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Protective effects of methane-rich saline on mice with allergic asthma by inhibiting inflammatory response, oxidative stress and apoptosis*

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Abstract: Background: Asthma is a common cause of breathing difficulty in children and adults, and is characterized by chronic airway inflammation that is poorly controlled by available treatments. This results in severe disability and applies a huge burden to the public health system. Methane has been demonstrated to function as a therapeutic agent in many diseases. The aim of the present study was to explore the effect of methane-rich saline (MRS) on the pathophysiology of a mouse model of asthma and its underlying mechanism. Methods: A murine model of ovalbumin (OVA)-induced allergic asthma was applied in this study. Mice were divided into three groups: a control group, an OVA group, and OVA-induced asthmatic mice treated with MRS as the third group. Lung resistance index (RI) and dynamic compliance (Cdyn) were measured to determine airway hyper-responsiveness (AHR). Haematoxylin and eosin (H&E) staining was performed and scored to show histopathological changes. Cell counts of bronchoalveolar lavage fluid (BALF) were recorded. Cytokines interleukin (IL)-4, IL-5, IL-13, tumor necrosis factor α (TNF-α), and C-X-C motif chemokine ligand 15 (CXCL15) from BALF and serum were measured by enzyme-linked immunosorbent assay (ELISA). The oxidative stress indexes, including malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), myeloperoxidase (MPO), and 8-hydroxydeoxyguanosine (8-OHdG), were determined using commercial kits. Apoptosis was evaluated by western blot, quantitative real-time polymerase chain reaction (qRT-PCR), and biochemical examination. Results: MRS administration reversed the OVA-induced AHR, attenuated the pathological inflammatory infiltration, and decreased the cytokines IL-4, IL-5, IL-13, TNF-α, and CXCL15 in serum and BALF. Moreover, following MRS administration, the oxidative stress was alleviated as indicated by decreased MDA, MPO, and 8-OHdG, and elevated SOD and GSH. In addition, MRS exhibited an anti-apoptotic effect in this model, protecting epithelial cells from damage. Conclusions: Methane improves pulmonary function and decreases infiltrative inflammatory cells in the allergic asthmatic mouse model. This may be associated with its anti-inflammatory, antioxidative, and anti-apoptotic properties.

Key words: Asthma; Methane-rich saline; Antioxidation; Anti-inflammation; Anti-apoptotic
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1 Introduction

Asthma is a chronic respiratory disease, characterized by airway hyper-responsiveness (AHR), inflammatory infiltration, and mucus over-secretion (Nakagome and Nagata, 2011). Its increasing prevalence results in a heavy burden on public health. Asthma is closely related to over-production of various

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inflammatory mediators, cytokines, and adhesion molecules (Pawankar, 2002; Poivre and Duez, 2017). Interleukin (IL)-4, IL-5, and IL-13, cytokines secreted from T helper type 2 (Th2) cells, along with eosinophilia in allergic airway may ultimately contribute to AHR in allergic asthma (Lloyd and Hessel, 2010; Zhao and Wang, 2018). Moreover, oxidative stress has been reported to contribute to airway inflammation, pulmonary function reduction, excessive mucus secretion, tissue injury, and airway remodeling in animal and human studies (Andreadis et al., 2003; Al-Afaleg et al., 2011). Additionally, epithelial cell apoptosis is one of the main consequences of oxidative stress. An elevated rate of epithelial apoptosis in the airway was determined in asthma, and was correlated with increased disease severity (Zhou et al., 2011). This indicated that excessive epithelial apoptosis may be involved in the pathogenesis of asthma.

Methane, a by-product of carbon dioxide and hydrogen by methanogens in the human colon, is conventionally considered biologically inert. However, the application of methane as a therapeutic gas has drawn much attention recently. Ye et al. (2015) reported that methane protected against hepatic ischemia/reperfusion (I/R) injury by reducing reactive oxygen species (ROS) production and inflammation. Most recently, Sun et al. (2017) found that intraperitoneal injection of methane-rich saline (MRS) exerted a protective effect against LPS-induced acute lung jury in mice. Our previous studies also demonstrated that methane attenuated myocardial ischemia injury through its anti-inflammatory, antioxidative, and anti-apoptotic properties (Chen et al., 2016). Although many studies have been performed on the medical use of methane, the therapeutic effect of methane on asthma has not been reported.

In this study, we applied an established allergic asthmatic mouse model employing immunological challenge with ovalbumin (OVA) to investigate whether methane can exhibit a protective effect against asthma and to further explore its underlying mechanism.

2 Materials and methods

2.1 Animals

Female BALB/c mice (seven weeks old) were purchased from the Experimental Animal Center of Naval Medical University, Shanghai, China, and acclimated for one week before experiment. Thirty-six mice were randomly divided into three groups: a control group (Ctrl), an OVA-challenged group (OVA), and OVA-challenged mice treated with MRS as the third group (OVA+MRS).

2.2 Establishment of asthmatic mouse model

A schematic diagram of the challenge and administration schedule is presented in Fig. 1. Mice from the OVA and OVA+MRS groups were sensitized and challenged with OVA according to the previous studies (Yang et al., 2011; Zemmouri et al., 2017). Briefly, mice were actively sensitized by intraperitoneal injection of OVA (1 mg/mL) with alum (1 mg/mL in saline) as an adjuvant on Days 0, 7, and 14. On Days 21, 22, and 23, mice were challenged for 20 min with an aerosol of saline alone (Ctrl group) or 0.01 g/mL OVA in phosphate-buffered saline (PBS) (OVA and OVA+MRS groups) via an ultrasonic nebulization (DeVibiss, USA) coupled to a plastic box.

Fig. 1 Experimental protocol for the induction of allergic asthma and administration of MRS

Mice were divided into three groups (n=12 per group) and sensitized using a 1 mg/mL OVA solution on Days 0, 7, and 14. The mice from the OVA and OVA+MRS groups were exposed and challenged with a 0.01 g/mL OVA solution on Days 21 to 23. Intraperitoneal injection of MRS or PBS was performed on Days 21 to 23. Control mice were sensitized and challenged with the equivalent of PBS. AHR estimation and sampling were performed 24 h after the last challenge. OVA: ovalbumin; MRS: methane-rich saline; PBS: phosphate-buffered saline; AHR: airway hyper-responsiveness

2.3 Methane-rich saline administration

MRS was produced and administered according to published methods (Ye et al., 2015). Briefly, pure methane (>99.9%) was dissolved in saline for 3 h under 0.4 MPa to a super-saturated solution. The supersaturated MRS solution was prepared the day before administration and stored under atmospheric pressure at 4 °C to optimize a high concentration of methane before administration (i.e. to minimize methane losses by evaporation). Gas chromatography (Gas Chromatography-9860, Qiyang, Shanghai, China)

was applied to measure the concentration of methane in the saline. Mice in the OVA+MRS group were injected intraperitoneally with 1 mL of MRS at Days 21, 22, and 23 after airway challenge. Meanwhile, mice in the OVA group received 1 mL PBS intraperitoneally.

2.4 Measurement of airway hyper-responsiveness

At Day 24, mice were anesthetized and intubated with an 18-gauge tube into the trachea and ventilated at the rate of 120 breaths per minute. Then mice were put into a chamber connected with four channels of biological signal system (Model SUMUP-PC, Fudan University, Shanghai, China). A Fleisch air flow transducer was used to detect the pulmonary function of the mice though the cannula. Lung resistance index (RI) and dynamic compliance (Cdyn) of the respiratory system in response to increasing doses of aerosol-inhaled methacholine (Mch; Sigma-Aldrich, Missouri, USA) were recorded and calculated as described (Bao et al., 2007).

2.5 Bronchoalveolar lavage fluid collection and cell counts

Bronchoalveolar lavage fluid (BALF) was collected from anesthetized mice through the tracheal cannula. A total of 500 μL of sterile and cold PBS was instilled into the lungs and retrieved three times and placed on ice. Afterwards, the BALF samples were centrifuged at 3000 r/min for 10 min at 4 °C. Supernatants were stored at −80 °C for further assessment. BALF cell pellets were resuspended in 100 μL PBS and the proportions of inflammatory cells (neutrophils, eosinophils, macrophages, and lymphocytes) were determined by morphology after Wright-Giemsa staining. At least 200 cells were counted on each slide.

2.6 Blood sample collection and measurement of cytokines

A 20-gauge needle was used to perform cardiac puncture for blood collection. After collection, the blood samples were placed on ice. Serum was separated by centrifugation (4 °C, 3000 r/min, 10 min). Enzyme-linked immunosorbent assay (ELISA) was applied to measure the levels of cytokines, IL-4, IL-5, IL-13, tumor necrosis factor α (TNF-α), and C-X-C motif chemokine ligand 15 (CXCL15), in serum and BALF supernatants according to the manufacturer's instructions (R&D System, Minneapolis, MN, USA).

2.7 Histological analysis

After mice were euthanized, the lung tissue was isolated from mice and fixed in 4% formaldehyde for 24 h. Tissues were embedded in paraffin, and 5 μm-thick paraffin sections were stained with hematoxylin and eosin (H&E) for histological assessment. The degrees of inflammation were evaluated by two blinded investigators according to a scale as follows: (0) normal, (1) mild, (2) moderate, (3) severe, and (4) very severe inflammation.

2.8 Oxidative stress index assessment

A total of 100 μg of fresh lung tissue was collected from mice and immediately homogenized with 1 mL of PBS. Tissue debris was removed by centrifugation at 3000 r/min for 10 min at 4 °C and the supernatant was used for assessment. The concentration of protein in the supernatant was assessed by bicinchoninic acid (BCA) assay. The levels of malondialdehyde (MDA), myeloperoxidase (MPO), superoxide dismutase (SOD), glutathione (GSH), catalase (CAT), and 8-hydroxydeoxyguanosine (8-OHdG) were measured via commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9 Detection of caspase-3 and caspase-9 activity

The lung tissues obtained were used to determine caspase-3 and caspase-9 activity using the colorimetric assay kits (Beyotime Institute of Biotechnology, Jiangsu, China). The absorbance at 405 nm was determined using a spectrophotometer (ELx800, BioTek Instruments, Winooske, VT, USA).

2.10 Western blot

The expression of apoptosis-related proteins (Bax, Bcl-2, cytochrome *c*, caspase-3, and caspase-9) in the isolated right lung tissue was measured via western blotting with antibodies (Abcam, Cambridge, MA, USA). The total protein content in each sample was determined. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins, which were then transferred to membranes (Bio-Rad, CA, USA). Blots were blocked in blocking solution for 1 h and then incubated with primary antibodies (1:1000, v/v) for 2 h. Afterwards, membranes were washed and incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies (1:1000,

v/v) for 50 min at room temperature. The expression of protein was normalized to β-actin.

2.11 RNA preparation and qRT-PCR

Total RNA was extracted from lung tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and processed for quantitative real-time polymerase chain reaction (qRT-PCR) according to the manufacturer's instructions. Expression of each sample was normalized to its β-actin messenger RNA (mRNA) content. The primers used were as follows: Bcl-2, 5'-CCT GTG GAT GAC TGA GTA CCT-3' (forward) and 5'-GAG CAG GGT CTT CAG AGA CA-3' (reverse); Bax, 5'-CTG AGC TGA CCT TGG AGC-3' (forward) and 5'-GAC TCC AGC CAC AAA GAT G-3' (reverse); β-actin, 5'-CAC TAT CGG CAA TGA GCG GTT CC-3' (forward) and 5'-CAG CAC TGT GTT GGC ATA GAG GT-3' (reverse).

2.12 Statistical analysis

Data are presented as mean±standard error of the mean (SEM). The statistical differences among groups were calculated using SPSS software (SPSS 21.0, Chicago, USA). For comparisons of quantitative data, one-way analysis of variance (ANOVA) followed by the Newman-Keuls post hoc test was used to determine significant differences (P<0.05).

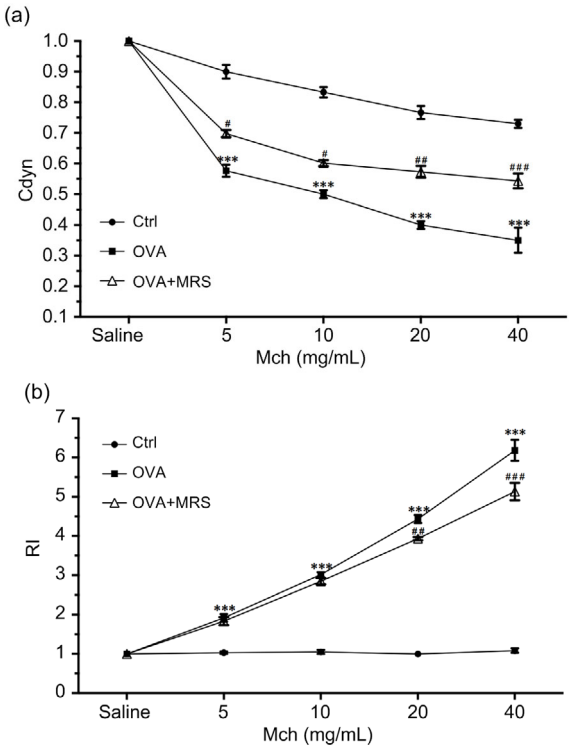

Fig. 2 Effects of MRS on OVA-challenged AHR in mice
The reduction of Cdyn (a) and the elevation of lung RI (b) were measured by a Mch dose-responsive curve for AHR. All data are represented as mean±SEM (n=6 in each group). $^{***}P$<0.001, vs. the control group; $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, vs. the OVA group. Cdyn: dynamic compliance; RI: resistance index; Mch: methacholine; AHR: airway hyper-responsiveness; MRS: methane-rich saline; OVA: ovalbumin; Ctrl: control

3 Results

3.1 Effect of methane-rich saline on the airway hyper-responsiveness in asthmatic mice

Lung RI and Cdyn were assessed to determine the AHR. Mch was applied via the airway to challenge the ventilated mice airway function.

Compared to the control group, RI increased and Cdyn decreased in the OVA-induced mice with increasing dose of Mch administered, confirming the establishment of AHR (Fig. 2). However, administration of MRS significantly ameliorated the changes in both RI and Cdyn compared with OVA-induced mice (Fig. 2).

3.2 Effect of methane-rich saline on inflammatory cell infiltration in asthmatic mice

Histological changes of the lung tissues collected from the different groups were assessed through H&E staining. As shown in Fig. 3a, histopathological features of asthma were observed in mice from the OVA group, characterized by heavy peri-bronchial and perivascular inflammatory cell infiltration and airway wall thickening. MRS significantly suppressed the accumulation of infiltrated cells and mucus overproduction (Figs. 3a and 3b).

We analyzed the number of inflammatory cells in BALF (Fig. 3c). There was a notable increase in the numbers of total cells, neutrophils, eosinophils, lymphocytes, and macrophages in BALF collected from OVA-challenged mice compared with the control. The administration of MRS resulted in a reduction in the numbers of total cells, neutrophils, eosinophils, and macrophages but no significant difference in the number of lymphocytes of MRS-treated mice compared with the asthma group.

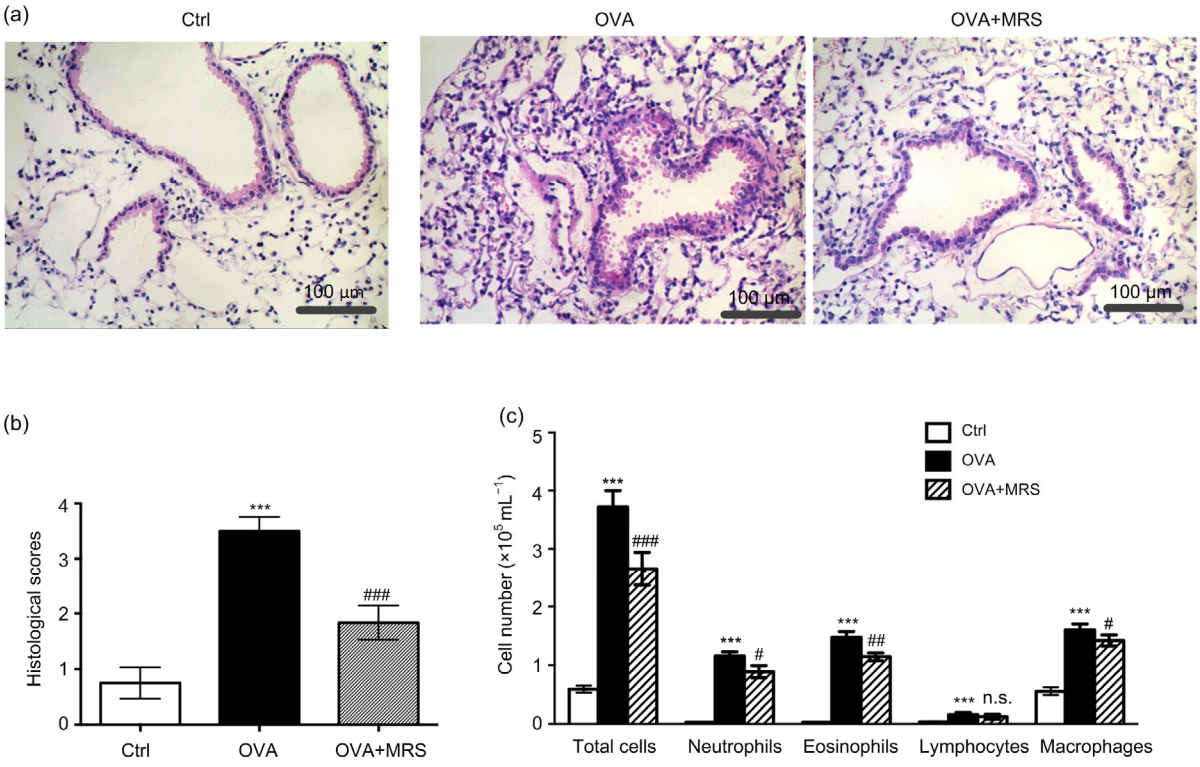

Fig. 3 Effects of MRS on airway inflammation and cell counts in BALF in a mouse model of OVA-induced asthma
(a) Representative haematoxylin and eosin (H&E) staining from each group; (b) Histological scoring was performed to give a quantitative measure; (c) The numbers of total cells, neutrophils, eosinophils, lymphocytes, and macrophages in BALF obtained from each group. All data are expressed as mean±SEM (n=6 in each group). $^{***}P<0.001$, vs. the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, vs. the OVA group. BALF: bronchoalveolar lavage fluid; Ctrl: control; OVA: ovalbumin; MRS: methane-rich saline; n.s.: not significant

3.3 Effect of methane-rich saline on inflammatory cytokines in asthmatic mice

BALF and serum samples were obtained from mice at 24 h after the OVA challenge to determine the effects of MRS on inflammatory cytokines.

As shown in Fig. 4a, mice from the OVA group exhibited strongly increased levels of IL-4, IL-5, IL-13, TNF-α, and CXCL15 in BALF compared with the control group. The administration of MRS resulted in significantly decreased levels of IL-4, IL-5, IL-13, TNF-α, and CXCL15.

Additionally, there was a notable increase in IL-4, IL-5, IL-13, TNF-α, and CXCL15 in serum obtained from the OVA group compared with the control. However, the increase of inflammatory cytokines was abolished through MRS treatment (Fig. 4b).

3.4 Effect of methane-rich saline on oxidative stress in lung tissues

Lung tissue was collected for assessment of oxidative stress products, including MDA, SOD, GSH, CAT, MPO, and 8-OHdG (Fig. 4c).

Mice from the OVA group produced elevated levels of MDA, MPO, and 8-OHdG in lung tissue. Levels of SOD, GSH, and CAT activity were lower than those of the control, further confirming the production of oxidative stress in this model. However, the concentrations of MDA, MPO, and 8-OHdG reduced significantly after treatment with MRS. Moreover, the GSH content and SOD activity were elevated in the OVA+MRS group compared with the OVA group. However, the level of CAT did not change significantly after MRS administration.

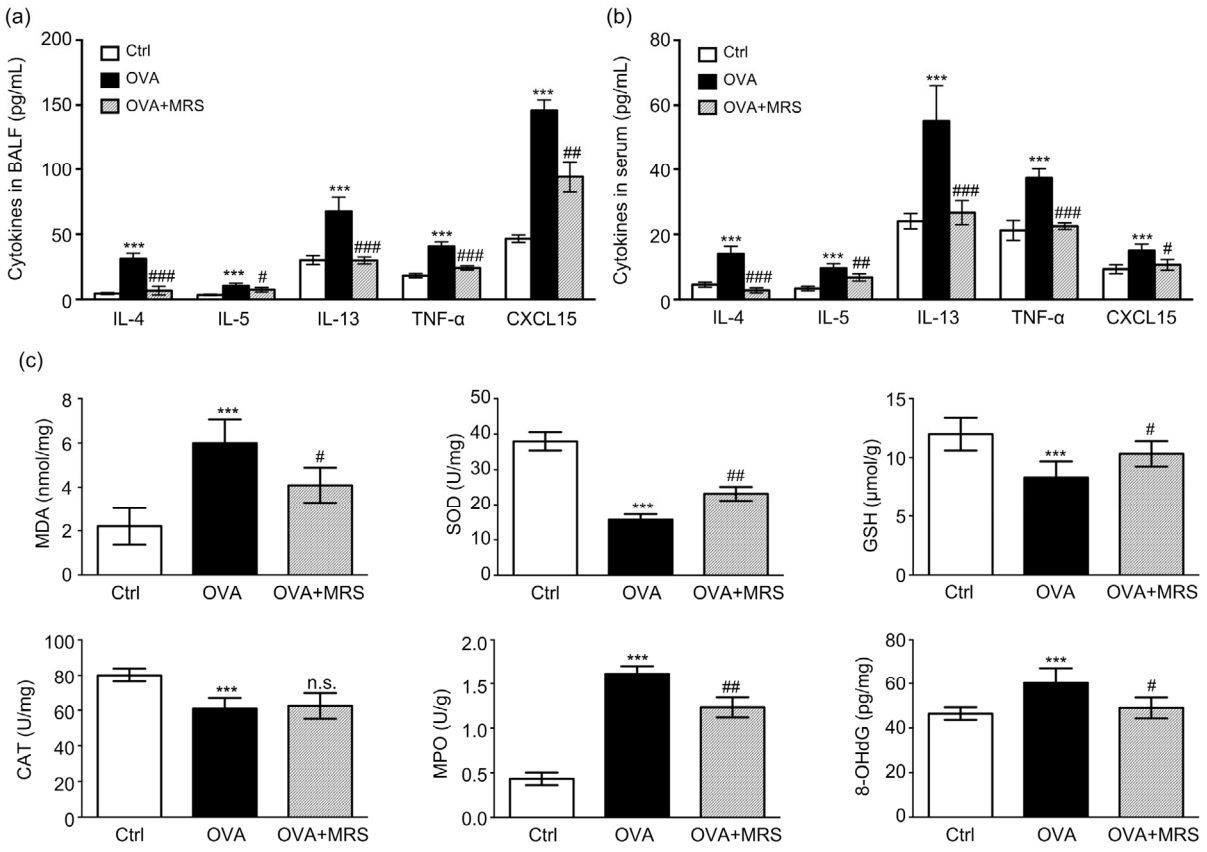

Fig. 4 Effects of MRS on inflammatory cytokines and oxidative stress indexes in asthmatic mice

The concentrations of cytokines obtained from BALF (a) and serum (b) were determined by ELISA. (c) The levels or activities of MDA, SOD, GSH, CAT, MPO, and 8-OHdG were determined via biochemical examination. All data are represented as mean±SEM (n=6 for each group). *** P<0.001, vs. the control group; $^{\#}$ P<0.05, $^{\#\#}$ P<0.01, $^{\#\#\#}$ P<0.001, vs. the OVA group. Ctrl: control; OVA: ovalbumin; MRS: methane-rich saline; n.s.: not significant; BALF: bronchoalveolar lavage fluid; MDA: malondialdehyde; SOD: superoxide dismutase; GSH: glutathione; CAT: catalase; MPO: myeloperoxidase; 8-OHdG: 8-hydroxydeoxyguanosine

3.5 Anti-apoptotic effects of methane-rich saline in asthmatic mice

Western blot assays revealed increased expression of Bax, caspase-3, and caspase-9, and a reduced Bcl-2 in the OVA group compared with the control, but the MRS treatment ameliorated these changes (Figs. 5a–5e).

To further confirm the anti-apoptotic effects of MRS, qRT-PCR and biochemical examinations were performed, and the data are illustrated in Figs. 5f–5i. Compared with the control, increased mRNA expression of *Bax* and a reduced expression of *Bcl-2* were observed in the OVA-induced mice, whereas the levels of caspase-3 and caspase-9 activity were amplified. However, following MRS treatment, reduced mRNA expression of *Bax* and increased expression of *Bcl-2* were detected, and the levels of caspase-3 and caspase-9 activity were reduced markedly.

4 Discussion

Methane, as the simplest aliphatic hydrocarbon, can be produced by bacteria within the human body. The biological synthesis occurs in the colon, where methanogens can convert CO_2 and H_2 into methane (Pimentel et al., 2006). Previous studies mainly focus on the involvement between intestinal diseases and methane production including irritable bowel syndrome, colonitis, and cancer (Roccarina et al., 2010; Sahakian et al., 2010). It was considered that methane was an inert or biologically inactive gas. However,

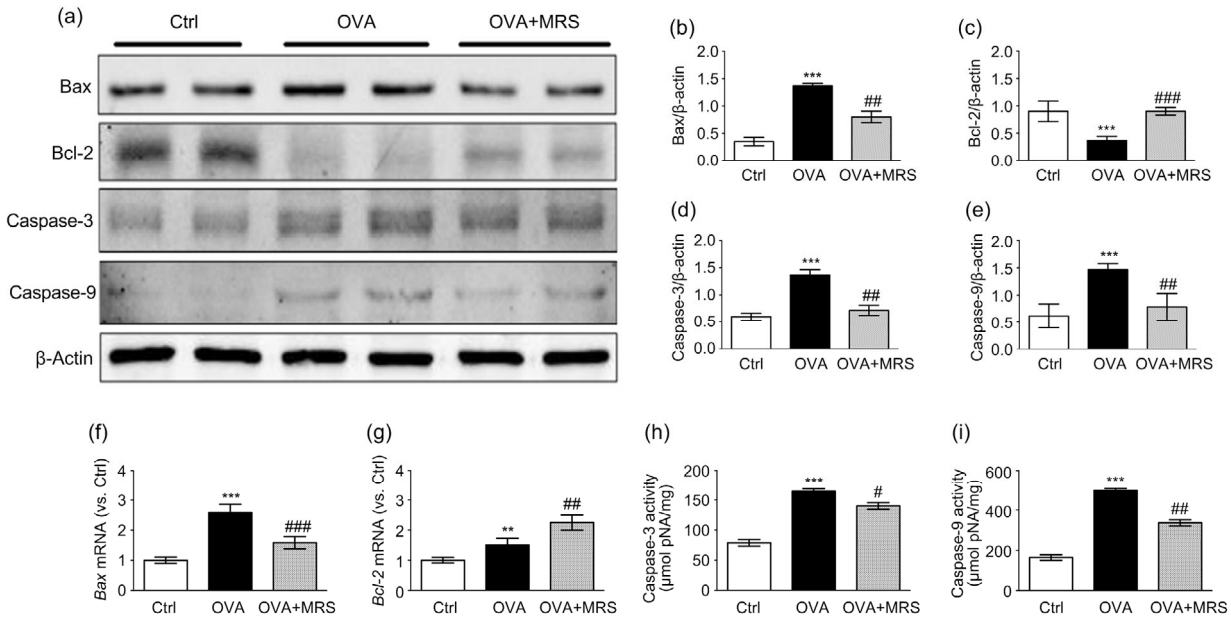

Fig. 5 Effects of MRS on apoptosis in asthmatic mice

The protein expression of Bax, Bcl-2, caspase-3, and caspase-9 (a–e) obtained from different groups was detected using western blot assay. The mRNA expression of *Bax* (f) and *Bcl-2* (g) was assessed using qRT-PCR. The levels of caspase-3 (h) and caspase-9 (i) activity were detected using commercial kits according to the manufacturer's instruction. All data are expressed as mean±SEM (n=6 in each group). $^{**}P<0.01$, $^{***}P<0.001$, vs. the control group; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, vs. the OVA group. Ctrl: control; OVA: ovalbumin; MRS: methane-rich saline; pNA: *p*-nitroaniline

considerable evidence has accumulated indicating the potential protective effects of methane in many diseases, such as spinal cord ischemia-reperfusion injury, concanavalin A (Con A)-induced hepatic injury, and myocardial ischemia injury (Chen et al., 2016; Wang et al., 2017; Xie et al., 2017). In the present study, we investigated the protective effects of methane in an asthmatic mouse model.

Despite great advances in pharmacology and technology, increasing asthma prevalence remains a common clinical challenge. It was found in our present study that intraperitoneal injection of MRS ameliorated pathological features of an OVA-induced asthmatic mouse model, as evidenced by decreased AHR and infiltrate inflammatory cells in lung tissue. The anti-inflammatory, antioxidant and anti-apoptotic effects likely also contributed to the protective effects of MRS treatment.

Allergic asthma is one of the most common respiratory disorders, characterized by AHR and caused by infiltrative inflammatory cells and mucus hypersecretion (Yuda et al., 2004). Our results indicated that MRS administration could ameliorate AHR in the OVA-challenged mice. Many studies have also reported a correlation between asthma and eosinophilia in lung tissue, as well as a correlation with the level of eosinophils in the BALF (Barrett and Austen, 2009). Thus, histopathologically less accumulation of infiltrative inflammatory cells, as well as a decreased number of eosinophil cell counts in BALF, also confirmed the protective effects of MRS in this allergic asthma mouse model.

Early studies documented that a Th2 type-dominated cytokine profile exhibits a profound role in allergic asthma (Yuda et al., 2004; Barret and Austen, 2009). Modulation of Th2 cytokines, IL-4, IL-5, and IL-13, is closely related to the pathophysiological process of asthma. These cytokines initiate the progression of inflammatory cascades, which consequently result in inflammatory cell recruitment, mucus hypersecretion, and AHR (León, 2017). It is reasonable to assume that inhibition of inflammation might contribute to the amelioration of asthma. In our study, we found that the intraperitoneal injection of MRS could decrease the levels of Th2-oriented cytokines IL-4, IL-5, and IL-13 in BALF and serum. Reduction of these cytokines

could be partially explained by the attenuated lung RI and less accumulation of inflammatory cells. Additionally, TNF-α has been demonstrated to be associated with asthmatic pathogenesis, including AHR, inflammatory cells recruitment and airway remodeling (Whitehead et al., 2017). CXCL15, a small cytokine belonging to the CXC chemokine family, recruits neutrophils during inflammation of lungs (Nakagome and Nagata, 2011). Administration of MRS also decreased the expression of TNF-α and CXC15 in asthmatic mice, suggesting that MRS could exert anti-inflammatory effects.

Another announced pathological factor associated with the progression of allergic asthma is oxidative stress (Andrianjafimasy et al., 2017). When ROS are produced in excess of the antioxidant defense capability, oxidative stress and cellular injury occur. Excessive production of ROS and their by-products directly damages the epithelial cells, and leads to the activation of apoptosis. Moreover, accumulation of ROS around alveolar epithelial cells and pulmonary vascular endothelial cells damages the blood–air barrier integrity, resulting in increased permeability and pulmonary edema (Andreadis et al., 2003). MDA is a key indicator of oxidative balance and reflects the amount of ROS directly (Gwarzo and Muhammad, 2010). MPO, a heme-containing protein secreted during neutrophil activation, is thought to stimulate MDA production and also participate in neutrophilic airway inflammation in asthma (Seys et al., 2013). CAT, SOD, and GSH, three key antioxidant enzymes, are decreased in patients with allergic asthma (Mak et al., 2004; Al-Afaleg et al., 2011). Significantly, we recorded increased levels of MDA and MPO, and decreased levels of CAT, SOD, and GSH in the lung tissue of OVA-challenged mice compared with the control mice, confirming this animal model as a relevant tool for evaluating therapeutic interventions for allergic asthma. Following MRS administration, the imbalance was partially attenuated, with decreased levels of MDA and MPO and increased levels of SOD and GSH compared with the asthma mice. However, MRS had no effects on the levels of CAT activity, suggesting that MRS protected against oxidative stress but with some limitations.

Accumulation of ROS not only damages the structure of cells but also activates the mitochondrial apoptotic pathway (Tesfaigzi, 2006; Baskaran et al., 2018). Elevated pro-apoptotic protein Bax and decreased anti-apoptotic protein Bcl-2 trigger cascades of apoptosis (James and Martinou, 2008). Previous studies have reported excessive apoptosis of airway epithelial cells is in asthma (Tesfaigzi, 2006; Hackett and Knight, 2007). This is readily demonstrable in both human and animal models of asthma (Cohen et al., 2007; Li and Shang, 2014). Therefore, inhibition of apoptosis can exhibit a profound beneficial effect on allergic asthma. Consistently, our results showed that the expression of Bax, pro-apoptotic protein, was remarkably increased, coinciding with an up-regulation of caspase-3 and caspase-9. The expression of Bcl-2, an anti-apoptotic protein which can inhibit Bax-induced events (Antonsson, 2004), was decreased in our asthma mouse model when compared with controls, thus implying activation of apoptosis. Following administration of MRS, the pro-apoptotic proteins Bax, caspase-3, and caspase-9 were down-regulated, whereas Bcl-2 was up-regulated. Therefore, MRS administration may exert anti-apoptotic effects.

However, the exact mechanism underlying how methane exhibits its antioxidative, anti-inflammatory, and anti-apoptotic properties has not been clarified. Several hypotheses have been proposed, including moderation of G-coupled receptors, such as acetylcholine-activated ion channel (Kai et al., 1998; Boros et al., 2012). Wang et al. (2017) reported that methane can activate the anti-apoptotic effects of Nrf2, along with its downstream antioxidants, to reduce spinal cord IR injury. This indicates that the Nrf2 pathway is involved during the methane treatment, although more pathways need to be detected.

Methane, a lipid-soluble gas, can rapidly penetrate membranes and diffuse into organelles. Although methane is non-toxic in vivo, the fact that it is flammable and explosive limits its use via administration of methane gas (Boros et al., 2012). Therefore, intraperitoneal injection of MRS is considered to be safe and precise. Of note, no study has been conducted to compare the therapeutic effects of inhalation and intraperitoneal injection. Further research on the mechanism of therapeutic effects of methane should be explored before it is widely applied to clinical practice.

Overall, our study demonstrated that intraperitoneal injection of MRS could ameliorate pathological

effects of an allergic asthmatic mouse model via its anti-inflammatory, antioxidative, and anti-apoptotic effects, and may prove to be a promising method for treatment of asthma.

Contributors

Ning ZHANG conducted the study and wrote the manuscript. Xue-jun SUN designed the project and supervised the process. Hong-tao LU participated in histological and western blot procedures. Rong-jia ZHANG gathered all data and performed statistical analysis. All authors have approved the final manuscript. Therefore, all authors have full access to all the data in the study and take responsibility for the integrity and security of the data.

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Compliance with ethics guidelines

Ning ZHANG, Hong-tao LU, Rong-jia ZHANG, and Xue-jun SUN declare that they have no conflict of interest.

All procedures including mice were approved by the Ethical Committee for Animal Studies of Naval Medical University, Shanghai, China. All institutional and national guidelines for the care and use of laboratory animals were followed.

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中文概要

题　目：甲烷生理盐水通过抗氧化、抗炎症和抗凋亡发挥对哮喘小鼠的保护作用

目　的：研究通过腹腔注射甲烷生理盐水对哮喘动物模型的保护作用及其可能的机制。

创新点：通常我们认为甲烷生理盐水对人体并不发挥生理性的影响，但近年来涌现出的研究发现甲烷生理盐水可以发挥对多种脏器缺血再灌注损伤的保护作用。我们采用卵清蛋白刺激的小鼠哮喘模型，发现腹腔注射甲烷生理盐水的方式可以发挥对哮喘小鼠的保护作用，减轻哮喘小鼠氧化应激指标，缓解炎症和凋亡水平。

方　法：通过卵清蛋白刺激诱导小鼠气道高反应性的方式建立小鼠哮喘模型，治疗组小鼠给予甲烷生理盐水腹腔注射。通过测量小鼠气道阻力指数（RI）和动态肺顺应性（Cdyn）来检测小鼠气道高反应性；通过苏木精-伊红染色法（H&E）检测小鼠肺组织形态学；对小鼠肺泡灌洗液进行细胞测量；通过酶联免疫吸附试验（ELISA）测定灌洗液和采集的血清中白介素 4（IL-4）、IL-5、IL-13 和肿瘤坏死因子（TNF-α）；通过生物化学的方式检测氧化应激指标（如丙二醛（MDA）、超氧歧化酶（SOD）、过氧化氢酶（CAT）、谷胱甘肽还原酶（GSH）、髓过氧化物酶（MPO）和 8 羟基脱氧鸟苷（8-OHdG））；通过蛋白免疫印迹实验、实时定量聚合酶链式反应（qRT-PCR）和生化检测试剂盒检测凋亡相关蛋白。

结　论：甲烷生理盐水可以改善哮喘小鼠的气道功能，减少肺组织中浸润的炎性细胞。其保护作用有可能是通过甲烷抗氧化、抗炎和抗凋亡的生物学特性发挥的。

关键词：哮喘；甲烷生理盐水；抗氧化应激；抗炎症；抗凋亡