leading to increased morbidity and mortality[8]. Meanwhile, the anti-influenza effects of many natural products were effectively evaluated using the restraint stress mouse model[8,10]. The WHT has the potential to modulate immunity against viral epidemics. However, experimental data supporting the pharmacological activities of WHT are limited. This study would provide information on the pharmacodynamics of WHT. Therefore, the current study aimed to investigate the protective effects and related mechanisms of WHT against influenza-induced pneumonia utilizing the restraint stress mouse model.

MATERIALS AND METHODS

Preparation of WHT: Extract of Wanglaoji herbal tea (batch number 1402048) was provided by Guangzhou Wanglaoji Pharmaceutical Company Limited.

Virus and animals: The influenza virus A/FM/1/47 (H1N1) was provided by the College of Veterinary Medicine, South China Agricultural University (Guangzhou, China). Allantoic fluid containing the virus was stored in aliquots at -80°C. The LD_{50} was determined in mice after a serial dilution of the stock. The amount of virus used for the viral challenge in all animal experiments was $LD_{50}\times 2$. Infection was established by intranasal inoculation in mice anesthetized with ethyl ether. All experiments related to the influenza virus were performed in a Biosafety Level 2 Laboratory at Jinan University. Male Kunming mice, 13-16 g, were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The mice were acclimated in a pathogen-free animal room and feeding environment where the relative humidity was 75±10%, the temperature was 23±2°C and the lighting time of the light-dark cycle was 12 h/day. The mice were provided with a standard laboratory diet and water. All animal care and experimental procedures were approved by the Laboratory Animal Ethics Committee of Jinan University (20131011017). All studies were conducted on the basis of the guidelines set by the National Institutes of Health (7th Edition, USA).

Experimental design: The study was carried out during late 2015. The mice were randomly divided into six groups: normal control, virus control (virus only), model control (restraint+virus), positive control (restraint+virus+50 mg kg^{-1} ribavirin) and two WHT groups (restraint+virus+500 or 125 mg kg^{-1} WHT), which were named WHT-H (500 mg kg^{-1} WHT) and WHT-L (125 mg kg^{-1} WHT). The WHT and ribavirin were orally administered before restraint, while the rest of the groups received water only. On the 2nd day of administration, all mice except the normal control group and the virus control group were put into 50 mL polypropylene centrifuge tubes

with holes to restrict movement for 18 h (15:00 pm-9:00 am). All the mice had no food or water during the time of restraint stress. After recovery for 3 days, the animals were anesthetized by inhalation of ether vapor and then given an approximate $2\times LD_{50}$ amount of virus (35 μL) through a nasal drip. The mice in the normal group were given 35 μL of normal saline. On the 4th day post-inoculation, the mice were weighed and sacrificed to harvest their lung tissues.

Histopathologic analysis: To monitor the histological changes in the lung tissues of influenza virus-infected animals, lung tissues were removed and washed with normal saline on the 4th day after viral infection. Lung tissues were immersed in 4% paraformaldehyde (analytical grade) and embedded in paraffin wax[9]. Lung sections were sliced and the thickness of every paraffin section was 5 μm. The paraffin sections of lung tissues were stained with Hematoxylin and Eosin (HE). Histopathological changes were examined under a light microscope (Olympus, Tokyo, Japan).

Quantitation of TNF-α and IL-1β: Lung tissues were removed and washed with normal saline. The concentration of tumor necrosis factor α (TNF-α) and interleukin-1β (IL-1β) in lung tissue were determined using a mouse TNF-α ELISA Kit and a mouseIL-1β ELISA Kit (Excell, Shanghai, China). The specific methods employed were those provided in the manufacturer's instructions.

Determination of MDA and SOD: Lung tissues were removed and washed with normal saline. This experiment used commercial kits to measure the content of malonaldehyde (MDA) and the activity of superoxide dismutase (SOD, Nanjing Jiancheng Biological Technology Company, Nanjing, China). The specific method employed were those provided in the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR): Total RNA in lung tissue was extracted as He *et al.*[8] described. The concentration of the extracted RNA was determined by optical density measurement at 260 nm using a spectrophotometer (Thermo, USA). One microgram of RNA was reverse transcribed to cDNA. The sequences of the primer pairs used for nucleoprotein (NP) and internal control 18S were listed in Table 1. Viral NP gene and internal control 18S gene mRNA levels in lung tissues were determined by separating cDNA on a 1% agarose gel and visualizing the bands with ethidium bromide staining. The band intensity of ethidium bromide fluorescence of the viral NP gene was measured, quantified with Quantity One analysis software (Bio-Rad, Hercules, CA)and expressed as the ratio to 18S fluorescence.

Western blotting: Lung tissues were lysed in lysis buffer (pH = 7.5) on ice and the supernatant of lung tissues was collected after centrifugation. The total protein content of the supernatant was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, USA). Samples were separated by SDS-PAGE on a 10% gel and electroblotted onto nitrocellulose membranes. Proteins were detected using monoclonal antibodies to mitochondrial antiviral signaling protein (MAVS; Proteintech Group, IL, USA), phosphorylated interferon regulatory factor 3 (p-IRF3; Cell Signaling Technology, MA, USA), inducible Nitric Oxide Synthase (iNOS; Santa Cruz Biotechnology, TX, USA), interferon-β (IFN-β; OriGene Technology, MD, USA) and NF-κB (Cell Signaling Technology, MA, USA). The immunodetection was performed using an enhanced chemiluminescence detection kit (MultiSciences Biotech, Hangzhou, China). The density of the bands was quantified using Quantity One analysis software (Bio-Rad, Hercules, CA).

Statistical analysis: The data are expressed as the Mean±Standard Deviation (SD). The statistical analysis of data was performed by one-way analysis of variance (ANOVA) using the Statistical Package for Social Sciences (SPSS) software (Version 19.0 for Windows, SPSS Inc., Chicago, IL). Multiple Tukey's *post hoc* test was used to determine the statistical significance ($p<0.05$) of multiple comparisons. Survival curves were estimated using the Kaplan-Meier method[18]. Differences in morbidity (time to sickness) and mortality (time to death) between groups were determined using GraphPad Prism 5 (GraphPad Software, La Jolla, CA) with the log-rank test.

RESULTS

Effects of WHT on influenza-induced morbidity and mortality in restraint-stressed mice: Mice were intranasally inoculated with 35 μL of viral suspension under anesthesia

Table 1: Sequences of primers used in RT-PCR

Name	Sequence of primer	Amplicon (bp)	Annealing temperature (°C)
18S	Forward Primer (5'-3') AGGGGAGAGCGGGTAAGAGA	241	60
	Reverse Primer (5'-3') GGACAGGACTAGGCGGAACA		
NP	Forward Primer (5'-3') CAGGTACTGGGCCATAAGGAC	330	55
	Reverse Primer (5'-3') GCATTGTCTCCGAAGAAATAAG		

and were monitored daily for body weight and behaviors until the 21st day post-viral infection. In the virus group, only half of the mice presented a severe pattern of illness signs and death, which suggested that 50.00% of them were relatively resistant to the influenza virus as shown in Fig. 1. Mice underwent restraint stress before viral infection to establish a susceptible animal model. The model control group experienced a 100.00% incidence of morbidity, while only 83.33% did in the virus control group. The survival also decreased in the mice exposed to "restraint stress+virus". The mice in model group had a higher morbidity rate (100.00% vs. 83.33%) compared to those that received the virus alone group ($p<0.05$). The survival rate of mice in the virus alone group was 50.00% and the survival rate of mice in the model group decreased to 33.33%. Compared with the model group, WHT-H treatment significantly decreased the morbidity (100.00 vs. 81.81%, $p<0.01$), the survival rate with WHT-H treatment was not significantly different. Compared with the model group, WHT-L treatment alleviated influenza symptoms. The morbidity rate was decreased (100.00 vs. 83.33%, $p<0.01$), while the survival rate was significantly improved (33.33 vs. 75.00%, $p<0.05$). These results demonstrated a protective effect of WHT against the virus and stress-induced death.

Effects of WHT on influenza-induced pneumonia in restraint-stressed mice: Tissue destruction induced by a cytokine storm and excessive inflammatory infiltrates contributes to high morbidity and mortality after influenza virus infection. In the present study, the lung tissues of mice were removed and washed with normal saline on the 4th day post-viral infection. In the virus control group, the morphology of intact lung tissue was characterized by slight edema and lesions in a small area, as shown in Fig. 2a. Large lesions and severe edema on intact lung tissues were seen in the model control group. Pulmonary pathological changes were observed by HE staining. The lung tissues of mice in the model group showed significant inflammatory cell infiltration, red blood cell extravasation and bronchial epithelial hyperplasia around the lesions compared with the virus control group, as shown in Fig. 2b. Alveoli were filled with hemorrhage exudates and alveolar walls were thickened compared with the virus control group. WHT treatment significantly alleviated $p<0.05$ the pulmonary morphology and pathological changes associated with restraint stress and the virus compared with the model group.

On the 4th day post-viral infection, lung weights were recorded to calculate the lung index, which was used to evaluate the inflammation and edema level. The average lung index of the normal control group was 6.81 ± 0.44 mg g^{-1}. It increased to 10.99 ± 1.14 mg g^{-1} in the virus control group, which was significantly worse than the normal control group ($p<0.01$), as shown in Fig. 2c. It further increased to 11.45 ± 1.77 mg g^{-1} in the model control group, which was also significantly higher compared with the normal control group ($p<0.01$). In comparison to the model control group, administration of WHT (500 or 125 mg kg^{-1}/day) recovered the lung index to 9.25 ± 1.13 and 9.08 ± 0.75 mg g^{-1} ($p<0.05$), respectively.

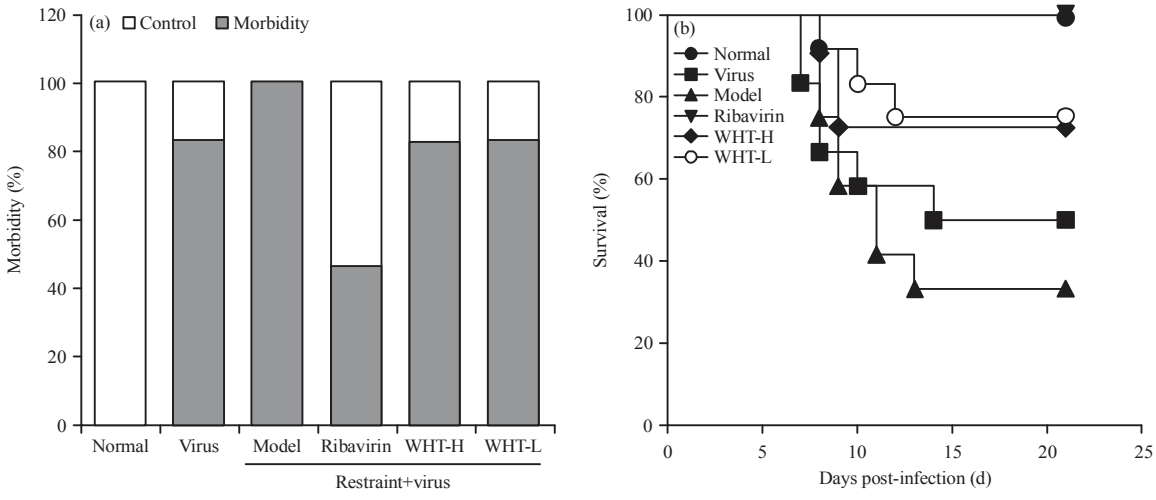

Fig. 1(a-b): Effects of WHT on the (a) Morbidity and (b) Survival rate of restraint-stressed mice post-viral infection

The time course of morbidity and survival of each mouse was recorded until the 21st day after viral infection (n = 12)[10]

Pro-inflammatory markers were analyzed on the 4th day post viral infection. After infection with the influenza virus, the expression level of TNF-α in lung tissues increased from 24.44±2.25-105.44±23.14 pg mL^{-1} ($p<0.01$). The expression level of TNF-α in lung tissues of the model mice increased to 126.32±15.31 pg mL^{-1} and was significantly higher compared with the normal control mice ($p<0.01$). In comparison to the model control group, the expression levels of TNF-α decreased to 102.28±11.41 and 90.47 ± 8.46 pg mL^{-1} ($p<0.05$) after WHT treatment (500 or 125 mg kg^{-1}/day, respectively, Fig. 2d). The content of IL-1β in lung tissues of the model group was 625.68±45.36 pg mL^{-1}, which was significantly higher than that in the normal group (516.30±37.56 pg mL^{-1}, $p<0.05$). Both WHT-H (515.76±46.35 pg mL^{-1}) and WHT-L (453.34±91.19 pg mL^{-1}) treatments obviously decreased the content of IL-1β compared with the model group ($p<0.05$, Fig. 2e).

Influenza virus NP indicates the state of viral replication and clearance during an influenza infection. As shown in Fig. 2f, NP gene expression in the lung tissues of the model control group was nearly three-fold higher than that in the virus control group ($p<0.01$). The WHT treatment (500 or 125 mg kg^{-1}/day) significantly decreased NP gene expression ($p<0.01$). These results revealed that the WHT

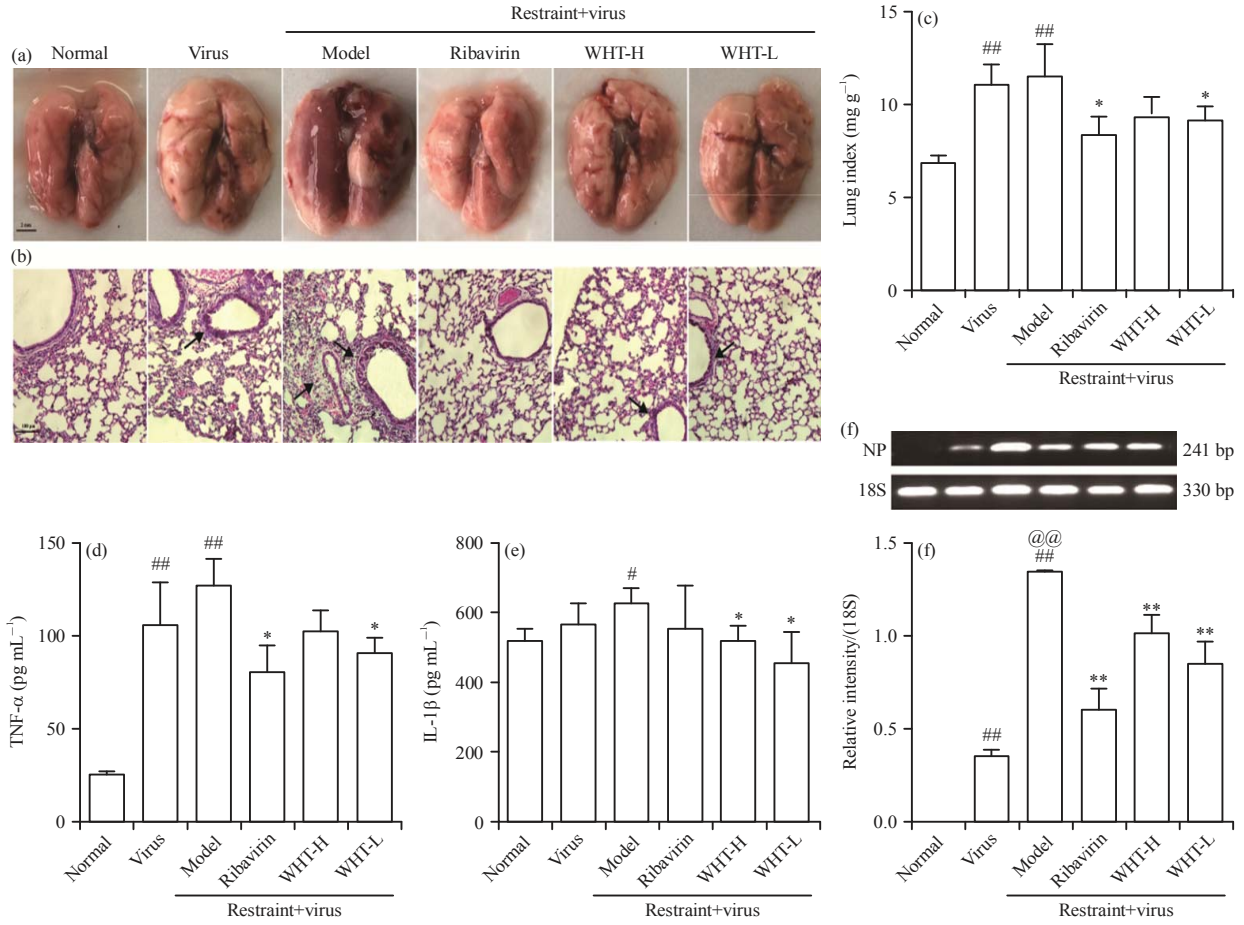

Fig. 2(a-f): Effects of WHT on influenza-induced pneumonia in restraint-stressed mice, (a) Intact lungs, bar = 2 mm, n = 5, (b) Histopathological changes in lung tissues. Representative histological sections of lung tissues from experimental mice were stained with HE, bar = 100 μm, n = 5, (c) The lung index was calculated according the following formula: Lung index = lung weight (mg)/body weight (g), n = 4, (d) TNF-α expression in lung tissues was determined using an ELISA kit, n = 3, (e) IL-1β expression in lung tissues was determined using an ELISA kit, n = 3 and (f) NP gene expression in lung tissues was determined by RT-PCR and normalized to 18S expression, n = 3

The significant differences are compared to the normal control group at $^{\#}p<0.05$ and $^{\#\#}p<0.01$, to the virus control group at $^{@@}p<0.01$ and to the model control group at $p<0.05$ and $p<0.01$, values are the Mean±SD

treatment decreased the susceptibility to influenza virus and alleviated influenza virus pneumonia in restraint-stressed mice.

Effects of WHT on influenza-induced oxidative status in restraint-stressed mice: In the body's normal metabolism process, SOD effectively removes oxygen free radicals. The MDA reflects the degree of oxidation of lipids. The SOD and MDA maintain the body's oxidation/antioxidation dynamic balance.

The SOD activity indicates the body's ability to clean oxygen radicals. After infection with the influenza virus, the activity of SOD in lung tissues decreased from 10.54±2.12-7.49±1.09 U mg^{-1} protein ($p<0.05$). The activity of SOD in the lung tissues of the model mice decreased to 2.37±1.25 U mg^{-1} protein and was significantly lower than that in the virus control mice ($p<0.01$). In comparison to the model control, the activity of SOD increased to 10.31±0.91 U mg^{-1} protein ($p<0.01$) and 6.64±1.05 U mg^{-1} protein ($p<0.01$) after WHT treatment (500 or 125 mg kg^{-1}/day, respectively, Fig. 3a).

After infection with the influenza virus, the content of MDA in lung tissues increased from 6.57±0.23 nmol mg^{-1} protein to 7.38±1.93 nmol mg^{-1} protein, meanwhile, the

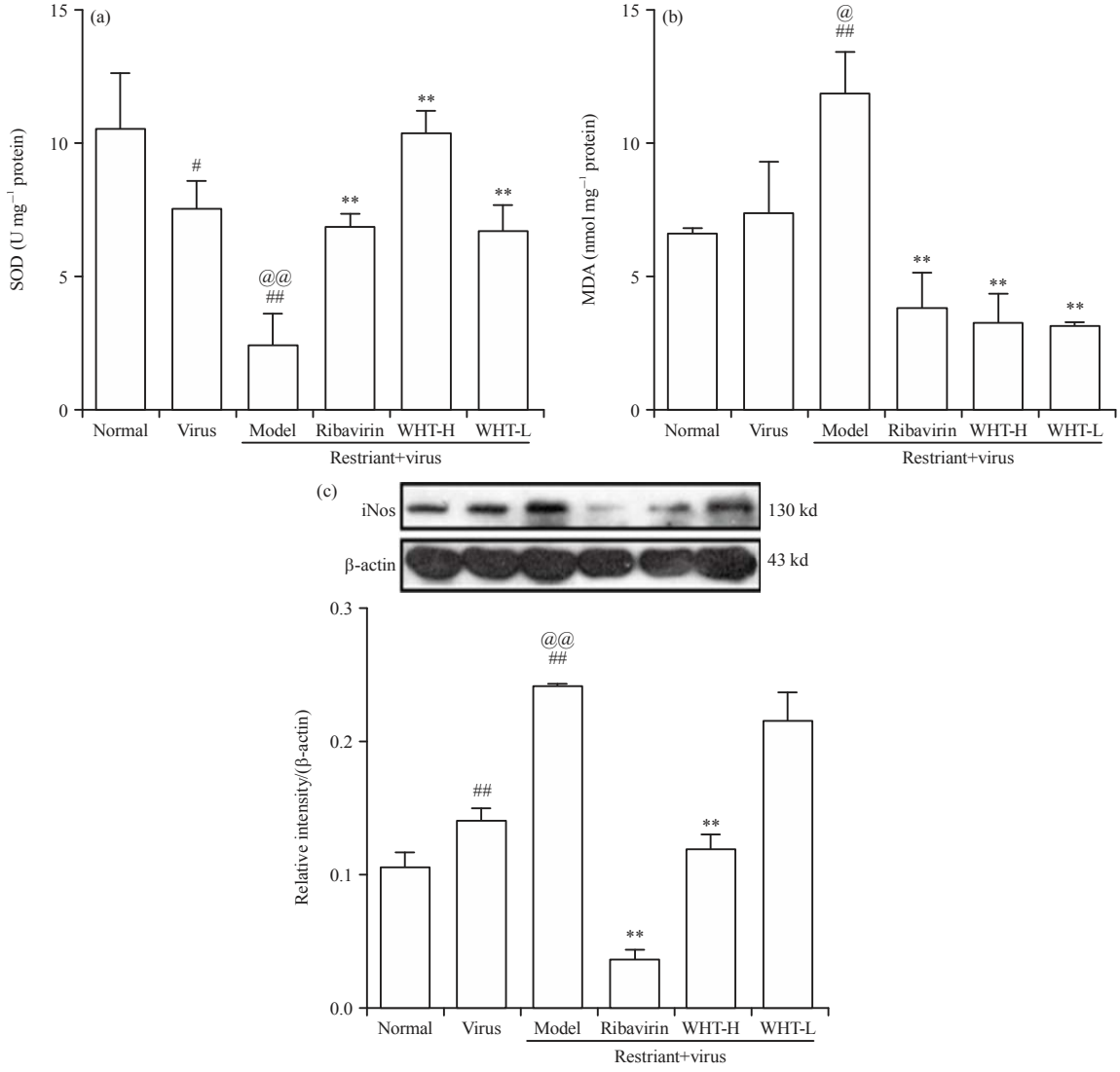

Fig. 3(a-c): Effects of WHT on the antioxidant capacity of restraint-stressed mice post-viral infection, (a) The activity of SOD in lung tissues was determined using a kit, n = 4, (b) The content of MDA in lung tissues was determined using a kit, n = 3 and (c) iNOS expression in lung tissues was determined by Western blotting and normalized to β-actin expression, n = 4
The significant differences are compared to the normal control group at $^{\#}p<0.05$ and $^{\#\#}p<0.01$, to the virus control group at $^{@}p<0.05$ and $^{@@}p<0.01$and to the model control group at $p<0.01$, Values are the Mean±SD

mice subjected to restraint stress and infected with the influenza virus showed an even greater increase to 11.83±1.60 nmol mg^{-1} protein, significantly greater than the virus control ($p<0.05$). The more MDA in lung tissues means a greater degree of lipid oxidation, which is harmful to the body. In comparison to the model control group, the content of MDA was significantly decreased to 3.24±1.08 nmol mg^{-1} protein ($p<0.01$) and 3.13±0.13 nmol mg^{-1} protein ($p<0.01$) after WHT treatment (500 or 125 mg kg^{-1}/day, respectively, Fig. 3b).

Meanwhile, the expression levels of iNOS in lung tissues were detected. After infection with the influenza virus, the expression level of iNOS in lung tissues was significantly increased compared with the normal control group ($p<0.01$). In comparison to the virus control group, the level of iNOS in the model control group further escalated ($p<0.01$). In comparison to the model group, the expression level of iNOS decreased after WHT treatment (500 or 125 mg kg^{-1}/day). Among the WHT treatments, the high dose had a significant effect ($p<0.01$), as shown in Fig. 3c. These results revealed that the treatment with WHT alleviated the oxidative state in restraint-stressed mice infected with the influenza virus.

Mechanism of WHT against influenza-induced pneumonia in restraint-stressed mice: Innate immunity is an essential and ubiquitous system that defends organisms from infectious pathogens. The mitochondrial antiviral signaling pathway plays an important role in innate immunity. Once viral RNA is recognized by retinoic acid-inducible gene-I (RIG-I), RIG-I interacts with MAVS, which induces the production of type I interferons (IFNs) and releases pro-inflammatory cytokines by triggering the activation of interferon regulatory factor 3 (IRF3) and NF-κB[19]. Appropriate pro-inflammatory cytokines are made for the body. Therefore, the protein expression levels of NF-κB, IFN-β, p-IRF3 and MAVS were examined in the lung tissues.

The expression level of NF-κB p65 in the virus group was increased compared with the normal control group ($p<0.01$). Meanwhile, NF-κB p65 expression level in the model group was further increased ($p<0.01$). The WHT treatment (500 or 125 mg kg^{-1}/day) lowered the expression level of NF-κB p65 compared to the model control mice ($p<0.01$), as shown in Fig. 4.

The effect of WHT on the production of IFNs was examined. The expression level of IFN-β in the virus control group was increased compared with that of the normal control group ($p<0.05$) but the expression level of IFN-β in the model group was decreased compared with the virus control

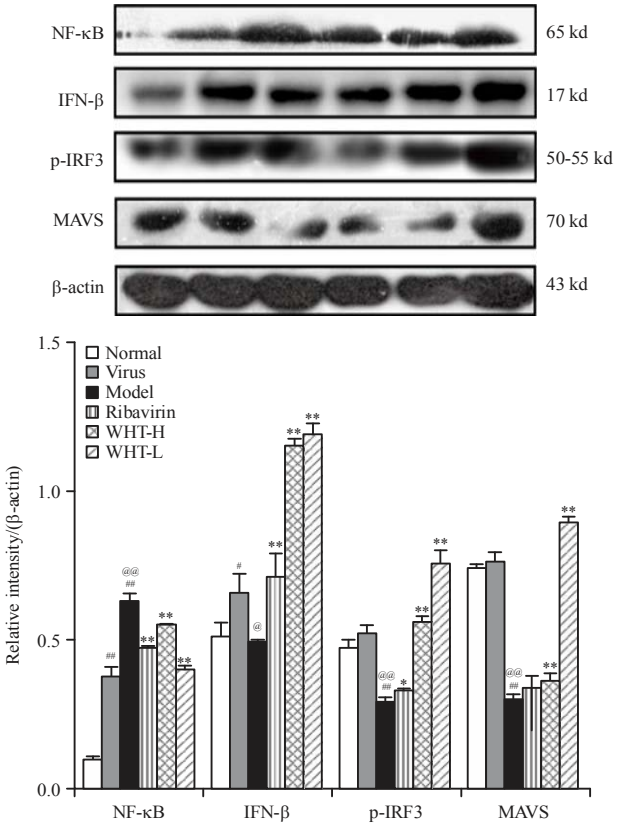

Fig. 4: Effects of WHT on NF-κB, IFN-β, p-IRF3 and MAVS expression in lung tissues of restraint-stressed mice post-viral infection

The significant differences are compared for the normal control group at $^{\#}p<0.05$ and $^{\#\#}p<0.01$, for the virus control group at $^{@}p<0.05$ and $^{@@}p<0.01$ and for the model control group at $p<0.05$ and $p<0.01$, n = 3, Values are the Mean±SD

group ($p<0.05$). In comparison to the model control group, administration of WHT (500 or 125 mg kg^{-1}/day) increased the expression level of IFN-β ($p<0.01$).

RIG-I interacts with MAVS and IRF3 is transformed into p-IRF3. p-IRF3 is crucial in host defense, it can affect viral replication and clear viruses from the lung. Therefore, the expressions levels of MAVS and p-IRF3 in lung tissues were examined to further investigate the protective mechanism of WHT against influenza-induced pneumonia in restraint-stressed mice. As shown in Fig. 4, the expression levels of MAVS and p-IRF3 were increased in the virus control group were increased and the expressions of MAVS and p-IRF3 were significantly decreased in the model control group in comparison to the normal control group and the virus control group ($p<0.01$). Administration of WHT (500 or 125 mg kg^{-1}/day) significantly increased the expressions levels of MAVS and p-IRF3 in comparison to the model control

group ($p<0.01$). These results revealed that treatment with WHT alleviated and protected against influenza-induced pneumonia in restraint-stressed mice via the mitochondrial antiviral signaling pathway.

DISCUSSION

The influenza pandemic seriously threatens the length and quality of human life. Secondary influenza pneumonia is the leading cause of death. Corticosteroids and cyclooxygenase-2 inhibitors have been proven to play a crucial role in limiting influenza virus-induced pneumonia by suppressing inflammation but their effectiveness has been limited due to excessive side effects[20,21]. The TCM has a long history of fighting the influenza pandemic. It performs well in clinical practice and is important in the therapy of influenza-induced pneumonia[22,23]. The TCM and chemical antiviral drugs work in very different ways in the human body. TCM might target both the virus and the organism itself by keeping the pathogens and the body in a fine balance[22,23]. Moreover, research has found that in industrialized countries, the majority of deaths occur among people over the age of 65, in the very young or in people with a weakened immune system[24]. Thus, in the evaluation of the effects of TCM on influenza-induced pneumonia, host factors cannot be ignored. In daily life, stress exists everywhere and reduces the body's immunity. Previous studies have demonstrated that restraint stress enhanced the susceptibility of the host to the influenza virus and eliminated individual differences in mice[8,17]. In southern China, herbal tea is usually prepared to prevent disease and enhance the resistivity of the organism. The WHT has been consumed for more than 200 years and is still a popular herbal beverage. In this study, the results showed that illness was observed in all restraint-stressed mice infected with the influenza virus. Meanwhile, the mortality of the restraint-stressed mice infected with influenza virus was higher compared with that of the virus group mice. A slightly better physiological condition was found after the oral administration of WHT. The WHT treatment reduced morbidity and mortality in restraint-stressed mice infected with the influenza virus. Therefore, the effects of WHT on influenza-induced pneumonia were evaluated and the related mechanism was investigated using the restraint stress model. Severe influenza infection induces a large range of cytokines and chemokines[11]. Studies have shown that inflammatory cytokines and chemokines make critical contributions to the control of virus replication. However, excessive inflammatory cytokines and chemokines exacerbated morbidity and tissue injury[25-27]. The intact lung tissues of the virus control group displayed edema and substantial damage. The intact lung tissues of the model control group presented more serious damage. The same was observed for the lungs in the pathological images. In addition, an exaggerated pro-inflammatory response was observed in the lung tissues of stressed-mice infected with the influenza virus, with high contents of inflammatory markers such as TNF-α and IL-1β. From the view of intact lung tissues, WHT treatment alleviated extensive edema and inflammation. Lung tissue pathological images showed that oral administration of WHT mitigated the influenza virus-stimulated pulmonary morphology, including that observed in the blood vessels, alveoli and bronchi. The expression levels of TNF-α and IL-1β decreased after WHT treatment in restraint-stressed mice infected with the influenza virus. The mRNA level of NP was examined to detect viral replication and degree of clearance. In this study, the mRNA level of NP in lung tissues of the model mice was significantly ($p<0.05$) higher than that of the virus mice. The WHT treatment decreased the mRNA level of NP in restraint-stressed mice infected with the influenza virus. These results indicated that WHT reduced the susceptibility of the body to the influenza virus and alleviated influenza-induced pneumonia in restraint-stressed mice infected with the influenza virus.

Previous results suggested a correlation between the immunological response and the antioxidative capacity of immunocytes[28]. In addition, studies have shown that stress can induce the production of Reactive Oxygen Species (ROS)[29]. A state of peroxide was found in the restraint-stressed mice infected with the influenza virus by determining the MDA content and SOD activity. WHT treatment reduced the content of MDA and increased the activity of SOD in restraint-stressed mice infected with the influenza virus. Increased production of NO is related to lung tissue pathology following influenza infection. WHT treatment reduced the protein expression of iNOS. The results showed that oral administration of WHT alleviated the peroxide state in restraint-stressed mice infected with the influenza virus.

Influenza virus infection in the host can be divided into five stages: Adsorption, invasion, replication, maturation and release[30]. IFN-β expression induced by influenza virus depends on the recognition of RNA viral genomes by either the cytosolic receptors for RIG-I or the Toll-like receptor (TLR) systems[31]. When host cells are infected with an influenza virus, RIG-I recognizes replicating viral RNA. The helicase domain of

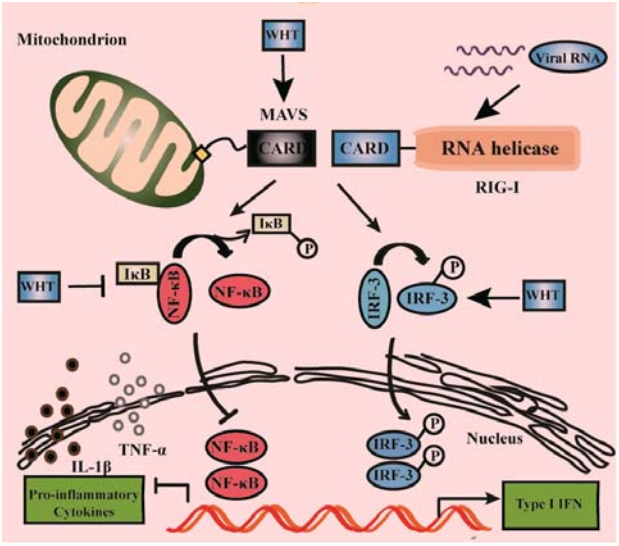

Fig. 5: Mechanism of WHT against influenza-induced pneumonia in restraint-stressed mice[32]

RIG-I binds to adenosine triphosphate (ATP), which facilitates conformational changes that enable its caspase-recruitment domains to bind to MAVS. Then, IRF3 is phosphorylated and NF-κB is activated, which results in the production of type I IFNs and inflammatory cytokines. Type I IFNs promote the subsequent development of adaptive antiviral immunity and protect the host. In this study, the expression of MAVS and p-IRF3 was decreased in restraint-stressed mice infected with the influenza virus. However, oral administration of WHT increased the expression of MAVS and p-IRF3 in restraint-stressed mice infected with the influenza virus. In addition, the production of IFN-β increased after WHT treatment in restraint-stressed mice infected with the influenza virus. Fatal injury to lung tissue injury induced by a cytokine storm is the main factor of high mortality during an influenza pandemic[12,19]. Excessive expression of NF-κB mediates the inflammation and leads to lung tissue injury. In this study, an increased expression of NF-κB was observed in stressed mice infected with the influenza virus. Oral administration of WHT decreased the expression of NF-κB. These results indicated that WHT exerted a protective effect in restraint-stressed mice infected with the influenza virus by regulating the MAVS antiviral signaling pathway, as shown in Fig. 5.

CONCLUSION

These data indicated that WHT reduced the susceptibility and severity of influenza in restraint-stressed mice by activating the mitochondrial antiviral signaling pathway to increase the host defense system and ameliorate influenza-induced pneumonia.

SIGNIFICANCE STATEMENT

This study provides information on the pharmacodynamics of WHT. It can modulate immunity against viral epidemics by activating the MAVS pathway.

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