

Xing Su San and Sang Xing Tang attenuate PM2.5-induced lung injury and inflammatory marker expression in rats

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Abstract: Background: Fine particulate matter (PM2.5) induces lung inflammation through pathways involving receptor for advanced glycation end-products (RAGE), S100A9 and nuclear factor-kappa B (NF-κB). Xing Su San and Sang Xing Tang are traditional Chinese medicine formulas used for respiratory disorders associated with cool-dry and warm-dry conditions, respectively. **Objectives:** To investigate whether treatment with these formulas is associated with reduced expression of RAGE, S100A9 and NF-κB in lung tissue of rats exposed to PM2.5 under simulated warm-dry and cool-dry conditions. **Methods:** Forty male Wistar rats were divided into five groups (n=8 each): blank control, warm-dry model, cool-dry model, Sang Xing Tang treatment and Xing Su San treatment. Exposed groups received PM2.5 (500 µg/m³, 3 hours/day, 28 days) under warm-dry (20°C, 33% humidity) or cool-dry (8°C, 33% humidity) conditions. Treatment groups received oral gavage of Sang Xing Tang (8.5 g/kg) or Xing Su San (12.5 g/kg) before exposure. RAGE messenger RNA was measured by reverse transcription quantitative polymerase chain reaction, NF-κB p65 protein by Western blot and S100A9 by immunohistochemistry. **Results:** Both PM2.5 exposure conditions were associated with significantly increased RAGE (2.8-fold and 2.5-fold), NF-κB p65 (2.4-fold and 2.2-fold) and S100A9-positive cells (3.45% and 3.13%) compared to controls (0.82%; all P < 0.05). Sang Xing Tang treatment was associated with reduced markers in the warm-dry model (RAGE: 1.6-fold; NF-κB: 1.5-fold; S100A9: 2.25%). Xing Su San treatment was associated with reduced markers in the cool-dry model (RAGE: 1.5-fold; NF-κB: 1.4-fold; S100A9: 2.08%; all P < 0.05). **Conclusion:** Xing Su San and Sang Xing Tang attenuate PM2.5-induced upregulation of RAGE, S100A9 and NF-κB in rat lung tissue in a pattern-specific manner. These hypothesis-generating findings provide preliminary evidence warranting further mechanistic validation.

Keywords: Xing Su San; Sang Xing Tang; PM2.5; Lung injury; RAGE; S100A9; NF-κB; Traditional Chinese Medicine

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INTRODUCTION

The main pathogenic component of smog is fine particulate matter PM2.5. Studies have shown that PM2.5 mainly causes disease by inducing oxidative stress and inflammatory responses in cells, altering disease-related gene polymorphisms and DNA methylation, regulating the expression of related genes and altering signaling pathways (Rundblad *et al.*, 2025). Haze weather often occurs in cold, dry autumn and winter and traditional Chinese medicine holds that smog is closely related to dryness.

Among the key molecular pathways implicated in PM2.5-induced lung injury is the receptor for advanced glycation end-products (RAGE) signaling axis. RAGE is a pattern recognition receptor that, upon binding to damage-associated molecular patterns such as S100A9, activates nuclear factor-kappa B (NF-κB), a master transcription factor regulating pro-inflammatory gene expression (Ji *et al.*, 2024; Salehi, Amiri, Ilghari, Hasham, & Piri, 2023). This RAGE/S100A9/NF-κB pathway represents a critical

mechanism linking environmental particulate exposure to pulmonary inflammation.

Despite evidence that this pathway is activated by particulate exposure, it remains unknown whether traditional Chinese medicine formulas historically used for dryness-related respiratory conditions affect these molecular markers. Specifically, no study has examined Xing Su San (traditionally indicated for cool-dry conditions) or Sang Xing Tang (traditionally indicated for warm-dry conditions) in the context of PM2.5 exposure.

In TCM terminology, "warm-dry" and "cool-dry" refer to patterns of respiratory illness associated with different environmental conditions warm-dry typically involves higher ambient temperatures, with symptoms such as a dry cough and a feverish sensation, while cool-dry involves lower temperatures, with chills and nasal congestion. For the purposes of this study, these concepts were operationalized as exposure chamber conditions: warm-dry (20°C, 33% RH) versus cool-dry (8°C, 33% RH).

The present study was designed to test whether

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administering these formulas is associated with altered expression of RAGE, S100A9 and NF- κ B in lung tissue of rats exposed to PM_{2.5} under these two environmental conditions. We hypothesized that (1) exposure to PM_{2.5} under both environmental conditions would be associated with upregulation of these markers; (2) treatment with Sang Xing Tang would be associated with reduced markers in the warm-dry model; (3) treatment with Xing Su San would be associated with reduced markers in the cool-dry model; and (4) pattern-specific effects would align with classical TCM indications.

MATERIALS AND METHODS

Experimental animals

40 SPF-rated healthy Wistar male rats, weighing 200~250g, were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (license number: SCXK (Liao) 2015-0001). The sample size (n=8 per group) was determined based on previous studies in the field and the 3Rs (Replacement, Reduction, Refinement) principles to minimize animal use while ensuring sufficient statistical power to detect meaningful differences between groups. All animals were housed at the Institute of Acupuncture and Moxibustion, Hubei University of Traditional Chinese Medicine (license number: SYXK (E) 2017-0067) under controlled environmental conditions: temperature $22 \pm 2^\circ\text{C}$, relative humidity $50 \pm 10\%$ and a 12-hour light/dark cycle. Rats were provided with standard laboratory chow and sterilized water ad libitum and were acclimatized for one week prior to the start of the experiment. Inclusion criteria: rats with body weight 200-250g and no signs of illness during acclimatization. Exclusion criteria: animals showing signs of respiratory distress, weight loss $>20\%$, or failure to complete the 28-day exposure protocol. No animals met exclusion criteria during the study.

Ethical statement

This study was conducted in accordance with the ethical standards of the institutional and national research committees and the ARRIVE guidelines for the reporting of animal experiments.

Instruments and reagents

The following instruments and reagents were used in this study Table 1:

Preparation of experimental drugs

Xing Su San and Sang Xing Tang were prepared according to their classical formulas as documented in traditional Chinese medicine texts. The crude herbal materials were purchased from the outpatient pharmacy of Hubei Provincial Hospital of Traditional Chinese Medicine and authenticated by a licensed pharmacist. Voucher specimens have been retained at the School of Basic Medical Sciences, Hubei University of Traditional Chinese Medicine.

Preparation method: For each formulation, one standard dose of the herbal mixture was immersed in 3 volumes (v/w) of distilled water for 1 hour at room temperature. The mixture was then brought to a boil and simmered gently until the volume was reduced to 100 mL. This decoction process was repeated three times and the resulting liquids from all three decoctions were combined, filtered through sterile gauze and concentrated in a constant-temperature water bath to a final volume of 100 mL. The final concentrations of the crude drug were 0.72 g/mL for Xing Su San and 0.255 g/mL for Sang Xing Tang. The prepared decoctions were stored at 4°C and used within one week.

Preparation of particulate matter (PM) exposure model

The exposure system was designed to simulate complex environmental conditions involving particulate matter. A mixture of natural materials (wood chips, straw and coal in a 1:1:1 ratio by volume) was combined with a small amount of gasoline (5% of total volume) as a combustion initiator. The mixture was ignited in a controlled combustion chamber and the resulting smoke was combined with water mist generated by a spray fan to create a particulate-laden atmosphere. This aerosol mixture was passed through a PM₁₀ pre-filter to remove larger particles before entering the exposure chamber.

PM concentration monitoring: A real-time PM_{2.5} monitor (DustTrak II, TSI Incorporated) was used to measure particulate matter concentration in the exposure chamber continuously. Based on historical air quality data from Wuhan during autumn haze events, PM_{2.5} concentrations were approximately $500 \pm 50 \mu\text{g}/\text{m}^3$ throughout the exposure periods.

All personnel involved in the PM generation and animal exposure procedures wore appropriate personal protective equipment, including N95 respirators, chemical-resistant gloves and laboratory coats. The exposure system was operated within a fume hood to prevent environmental contamination and ensure investigator safety.

Methods

Establishment and processing of animal models

Forty rats were equally and randomly divided into five groups: a blank control, a warm-dry model, a cold-dry model, a Sangxing Decoction treatment group and a Xingsu San treatment group (8 rats/group). Beginning on day one, four groups were exposed to a simulated haze environment in a climate-controlled chamber for 3 hours per day (17:00–20:00) over 4 weeks, while the control group was raised under routine conditions. To reflect recent autumn haze conditions in Wuhan, the chamber was set to a warm-dry climate (20°C , $33\% \pm 2\%$ RH) for the warm-dry model and Sangxing Decoction groups and a cold-dry climate ($\sim 8^\circ\text{C}$, $33\% \pm 2\%$ RH) for the cold-dry model and Xingsu San groups. Outside exposure periods, all rats received standard care.

Table 1: Instruments details.

Instrument/Reagent	Source	Catalog No.	Application	Dilution
Poison Chamber	Antowan (antuowan-8100)	-	PM exposure	-
Anti-S100A9 antibody	Wuhan Purity Bio	PB0123	Immunohistochemistry	1:200
Anti-NF- κ B p65 antibody	Wuhan Purity Bio	PB0456	Western blot	1:1000
Anti- β -actin antibody	Wuhan Miying	MY1024	Western blot (loading control)	1:5000
Sheep anti-rabbit HRP	Wuhan Bode	BD2105	Secondary antibody (WB/IHC)	1:5000 (WB), 1:200 (IHC)
Reverse first strand cDNA synthesis kit	Thermo Scientific	K1622	RT-qPCR	-
BCA protein detection kit	cwbiotech	CW0014	Protein quantification	-
Protein ladder	Biomed	BM161	Western blot molecular weight marker	-
Acrylamide	Amresco	0341	SDS-PAGE gel preparation	-
PVDF membrane	Millipore	IPVH00010	Protein transfer	-
TRIzol reagent	Invitrogen	15596026	RNA extraction	-
SYBR Green Master Mix	Applied Biosystems	4309155	qPCR amplification	-

All reagents were stored and used according to the manufacturers' instructions.

Route of administration

Post-exposure, rats received once-daily oral gavage (08:00) for four weeks. Using standard dose conversion methods, the Xingsu San group received 12.5 g/kg BW and the Sangxing Decoction group received 8.5 g/kg BW. Doses were calculated using standard body surface area conversion from human equivalent doses based on classical TCM texts (Xing Su San: 18 g/day for an adult; Sang Xing Tang: 12 g/day for an adult). The difference in administered doses (12.5 g/kg vs. 8.5 g/kg) reflects differences in composition and recommended daily dosages between the two classical formulas rather than biological equivalence. No prior dose-response studies were available to optimize or equalize these doses. This represents a significant limitation, as the lack of dose-optimization studies means the selected doses may not reflect equipotent or therapeutically optimal concentrations for comparing the two formulas. No prior dose-response studies were available to optimize or equalize these doses. All model groups and the blank control received an equivalent volume of normal saline.

Sample collection

Twenty-four hours after the final PM exposure and drug administration (day 29), all rats were euthanized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Lung tissues were rapidly excised and processed as follows:

For RT-qPCR: A portion of the left lung (approximately 50 mg) was immediately placed in RNase-free tubes, snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

For Western blot: A portion of the right lung (approximately 100 mg) was snap-frozen in liquid nitrogen and stored at -80°C for protein analysis.

For immunohistochemistry: The remaining lung tissue was fixed in 4% paraformaldehyde for 24 hours, then embedded in paraffin for sectioning.

RT-qPCR for RAGE mRNA expression

Total RNA was extracted from frozen lung tissue (approximately 30 mg) using TRIzol reagent according to the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific), with A260/A280 ratios of 1.8-2.0 considered acceptable. RNA integrity was verified by agarose gel electrophoresis. One microgram of total RNA was reverse transcribed to complementary DNA (cDNA) using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. The reaction conditions were: 25°C for 5 min, 42°C for 60 min and 70°C for 5 min. The resulting cDNA was stored at -20°C until analysis.

Quantitative PCR: RT-qPCR was performed using PowerUp SYBR Green Master Mix on a QuantStudio 3 Real-Time PCR System (Applied Biosystems). Each reaction (20 μ L total volume) contained 10 μ L SYBR Green Master Mix, 1 μ L cDNA template, 0.5 μ L each of forward and reverse primers (10 μ M) and 8 μ L nuclease-free water (Table 2).

Table 2: Primer sequences.

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Product Size (bp)
RAGE	GGACCCTTAGCTGGCACTTAG	GAGTCCCGTCTCAGGGTGTCT	142
β-actin	CCCATCTATGAGGGTTACGC	TTTAATGTCTACGCACGATTTC	150

Each sample was run in triplicate. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as fold change relative to the blank control group. Non-template controls were included in each run to verify the absence of contamination.

Western blot analysis for NF-κB p65 protein expression

Frozen lung tissue samples (approximately 50 mg) were homogenized in ice-cold RIPA lysis buffer (Beyotime Biotechnology) containing protease and phosphatase inhibitor cocktails (Roche). The homogenates were incubated on ice for 30 min and centrifuged at $12,000 \times g$ for 20 min at 4°C. The supernatant was collected and protein concentration was determined using the BCA Protein Assay Kit (cwbiotech) with bovine serum albumin as the standard.

Gel electrophoresis and transfer: Equal amounts of protein (30 µg per lane) were mixed with 5× loading buffer, heated at 95°C for 5 min and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 90 min. Proteins were then transferred electrophoretically to PVDF membranes (Millipore) at 300 mA for 90 min at 4°C. Transfer efficiency was verified by Ponceau S staining.

Immunodetection: Membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature to prevent non-specific binding. Membranes were then incubated overnight at 4°C with primary antibodies:

Rabbit anti-NF-κB p65 antibody (1:1000 dilution, Wuhan Purity Bio)

Mouse anti-β-actin antibody (1:5000 dilution, Wuhan Miying) as loading control

After washing three times with TBST (10 min each), membranes were incubated with horseradish peroxidase (HRP)-conjugated sheep anti-rabbit secondary antibody (1:5000 dilution, Wuhan Bode) for 1 hour at room temperature. After three additional washes with TBST, protein bands were visualized with an enhanced chemiluminescence (ECL) substrate (Thermo Scientific) and imaged with a ChemiDoc XRS+ system (Bio-Rad).

Immunohistochemistry for S100A9 protein expression

Lung tissue specimens fixed in 4% paraformaldehyde were embedded in paraffin and sectioned at 4 µm thickness using a microtome (Leica RM2235). Sections were mounted on poly-L-lysine-coated slides and dried overnight at 37°C.

Deparaffinization and rehydration: Sections were deparaffinized in xylene (three changes, 10 min each) and rehydrated through a graded ethanol series (100%, 95%,

80%, 70%; 5 min each) followed by washing in distilled water.

Antigen retrieval: Heat-induced epitope retrieval was performed by immersing slides in 10 mM sodium citrate buffer (pH 6.0) and heating in a microwave oven at 95°C for 15 min. Slides were allowed to cool to room temperature for 30 min, then washed with phosphate-buffered saline (PBS, pH 7.4) for 5 min (three times).

Immunostaining: Endogenous peroxidase activity was blocked by incubating sections with 3% hydrogen peroxide in methanol for 15 min at room temperature. After washing with PBS, sections were incubated with 5% normal goat serum for 30 min to block non-specific binding. Sections were then incubated overnight at 4°C with rabbit anti-S100A9 primary antibody (1:200 dilution, Wuhan Purity Bio) in a humidified chamber.

After three washes with PBS (5 min each), sections were incubated with HRP-conjugated sheep anti-rabbit secondary antibody (1:200 dilution, Wuhan Bode) for 1 hour at room temperature. Following additional washes, antibody binding was visualized using 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories) for 5-10 min, with brown color development monitored under a microscope. Sections were counterstained with hematoxylin for 30 sec, dehydrated through graded ethanol and xylene and mounted with neutral resin.

Statistical methods

The experimental data were statistically analyzed using SPSS 22.0 and after normalization and homogeneity, the one-way ANOVA was used to compare multiple groups and the LSD-t test was used to compare two groups. If the data do not meet the normality or homogeneity of variance assumptions, the test at level $\alpha=0.05$ and $P<0.05$ indicate that the difference is statistically significant.

RESULTS

Expression in lung tissue

RT-qPCR analysis revealed that RAGE mRNA expression in lung tissue was significantly elevated in both the warm-dry and cold-dry model groups compared to the blank control group ($P < 0.05$). This elevation was significantly reduced by treatment; expression was lower in the Sangxing Decoction group than in the warm-dry model and in the Xingsu San group than in the cold-dry model (both $P < 0.05$; see Fig. 1).

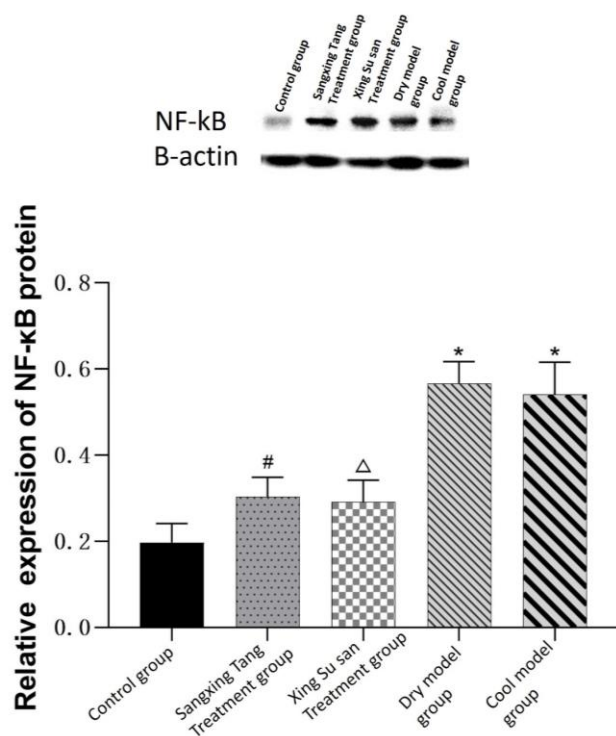


Fig. 1 Relative expression of RAGE mRNA (normalized to β -actin) in the lung tissues of rats in each group. Note: * Compared with the blank control group, $P < 0.05$; # Compared with the warm-dry model group, $P < 0.05$; Δ Compared with the cool-dry model group, $P < 0.05$.

Xingsusan treatment reduces NF- κ B expression in lung injury models

Western blot analysis showed that NF- κ B protein expression in lung tissue was significantly higher in both the warm-dry and cold-dry model groups than in the blank control group ($P < 0.05$). Treatment significantly reduced this expression; NF- κ B levels were lower in the Sangxing Decoction treatment group than in the warm-dry model and in the Xingsu San treatment group than in the cold-dry model ($P < 0.05$ for both; see Fig. 2).

Immunohistochemical analysis of S100A9 expression in lung tissue

Immunohistochemistry showed that S100A9 protein was expressed in the cytoplasm with a small amount of brownish-yellow color in the lung tissue of the blank control group. S100A9 protein was predominantly expressed in the cytoplasm and exuded from the lesion in the treatment group and the Sangxing Decoction and Xingsusan group. S100A9 in the lung tissue of the warm and dry model group and the cold model group was mostly strongly positive in the cytoplasm of the lesion and weakly positive in a small amount of cytoplasm, as shown in figure. 3.

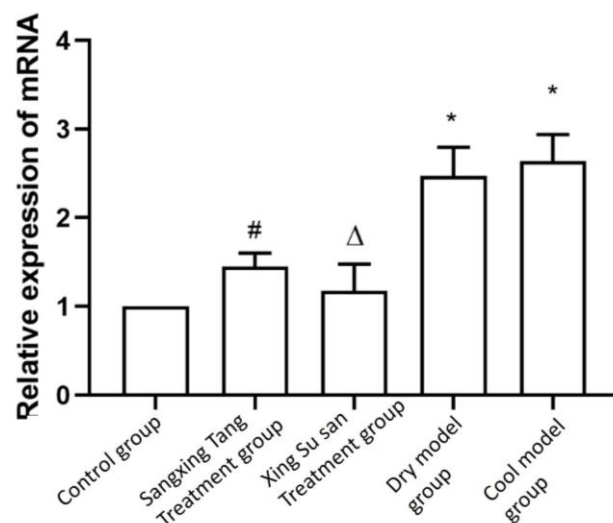


Fig. 2 Relative protein expression of NF- κ B P65 (NF- κ B/ β -actin) in the lung tissues of rats in each group. Note: * Compared with the blank control group, $P < 0.05$; # Compared with the warm and dry model group, $P < 0.05$; Δ Compared with the cool and dry model group, $P < 0.05$.

The positive expression rate of S100A9 in the lung tissue of the rats in the warm and dry model group and the cold dry model group was higher than that in the blank control group ($P < 0.05$) and the positive expression rate of S100A9 in the lung tissue of the rats in the treatment group was lower than that in the warm and dry model group ($P < 0.05$). The positive expression rate of S100A9 in the lung tissue of rats in the treatment group was lower than that in the cool and dry model group ($P < 0.05$). See table 3.

DISCUSSION

This study found that administration of Xing Su San and Sang Xing Tang was associated with reduced expression of RAGE, S100A9 and NF- κ B in lung tissue of PM2.5-exposed rats, with patterns of specificity corresponding to their traditional indications. However, it is critical to emphasize that these findings demonstrate associations, not causality. We did not perform pathway validation experiments such as RAGE blockade, S100A9 neutralization, or NF- κ B inhibition. Therefore, we cannot conclude that the formulas act directly on the RAGE/S100A9/NF- κ B pathway or that changes in these markers are causally related to lung protection. The term "pathway" is used descriptively throughout this discussion to frame our results within the known biological literature, not to imply that we have verified causal relationships.

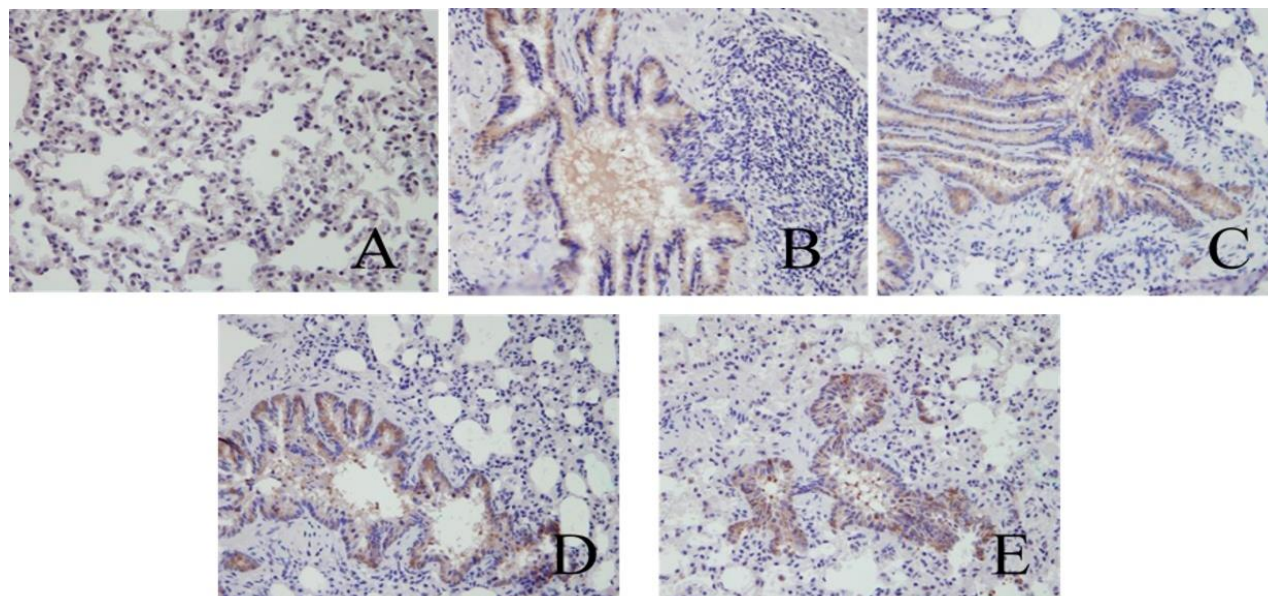


Fig. 3: Immunohistochemistry results of S100A9 protein expression in lung tissue of rats in each group (IHC, $\times 200$)
 Note: A blank model group, B Sangxing decoction treatment group, C Xingsu San treatment group, D warm and dry model group, E cold and dry model group

Table 3: Expression of S100A9 content in rat lung tissue ($\bar{x} \pm s$)

Group	Sample size (n)	S100A9 (%)
Blank control group	8	0.82 $\pm$ 0.16
Sangxing Decoction Treatment Group	8	2.25 $\pm$ 1.43 [#]
Apricot and Soothe Powder Treatment Group	8	2.08 $\pm$ 1.41 ^Δ
Warm and Dry Model Group	8	3.45 $\pm$ 0.44 [*]
Cool and Dry Model Group	8	3.13 $\pm$ 1.10 [*]

Note^{*} The ratio with the blank control group is $P < 0.05$, [#] is $P < 0.05$ with the warm and dry model group, and the ratio is $P < 0.05$ with the cool and dry model group

When positioned within the existing literature, our results suggest several points of consistency and one important methodological divergence from prior work. Our finding that PM_{2.5} exposure is associated with increased RAGE mRNA expression is consistent with Zou *et al.* (Chen *et al.*, 2026; Zhou *et al.*, 2025), who reported activation of HMGB1-RAGE signaling in human airway epithelial cells exposed to PM_{2.5}. Similarly, (Oliveira *et al.*, 2023; Zou *et al.*, 2018) (Lazarian *et al.*, 2020) demonstrated that S100A9 promotes fibroblast activation through RAGE-dependent pathways, supporting the biological plausibility of our observations. However, unlike the present study, those investigations included pathway validation experiments (e.g., RAGE knockdown) that permitted causal inferences (Marinkovic *et al.*, 2019; Raquil, Anceriz, Rouleau, & Tessier, 2008).

The observed associations between herbal formula administration and reduced inflammatory markers align with previous work on related formulations. (Chulrik *et al.*, 2026; Meng *et al.*, 2022; Sun *et al.*, 2022; Wang *et al.*, 2025) reported that Sang Xing Decoction modulated

protein and metabolic profiles in smoke-induced acute bronchitis. The present study extends these findings by examining specific components of the RAGE/S100A9/NF- κ B pathway and directly comparing two formulas under distinct environmental conditions.

The pattern-specific effects observed—Sang Xing Tang associated with reduced markers in warm-dry conditions and Xing Su San in cool-dry conditions—suggest that environmental temperature may modify the molecular response to PM_{2.5} or the pharmacological activity of these herbal formulations (Yang, Li, & Tang, 2020). From a biological perspective, temperature can influence inflammatory signaling through multiple mechanisms, including heat shock protein expression and membrane fluidity (Li *et al.*, 2021; Miura *et al.*, 2024; Raveendran, AlQattan, & AlMutairy, 2025; Xing, Xu, Shi, & Lian, 2016). Whether these mechanisms explain the differential effects observed here requires further study.

It is important to note what this study does not demonstrate. We did not perform RAGE blockade,

S100A9 neutralization, or NF- κ B inhibition experiments. Therefore, we cannot conclude that the formulas act directly on this pathway or that changes in these markers are causally related to lung protection. The findings demonstrate associations, not causality. The term 'pathway' is used descriptively to frame our results within the known biological literature, not to imply that we have verified causal relationships.

When positioned within the existing literature, our results suggest that these TCM formulas warrant further investigation using designs capable of establishing causality (Ding *et al.*, 2021; Drexler *et al.*, 2013; Guan *et al.*, 2025; Mitranovici *et al.*, 2025; Sejersen *et al.*, 2025; Xiang *et al.*, 2021). Future studies should include treatment-only control groups, dose-response analyses, chemical characterization of herbal decoctions and targeted pathway inhibition experiments. Additionally, female animals should be included to assess sex differences and functional outcomes such as pulmonary function tests should be measured.

Limitations of the study

Several important limitations of this study must be acknowledged. First, the PM2.5 exposure model utilized a complex mixture generated from combustion of natural materials combined with gasoline, without chemical characterization of the resulting particulate matter; while this approach may better simulate real-world environmental exposures than purified carbon particles, the lack of compositional analysis prevents identification of specific components responsible for the observed biological effects and limits reproducibility across studies. Second, the herbal decoctions were not subjected to quality control analysis such as high-performance liquid chromatography (HPLC) fingerprinting, making it impossible to verify batch-to-batch consistency, identify active compounds, or establish pharmacokinetic-pharmacodynamic relationships. Third, our experimental design lacked treatment-only control groups (rats receiving herbal formulas without PM2.5 exposure). This omission means we cannot distinguish between true therapeutic effects (reversal of PM2.5-induced changes) and nonspecific physiological effects of the formulas (e.g., baseline suppression of these markers). Consequently, our findings demonstrate association, not causation and should be interpreted as hypothesis-generating. Future studies must include both treatment-only and vehicle-only control groups to establish true therapeutic efficacy. Fourth, the dosages of Xing Su San and Sang Xing Tang were based on standard human-to-rat conversion formulas derived from classical texts without prior dose-response or toxicity studies and the different doses for the two formulations (12.5 g/kg vs. 8.5 g/kg) were not selected based on biological equivalence or optimized therapeutic windows. Fifth, the use of different

detection methods for the three molecular markers (RT-qPCR for RAGE mRNA, Western blot for NF- κ B protein, immunohistochemistry for S100A9 protein) was necessitated by antibody availability and technical considerations for each target. While this approach provides complementary information at different levels of regulation (transcript vs. protein, quantitative vs. spatial), it complicates direct quantitative comparisons among markers. An ideal design would measure all three markers by the same method (e.g., all by Western blot or all by immunohistochemistry), but technical constraints prevented this. Sixth and most importantly, our study design does not permit conclusions about causal relationships within the RAGE/S100A9/NF- κ B pathway; the term "axis" is used descriptively to frame our results within the known biological literature, but we did not perform pathway verification experiments such as RAGE blockade, S100A9 neutralization, or NF- κ B inhibition, meaning the findings demonstrate associations rather than causality. Finally, this study examined only three molecular markers at a single time point (28 days), did not assess functional outcomes such as pulmonary function tests or arterial blood gases and used exclusively male rats, limiting generalizability to female populations and translational relevance. These limitations should be carefully considered when interpreting the findings and future studies addressing these issues are necessary to validate and extend our conclusions.

CONCLUSION

In conclusion, this study demonstrates that Xing Su San and Sang Xing Tang are associated with reduced PM2.5-induced upregulation of RAGE, S100A9 and NF- κ B in rat lung tissue in a pattern-specific manner consistent with their classical TCM indications. These hypothesis-generating findings provide preliminary molecular evidence supporting further investigation of these formulas for dryness-related respiratory disorders. However, given the significant limitations of this study, particularly the lack of pathway validation, the absence of treatment-only controls and the associative study design, these conclusions should be considered exploratory rather than definitive. Rigorous mechanistic studies including targeted pathway inhibition experiments and well-designed clinical trials are needed to confirm these findings and establish therapeutic potential.

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Author's contributions

Meijing Kou: Conceptualization, Investigation, Formal analysis, Writing – original draft. Youqiong Xie:

Methodology, Investigation, Data curation, Writing – review & editing. Ruixue Jiang: Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing. All authors read and approved the final manuscript.

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Data availability statement

The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

Ethical approval

This study was conducted in accordance with the ethical standards of the institutional and national research committees and the ARRIVE guidelines for the reporting of animal experiments. All animal procedures were approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, Hubei University of Traditional Chinese Medicine (Approval No. SYXK (E) 2017-0067) and complied with the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978). Consent to participate is not applicable as this study did not involve human subjects. This study was performed in adherence with the ARRIVE guidelines. See supplementary file for the ARRIVE checklist.

Conflict of interests

The authors declare that they have no competing interests, financial or non-financial, that could have appeared to influence the work reported in this paper.

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