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Recuperative herbal formula Jing Si maintains vasculature permeability balance, regulates inflammation and assuages concomitants of "Long-Covid"

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ABSTRACT

Coronavirus disease 2019 (COVID-19) is a worldwide health threat that has long-term effects on the patients and there is currently no efficient cure prescribed for the treatment and the prolonging effects. Traditional Chinese medicines (TCMs) have been reported to exert therapeutic effect against COVID-19. In this study, the therapeutic effects of Jing Si herbal tea (JSHT) against COVID-19 infection and associated long-term effects were evaluated in different in vitro and in vivo models. The anti-inflammatory effects of JSHT were studied in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and in Omicron pseudotyped virus-induced acute lung injury model. The effect of JSHT on cellular stress was determined in HK-2 proximal tubular cells and H9c2 cardiomyoblasts. The therapeutic benefits of JSHT on anhedonia and depression symptoms associated with long COVID were evaluated in mice models for unpredictable chronic mild stress (UCMS). JSHT inhibited the NF-ƙB activities, and significantly reduced LPS-induced expression of TNFα, COX-2, NLRP3 inflammasome, and HMGB1. JSHT was also found to significantly suppress the production of NO by reducing iNOS expression in LPS-stimulated RAW 264.7 cells. Further, the protective effects of JSHT on lung tissue were confirmed based on mitigation of lung injury, repression in TMRRSS2 and HMGB-1 expression and reduction of cytokine storm in the Omicron pseudotyped virus-induced acute lung injury model. JSHT treatment in UCMS models also relieved chronic stress and combated depression symptoms. The results therefore show that JSHT attenuates the cytokine storm by repressing NF-κB cascades and provides the protective functions against symptoms associated with long COVID-19 infection.

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1. Introduction

According to the World Health Organization, more than 560 million people are affected and more than 6 million people have died due to SARS-CoV-2 (source: World Health Organization). Unfortunately, there are still no comprehensive treatments available against the viral infection and the associated pot-infection abnormalities. Evidences suggest that proper adaptive immune responses help to control and subsequently neutralize the viruses. Specialized immune cells such as the Cytotoxic T lymphocytes effectively and specifically kill the infected cells and further eliminate viruses. However, in spite of wide spread vaccine administrations, lasting COVID-19 pandemic has led to negative heterogeneous post-recovery impacts in several patients [1]. The current trends suggest that the "Long COVID" is potentially the next public health disaster that is brewing [2].

Many studies have confirmed that excess inflammatory responses seem to be highly associated with the disease severity and mortality rate in COVID-19 patients [3]. Reduced or delayed type I interferon (IFN-I) is known to be a key immunopathological manifestation that hampers viral clearance and induces paradoxical hyperinflammation[4–6]. Moreover, epithelial-immune cell interactions and abrupt expression of chemokines and cytokines trigger highly over-active immune response [7]. This causes to massively release of many proinflammatory cytokines, such as interleukin (IL)−1, IL-6, IL-18, tumor necrosis factor α (TNFα), G-CSF, and interferon (IFN)-γ in the blood of severely ill COVID-19 patients [3,7,8]. Long lasting signature of these elevated cytokine levels potentially correlates with clinical symptoms post-acute COVID-19, a condition with persistent symptoms and/or delayed or long-term complications lasting over 4 weeks[9].

Various reports point out that the levels of $CD4^+$, $CD8^+$ T lymphocyte population and B cells were all reduced in COVID-19 patients [6,10–12]. Therefore, both the inhibition of COVID19 infection and the dampening of the inflammatory reaction before a cytokine storm is an important way to fight against COVID-19.

While most affected by COVID-19 recovered, 10%−30% still experience a variety of mid-, long-term and delayed effects amid neuropsychiatric complications of “long COVID” been reported widely [1,13–15]. Fatigue, depression and associated anhedonia are often reported as the common Post-COVID-19 manifestations after recovery from this novel viral pandemic [16–19]. Such long COVID associated psychological effects and brain clouding is been attributed to damages to CNS caused by viral infection, persistent inflammation and oxidative stress in addition to impairment in renin-angiotensin system, coagulation and immune disturbances that normally affect proper vascular functions and modulations in neurotransmitter secretion [20–23]. Depression and anxiety symptoms in Long COVID patients may result from the viral infection itself or psychological stress. Other than the hyperimmune responses [24,25], coronavirus infection can directly cause neurological damage through hypoxic injury, altered vascular permeability, and neuroinvasion [26].

In the face of the complex biological system of the "neuroendocrine and immune network," it is not easy for antidepressants with a single target to achieve a remarkable curative effect [27]. On the other hand, pleiotropic and multi-targeted Chinese herbal medicine has great potential in assisting the development of new treatments for improving depression systemically. Various studies on COVID-19 treatment approaches have shown TCMs as an effective complementary therapy along with conventional treatment by reducing the hospitalization period, easing symptoms, decreasing mortality and relieving adverse reactions [28–32]. Extracts from *Artemisia* species like *A. annua*, *A. afra*, *A. argyi* have shown alleviating effects on COVID symptoms regulate innate and adaptive immunity [33–35]. Jing Si Herbal Tea (JSHT), is an eight herb (*Artemisia argyi*, *Ohwia caudate*, *Ophiopogon japonicas*, *roots of Houttuynia cordata*, *Platycodon grandifloras*, *Glycyrrhiza uralensis*, *Perilla frutescens*, and flowers of *Chrysanthemum* × *morifolium*) formula containing leaves of *A. argyi,* with demonstrated potential as an adjuvant treatment for COVID-19 infection [31,32,36]. The constituent herbal extracts and the JSHT have been previously characterized by HPLC finger printing and were verified in the present study. In this study we have evaluated the effect of JSHT on long term COVID-19 associated effects such as abrupt imflammation, immune regulation, Fatigue, depression and associated anhedonia. We have established a standard depression animal model and examined the antidepressant effect of JSHT.

2. Materials and methods

2.1. Cell culture and treatment

The mouse macrophage cell line RAW264.7 (BCRC number 60001) was obtained from the Food Industry Research and Development Institute (FIRDI, Hsinchu, Taiwan). Cells were maintained in high-glucose DMEM (Gibco) with 10% FBS and 1% penicillin/streptomycin (Gibco) in a humidified 5% CO_2 incubator at 37 °C. Before treatment, RAW264.7 cells were seeded into 96-well plates at a density of 5×10^4 cells/mL and incubated overnight. After overnight culture, the culture medium was replaced with serum-free medium and treated with different concentrations (12.5, 25, 50, 100, 200 and 400 μg/mL) of JSHT for 3 h. And then, the cells were treated with 10 μg/mL lipopolysaccharide (LPS, Sigma) additional 24 h.

2.2. Animal experiments

Eight weeks old SKH1/J mice were used and randomly separated into 3 groups, PBS only, Omicron pseudo lentivirus, Omicron pseudo lentivirus with JSHT. Mice received PBS 0.05 mL, Omicron pseudo lentivirus 120,000 particles/0.05 mL (RNAicore facility, Academia Sinica, Taiwan) via trachea and oral JSHT 48.66 mg/mice/day for 3 days.

2.3. Cytokine array

Sera were collected from SKH1/J mice with trachea. Serum levels of inflammatory cytokines were detected by using a Mouse inflammation antibody array (Abcam, UK) following the manufacturer’s instructions. Images were acquired by iBright Imaging Systems (Thermo Fisher Scientific) and quantified by Image J.

2.4. Endothelial cell permeability assay

Endothelial cell permeability was studied as previously described [37]. HUVEC were cultured on Transwell filters (Corning; 12 mm diameter, 0.4 μm pore size) and detected the flux of FITC–dextran (Sigma, St Louis, MO, USA) across the endothelial monolayer. After 2 days, HUVECs were grown to confluence on the transwell inserts and medium was switched to serum-free conditions. In separate experiments, HUVECs were treated JSHT (100 or 200 μg/mL), the cells were treated with 10 μg/mL and FITC-conjugated dextran (1 μg/mL) was added to the upper chambers. Samples of the medium from the lower chamber were removed after 2 h and the amount of FITC-dextran that diffused across the endothelial monolayer into the lower chamber was measured using a microplate reader (Molecular Devices, Sunnyvale, CA) at 485 nm excitation wavelengths and using a 525 nm emission bandpass filter.

2.5. Immunoglobulin measurements and immunophenotyping

Serum IgA, IgG and IgM were quantified using commercial colorimetric ELISA assay kit following manufacturer’s protocol and the quantification was carried out by comparing with a standard curve derived from reference serum supplied by the manufacturers. For lymphocyte phenotyping flowcytometric analysis was performed

following methods mentioned previously [38].

2.6. Cell viability analysis

CCK-8 assay was used to determine the cell viability of RAW264.7 cells which were treated with various concentrations of JSHT in the absence or presence of 10 μg/mL LPS for 24 h. Thereafter, culture termination, 10 μL of CCK-8 was added to each well, and then incubated for 2 h at 37 °C. in a 5% CO_2 incubator. Finally, the absorbance of each well was recorded at450 nm using a microplate reader.

2.7. Nitric oxide (NO) production analysis

RAW264.7 cells were seeded into a 96-well plate and incubated with or without LPS (10 μg/mL) in the absence or presence of various concentrations of JSHT (6.25, 12.5, 25, 50, 100 and 200 μg/mL) for 24 h. The concentration of nitric oxide (NO) in the culture medium was measured using the Griess reagent method (Biovision, Catalog #: K544), according to the manufacturer's protocol. Absorbance at 540 nm was measured using a microplate reader.

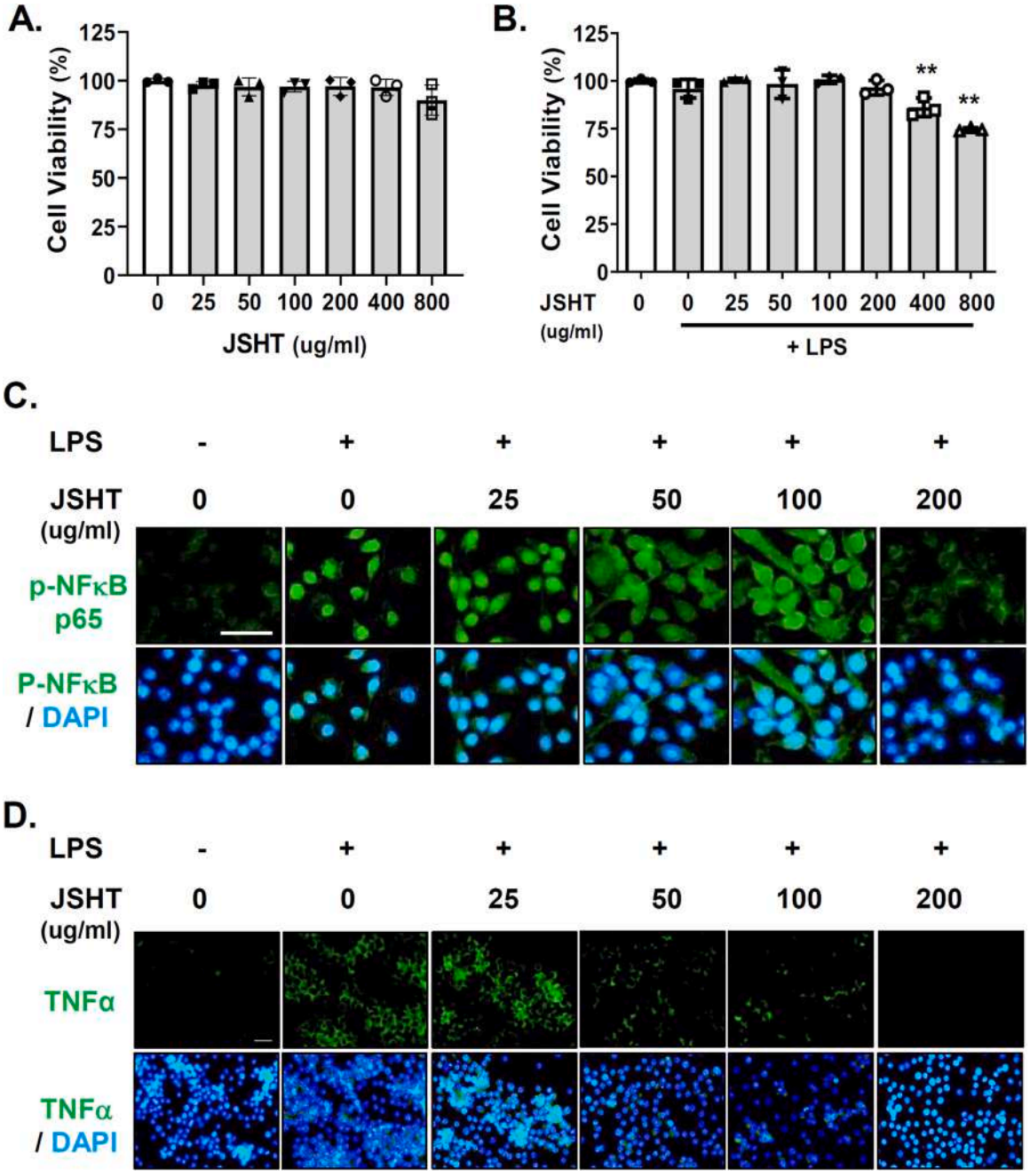

Fig. 1. JSHT abates LPS-induced inflammation by reducing NFκB activities. and TNFα expression in macrophages. (A) Dose-dependent effects of JSHT (25–800 μg/mL) on the cell viability for 24 hr in RAW264.7 cells. (B) The viability of RAW264.7 cells treated with PBS or different concentrations of JSHT and/or LPS for 24 hr. The effects of JSHT on (C) NF-κB activities (phospho-NF-κB p65 in green) and (D) TNFα (in green) expression in LPS- stimulated RAW264.7 cells by immunofluorescence staining. The cell nuclei were stained with DAPI (blue). The concentration of LPS is 10 ng/mL. PBS was used as control. Data are representative of three independent experiments (n = 4/group per experiment) and showed as mean ± SEM from triplicates. Asterisks indicate statistical significance versus control (**$p < 0.001$ by unpaired *t* test); Scale bars, 50 μm.

2.8. TUNEL assay

Cell apoptosis and damage were also analyzed by TUNEL staining assay. Cells from different groups were fixed by 4% paraformaldehyde. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions.

3. Results

3.1. JSHT abates LPS-induced inflammation by reducing NF-κB and TNFα expression in macrophages

In order to find an appropriate concentration of JSHT, the toxic dosage of JSHT was determined by CCK-8 viability assay. And the results showed that RAW264. 7 cells treated with 400 μg/mL JSHT did not exhibit any toxic effect (Fig. 1A). However 800 μg/mL of JSHT showed slight toxic effects. Moreover, while the LPS challenge itself did not cause any reduction in cell viability, concentrations of JSHT above 400 μg/mL along with 10 μg/mL LPS challenge showed reduction in the viability of RAW264. 7 cells (Fig. 1B). Further in order to expound the

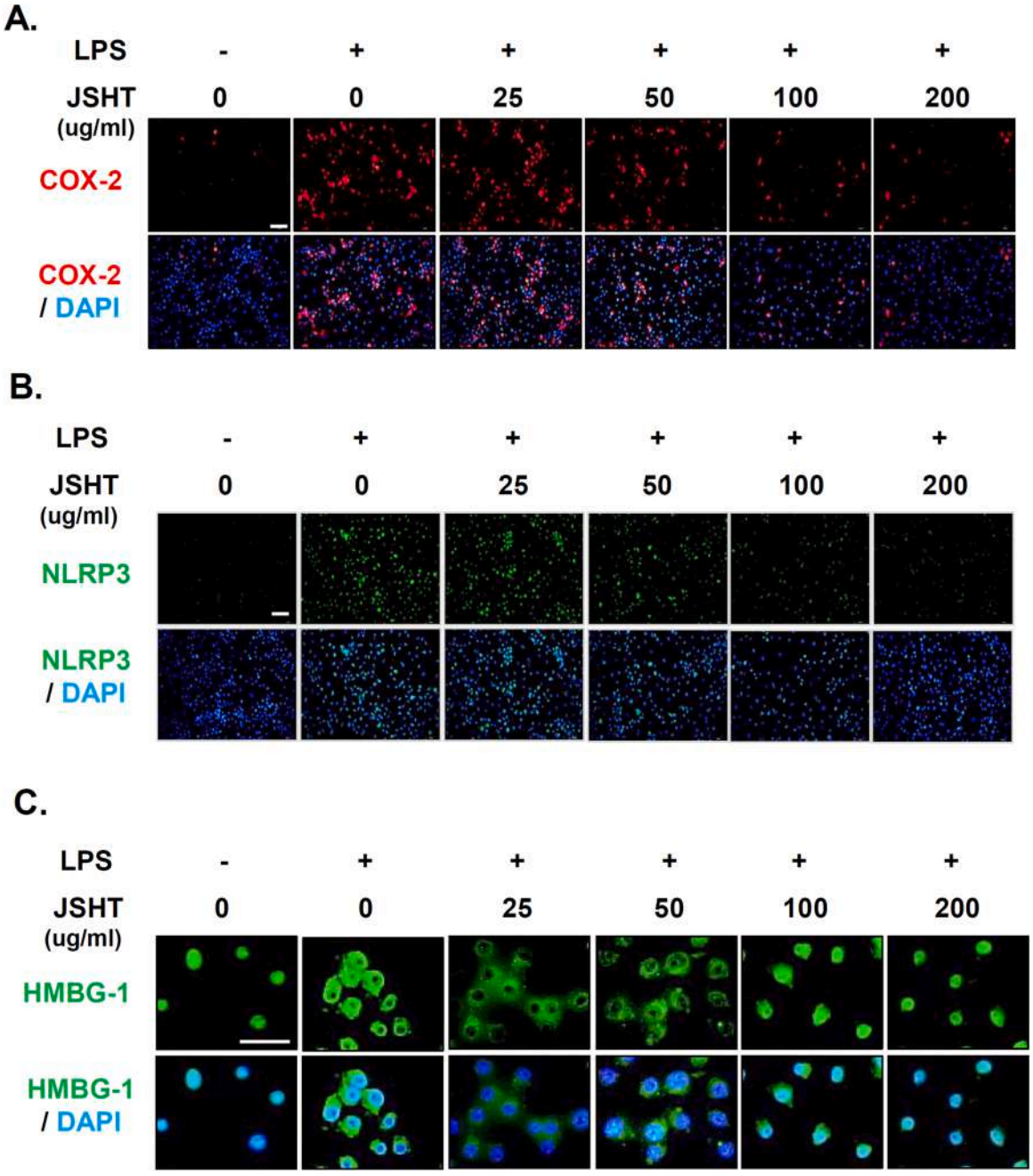

Fig. 2. JSHT Weakens LPS-Induced COX-2, NRRP3 Inflammasome Formation and HMGB-1 Activities in Macrophage. The effects of JSHT (0–200 μg/mL) on (A) COX-2 (in green) expression and (B) the formation of NLRP3 (in green) and (C) the activities of HMBG-1 (in green) in LPS- stimulated RAW264.7 cells by immunofluorescence staining. The cell nuclei were stained with DAPI (blue). The concentration of LPS is 10 ng/mL. PBS was used as control. Data are representative of three independent experiments (n = 4 /group per experiment); Scale bars, 50 μm.

possible inhibitory action of JSHT on LPS-induced pro-inflammatory mechanisms, their effect on LPS-induced activation of inflammation mediator NF-κB and inflammatory cytokine TNFα were determined by immunofluorescence analysis. The results show that LPS activated the levels of p-NF-κB p65 (Fig. 1C) and TNFα (Fig. 1D) in a dose dependent manner whereas, treatment with JSHT reduced the levels of p-NF-κB p65 and TNFα. Therefore, as seen in Fig. 1, JSHT reduces inflammatory cytokines in LPS-challenged RAW264. 7 cells.

3.2. JSHT diminishes LPS challenge associated COX-2, NLRP3 Inflammasome formation and HMGB-1 functions in RAW264. 7 cells

Various inflammatory mediators such as COX-2 are known to be induced upon elevation in the levels of p-NF-κB p65 [39–41]. HMBG-1 cytoplasmic translocation has been considered as a critical driving factor of inflammatory cascades. LPS induced inflammations is also mediated by protein complexes called inflammasomes. The formation of NLRP3 inflammasomes, a central innate immune sensor that connects inflammatory response to pathological effects, is also known to mediate LPS induced inflammatory processes [42]. Moreover, excessive Nitric oxide (NO) promotes inflammation by reacting with superoxide anions and generates peroxide nitrite. The main enzyme that catalyzes the production of NO in LPS-treated cells is iNOS and evaluation of iNOS and other mediators may help in evaluating drugs that target LPS induced inflammatory mediator. Therefore, to further assess the effect of JSHT on the mechanisms involved in the LPS induced inflammation the modulations in the levels of COX-2, HMBG-1, NLRP3 inflammasomes and iNOS levels were analyzed by Immunofluorescence staining. The results show that LPS greatly enhanced the expression of COX-2 (Fig. 2A), the formation of NLRP3 inflammasome (Fig. 2B), cytoplasmic translocation of HMBG-1 (Fig. 2C) and iNOS levels (Fig. 3). Treatment with JSHT effectively suppressed the LPS induced increase in COX-2 levels, formation of NLRP3 inflammasome, cytoplasmic HMBG-1 levels and iNOS (Figs. 2 and 3).

3.3. JSHT attenuates Omicron SARS-CoV2-S-pseudotyped lentivirus induced pulmonary inflammation and associated Edema

To explore the protective effects of JSHT on COVID-19 virus infection, lung histopathological changes were examined in lung lesions of Omicron SARS-CoV2-S-pseudotyped lentiviruses induced acute lung injury animal model. Hematoxylin and eosin (H&E) staining showed that the control group mice displayed no notable injury and had normal morphology. However, the trachea and alveolar insterstitium infected by SARS-CoV2-S-pseudotyped lentiviruses showed notable damages including increased influx of inflammatory cells in the alveolar interstitium, interstitial edema, thickened alveolar walls and hemorrhage in the intraalveolar tissues compared with the PBS-treated groups (Fig. 4A). However, SARS-CoV2-S-pseudotyped lentiviruses challenged mice that received oral JSHT showed reduction in these histopathological changes (Fig. 4A). In addition, the expression levels of TMPRSS2 (Fig. 4B), a protease that facilitates viral entry into host cells, and that of HMGB-1(Fig. 4C) were elevated in the trachea and alveolar insterstitium as seen from immunofluorescence staining.

3.4. JSHT ameliorates cytokine immune storm following omicron SARS-CoV2-S-pseudotyped lentiviruses infection associated acute lung inflammation

According to previous studies COVID-19 significantly enhanced the expressions of IL-1β, IL-6, IFN-γ, IL-12 and several proinflammatory cytokines to induce cytokine storm which is associated with the COVID-19 severity. To explore the effects of JSHT on systemic cytokine levels in SKH1/J mice models with Omicron SARS-CoV2-S-pseudotyped lentivirus induced acute lung inflammation, serum from the mice were collected and analyzed for their cytokines expression using mouse inflammation antibody array. The results showed that expressions of IL-1α, IL-1β, IL-2, IL-6, IL-9, IL-10, IFNγ and TNFα were markedly increased in SKH1/J mice upon SARS-CoV2-S-pseudotyped lentivirus infection (Fig. 5). However, JSHT significantly reduced these cytokines-induced by Omicron pseudotyped virus in acute lung inflammation animals

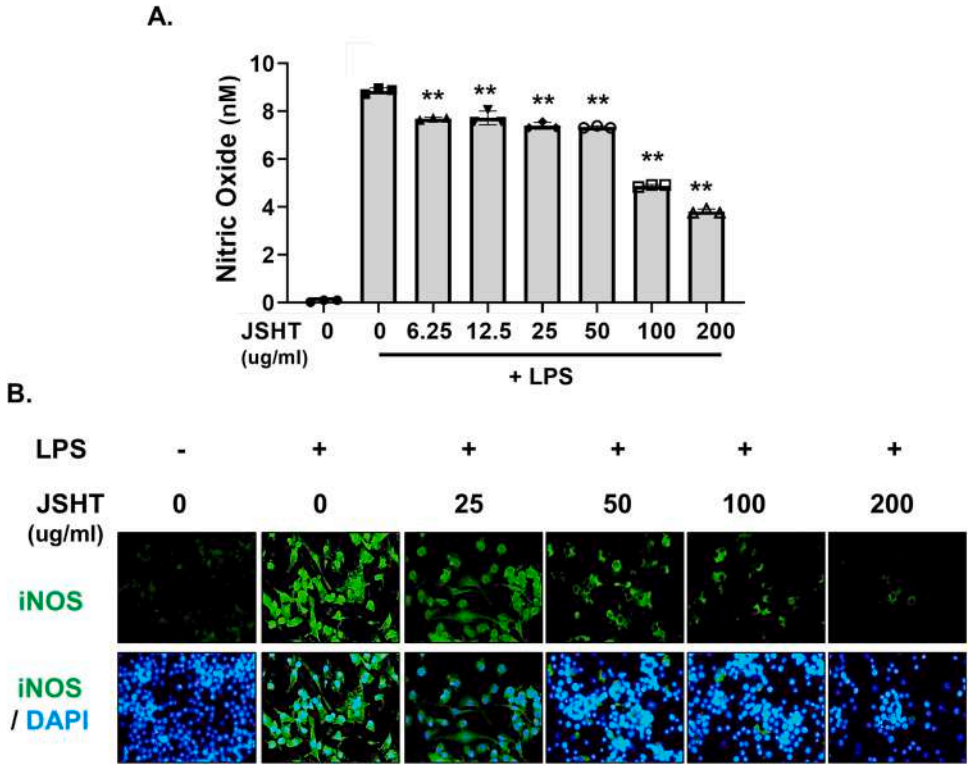

Fig. 3. JSHT impairs LPS-enhanced nitric oxide production by inhibiting iNOS expression in macrophages. (A) Dose-dependent effect of JSHT (6.25–200 μg/mL) on the nitrite levels in LPS- treated RAW264.7 cells for 24 hr. (B) The effects of JSHT on iNOS (in green) expression in LPS-stimulated RAW264.7 cells for 24 hr. The cell nuclei were stained with DAPI (blue). The concentration of LPS is 10 ng/mL. PBS was used as control. Data are representative of three independent experiments ($n = 3$ /group per experiment) and showed as mean ± SEM from triplicates. $^{**}p < 0.001$ indicate statistical significance versus control determined by unpaired *t* test; Scale bars, 50 μm.

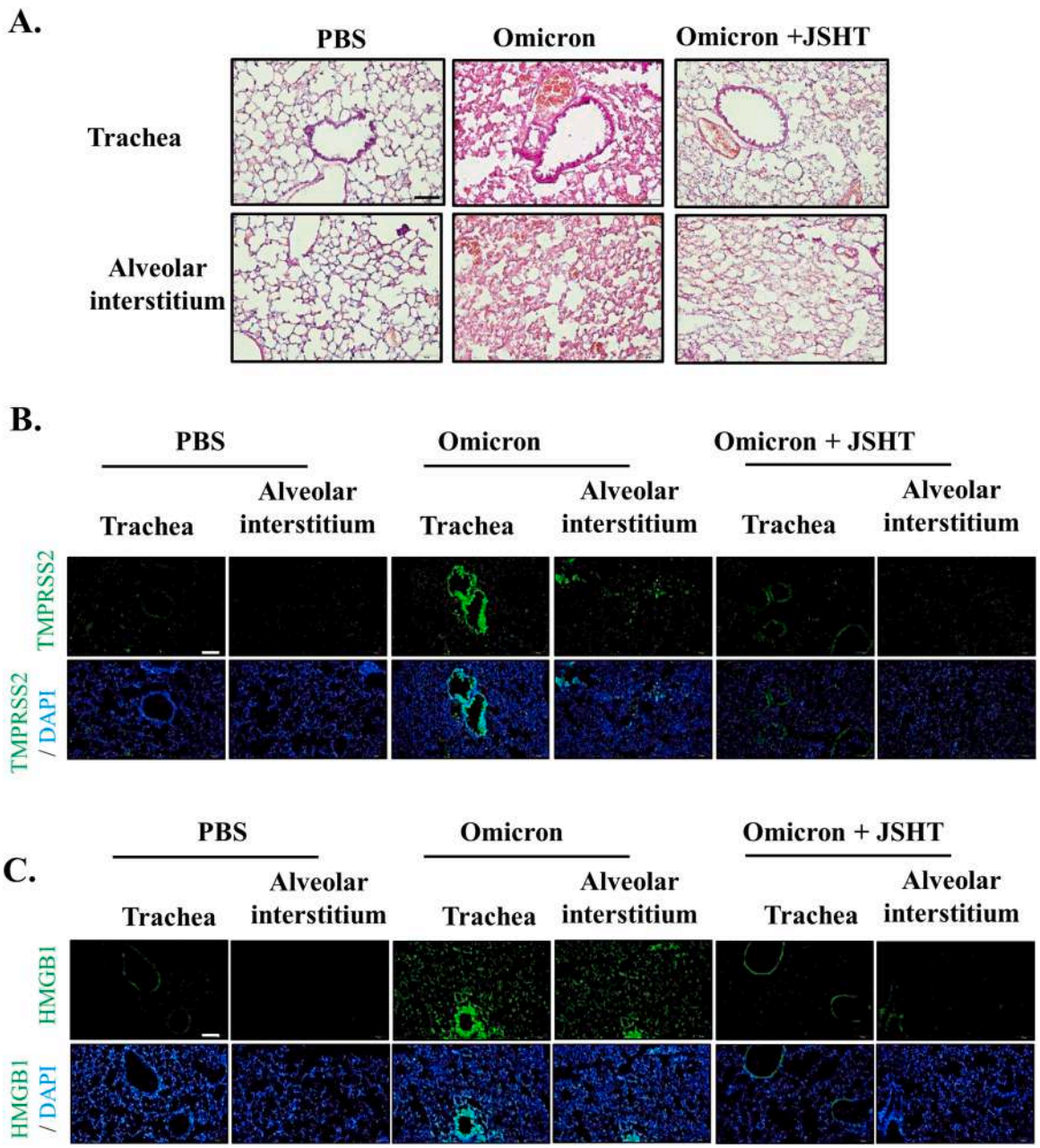

Fig. 4. JSHT mitigates pulmonary inflammation and edema in acute lung. injury-induced by Omicron pseudotyped virus. (A) Acute pulmonary injury was induced by the Omicron pseudotyped lentivirus via trachea infection. One hour prior to Omicron pseudotyped lentivirus infection, the mice were orally treated with PBS or JSHT for 3 days. After 3 days infection, the mice were sacrificed and collected their lungs to fix and prepare the histological sections for staining with H&E. The representative lung images of PBS, and Omicron pseudotyped lentivirus and Omicron pseudotyped lentivirus combined with JSHT-treated are shown (upper panel was the region of Trachea; lower panel was the region of alveolar interstitium). (B) The lung tissues of control, Omicron pseudotyped lentivirus and Omicron pseudotyped lentivirus combined with JSHT-treated mice were subjected to immunofluorescence staining to determine TMPRSS2 and HMGB-1 expression. The representative images of TMPRSS2 and HMGB-1 staining in the trachea and alveolar interstitium of control, Omicron pseudotyped lentivirus - and Omicron pseudotyped lentivirus combined with JSHT-treated mice are shown. The photographed section shows a representative view of each group. n = 6. Scale bar, 200 μm (A). 50 μm (B andC).

(Fig. 5).

3.5. JSHT helps improve endothelial barrier function

Endothelial cell permeability for FITC-dextran into the lower chambers through monolayer cells was determined by the flux expressed as a percentage of the amount of FITC-dextran added to the upper chamber (inserts). The significant increase in paracellular permeability of FITC-dextran flux observed with LPS challenge significantly reduced when treated with 100 μg/mL and 200 μg/mL (Fig. 6). Therefore the results point out that JSHT treatment can improve the endothelial barrier function.

3.6. JSHT enhances the humoral response and cellular immunity

Immune responses against SARS-CoV-2 infection comprises of humoral (immunoglobin IgA and IgG) immunity and the T cell responses and the later plays an essential role in controlling SARS-CoV-2 infection [43,44]. Therefore humoral and cell mediated immune responses to delta pseudotyped lentivirus and the effect of JSHT treatment were

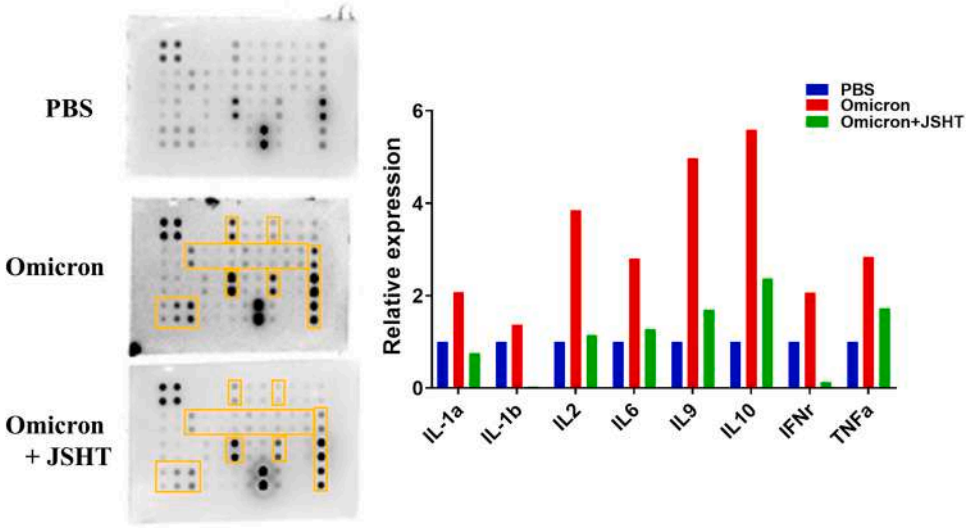

Fig. 5. JSHT reduces the Cytokine Immune Storm Caused by Omicron. pseudotyped lentivirus infection in the Acute Lung Inflammation. The effects of JSHT on serum cytokines in the acute lung inflammation-induced by Omicron pseudotyped lentivirus. Acute pulmonary injury was induced by Omicron pseudotyped lentivirus via trachea infection. After 3 days infection and orally treated with PBS or JSHT, the serum of each group was collected and mixture to analysis the cytokine expression by mouse inflammation antibody array. N = 3 per group. Images were acquired by iBright Imaging Systems and quantified by Image J.

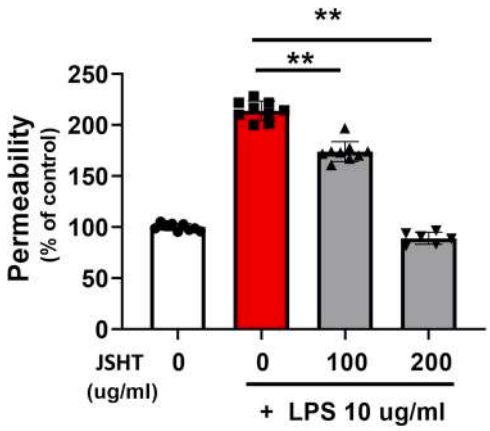

Fig. 6. JSHT enhances endothelial barrier function. Endothelial cell permeability was determined using transwell assay. HUVECs were treated with 100 μg/mL and 200 μg/mL of JSHT and were challenged with LPS and the changes in FITC-dextran flux was determined from the levels of dextran that diffused across the endothelial monolayer into the lower chamber measured at at 485 nm excitation. (**$p < 0.001$ represent significant difference when compared to the LPS challenged group).

analyzed. As observed previously from COVID-19 patient samples [45], delta pseudotyped lentivirus elicited higher levels of IgG, IgM, and IgA in SKH1 mice. The increase in baseline immunoglobulins in SKH1 mice indicated the humoral response; interestingly JSHT treatment showed an improvement in the IgA and IgG levels but not the IgM levels (Fig. 7A). It is known that among the memory B cell responses, those that of IgM are short lived, IgG is the dominant isotype and IgA is a minor population [46]. Increase in IgA and IgG potentially confer a longer memory to the viral infection. Similarly the $CD3^+/CD4^+$ population that represents Th cell and the CD3 + /CD8 + population representing Tc cells that increased with delta pseudotyped lentivirus infection increased drastically in the JSHT treatment groups (Fig. 7B). Therefore, the results show that JSHT enhances the immune response and improve the cellular memory in SKH1 mice.

3.7. JSHT alleviates cellular stress

COVID-19 infection is associated with persisting multisystem abnormalities and cardio-renal inflammation is known to contribute to pathogenesis of multi-organ damages [47–50]. Host cell stress has been correlated with various comorbidities of COVID-19 such as multi-organ damages [51]. In our studies, JSHT treatment showed amelioration of H_2O_2 induced accumulation of mitochondrial ROS in HK-2 human proximal tubular cells. While H_2O_2 challenge increased ROS in the mitochondria (Red), treatment with JSHT (50 μg/mL and 100 μg/mL) attenuated the effect (Fig. 8A). In addition, in H9c2 cells, H_2O_2 induced apoptosis as seen from the TUNEL staining (green) however, JSHT treatment affectively reduced the stress induced apoptosis (Fig. 8B). Similarly infection with Delta pseudo lentivirus induced apoptosis in H9c2 cells and treatment with 45 μg/mL of JSHT inhibited the viral induced cellular damages in H9c2 cells (Fig. 8C). The result therefore demonstrates that JSHT exhibits potential anti-stress effects in viral infected cells.

3.8. Effect of JSHT on anhedonia and depression symptoms

Long-lasting, unresolved stress is one of the significant contributors to clinical depression. We utilized the unpredictable chronic mild stress (UCMS) model, a general and standard animal model of long-term depression, to simulate behavioral symptoms associated with clinical depression, such as anhedonia and behavioral changes. Following and modified rigorous procedures [52], we randomly exposed mice to 7 different stressors each day: damp bedding, bedding removal, cage tilting, changing light/dark cycles, confinement in confined spaces for 4 h, shallow water baths, and shaking environments (60 rpm). We used extended periods of restricted movement to simulate the real-world psychological state of people with COVID-19 who are quarantined for long periods. Anhedonia and behavioral despair were assessed by subjecting rodents to these mild stressors 3–4 h a day for eight weeks, followed by a sucrose preference test and a tail suspension test at week 8, respectively. The study's results indicated that UCMS could lead to anhedonia, while JSHT treatment (600 mg/kg/day, daily for eight weeks) effectively maintains the preference for sugar water in mice (Fig. 9A). On the other hand, the treatment of JSHT can also improve the behavioral despair of mice caused by UCMS stress and increase the survival instinct of mice (Fig. 9B) as seen from the tail suspension test. The experimental results demonstrate that JSHT has the effect of helping to relieve chronic stress and its potential to combat depression symptoms caused by Long COVID.

4. Discussion

In this study, the effects of JSHT on Long COVID associated effects were determined using different possible models. The anti-inflammatory

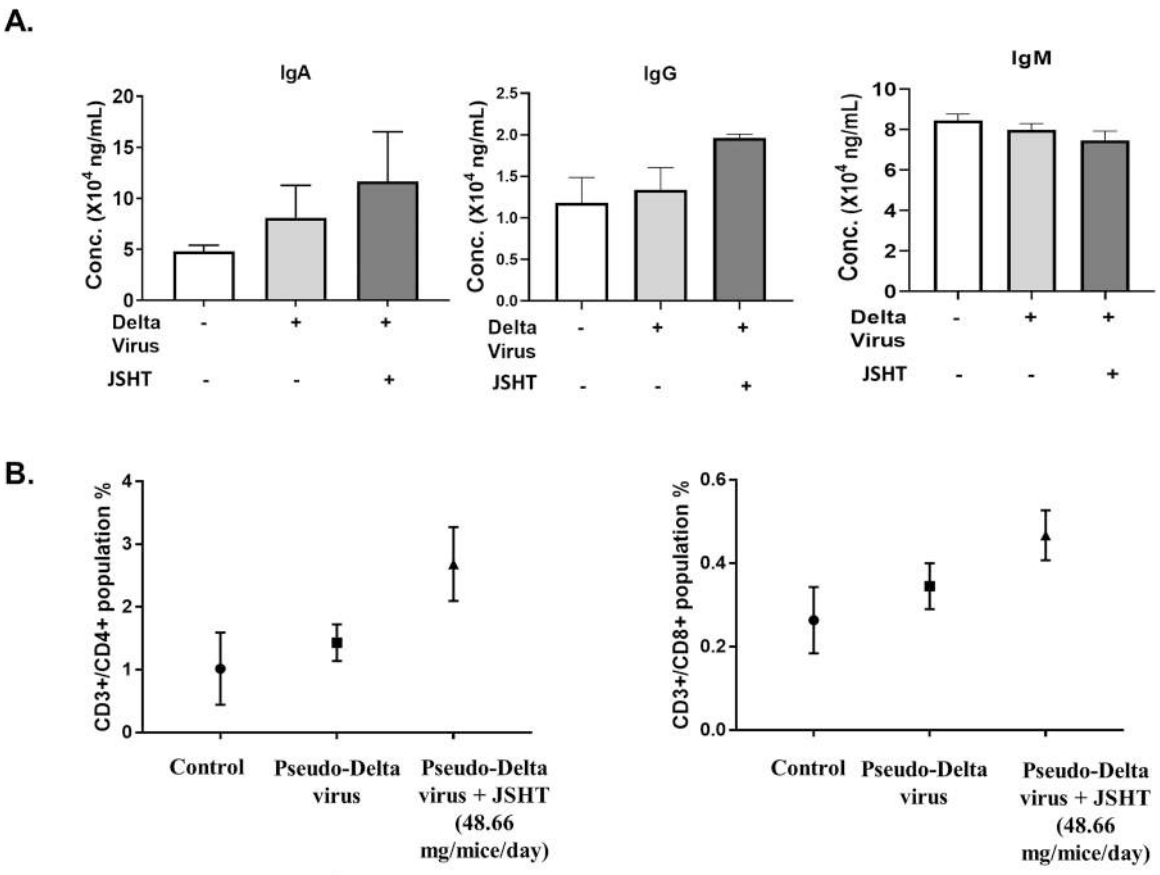

Fig. 7. JSHT enhances the cellular immunity by activating Th and Tc cells. The humoral response was determined with the levels of IgA and IgG levels in SKH1 mice. The mice were administered with omicron pseudo lentivirus 120,000 particles/0.05 mL via trachea and each control group mice received PBS. The treatment group received oral JSHT 48.66 mg/mice/day for 3 days. Levels of IgA, IgG and IgM were measured from serum using ELISA (A). Lymphocyte phenotyping to find the population of CD3 + /CD4 + Th cell and CD3 + /CD8 + Tc cells was performed by flowcytometry analysis (B).

effects of JSHT were verified with LPS-stimulated RAW264.7 cells in vitro, and further the efficacy of JSHT treatment on COVID-19-infection was explored using in vitro and in vivo models. The in vitro results showed that JSHT possess no cytotoxicity on macrophage cells but significantly repressed NF-κB and proinflammatory factors including TNFα and COX-2. Further, JSHT effectively reduced the activities of HMGB1, NLRP3 inflammasome formation and suppressed NO level associated iNOS. These findings show that JSHT has strong anti-inflammatory effects which may benefit in ameliorating the lasting effects of COVID-19. The in vivo experiments, we observed JSHT clearly attenuated COVID-19 induced pulmonary lesions and cytokine storm by reducing the expression of TMPRSS2 and the activities of HMGB-1. Taken together, our results reveal that JSHT exerts anti-inflammatory effects and attenuates the entry of COVID-19 into the host by regulating the NF-κB pathway, suggesting that JSHT could be a potential therapy for COVID-19 infection. Further studies also show that JSHT improves the endothelial barrier function and therefore potentially improves the Blood Brain Barrier stability and it also reduces the cell stress in kidney and cardiac cells. JSHT also enhance the cellular immunity and improves IgA and IgG that are associated with swift recovery from COVID-19 infection. Most notably, JSHT treatment reduced depression associated effects in UCMS models. Therefore, JSHT may reduce COVID-19 infection associated tissue damages and long COVID-19 associated depression effects as observed in the mice models.

Current TCM-based formulations known to treat COVID-19 patients were reported to act on multiple pathways and possess anti-viral, anti-inflammatory and immunoregulatory effects [32,53–56]. For example, Tsai et al., demonstrated the therapeutic effects of Taiwan Chingguan Yihau (NRICM101) [53] which include blocking the viral entry and cytokine production. The anti-viral effects of NRICM101 were due to inhibition of the viral spike protein-host ACE2 interaction, 3CL protease activity and the anti-inflammatory effects were attributed to their potential to suppress the production of cytokines interleukin (IL)−6 and tumor necrosis factor (TNF)-α [53].

A. argyi in the JSHT is already known for their antiviral effects. Ethanol extracts of *A. argyi* at a concentration of10 μg/mL exhibits antiviral effects against wild type and acyclovir- resistant HSV-1variant [57]. In addition, five major flavonoids Jaceosidin, Eupatilin, Apigenin, Eupafolin, and 5,6-Dihydroxy-7,3′,4′-trimethoxyflavone, of *A. argyi*, have been predicted to be the potential inhibitors the main protease Mpro of COVID-19 [58]. Other constituents of JSHT such as *H. cordata*, *O. caudate* and *G. uralensis* were found to block the ACE2-spike protein binding and thereby inhibit of COVID-19 [32,59–62]. Previous reports partially attribute the antiviral activity to of *O. caudata* to their flavonoid content [59]. Swertisin, is a flavonoid present in *O. caudata* with known anti-hepatitis B and influenza virus effect. Recent studies also highlights the potential effect of swertisin to inhibit SARS-CoV2 RNA--dependent RNA polymerase (RdRp) [63]. Molecular docking studies also show that 1,2,3,4,5-pentamethoxy-dibenzo-quinolin-7-one, 7-oxo-dehydroasimilobine, 1,2-dimethoxy-3-hydroxy-5-oxonoraporphine from *H. cordata* display high affinity to SARS-CoV-2 RdRp enzyme [64]. The anti-inflammatory and antiallergic actions of *G. uralensis* are known to provide preventive effects to treat COVID-19 [65]. Glycyrrhizin and enoxolone from *G. uralensis* have shown inhibitory effects on various viral pathogens belonging to different families such as Hepatits A [66,67].

O. japonicas are known to provide cardioprotection in diabetic conditions and therefore may help in preventing comorbidities associated

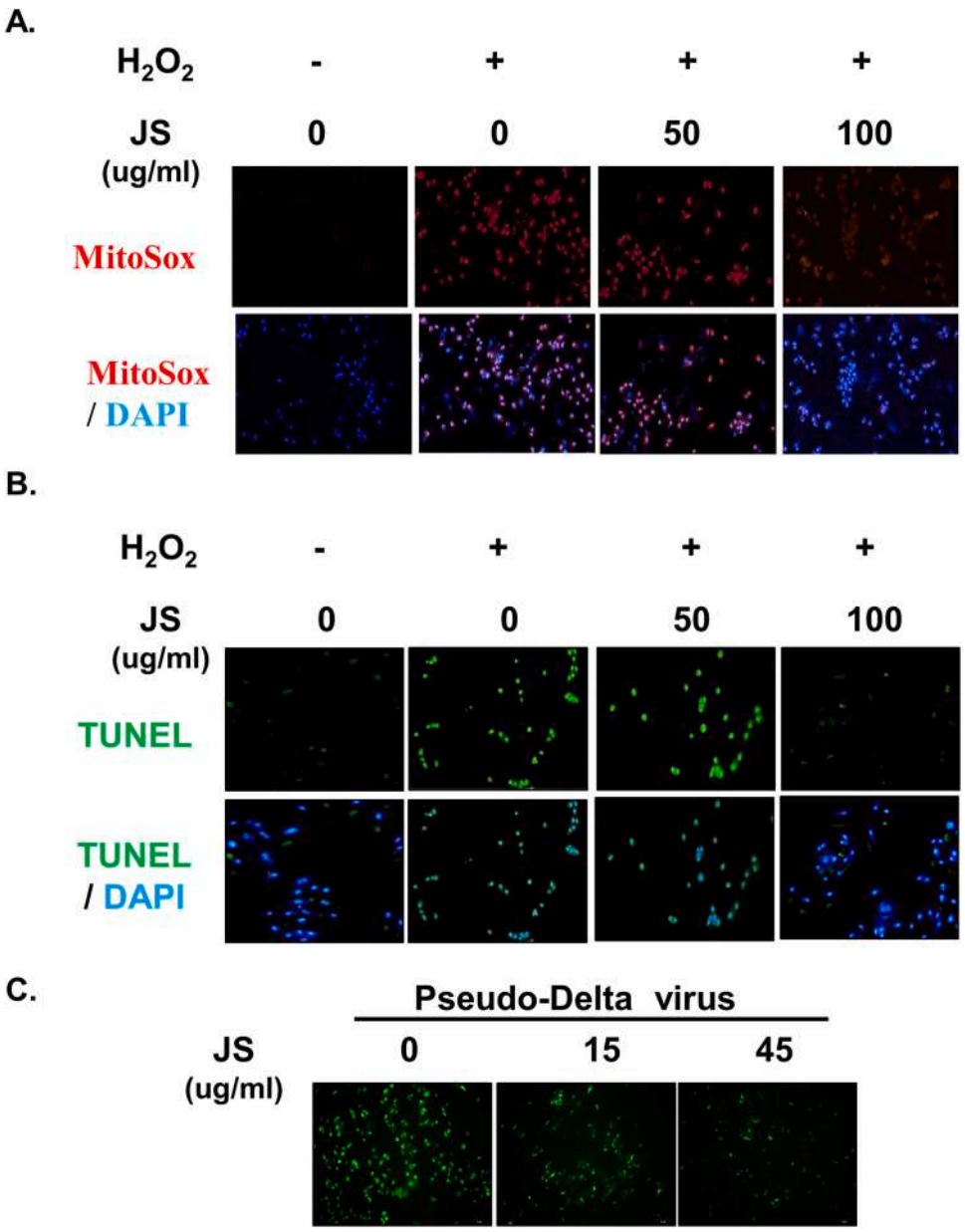

Fig. 8. JSHT mitigates cellular stress. Cellular stress induced by H_2O_2 (A and B) and by pseudo-delta virus infection (C) were determined by mitochondrial ROS accumulation in HK-2 human proximal tubular cells using MitoSox staining (A) and by apoptotic events in H9c2 cardiomyoblast cells using TUNEL assay (B). Treatment with 50 and 100 μg/mL of JSHT show ameliorative effects on H_2O_2 induced stress and pseudo-delta virus infection induced apoptosis (C).

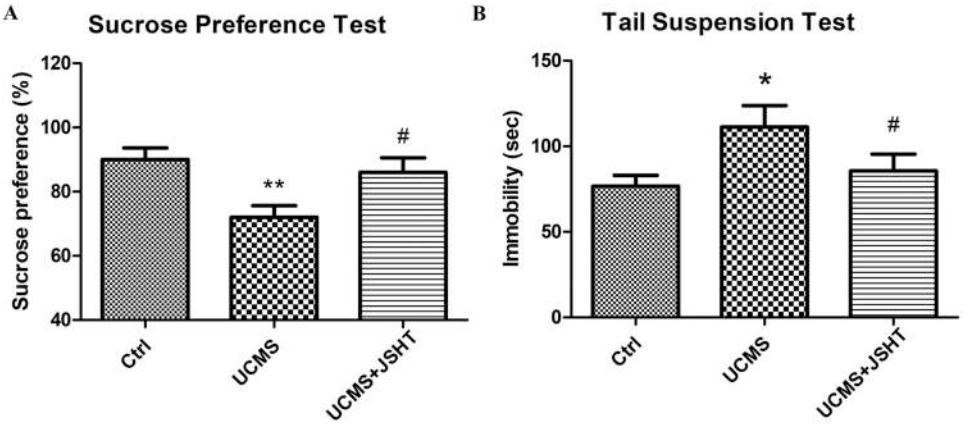

Fig. 9. JSHT improves UCMS-induced depressive behavior. The sucrose preference test (A) was performed in a free-bottle-based design. After eight weeks of UCMS, the mice show a lower preference for sucrose water (1%). Gavage treatment with the JSHT maintains the preference behavior after UCMS. The depression-like behavior was assessed by tail suspension test (B). The JSHT treatment mice show more activity and mobility in the UCMS experiment than the UCMS mice. *$p < 0.01$, **$p < 0.001$ represent significant difference when compared to control group. #$p < 0.01$ represents significant difference when compared to UCMS group.

with COVID-19 [68]. The roots of *P. grandiflorus* have previously known to provide treatment benefits against bronchitis, asthma, diabetes, and inflammatory disorders [69–72]. Recent findings show that platycodin D, a triterpenoid saponin abundant in *P. grandiflorum*, effectively blocks entry of SARS-CoV-2 by inhibiting spike glycoprotein cleavage by TMPRSS2 and cathepsin B/L [73].

Similarly the leaves of *Perilla frutescens* is also known to inhibit SARS-CoV-2 by blocking the viral RNA and protein synthesis [74]. Hypericin from *Chrysanthemum morifolium* has been recently found to possess higher affinity to the spike protein, the main protease 3CLpro that is necessary for viral replication, RdRp, and nucleocapsid (N) protein [75].

The infection of SARS-CoV-2 is predominantly in the lungs and with severity of infection it progresses to acute lung injury [76]. With worsening hypoxemia acute lung injury may develop into acute respiratory distress resulting in high mortality rate [77]. Acute respiratory distress in SARS-CoV-2 infection presents an "unique inflammatory signature" [78] and the cytokine storm that results from combined effects of many immune mediators like interferons, interleukins, chemokines and TNFα is culmination of a feedback loop recruiting monocytes, macrophages, and neutrophils to the infection site [79,80]. Severe COVID-19 symptoms has also been associated with hyper-activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway [81] which acts as a transcription factor for variety of cytokines, chemikines, adhesion molecules and inducible effectors such as iNOS and COX-2 [82]. Inhibition of NF-κB pathway has been considered as a potential therapeutic strategy in alleviating the severe form of COVID-19 [82]. In addition, in the studies conducted in SARS-CoV and the Middle East Respiratory syndrome coronavirus, viral proteins particularly the spike, and nucleocapsid proteins were found to cause excessive NF-κB activation which was correlated with the high disease severity and fatality rate [82,83]. Previous reports pointed out that *A. argyi* suppresses NF-κB/iNOS/TNFα cascades in pulmonary inflammation in vivo and in LPS-stimulated macrophages [84]. The present results show reduction in the inflammatory mediators upon treatment with JSHT in a dose dependent manner. *Ophiopogon japonicas* was showed to have antithrombotic effects through protecting endothelial cells and reducing leukocyte–endothelial cell adhesion [39]. Therefore, the constituent of JSHT may provide the potential therapeutic benefits of JSHT with antiviral, anti-inflammation and anti-thrombosis effects.

The role of NLRP3 inflammasome and HMGB-1 has been generally associated with inflammation disorders [85], and recent reports have also observed their activation in response to COVID-19 infection. Notably, the formation of NLRP3 inflammasome and HMGB-1 expression are also correlated to the severity of COVID-19 patients [86,87]. Besides, recent observations highly suggests that NLRP3 inflammasome activation, and prolonged existence of SARS-CoV-2 RNA in some patients after recovery, may heighten the susceptibility for long COVID and cause pulmonary, neurological and cardiovascular manifestations [88]. This study provides evidence to support that JSHT not only displays the anti-inflammatory effects in LPS-induced inflammation, but also significantly inhibited the entry of COVID-19 into the host by reducing the expression of TMPRSS2 in acute lung injury. In addition to the respiratory infection, evidences show that SARS-CoV-2 can infect central nervous system [89–91]. Reports on 2D static and 3D microfluidic in vitro models suggests that SARS-CoV-2 S protein may alter BBB function and reduce their integrity [92]. SARS-CoV-2 was been recently found alter to infect and cross through the brain microvascular endothelial cells in in the infected K18-hACE2 transgenic mice [22]. In addition to the long term inflammation caused by the infection, SARS-CoV-2 associated brain damages are often independent of their respiratory symptoms [93] and present a long term effect on patients with conditions such as anxiety and depression [94,95]. The present data show that JSHT can reduce LPS-enhanced BBB permeability in the endothelial cells and therefore could be a potential therapeutic choice for SARS-CoV-2 associated neurological effects. The results also show that JSHT treatment in UCMS mice models ameliorated anhedonia and behavioral despair.

In conclusion, our current finding elucidated that JSHT significantly alleviated LPS-induced NF-κB activities and repressed the expression of pro-inflammatory factors such as TNFα, COX-2, iNOS, NLRP3 inflammasome and HMBG-1 in vitro. Besides, JSHT could obviously reduce the COVID-19 related inflammatory cytokine storm and alleviate lung immunopathology in the acute lung injury animal model. Our results support that JSHT has an anti-inflammatory effect through NF-κB cascades signaling and thus contributes to reducing systemic inflammation and cytokine storm in vitro and in vivo. By reducing the inflammation and improving the endothelia integrity JSHT potentially reduce the brain damage associated with SARS-CoV-2 S protein and may provide relief to patients with long term COVID from depression.

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CRediT authorship contribution statement

C-Y C, M.A.S, wrote the manuscript draft. Y-J.L, W-T.W, H-D.L, C-Y. L, C-Y.S, P-Y.L, T-J.H and C-Y.H corrected the draft. W-J.C, C-Y.L, S-Z.L supervised the experiments. C-Y.C and W-T.W, H-D.L performed the experiments. All data were generated in-house, and no paper mill was used. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

The authors declare that there is no conflict of interest exists.

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