Suez Canal University Medical Journal Vol. 26 (12), 2023
Pages 12-25

Evaluation of the Effect of Spirulina and Pioglitazone Individually and Combined in Ovalbumin-Induced Bronchial Asthma in Male Albino Rats

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Abstract

Background: **Peroxisome proliferator-activated receptor gamma (PPAR-γ) transcriptional pathway is crucial in regulating inflammation, including allergic subtypes. In experimental asthma, pioglitazone (PIO), a PPAR-γ agonist, exhibits protective and remodeling effects. *Spirulina platensis* extract (SPE), an extract from a type of blue-green algae, is a popular adjunctive therapy for allergic asthma. However, SPE's molecular targets in eosinophilic allergic asthma remain debatable. *Aim:* The current study looked at alterations in the PPAR-γ/nuclear factor (NF)-κβ pathway in ovalbumin (OVA)-induced allergic airway inflammation following treatment with SPE and PIO alone or together. *Materials and Methods:* A randomized controlled experimental study was held using fifty-six male Wistar rats. Sensitization and challenge by OVA were performed. SPE (500 mg/kg/day) and PIO (30 mg / kg / day) were given orally (from day 15 to day 28). *Results:* The severity of OVA-induced airway inflammation, as measured by elevated levels of total serum immunoglobulin (Ig)E, total and differential leukocytic counts in bronchoalveolar lavage (BAL) fluid, lung PPAR-γ gene expression, and NF-κβ expression in lung tissue, was significantly reduced by either SPE alone or in combination with PIO. Furthermore, the histological inflammatory and fibrotic lung scores decreased significantly following SPE and PIO, both individually and in combination. Of notice, the combination regimens displayed the highest improvements above individual SPE or, PIO. *Conclusion:* Spirulina's ability to stimulate PPAR-γ means it can mitigate the allergic airway inflammation caused by OVA.**

Keywords: Allergy, *Spirulina platensis,* Peroxisome proliferator-activated receptor-gamma, Airway inflammation, pioglitazone, Nuclear factor-κβ.

Introduction

Asthma is a chronic inflammatory disorder characterized by airway inflammation and airway remodeling, increasing the risk of progressive loss of lung function[1]**.** Three hundred and thirty-four million individuals worldwide have asthma. In Egypt, it considers about 6.7 percent of the adult population[2]. To assess potential treatments for this condition, a variety of animal models of bronchial asthma (BA) have been established. Animals exposed to and challenged with OVA are often employed as asthma models. Epithelial hypertrophy, goblet cell hyperplasia, airway hyperresponsiveness (AHR), and inflammation are shared features with those who have

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human asthma[3]. Allergic asthma's pathological and clinical symptoms result from an inflammatory process initiated by a subset of CD4+ T cells called T helper 2 (Th2) cells. Th2 cells produce proinflammatory cytokines that trigger a cascade of subsequent events such as airway remodelling, smooth muscle hypertrophy, mucous cell hyperplasia, and stimulate type 2 immunity, characterized by a high immunoglobulin (Ig)E antiby titer and eosinophilia[4,5]. The superfamily of receptors that contains transcription factors that influence gene expression is one of the many strategies used by pulmonary cells to control the inflammatory process[6]. Receptors for peroxisome proliferator-activated protein are a subgroup of these receptors (PPARs). They are ligand-activated transcription factors belonging to the nuclear hormone receptor superfamily. There are three varieties of PPARs, each encoded by a different gene: PPAR-alpha (α), PPAR-beta (β), and PPAR-gamma (γ).PPAR-γ was discovered to be a regulator of fatty acid production, glucose metabolism, and an adipocyte differentiation factor[7]. Over time, it has become abundantly evident that PPAR-γ is essential for the immune system. PPAR-γ is expressed in a wide variety of immune cells. These include monocytes/macrophages, dendritic cells (DCs), T lymphocytes, B lymphocytes, and platelets. PPAR-γ may regulate the inflammatory immune response due to its anti-inflammatory properties. In studies, PPAR-agonists were found to operate as negative regulators of monocytes and macrophages, reducing the production of proinflammatory cytokines, including tumor necrosis factor (TNF)-α,, interleukin (IL)-1β, and IL-6 in a dose-dependent manner[8]. Additionally, agonists of PPAR-γ suppress NF-κβ activity[9]. By controlling the production of cytokines, chemokines, and cell adhesion molecules, NF-kβ plays a crucial role in airway illness. In BA, the airway tissue is invaded by inflammatory cells of a different kinds and in different numbers due to the actions of these inflammatory mediators. Most of the time, inflammatory mediators cause NF-kβ to become active in asthma[10]. The activation of NF-kβ negatively regulates PPAR-γ[11]. A diverse set of natural and synthetic lipophilic ligands interact with PPAR-γ. These ligands inhibit inflammation in immune cells and particular lung cells. Also, several researchers have shown that when rosiglitazone and PIO bind to PPAR-γ, they have protective and reshaping effects on asthma. These agents mitigate antigen-induced hyperreactivity, airway inflammation, eosinophilia, and type 2 cytokine and immunoglobulin E (IgE) production[6]. Long-acting beta-agonists and inhaled corticosteroids are two treatments for BA. Even though these medications relieve most patients' symptoms and improve their lung function, some people experience worsening or dangerous side effects due to the treatment. As a result, alternative treatments are required[12]. *Spirulina*, a blue-green alga, is one of the suggested alternative remedies. It is produced and utilized as a dietary supplement to modulate the immunological function and treat oxidative and inflammatory illnesses such as BA, allergic rhinitis, and pulmonary fibrosis. Humans have traditionally consumed Spirulina platensis, a cyanobacterium[13]. By decreasing NF-kβ translocation into the nucleus, the organic SPE might decrease the production and release of proinflammatory cytokines in macrophages[11]. Previous research has shown that spirulina, through its antioxidant and anti-inflammatory properties, can help treat BA in humans. No investigation has proven that its therapeutic impact is attributable to its effect on PPAR-γ gene expression. As a result, in OVA-induced BA, we studied the role of SPE and

PIO, a PPAR-agonist, alone or in combination in the modulation of the allergic inflammatory response associated with BA via the effect on lung PPARγ/NF-κβ gene expression.

Methods

Experimental animals

In this study, 56 mature male albino rats weighing 150-180 grammes were employed. Animals were obtained from Egypt's National Centre of Research in Cairo. The rats were housed in plastic polyethylene cages with a normal light/dark cycle and a temperature of 25℃ 2 in a controlled environment, with unrestricted access to food and water, for one week before the commencement of the investigation. Ethical approval for the experimentation was granted by Suez Canal University's Institutional Animal Care and Use Committee, and all procedures followed National Institutes of Health guidelines for the care and use of laboratory animals (MD, USA).

Chemicals and drugs

All drugs, chemicals, and solvents were obtained from Sigma Aldrich *(St. Louis, Missouri, USA)*, except pure spirulina powder (*Arthrospira platensis*) was purchased from nutrex Hawaii, USA, in the form of green-powder. While PIO was obtained in the form of white powder. One gram of lyophilized powder, composed of albumin from hen egg white at a concentration of 90%, was included in the vial holding the OVA. Al (OH)3 (aluminium hydroxide) was purchased from a store(1 g of powder).

Experimentation procedures

A total of 56 rats were utilized in this experiment. Rats were divided among seven groups of eight at random. Group I (normal control): Rats without BA and did not receive any medications. Group II (Al $(OH)_3$ and CMC control group): On days 0, 7, and 14, rats without BA received 20 mg Al (OH)3 gelatinous I.P., followed by 20µL normal saline by intratracheal instillation on day 21. From day 15 to day 28, rats were given 1 percent carboxy-methyl-cellulose (CMC) orally in a dose of 2.5ml/kg. Group III (Spirulina control group): On days 0, 7, and 14, rats without BA received 20 mg Al (OH)3 gelatinous I.P., followed by 200µL normal saline by intratracheal instillation on day 21. Rats received Spirulina 500mg/kg/day suspended in normal saline (0.9% NaCl;5ml/kg) orally by gastric gavage from day 15 to day 28[14]. Group IV (OVA control group): Rats were sensitized with 1mg OVA adsorbed on 20mg $AL(OH)_3$ at 0, 7, 14 days I.P. and were challenged with 1.1% OVA in 200ml normal saline (0.9% NaCl) by intratracheal instillation at day 21, 23, 27 [15]. Group V (Spirulina treated group): Rats with BA were treated with spirulina 500mg/kg/day dissolved in normal saline (0.9% NaCl;5ml/kg) orally by gastric gavage from day 15 to day 28 [14]. Group VI (Pioglitazone treated group): Rats with BA were treated with pioglitazone 10mg/kg/day dissolved in CMC 1% orally by gastric gavage from day 15 to day 28 [14]. Group VII (Spirulina and Pioglitazone treated group): Rats with BA were treated with spirulina 500 mg/kg/day suspended in normal saline (0.9% NaCl; 5ml/kg) and pioglitazone 10 mg/kg/day dissolved in CMC 1% orally by gastric gavage from day 15 to day 28. The experimental timeline was sketched in Figure 1.

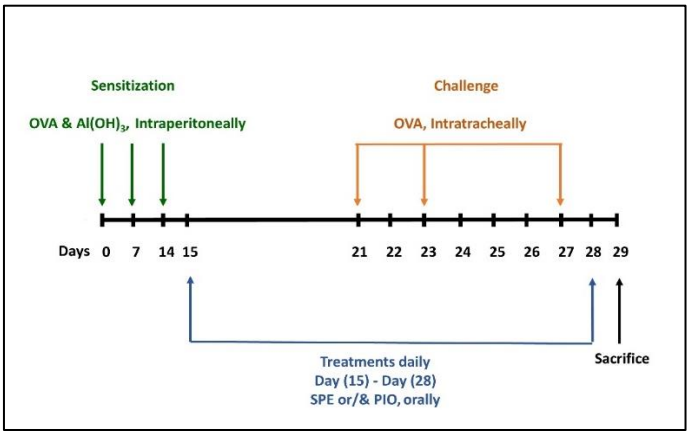

Figure 1. Diagrammatic outline of the experimental and treatment plans for asthmatic rats. Al (OH)3, Aluminum hydroxide; OVA, Ovalbumin; PIO, Pioglitazone; SPE, *Spirulina platensis* extract.

*Enzyme immunoassay for determining serum total IgE levels***.**

Blood samples from anesthetized rats were collected via retrobulbar venous plexuses on day 28, the day following the final injection of treatment/vehicle. Blood was centrifuged (x323g / 20 minutes at 4 ℃). Samples were kept at 80 ℃ after total serum immunoglobulin (Ig)E testing. Total blood IgE levels were measured with an automated ELISA reader (Metertech, M960) and a rat IgE ELISA kit (ab157736; Abcam®, UK).

Determining the overall number and kind of leukocytes in bronchoalveolar lavage fluid (BALF):

Rat lungs were carefully inspected for morphological abnormalities after euthanasia and opening of the chest cavity. The canula was then placed in the trachea and the airway lumen was cleaned with 2-5 ml of normal saline. Gentle aspiration was used to collect the fluid, and the operation was repeated three times. For 10 minutes, the collected fluid was centrifuged at 400g. The BALF cell pellet was spinned down and resuspended in 1 ml of N.S. A Neubauer counting chamber was used to determine the leukocyte count. Following Giemsa staining, the eosinophil cell count was measured using a cytospin centrifuge, and eosinophils were recognised using a light microscope[15].

Investigation of airway inflammation and subepithelial and peri-airway fibrotic changes by histopathology

The right lung was removed for histological investigation, fixed in formalin (10%) overnight, postfixed, dehydrated, clearing, and embedded in paraffin. At a thickness of 4 m, the tissues were sectioned and stained with H&E and Masson's Trichrome. All H&E-stained slides captured at original magnification 100x and 400x. A semi-quantitative scoring method with a grading scale ranging from 0 to 3 was used to assess lung inflammation's degree, amount, and distribution. When no inflammation could be detected, a value of (0) was given. Sporadic cuffing with inflammatory cells was assigned a rating of (1). For example, if there was a very thin layer of inflammatory cells (between one and five cells) around the majority of the bronchi or arteries, that would be worth a value of (2). When there was a thick coating of inflammatory cells around the majority of the bronchi or arteries (more than 5 cells), a value of (3) was assigned[16,17].

The immunohistochemical expressions for Lung NF-kβ proteins

Cut into 4 μm thick pieces, paraffin-embedded, formalin-fixed tissue blocks were placed on glass slides. They were deparaffinized in xylene for two hours after mounting and rehydrated. Three percent hydrogen peroxide was applied for 10 minutes to suppress the endogenous peroxidase activity. The microwave method of antigen retrieval was used. Overnight, a 1:80 dilution of a polyclonal NF-κβ antibody was incubated in 1% PBS-bovine serum albumin. After incubation with biotin-labeled anti-rabbit IgG at room temperature (1 hour), the slices were washed three times for 2 minutes in PBS. The sections were stained with a streptavidin-peroxidase detection system after being washed three times for two minutes in PBS[18].

Lung PPAR-γ gene expressional levels following SPE and PIO

Tissue was lysed in RLT lysis buffer and homogenised (40 seconds) with a tissue homogenizer. 700 l of the sample was placed in a 2 ml collecting tube and spun (8000 rpm / 15 seconds) in an RNeasy spin column. The supernatant was removed with care and transferred to a new microcentrifuge tube. The cleaned lysate was treated with one volume (350 μ l) of 70% ethanol. The PPAR-γ primer sequence was shown as forward: 5'- TGGTTATTTTGTAGGTTGGTTT-3' and reverse: 5'-TTGATCGCACTTTGG-TATTCTTGG-3'. A Bio-Rad iCycler PCR machine performed qRT-PCR using the PCR Kit (SYBR Green) and RN easy Mini Kit (Bio-Rad, Hercules, CA, USA). The PPAR-γ expression measurement kits were made available by (Qiagen, Valencia, CA, USA).

Statistical Analysis

Results were reported using the mean and SEM. The results were analysed using SPSS version 23. (SPSS Software, SPSS Inc., Chicago, USA). A one-way ANOVA was performed to test for statistically significant differences between the quantitative variables, and then a Bonferroni post hoc test was applied. Specific qualitative values were analysed using Fisher's exact test. Statistical significance was determined to exist when the p-value was <0.05.

Results

Changes in mean S. total IgE levels after individual and concurrent treatment of spirulina and pioglitazone to rats exposed to OVA

The levels of total serum IgE were not different (p>0.05) between the vehicle and normal control groups. The total IgE in the blood of OVA-exposed mice was substantially higher than that of the untreated control group ($p<0.05$). Total serum IgE levels were significantly decreased ($p<0.05$) when animals were treated with spirulina, pioglitazone, or both. The combined treatment significantly lowered serum IgE levels ($p< 0.05$) to spirulina or pioglitazone alone; Figure 2.

Total and differential leukocyte cell count in BALF obtained from different study groups

Figure 3 demonstrates that BALF's total and differential cell counts did not differ substantially ($p>0.05$) between the control group and the groups administered vehicles intraperitoneally and intratracheally.

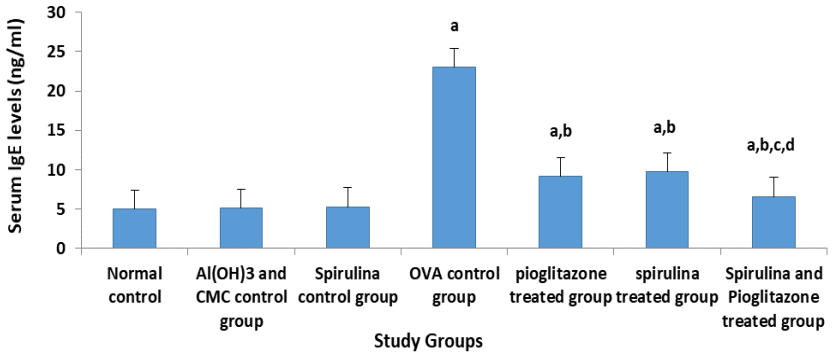

Figure 2: Serum immunoglobulin E (IgE) levels in OVA-confronted rats before and after spirulina and pioglitazone treatment alone and in combination. *The statistical analysis was performed using one-way ANOVA, and the results were presented as Mean ± SEM (n=56). Statistically comparable to the untreated control group [a], to the OVA-control group [b], to the pioglitazone-treated group [c], and to the spirulina-treated group [d].*

The OVA challenged and sensitized rats displayed significant augmentation of the total and differential leucocytics count when compared with the norml rat ($p<0.05$). All OVA-induced elevations were significantly ($p<0.05$) reduced after treatment with spirulina or pioglitazone alone or in combination.

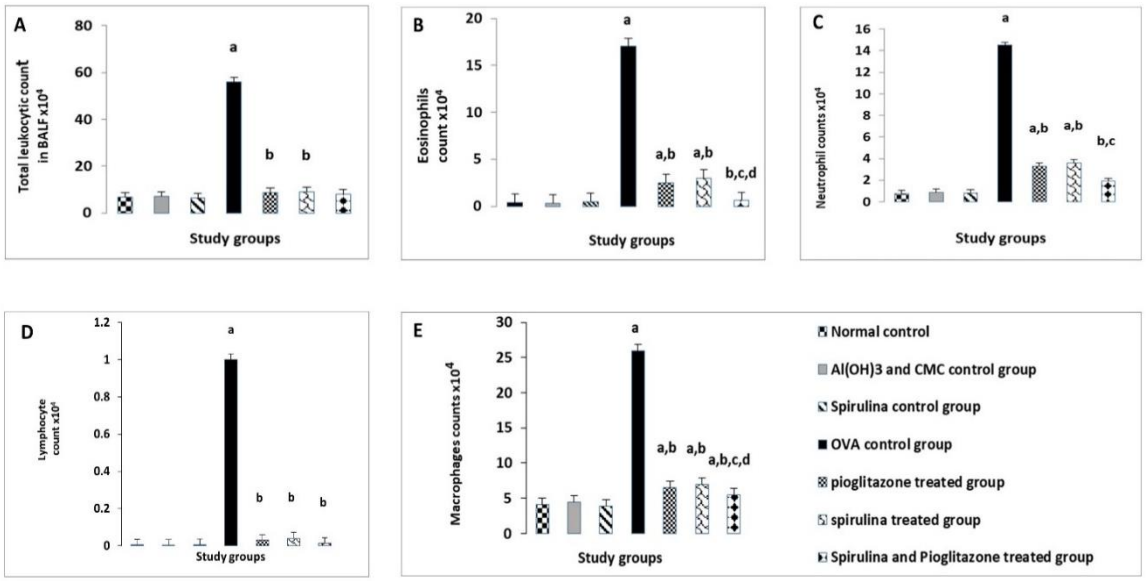

Figure 3: BALF counts of total leukocytes (A), eosinophils (B), neutrophils (C), lymphocytes (D), and macrophages (E) differed across groups. *One-way ANOVA was used for the statistical analysis, and the results were given as Mean ± SEM (n=56). Statistically comparable to the untreated control group [a], to the OVA-control group [b], to the pioglitazone-treated group [c], and to the spirulina-treated group [d].*

Histopathologic inflammatory and fibrotic alterations in the lung tissues

In both the control and vehicle groups, lung tissue architecture was preserved. The single layer of cells that lined the thin walls of alveoli left the inside of these air sacs open and visible. The bronchioles have a thin-walled muscle layer and pseudostratified columnar epithelium lining them. Masson trichrome staining revealed regular architecture free of fibrosis, Figure 4. By contrast, the lungs of the OVA-control group displayed architectural

deformation, alveolar and bronchiole constriction, bronchial wall thickening, and alveolar and bronchiole wall muscle layering. The bronchial epithelium was infiltrated by inflammatory cells, and blood vessels were swollen. Mild peribronchial and perivascular fibrosis was seen using the Masson trichrome stain. The OVA-induced histopathological alterations were alleviated by the administration of spirulina and pioglitazone either alone or in combination. There was a significant reduction in peribronchial and perivascular fibrosis as measured by Masson trichrome staining in both the spirulina- and pioglitazone-treated groups. No fibrosis was seen in the combination therapy group. Figure 3 clearly shows all of these alterations, Figure 4.

Variation in lung NF- κβ expression by immunohistochemistry among groups

The expression of NF-κβ did not alter significantly ($p > 0.05$) between the normal control group, CMC& Al (OH)3, and spirulina groups. When the OVA control group was compared to the normal control group, NF-κβ expression was considerably higher in the OVA group ($p<0.05$). Spirulina and pioglitazone, either alone or in combination, substantially reduced the increase in NF-κβ expression after oral administration of either compound ($p < 0.05$), Figure 5 A and B.

Lung PPAR-γ gene fold expression among the studied groups

The PPAR-γ gene fold expression did not differ substantially ($p > 0.05$) between the vehicle and normal control groups. In the OVA control group, PPAR-γ gene fold expression was considerably lower ($p<0.05$) than in the normal control group. At the conclusion of the trial, the PPAR-γ gene fold expression was reduced considerably after treatment with spirulina or pioglitazone alone or in combination ($p<0.05$), with significantly greater levels for spirulina and pioglitazone combined therapy ($p<0.05$), Figure 6.

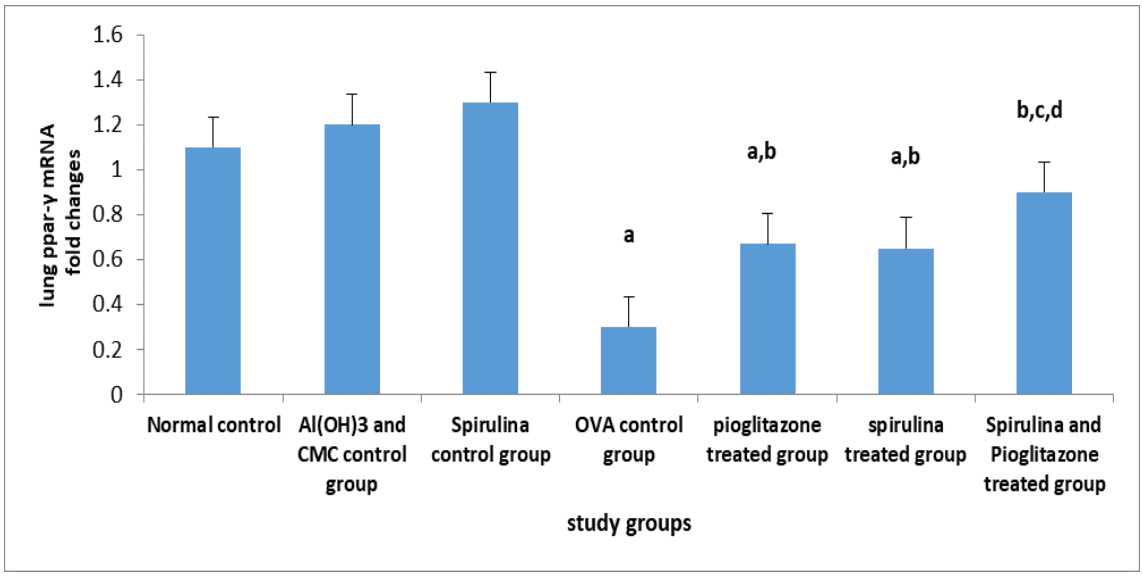

Figure 6: The levels of PPAR-γ gene expression in the lung in the different groups. *One-way ANOVA was used for the statistical analysis, and the results were given as Mean ± SEM (n =56). Statistically comparable to the untreated control group [a], to the OVA-control group [b], to the pioglitazone-treated group [c], and to the spirulina-treated group [d].*

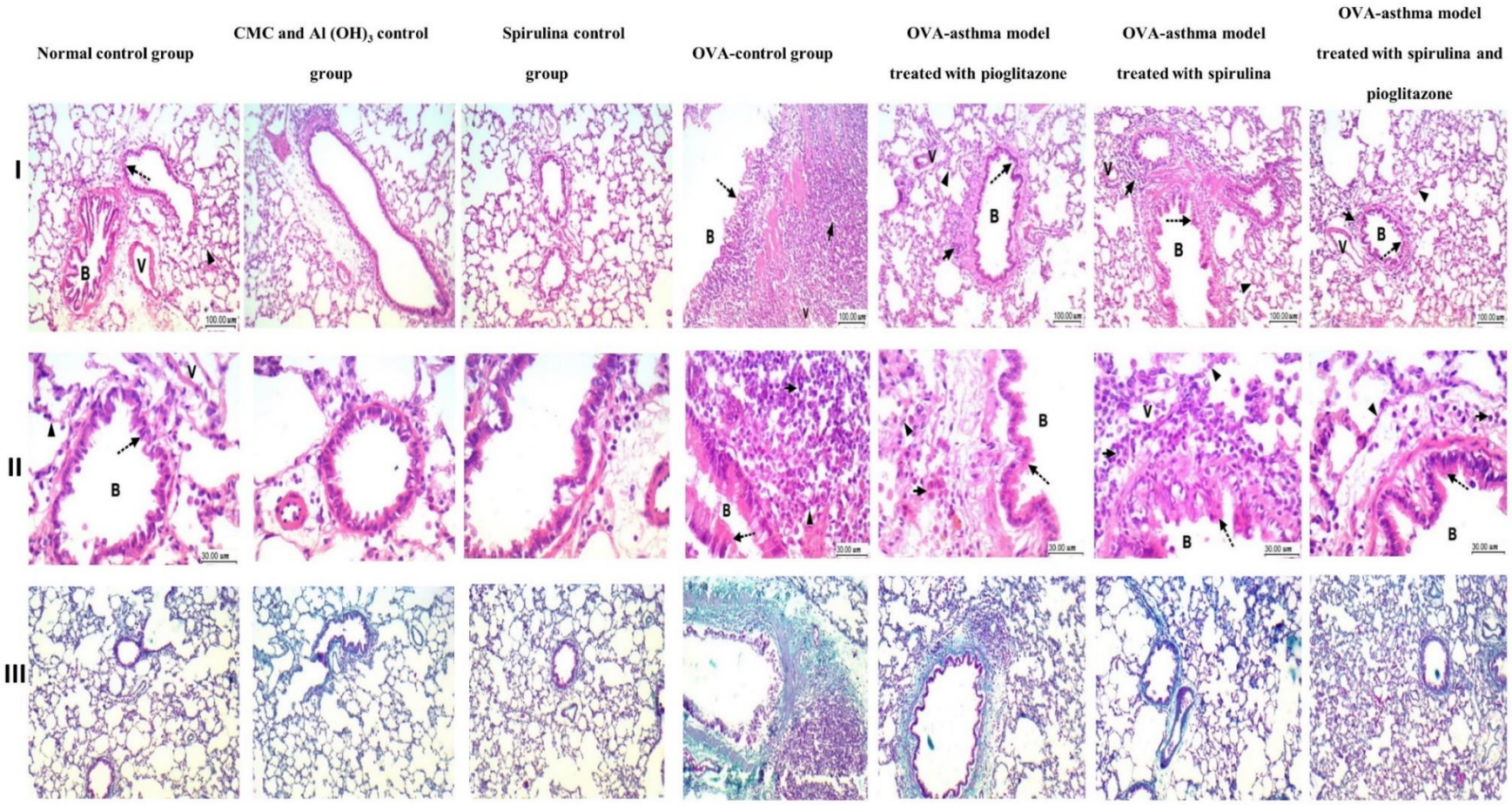

Figure 4: Lung histopathological alterations in several experimental groups; (I) H&E staining at 100x, (II) H&E staining at 400x, and (III) Masson's trichrome staining at 400x. *Lung structures were similar between the normal and vehicle control groups. The most significant lung abnormalities in the OVA control group were constriction and wall thickening of alveoli and bronchioles, widespread inflammatory infiltrates with shedding foci, a thicker muscular layer, and vascular congestion. These changes were mitigated by pioglitazone, and spirulina was taken together. Blood vessels (V), bronchi (B), and inflammatory infiltrates (black arrows) (B).*

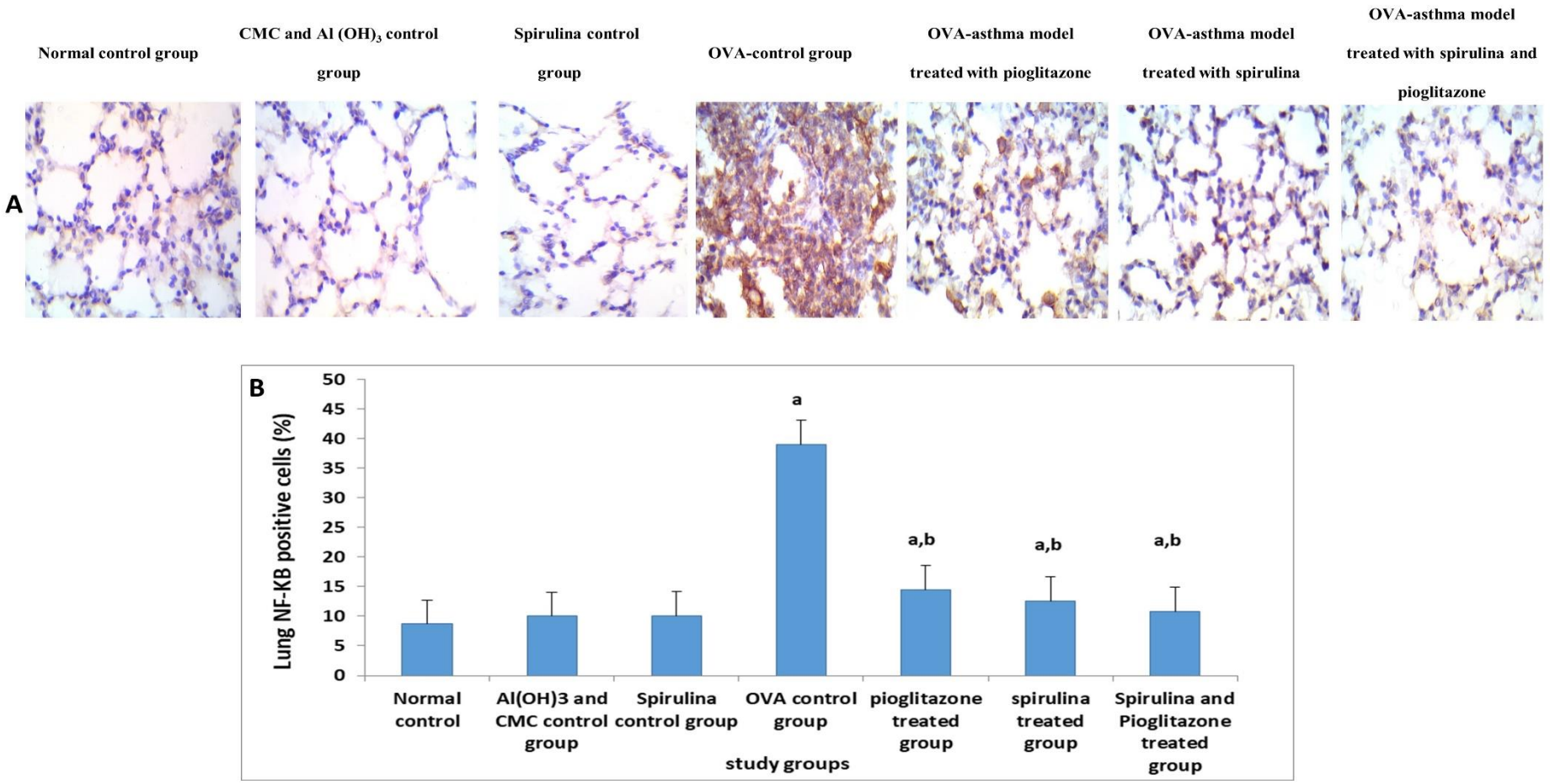

Figure 5: Immunohistochemical lung NF-kβ expression and allocation (A), and the percentage of lung NF-kβ positive cells (B) among the groups investigated. *The analysis was conducted using a one-way ANOVA, and the findings were presented as Mean ± SEM (n=56). Statistically comparable to the untreated control group [a], to the OVA-control group [b], to the pioglitazone-treated group [c], and to the spirulina-treated group [d].*

Discussion

The aims of treating asthma are to lessen the severity of symptoms and stop episodes from happening[19,20]. Beta-2 adrenergic agonists, corticosteroids, and leukotriene modifying are the most often prescribed groups of asthma drugs [21]. Some patients continue to have exacerbations and increasing impairment of respiratory function despite the success of standard asthma therapy[9]. One-way ANOVA was used for the statistical analysis, and the results were given as Mean SEM (n =56). Differences between groups were statistically significant when comparing [a] to the untreated control group (control-untreated), [b] to the OVA-control group (OVA-control), [c] to the pioglitazone-treated group, and [d] to the spirulina-treated group. To regulate the allergic inflammatory response associated with BA, we examined the effects of spirulina and pioglitazone on PPAR-γ gene expression in the lungs. IgE serves as a vital link between the adaptive immune system's antigen detection job and the effector capabilities of mast cells. It is well-recognized that individuals with atopic diseases, including asthma, have elevated IgE levels [22]. Overall serum IgE levels increased in the present study when OVA was administered to rats, as compared to unchallenged rats. This was consistent with research indicating an increased amount of serum IgE in an OVA-challenged mouse [23-25]. It is possible that IL-4 and IL-13, which are released as a consequence of Th2 cell activation, are to blame for stimulating B-cells to produce IgE[22]. In BAL fluid, eosinophilic inflammation and enhanced epithelial shedding were linked to asthma regardless of disease severity[26]. When comparing our normal control group with the OVA control group, we found that the latter had significantly more BALF total and differential leukocytes. Similar findings were seen in[23,27,28]. The recruitment of inflammatory cells in response to cytokines generated by Th2 cells could explain the increased absolute and relative number of inflammatory cells in BALF[29]. When compared to the OVA control group, the number of serum total IgE levels and inflammatory cells in BALF was significantly reduced in the pioglitazone group. This finding is in line with other studies [30-33]. Pioglitazone has an agonistic impact on PPAR-γ, which may explain why serum IgE levels dropped. T cells, macrophages, dendritic cells, mast cells, and eosinophils are all regulated by PPAR-γ as part of the inflammatory response. PPAR-γ agonists inhibit monocyte and macrophage production of inflammatory cytokines. In vitro studies using anti-IgE-stimulated human mast cells found that PPAR-γ agonists suppressed granulocyte-macrophage colony-stimulating factor production (HCMC)[34]. Consistent with other research [35-38], the current study found that spirulina substantially lowered blood IgE level compared to the OVA control group. Since IL-4 promotes TH2 to B cell differentiation, resulting in IgE production, spirulina's impact of lowering IL-4 cytokine levels[13] may account for this drop[39]. When compared to the OVA-challenged group, the total and differential inflammatory cells in the BAL were significantly lower in the spirulina-treated group. Spirulina's anti-inflammatory effects might be to blame. Evidence displayed that spirulina significantly attenuated inflammation by decreasing mononuclear and polymorphonuclear cell infiltration[40]. Experiments in which allergens (such as ovalbumin or HDM extract) were inhaled through the nose revealed that NF-κβ activation was increased in airway tissue and inflammatory cells in vivo [10,41]. Our findings that NF-κβ expression was much higher in untreated asthmatic rats compared to healthy control rats were corroborated by these

previous researches. Concerning spirulina, our research shown that it reduced NF-κβ expression relative to the OVA challenged group. This was consistent with prior research showing that the spirulina components, C-phycocyanin and heptadecane, inhibited NF-κβ [42,43]. According to a recent study, beta-carotene, the major component of spirulina, has anti-inflammatory qualities [44]. It was postulated that it could serve as a redox-based NF-κβ activation inhibitor. According to studies, the anti-inflammatory impact of *Spirulina platenesis* mediated through the regulation of the NF-κβ pathway[11]. The PPAR-agonist PIO decreased the inflammatory response by decreasing NF-κβ and, as a result, the generation of TNF-α and IL-6[45-47]. These studies indicated that NF-κβ expression was considerably lower in the pioglitazone group compared to the OVA-challenged group, which was consistent with our findings. When it comes to BA, PPAR is helpful. The production of inflammatory cytokines such as TNF-α and IL-1β and IL-6 may be reduced using a PPAR-γ agonist. When compared to the effects of corticosteroids, the activation of PPAR-γ exerts extra anti-inflammatory benefits in smooth muscle cells. Eosinophils were shown to express PPAR-γ, and PPAR-γ agonists were found to decrease in vitro eosinophil chemotaxis and antibody-dependent cellular cytotoxicity. Furthermore, the administration of a PPAR-γ agonist suppressed the onset of allergic inflammation in a mouse model of asthma. Decreases in airway AHR and pulmonary eosinophilia were among the effects[48]. Our findings reveal that PPAR-γ gene expression is significantly lower in the OVA-challenged group compared to the control group. This is corroborated by data indicating that after segmental allergen exposure, the expression of the PPAR-γ gene in asthmatic patients is significantly lower than in healthy controls [49]. In contrast to our findings[50], found that OVA inhalation upregulated PPAR- γ expression. Consistent with previous studies[51-53], we found that pioglitazone dramatically boosted PPAR-γ gene expression. The current research confirmed previous findings that spirulina therapy significantly upregulated PPAR-γ gene expression in lung tissue. To put it another way, PPAR-γ is adversely regulated by active NF-κβ and vice versa[54]. Since PPAR-γ gene expression is increased if NF-κβ is suppressed, it follows that NF-κβ should be targeted for inhibition. Previous research has not looked into the expression of the PPAR-γ gene in lung tissue after spirulina intake. On the other hand, SPE suppressed PPAR-γ gene expression in liver cells[55]. Alveoli demonstrated minor dilatation and moderate vascular congestion with considerable decrease in the thickened hyperplastic epithelia in groups treated with PIO and/or SPE, as shown in the current study. Additive and synergistic reductions in OVA-induced allergy and inflammatory changes may account for the remarkable improvement to almost normal lung architectural patterns seen when PIO is co-administered with SPE. Lastly, the inflammatory pathways generated by OVA in rats may be modulated by co-administering PIO and SPE, as seen by lower levels of IgE, inflammatory cells in BALF, lung NF-κβ expressions, and improved PPAR-γ gene expression.

Declarations of interest: none.

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