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Original Research Article (Experimental)

Evaluation of anti-inflammatory and immunomodulatory activity of *Chyawanprash* on particulate matter-induced pulmonary disease in mice

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ABSTRACT

Background: Particulate matter (PM) is the major component of air pollution, which includes emissions from both anthropogenic and natural sources. PM, with aerodynamic diameter of 2.5 ± 10 μm can remain in the air for a long time and be deposited in the lungs through inhalation and hence, is a major threat to human health.
Objective: The objective of the present study was to examine the protective effect of *Chyawanprash* (CP) on PM-induced pulmonary disease through estimation of cytokines and immunoglobulins.
Materials and methods: CP, standard drug, and vehicle (Group G1 to Group G7) were administered orally at the dose volume of 10 ml/kg, for 28 consecutive days (Prophylactic treatment; i.e., Day 1 to Day 28) and next 10 days (i.e., Day 29 to Day 38) of co-treatment with inducing agent $PM_{2.5}$ intratracheally. Animals of group G6 (Inhalation + control) and G7 (Inhalation + CP) were exposed group-wise to $PM_{2.5}$ aerosol (2 mg/5 ml, 15 min) via inhalation in histamine chamber on Days 29, 31, 33, 35, and 37. On Day 38, animals were anesthetised and blood and broncho alveolar lavage fluid (BALF) were collected. Animals were sacrificed and lungs were collected for histology.
Results: Prophylactic benefit of CP against pulmonary pathology was evidenced by the inhibition of inflammatory cytokines (BALF: TNF a, IFN-g, IL-7, IL-6 and lung: TNFa, Histamine and IL-6), chemokines (Lung: MMP-9), inflammatory cell infiltration (cell counts in BALF), and histopatholoy in experimental mice model.
Conclusion: These findings suggest that CP has potential benefit in protecting from harmful effects caused by air pollutants such as $PM_{2.5}$.

1. Introduction

Acharya Caraka in Caraka Samhita described the epidemic diseases and termed them as *janapadodhwamsa vyadhis* (*janapada* = community, *dhwamsa* = destruction). One of the four major factors for such epidemic is vitiated atmospheric air or polluted air, water, land, and time being the other three. Some important characteristics of polluted air are the one mixed with *sikata* (particles), *dhuli* (dust), *dhuma* (smoke), *atisheeta* (too cold), and others. These references from Ayurveda suggest that air pollution was considered the first among causes of epidemics even millennia ago [1].

The situation has only worsened over time. Air pollution can cause miscarriages, early delivery, and low birth weight. It contributes to diseases that account for almost 1 in 10 of all deaths of children under the age of five. It can harm the healthy development of children's brains. It is a drag on economies and societies, already costing as much as 0.3 per cent of global GDP – and rising. Ultrafine, airborne pollutants, caused primarily by smoke and fumes can more easily enter and irritate children's lungs, causing and exacerbating life-threatening disease. Studies show that these tiny particles can also cross the blood–brain barrier [2].

Particulate matter (PM) is the major component of air pollution, which includes emissions from both anthropogenic and natural sources. Based on its aerodynamic diameter, PM is crudely categorized as coarse PM, which has an aerodynamic diameter of 2.5 ± 10 μm; fine PM, which has an aerodynamic diameter of <2.5 μm; and ultrafine PM, which has an aerodynamic diameter of <0.1 μm.

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Fine PM is small enough to penetrate alveoli and terminal bronchioles, while coarse PM is primarily deposited in large conducting airways. As industrialization and urbanization have increased, diesel exhaust particles (DEPs) have become a major source of ambient PM in modern cities.

DEPs are composed of an elemental carbon core to which hundreds of chemicals and transition metals are attached. Evidence suggests that PM is associated with increased pulmonary and cardiovascular morbidity and mortality [3].

In addition, $PM_{2.5}$ accumulates toxic heavy metals, acid oxides, organic pollutants, bacteria, and viruses in the air. $PM_{2.5}$ can also remain in the air for a long time and be deposited in the lungs through inhalation and hence, it is a major threat to human health.

Numerous previous studies have suggested that $PM_{2.5}$ can stimulate the production of reactive oxygen species (ROS) and certain inflammatory mediators, resulting in changes to vascular permeability, airway constriction, and tissue injury. Most previous studies investigating $PM_{2.5}$ have examined lung sections for histopathology [4].

PM has been associated with increased pulmonary and cardiovascular mortality and morbidity. Additionally, PM is known to exacerbate asthma. The findings in mice also support the hypothesis that PM may contribute to the onset of asthma as it can trigger both Th1/Th2 inflammatory responses. In this study, we have used a murine intratracheal sensitization model of size-fractionated PM to determine how PM contributes to the development of Th2 immune responses in healthy mice [5].

At present, no effective control measures have been developed for the treatment of $PM_{2.5}$-induced respiratory diseases apart from reducing $PM_{2.5}$ emissions, wearing a dust respirator and increasing the number of plants. Therefore, novel medicines with fewer side effects and a high efficacy for treating $PM_{2.5}$-induced respiratory diseases are required.

2. Material and methods

2.1. Ethics committee approval

The study was approved by the Institutional Animal Ethics Committee on 13th Feb 2018 (Approval No. IAEC/43/562) Animal husbandry was maintained as per Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) recommendations.

2.2. Study product

Chyawanprash (CP) is a well-known Ayurvedic classical formulation, which is used since ancient times. Chyawan was an aged sage who first used this preparation to regain vitality and longevity. *Prash* means a drug or food stuff that is suitable for consumption.

CP is a polyherbal traditional Ayurvedic medicine with the following ingredients: *Bilva* (*Aegle marmelos*), *Agnimantha* (*Premna integrifolia*), *Syonaka* (*Oroxylum indicum*), *Patala* (*Stereospermum suaveolens*), *Gambhari* (*Gmelina arborea*), *Shalaprani* (*Desmodium gangeticum*), *Prishniparni* (*Uraria picta*), *Brihati* (*Solanum indicum*), *Kantakari* (*Solanum xanthocarpum*), *Gokshura* (*Tribulus terrestris*), *Bala* (*Sida cordifolia*), *Mudgaparni* (*Phaseolus trilobus*), *Mashaparni* (*Teramnus labialis*), *Karkatshringi* (*Pistacia integerrima*), *Tamalaki* (*Phyllanthus niruri*), *Draksha* (*Vitis vinifera*), *Jivanti* (*Leptadenia reticulata*), *Pushkara* (*Inula recemosa*), *Haritaki* (*Terminalia chebula*), *Guduchi* (*Tinospora cordifolia*), *Varahi* (*Dioscorea bulbifera*), *Vidari* (*Pueraria tuberosa*), *Karchura* (*Curcuma zedoaria*), *Musta* (*Cyperus rotundus*), *Punarnava* (*Boerhaavia diffusa*), *Shatavari* (*Asparagus racemosus*), *Utpala* (*Nymphaea stellata*), *Vasa* (*Adhatoda vasica*), *Ashwagandha* (*Withania somnifera*), *Kakanasika* (*Maritima annua*), *Yasti* (*Glycyrrhiza glabra*), *Amalaki* (*Emblica officinalis*), *Chandan Saar* (*Santalum album*), *Nagakesara* (*Mesua ferrea*), *Pippali* (*Piper longum*), *Tvak* (*Cinnamomum zeylanicum*), *Tvakpatra* (*Cinnamomum tamala*), *Lavanga* (*Syzygium aromaticum*), *Sukshmaila* (*Elettaria cardamomum*), *Abhraka Bhasma*, *Akarakarabha* (*Anacyclus pyrethrum*), *Muktashukti pishti*, *Kumkuma* (*Crocus sativus*), and *Vamsha* (*Bambusa bambos*) along with *Til tail* (*Sesamum indicum*), *Ghrit*, crystal sugar (*Sharkara*) and honey (Madhu*)* along with preservatives and excipients. The study product was procured from Dabur India limited [6]. (Batch No.BM3194, Mfg. Aug 2017, Exp. Aug 2020).

2.3. Experimental procedure

Fifty-six healthy male Balb/c mice were acclimatized for 5 days. Animals were randomized based on the body weight and allotted to seven groups; each group comprised 8 animals [Table 1].

2.4. Induction procedure

Groups G2, G3, G4 and G5 received intratracheal (i.t.) instillation of $PM_{2.5}$ (100 μg/per mouse; 100μL/animal [Conc.1 mg/ml in Normal Saline]) on Day 29 and Day 35 under mild anesthesia. Animals of Group G6 and G7 were exposed group-wise to $PM_{2.5}$ aerosol (2 mg/5 ml, 15 min) via inhalation in histamine chamber on Days 29, 31, 33, 35 and 37.

2.5. Hematology and Ig estimation

On Day 38, all the animals were anesthetized, and blood was collected from all the animals, to perform hematology and IgE estimation. Soon after, broncho alveolar lavage fluid (BALF) was collected from all the animals for total and differential cell count

Table 1
Allocation of animals.

Group	Treatment	Treatment Regimen	Induction Regimen	Sample Size
G1	Distilled water (Normal Control) + Normal Saline	10 ml/kg, p.o., q.d.x38	–	8
G2	Distilled water (Disease Control) + $PM_{2.5}$ intratracheal	10 ml/kg, p.o, q.d.x38	100 μg/per mouse, i.t. Day 29 and Day 35	8
G3	CP + $PM_{2.5}$ intratracheal	2000 mg/kg, p.o, q.d.x38	100 μg/per mouse, i.t. Day 29 and Day 35	8
G4	CP + $PM_{2.5}$ intratracheal	500 mg/kg, p.o., q.d.x38	100 μg/per mouse, i.t. Day 29 and Day 35	8
G5	Dexamethasone + $PM_{2.5}$ intratracheal	0.1 mg/kg, p.o., q.d.x38	100 μg/per mouse, i.t. Day 29 and Day 35	8
G6	PM Inhalation control ($PM_{2.5}$ inhalation)	10 ml/kg, p.o, q.d.x38	2mg/5 ml, Inhalation, 15min, Day 29, 31,33,35 and 37	8
G7	CP + $PM_{2.5}$ inhalation	2000 mg/kg, p.o, q.d.x38	2mg/5 ml, Inhalation, 15min, Day 29, 31,33,35 and 37	8

and cytokine estimation. Animals were euthanized, and lungs were collected. Portion of lung was homogenized for cytokines estimation and another portion was fixed in 10% neutral buffered formalin (NBF) for histopathological investigation.

Body weight of each animal was recorded daily till end of the experimental period. Change in body weight (in %) was calculated from Day 1 for each group. Whole blood was collected and analyzed for hematology. Blood was collected and allowed to clot for 15 min; centrifuged; serum separated and stored at −20 °C. IgE was estimated in serum. IgE level was calculated for each animal and Mean and SEM value was tabulated for each group. The % change was calculated with respect to disease control group.

2.6. BALF collection and estimation

On Day 38, animals were euthanized, the thoracic cavity was partly dissected, and the trachea was cannulated with an 18-gauge needle which was secured with a suture thread and three in-and-out washes with 0.6 ml of isotonic saline solution. The BALF was immediately centrifuged at 800g for 10 min at 4 °C.

Cell count in BALF was performed by fixing the cells in methanol and staining them with hematoxylin and eosin (H&E). Inflammatory cells (neutrophils, lymphocytes, and eosinophils, especially alveolar macrophages) were counted. The production of cytokines (Th1 and Th2) such as IL-17A, IL-6, TNF-α, and IFN-γ were estimated on the first supernatant of BALF using specific enzyme-linked immunosorbent assay (ELISA) method as per manufacturer's instruction.

2.7. Lung homogenation and examination

Portion of lung lobe was weighed and homogenized with PBS to estimate MMP-9, histamine, IL-6, and TNF-α using ELISA method as per manufacturer's instruction. Another portion of lung lobe was fixed with 10% neutral buffered formalin, embedded in paraffin and stained with H&E for histopathological investigation.

2.8. Statistical analysis

Data on each parameter were expressed as Mean ± SEM. Data were analyzed by one-way ANOVA or two-way ANOVA followed by appropriate test. Differences were considered significant at $p < 0.05$.

3. Results

No clinical sign of toxicity and no mortality were observed in any of the test item, i.e., CP treated groups throughout the study. However, two mortalities were recorded during the experimental phase. One disease related mortality in PM inhalation control group (G6) and one treatment related mortality in dexamethasone treated group (G5) was observed. Treatment related mortality may have occurred because of prophylactic long-term treatment with dexamethasone.

3.1. Percentage change in body weight

Significant decrease in % body weight was observed during $PM_{2.5}$ exposure phase (Day 36 to Day 38) in disease control group (G2, 12.60% loss) when compared to normal control group (G1, 3.02% gain). It is evident from the data that 38 days daily oral administration of CP at the two tested dose levels of 2000 mg/kg and 500 mg/kg did not cause body weight reduction. It is worthwhile to note that there was no significant body weight loss after intratracheal and inhalation exposure of toxic pollutant $PM_{2.5}$ when compared with the disease control groups.

3.2. Inflammatory and immunomarkers in BALF

3.2.1. BALF: TNF-α

$PM_{2.5}$ disease control (G2) showed significant increase ($p < 0.001$) of 268.2% in the BALF TNF-α level (731.03 ± 23.36 pg/ml) when compared to normal control (G1) which was 198.54 ± 15.97 pg/ml. CP2000 mg/kg and 500 mg/kg treated group i.e., G3 and G4 showed significant decrease ($p < 0.001$) of 66.9% and 67.1% respectively in the BALF TNF- α level when compared to disease control group (G2). Dexamethasone 0.1 mg/kg treated group (G5) showed significant decrease ($p < 0.001$) of 70.0% in the BALF TNF- α level when compared to disease control Group (G2). PM Inhalation control (G6) showed significant increase ($p < 0.01$) of 28.2% in the BALF TNF- α level when compared to Normal control (G1). PM Inhalation CP2000 mg/kg treated group (G7) showed significant decrease ($p < 0.01$) of 14.7% in the BALF TNF-α level when compared to group G6 (Fig. 1a and b).

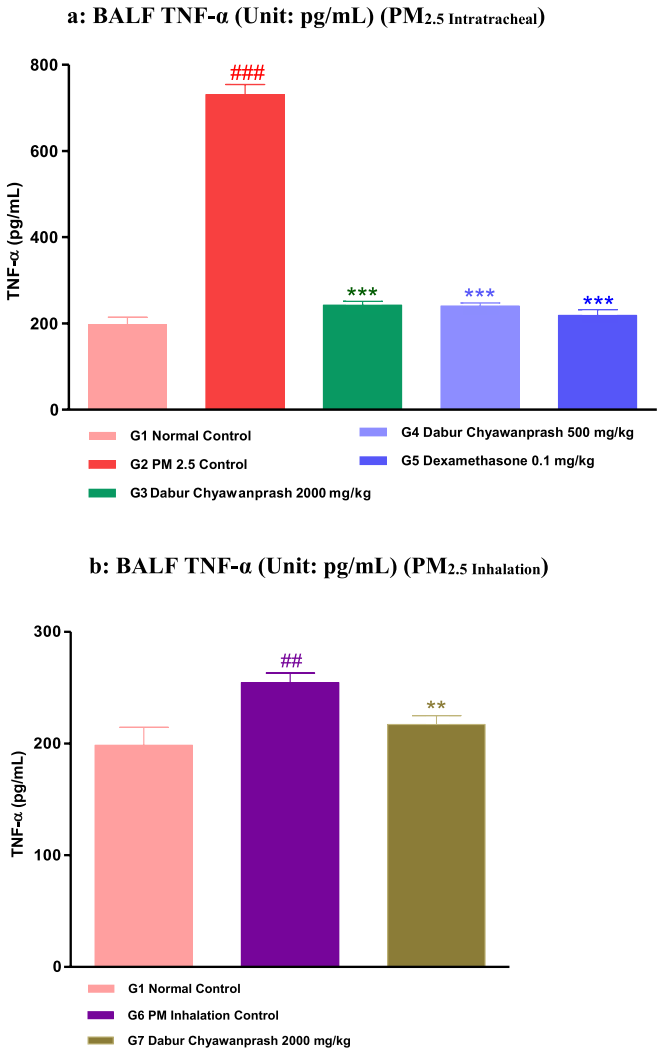

Fig. 1. a: BALF TNF-α (Unit: pg/mL) ($PM_{2.5}$ intratracheal). ###$p < 0.001$, values differ significantly from Normal control (G1). ***$p < 0.001$, values differ significantly from $PM_{2.5}$ control (G2). b: BALF TNF-α (Unit: pg/mL) ($PM_{2.5}$ Inhalation). ##$p < 0.0$, values differ significantly from Normal control (G1). **$p < 0.01$, values differ significantly from PM Inhalation control (G6).

3.2.2. BALF IFN- γ

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.01$) of 57.1% in the IFN-γ level when compared to normal control (G1). CP2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed non-significant decrease of 5.3% and 20.4% respectively in the BALF IFN-γ level when compared to disease control group (G2). Dexamethasone 0.1 mg/kg treated group (G5) showed non-significant decrease of 14.8% in the BALF IFN-γ level when compared to disease control group G2. PM Inhalation control (G6) showed significant increase ($p < 0.05$) of 26.7% in the BALF IFN-γ level when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed non-significant decrease of 16.1% in the BALF IFN-γ level when compared to group G6.

3.2.3. BALF IL-17

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.01$) of 116.1% in the BALF IL-17 level when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed non-significant decrease of 9.8% and 20.1% respectively in the BALF IL-17 level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed non-significant decrease of 23.1% in the BALF IL-17 level when compared to group G2. PM Inhalation control (G6) showed significant increase ($p < 0.01$) of 56.7% in the BALF IL-17 level when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed significant decrease ($p < 0.05$) of 25.7% in the BALF IL-17 level when compared to group G6 (Fig. 2a and b).

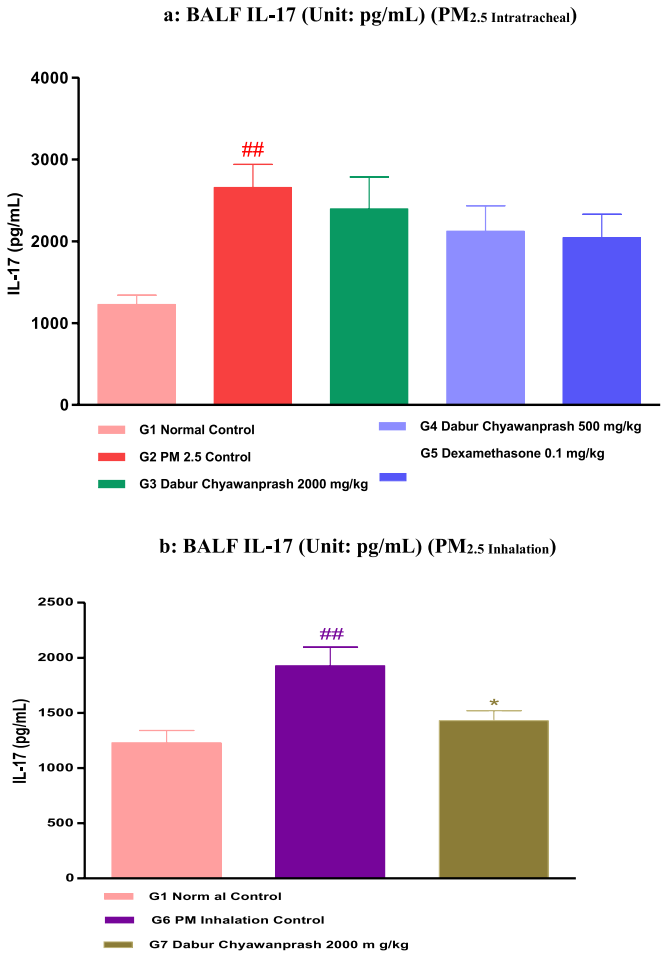

Fig. 2. a: BALF IL-17 (Unit: pg/mL) ($PM_{2.5}$ Intratracheal). ##$p < 0.01$, values differ significantly from Normal control (G1). b: BALF IL-17 (Unit: pg/mL) ($PM_{2.5}$ Inhalation). ##$p < 0.01$, values differ significantly from Normal control (G1). *$p < 0.05$, values differ significantly from PM Inhalation control (G6).

3.2.4. BALF IL-6

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.05$) of 98.2% in the BALF IL-6 level when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed non-significant decrease of 27.8% and 28.4% respectively in the BALF IL-6 level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed non-significant decrease of 11.9% in the BALF IL-6 level (27.69 ± 4.29 pg/ml) when compared to group G2. PM Inhalation control (G6) showed significant increase ($p < 0.001$) of 56.7% in the BALF IL-6 level when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed non-significant decrease of 16.5% in the BALF IL-6 level when compared to group G6.

3.3. Differential cell count in BALF

3.3.1. Neutrophils

Significant increase ($p < 0.001$) in %Neutrophils in BALF with 171.3% was observed in group G2 when compared to group G1 (28.75 ± 2.22), whereas groups G3 and G4 treated with CP at the doses of 2000 and 500 mg/kg showed non-significant decrease of 3.7% and 16.3%, respectively, when compared to $PM_{2.5}$ control group (G2). Dexamethasone treated group (G5) showed a significant decrease ($p < 0.05$) when compared to $PM_{2.5}$ control group (G2). Non-significant increase in %Neutrophils in BALF with 30.7% was observed in PM Inhalation control group G6 when compared to group G1, whereas PM Inhalation Group (G7) treated with CP at a dose of 2000 mg/kg showed non-significant decrease of 6.8% when compared to PM inhalation control group (G6).

3.3.2. Lymphocytes

Significant decrease ($p < 0.001$) in %Lymphocytes BALF with 70.3% was observed in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the doses of 2000 and 500 mg/kg showed non-significant increase of 10.1% and 59.5% respectively when compared to $PM_{2.5}$ control group (G2). Dexamethasone treated group (G5) showed a significant increase ($p < 0.05$, 106.1%) when compared to $PM_{2.5}$ control group (G2). Non-significant decrease in %Lymphocytes in BALF with 13.2% was observed in PM Inhalation control group (G6) when compared to group G1, whereas PM Inhalation Group (G7) treated with CP at a dose of 2000 mg/kg showed non-significant increase of 1.4% when compared to PM Inhalation control group (G6).

3.3.3. Eosinophils

Non-significant increase in %Eosinophils in BALF with 60.0% was observed in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the doses of 2000 and 500 mg/kg showed non-significant increase of 75.0% and 25.0% in %Eosinophils in BALF, respectively when compared to $PM_{2.5}$ control group (G2). Dexamethasone treated group (G5) showed a significant increase ($p < 0.05$, 85.7%) when compared to $PM_{2.5}$ control group (G2). Non-significant decrease in %Eosinophils in BALF with 31.4% was observed in PM Inhalation control group (G6) when compared to group G1, whereas PM Inhalation Group (G7) treated with CP at a dose of 2000 mg/kg showed non-significant increase of 75.0% when compared to PM Inhalation control group (G6).

3.4. Immunomarker in serum

3.4.1. Serum IgE

$PM_{2.5}$ control (G2) showed non-significant increase of 6.0% in the serum IgE level when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed non-significant decrease of 27.3% and 14.7% in the serum IgE level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed non-significant decrease of 10.0% in the serum IgE level when compared to group G2. PM Inhalation control (G6) showed non-significant decrease of 9.6% in the serum IgE level when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed non-significant increase of 2.0% in the serum IgE level when compared to group G6.

3.5. Inflammatory and immunomarkers in lung

3.5.1. Lung TNF-α

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.001$) of 59.7% in TNF-α level in the lung when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed significant decrease ($p < 0.01$) of 22.7% and 26.2% in the lung TNF- α level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed significant decrease ($p < 0.01$) of 23.7% when compared to group G2. PM Inhalation control (G6) showed significant increase ($p < 0.001$) of 48.6% when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed significant decrease ($p < 0.001$) of 34.1% when compared to group G6 (Fig. 3a and b).

3.5.2. Lung MMP-9

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.001$) of 67.1% in the lung MMP-9 level when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed significant decrease ($p < 0.001$) of 47.8% & 52.1% in the lung MMP-9 level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed significant decrease ($p < 0.001$) of 60.0% when compared to group G2. MMP-9 level from PM Inhalation control (G6) was found to be comparable to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed significant decrease ($p < 0.001$) of 46.1% when compared to group G6 (Fig. 4a and b).

3.5.3. Lung histamine

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.05$) of 38.5% in the lung histamine level when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed significant decrease ($p < 0.01$) of 31.7% and 30.0% in the lung histamine level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed non-significant decrease of 16.0% when compared to group G2. PM Inhalation control (G6) showed non-significant increase of 12.1% when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed non-significant increase of 5.9% when compared to group G6.

3.5.4. Lung IL-6

$PM_{2.5}$ control (G2) showed significant increase ($p < 0.001$) of 77.0% in the lung IL-6 levels when compared to normal control (G1). CP 2000 mg/kg (G3) and 500 mg/kg (G4) treated group showed non-significant decrease of 18.0% and 16.7% in the lung IL-6 level when compared to group G2. Dexamethasone 0.1 mg/kg treated group (G5) showed significant decrease ($p < 0.05$) of 24.8% when compared to group G2. PM Inhalation control (G6) showed significant increase ($p < 0.001$) of 116.3% when compared to normal control (G1). PM Inhalation CP 2000 mg/kg treated group (G7) showed significant decrease ($p < 0.01$) of 30.9% compared to group G6.

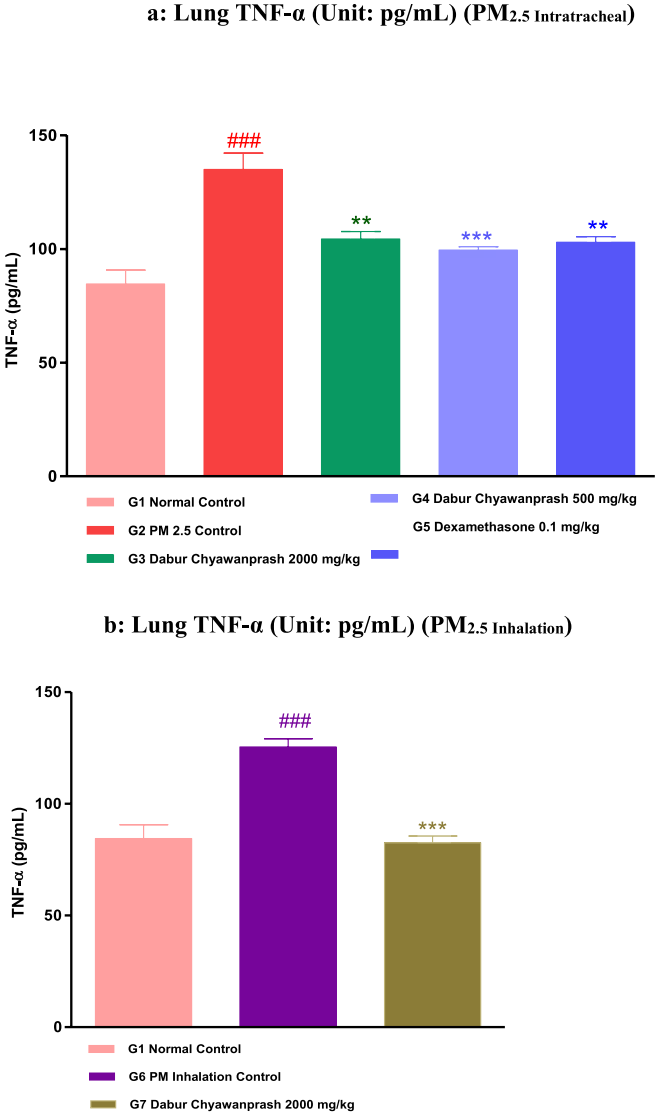

Fig. 3. a: Lung TNF-α (Unit: pg/mL) ($PM_{2.5}$ Intratracheal). ###$p < 0.001$, values differ significantly from Normal control (G1). **$p < 0.01$ & ***$p < 0.001$, values differ significantly from $PM_{2.5}$ control (G2). b: Lung TNF-α (Unit: pg/mL) ($PM_{2.5}$ Inhalation). ###$p < 0.001$, values differ significantly from Normal control (G1). ***$p < 0.001$, values differ significantly from PM Inhalation control (G6).

3.6. Hematology

3.6.1. Neutrophils

Significant increase ($p < 0.05$) in %Neutrophils was observed in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the doses of 2000 mg/kg, 500 mg/kg, and dexamethasone treated group (G5) showed non-significant decrease of 18.42%, 51.62%, and 6.93%, respectively when compared to group G2. Significant increase ($p < 0.05$) was observed in group G6 when compared to group G1, whereas PM Inhalation Group (G7) treated with CP at a dose of 2000 mg/kg showed significant decrease ($p < 0.05$) of 54.96% when compared to group G6.

3.6.2. Monocytes

Significant increase ($p < 0.05$) in %Monocytes was observed in group G2 when compared to group G1, whereas groups G3 and G4

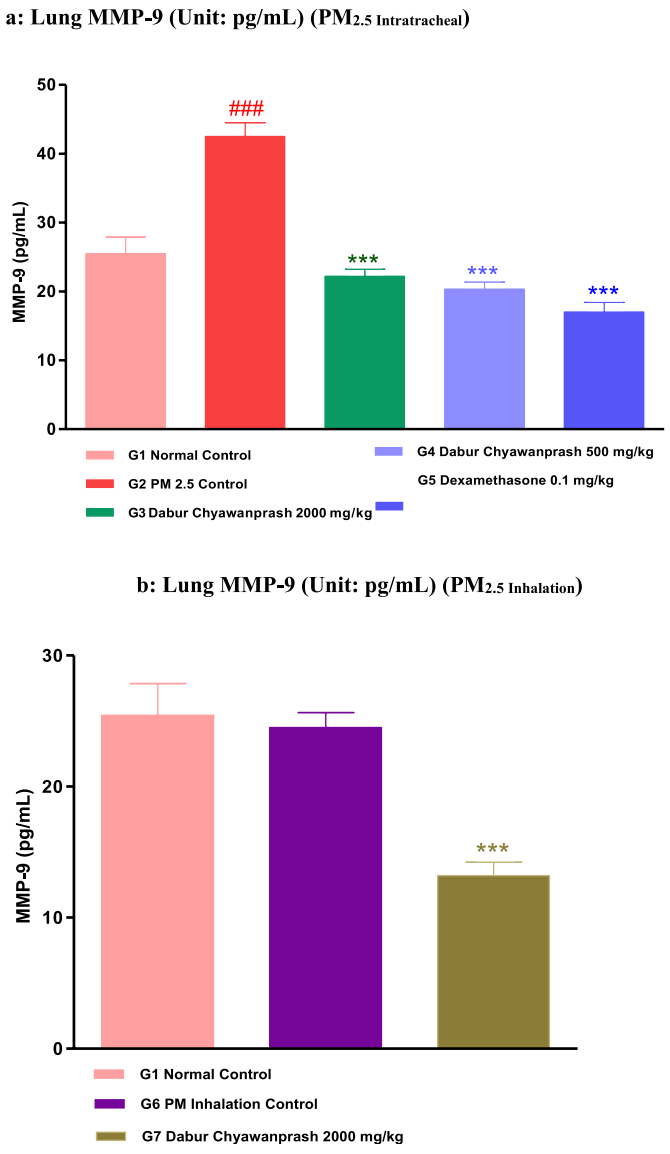

Fig. 4. a: Lung MMP-9 (Unit: pg/mL) ($PM_{2.5}$ Intratracheal). ###$p < 0.001$, values differ significantly from Normal control (G1). ***$p < 0.001$, values differ significantly from $PM_{2.5}$ control (G2). b: Lung MMP-9 (Unit: pg/mL) ($PM_{2.5}$ Inhalation). ***$p < 0.001$, values differ significantly from PM Inhalation control (G6).

treated with CP at the doses of 2000 and 500 mg/kg showed non-significant decrease of 20.51% and 3.0% respectively. Dexamethasone treated group (G5) showed significant decrease ($p < 0.05$) of 35.71% when compared to group G2. Non-significant increase was observed in group G6 when compared to group G1, whereas PM Inhalation Group (G7) treated with CP at a dose of 2000 mg/kg showed non-significant decrease of 32.06% when compared to group G6.

3.6.3. Eosinophils

Significant increase ($p < 0.05$) in %Eosinophils was observed in group G2 when compared to group G1, whereas groups treated with CP at a dose of 2000 mg/kg (G3) and dexamethasone treated group (G5) showed significant decrease ($p < 0.01$) of 47.58% and 56.22% respectively when compared to G2. Group 4 treated with CP at a dose of 500 mg/kg showed non-significant decrease of 14.11% when compared to group G2. No significant change was seen in PM Inhalation group G6 when compared to Group G1. PM Inhalation CP at a dose of 2000 mg/kg (G7) showed non-significant increase of 40.48% in %Eosinophils when compared to group G6.

3.6.4. Basophills

No change was observed in %Basophills in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the doses of 2000 and 500 mg/kg showed non-significant increase of 48.15% and 62.96% respectively. Dexamethasone treated group (G5) showed significant decrease ($p < 0.05$) of 65.08% when compared to group G2.

Non-significant increase in %Basophils was observed in PM Inhalation group G6 when compared to group G1, whereas PM Inhalation Group (G7) treated with CP at a dose of 2000 mg/kg showed non-significant decrease of 25.53% when compared to group G6.

Marginal changes were observed in the other hematological parameters like RBC, hemoglobin, HCT, and MCV across all the treatment groups (G3 to G5, and G7) including normal control (G1), disease control group (G2) and PM Inhalation control group (G6).

3.7. Histopathology

Total histological score of lungs showed a significant increase ($p < 0.01$) in group G2 when compared to group G1, whereas groups treated with CP at a dose of 2000 mg/kg (G3) and dexamethasone treated group (G5) showed non-significant decrease when compared to $PM_{2.5}$ control group (G2). Group G4, CP treated at dose of 500 mg/kg showed a significant decrease ($p < 0.05$) when compared to $PM_{2.5}$ control group (G2). CP treated at a dose of 2000 mg/kg (G7) showed non-significant decrease when compared to PM control group (G6).

Significant increase ($p < 0.05$) in alveolar infiltration of neutrophils was found in group G2 when compared to group G1, whereas groups treated with CP at a dose of 2000 mg/kg (G3) and dexamethasone treated group (G5) showed non-significant decrease when compared to $PM_{2.5}$ control group (G2). CP at dose of 500 mg/kg (G4) (0.00 ± 0.00) showed a significant decrease ($p < 0.05$) when compared to $PM_{2.5}$ control group (G2). No change was observed in PM Inhalation control group G6 when compared to when compared to group G1. PM Inhalation CP treated at a dose of 2000 mg/kg (G7) showed no change when compared to PM control group (G6).

Significant increase ($p < 0.05$) in Atelectasis was found in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the doses of 2000 mg/kg, 500 mg/kg, and dexamethasone treated group (G5) showed non-significant decrease when compared to $PM_{2.5}$ control group (G2). Non-significant increase in Atelectasis was found in PM Inhalation control group G6 when compared to group G1. No change was observed in PM Inhalation CP treated group (G7, dose of 2000 mg/kg) when compared to PM Inhalation control group (G6).

Non-significant increase in Foamy histocytes was found in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the doses of 2000 mg/kg, 500 mg/kg, and dexamethasone treated group (G5) showed non-significant decrease when compared to $PM_{2.5}$ control group (G2). No change was observed in Foamy histocytes in group G6 when compared to group G1. No change was observed in PM Inhalation CP treated at the dose of 2000 mg/kg (G7) when compared to PM Inhalation control group (G6).

Non-significant increase in perivascular fibrosis and alveolar edema was found in group G2 when compared to group G1, whereas groups G3 and G4 treated with CP at the dose of 2000 mg/kg, 500 mg/kg and dexamethasone treated group (G5) showed non-significant decrease when compared to $PM_{2.5}$ control group (G2). No change was observed in perivascular fibrosis and alveolar edema in group G6 when compared to group G1. No change was

observed in PM Inhalation CP treated group (G7, dose of 2000 mg/kg) when compared to PM Inhalation control group (G6).

Non-significant increase in peribronchial lymphoid hyperplasia was found in $PM_{2.5}$ control group G2 when compared to group G1. No change was observed in all test item treated groups when compared with disease control group G2. Non-significant decrease in peribronchial lymphoid hyperplasia was found in PM Inhalation group (G6) when compared to group G1. CP treated at a dose of 2000 mg/kg (G7) showed non-significant increase, when compared to PM control group (G6).

4. Discussion

The concept of primary prevention or prophylaxis has paramount importance in the science of Ayurveda. It is the primary of the two purposes of the medical field, second one being treatment of diseases. Ayurveda expounded a separate branch called *Rasayana* for this purpose. CP is one such formulation from the chapters on *Rasayana*. In addition to its rejuvenation effects, CP has special benefits on the respiratory system and hence, is used as specialized immune support in chronic conditions like *Kshaya* (tuberculosis). CP contains variety of herbs which possess multiple benefits and is used widely since ancient times as a health supplement for enhancing immunity and longevity [7].

Pre-clinical safety studies on CP suggest its safety in acute, subacute, and chronic toxicity studies. CP has been studied for its immunostimulatory activity on various pre-clinical models like estimation of phagocytosis in mice macrophages, effect on activity of natural killer cells (NK cells) and dendritic cells. These studies have shown potential immunomodulatory benefit of CP. CP was also evaluated for its anti-allergic activity by estimation of serum IgE and plasma histamines and showed beneficial effects [8].

Clinical studies on CP have elicited its benefits in both healthy volunteers and patients of tuberculosis [9]. These studies recorded benefits of CP through biochemical, endocrinal, and clinical parameters. Studies on radioprotective effect of CP showed protective role against radiation-induced reactions on skin and adjacent tissues in patients of different carcinomas receiving radiotherapy [10]. In a randomized, multicenter, open-label clinical study, CP also showed better percentage improvement in energy levels, physical fitness, strength, stamina, and quality of life in school children [11]. Evidence generated from such studies have opened new vistas for CP indications. Hence, it was deemed fit to evaluate the protective role of CP in PM-induced pulmonary diseases. Thus, the study was undertaken in experimental mice models as the evidence generated may be helpful for further clinical evaluation in this indication.

PM inhalation is a matter of grave concern currently in urban India. This contributes to the etiology of pulmonary diseases and can also trigger immune responses and lead to various systemic illness. In a published study, exposure to fine carbon black (FCB) resulted in early signs of lung injury. Effects were not enhanced in compromised animals when compared to healthy animals. Exposure to ultrafine carbon (UFC) particles at similar and higher number concentrations did not induce any biologically relevant changes. These data may indicate that at number concentrations occurring in ambient air, the size of the particles (in air) is more important than their number [12].

BALF is a very useful indicator in case of respiratory inflammations especially, in lower respiratory conditions. Even in clinical settings, wide array of systematic BALF analyses are often performed during bronchoscopic procedures (cell types, lipid-laden macrophages LLM, hemosiderin-laden alveolar macrophages HLAM, lymphocyte subsets, bacteria, atypical bacteria, viruses, fungi, parasites) as these values contribute in diagnosis of various baseline clinical conditions [13].

CP is regarded as a very good restorative and used in chronic illness for convalescence in Ayurvedic medicine. Prevention of weight loss in CP tested animals can be attributed to its *Rasayana* and *Brimhana* (nutritive) properties.

Anti-inflammatory effect of CP is evident by its effects on markers like TNFα. The effects are comparable to dexamethasone that suggest the prophylactic potential of the test drug. The findings with respect to immunological markers in BALF are similar to earlier studies on CP in experimental and clinical studies for immunity status on various markers in blood. In the current study, it was also found that the beneficial effects found in group G3 with 2000 mg/kg bw and G4 with 500 mg/kg bw feeding of CP showed comparable results to standard control. This suggests the wide therapeutic window of formulae under *Rasayana* category. The term *Rasayana* has a broader application with regard to adaptogenic, immunomodulator, restorative, and other tissue-supporting pharmacological functions [9,14]. In healthy mice, inhalation of urban coarse PM which presented with significant oxidative potential, induced a low grade inflammation that affected both the pulmonary and colonic mucosae [15].

Inflammation (*Sotha* in Sanskrit) is an important and preliminary pathogenesis in various diseases as per Ayurvedic pathophysiology. Hence, a *Rasayana* medicine is supposed to work as a prophylactic in inflammatory changes that occur due to air pollution [16]. Current study findings on effect of CP on inflammatory cytokines in BALF, serum and lung tissues suggest the positive potential of CP as *Rasayana* medicine. These evidences also add weightage to the concept of pre-*Rasayana* cleansing of gut (*sodhana* as *Rasayana poorvakarma*) as advocated in the science of Ayurveda [17].

CP has shown significant inhibition of inflammatory cytokines (TNFα, IFN-g, IL-7, IL-6) and inflammatory cells in BALF. CP has also shown inhibition of TNFα, Histamine and IL-6 and chemokines MMP-9 in lung tissue.

5. Conclusion

Prophylactic benefit of CP against pulmonary pathology was evidenced by the inhibition of inflammatory cytokines (BALF: TNFα, IFN-g, IL-7, IL-6 and lung: TNFα, histamine and IL- 6), chemokines (lung: MMP-9), inflammatory cell infiltration (cell counts in BALF and histopathology) in experimental mice model. These findings suggest that CP has potential in protecting from harmful effects caused by air pollutant such as $PM_{2.5}$.

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Conflict of interest

Authors 1, 2 and 4 are employed with Dabur India Limited, manufacturers of Chyawanprash formulation.

Author Contributions

Satyendra Kumar - Conceptualisation, Validation, Writing Original-draft, Project administration.

Padmanabha Rugvedi - Writing - review and editing, Visualisation.

Kamaraj Mani - Investigation, Resources.

Arun Gupta - Conceptualisation, Supervision, Writing- review editing.

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