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Mohamed L. Salem,

Editor in Chief

INTERNATIONAL JOURNAL OF CANCER AND BIOMEDICAL RESEARCH (IJCBR)

RESEARCH ARTICLE

Inflammatory mediator modulation by Short- and Long-Acting β_2 Agonists in Induced Bronchial Asthma in Rats

Nageh A. EL-Mahdy[1], Shady Allam[2] and Sara F. El Sayaad[1]

[1]Pharmacology and Toxicology Department, Faculty of Pharmacy, Tanta University, Egypt
[2]Pharmacology and Toxicology Department, Faculty of Pharmacy, Kafrelsheikh University, Egypt

ABSTRACT

Background: Bronchial asthma is an inflammatory lung disease characterized by hyper-responsiveness and bronchoconstriction. Beta2 agonists are bronchodilator drugs that possess anti-inflammatory properties. **Aim:** The present study aimed to investigate the anti-inflammatory and anti-oxidant effects of short-acting (salbutamol) and long-acting(bambuterol) as beta 2 agonists combination. Also, the effect of these drugs, singly and in combination with prednisolone was measured in asthma-induced rats in both lung tissue and broncho-alveolar lavage. **Materials and Methods:** Wistar rats were sensitized with intraperitoneal (I.P) administration of ovalbumin/$Al(OH)_3$, twenty days later, animals were treated orally with salbutamol 2mg/kg or bambuterol 10 mg/kg or prednisolone 3mg/kg or a combination. **Results:** Ovalbumin/$Al(OH)_3$-sensitized rats showed a significant elevation in inflammatory mediators (IL-4, MIP1α, PGE2, TNFα) and other oxidative parameters such as MDA, NO in both lung tissue and bronchial alveolar lavage (BAL). Moreover, salbutamol and bambuterol decreased inflammatory mediators and alleviated oxidative stress. These inhibitory effects were greatest when both short and long-acting β_2 agonists were used in combination. Also, co-administration of β_2 agonists with prednisolone revealed pronounced decreases in all parameters compared to β_2 agonists alone. **Conclusion:** The combined therapy of salbutamol and bambuterol has an anti-inflammatory and anti-oxidant effect on the experimentally induced asthma in both BAL fluids and lung tissues. Also, these drugs in combination with prednisolone possess greater inhibitory effects on inflammatory mediators.

Keywords: Bronchial asthma, β_2 agonists, inflammatory mediators, glucocorticoids

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Correspondence to:
Sara F. El Sayaad, PhD
Pharmacology and Toxicology Department,
Faculty of Pharmacy, Tanta University,
Tanta, Egypt
Tel: +02-01091020562
Email:
sara-elsayaad@hotmail.com

INTRODUCTION

Bronchial asthma is one of the most widely spread causes of morbidity and mortality in recent decades (Eder et al., 2006). It affects more than 300 million people worldwide, which are expected to reach 400 million people by 2025 (Cruz, 2007). It is considered as the most common chronic immunological inflammatory lung disease characterized by airway obstruction, increased mucus secretions, and bronchial hyper-responsiveness (Gauthier et al., 2015). Consequently, these problems may cause symptoms as recurrent episodes of wheezing, shortness of breath, cough, and chest tightness specially at night or in the early morning (Fergeson et al., 2017).

A complex network of cytokines plays a critical role in the pathophysiology of asthma. These cytokines are secreted mainly by the alveolar macrophages (AMs) in addition to other cells like lymphocytes, mast cells, eosinophils, neutrophils, dendritic cells, and some structural tissue components as smooth muscles and epithelial cells (Hamid et al., 2003; Kips, 2001)

After being exposed to causative agents such as allergens (pollen grains, house dust), exercise, cold air, and other inhaled irritants, allergens are taken up by antigen-presenting cells like dendritic cells and AMs where they present antigen to naive T helper type 0 (Th0) and stimulate their differentiation into T helper type 2 (Th2) lymphocytes. These Th2 cells can produce cytokines, e.g. IL-4, IL-5, and IL-13, which are responsible for the production of IgE

from B-cells (Chung, 2015; Draijer andPeters-Golden, 2017; Drazen et al., 1996; Holgate, 2008). Other mediators involved in the pathogenesis of asthma include chemokines as macrophages inflammatory protein 1α (MIP 1α), pro-inflammatory cytokines such as tumor necrotic factor α (TNF-α), arachidonic acid metabolites like prostaglandin E2 (PGE2), Reactive oxygen species and nitrogen intermediates like Nitric oxide (NO) which play an important role in asthma (Gillissen andPaparoupa, 2015). Systemic corticosteroids are the major therapy for the treatment of allergic inflammatory diseases including asthma and they can decrease rates of hospitalization among patients. Prednisolone is an oral corticosteroid with a direct anti-inflammatory effect on the airway (Liu et al., 2001)

Beta2 (β_2) agonists are the drug of choice for the treatment of bronchial asthma. They induce bronchodilatation that relieve the symptoms and improve the lung functions (Saharan et al., 2010). They are divided into short-acting β_2 agonists such as salbutamol which is a rescue medication with rapid onset and short duration and long-acting β_2 agonists, such as bambuterol that considered as a controller medication with long-period activity (Price andClissold, 1989; Singh et al., 2019) Also, the combination of β_2 agonists and glucocorticoid is usually used in the treatment of asthma as they have a synergistic effect together (Barnes, 2006).

Although β_2 agonists are frequently prescribed as the first line of treatment in asthmatic patients, little data are available to understand their effects on immune-modulating factors. This study aimed to further delineate the anti-inflammatory and anti-oxidant effects of the combination of both classes of β_2 agonists, Also the effect of these drugs in combination with prednisolone was measured in asthma-induced rats in both lung tissue and broncho-alveolar lavage.

MATERIALS AND METHODS

Experimental animals

64 male Wistar rats weighing (180-200g, 12-week age) were purchased from EL-NILE Company, Egypt. Rats were housed in pathogen-free wire cages at 20-24 °C, 60% humidity, and 12h light/dark cycle. All animals had a standard pellet diet and drinking water *ad libitum*. The animals were acclimatized at least one week before the start of the experiments. All procedures were conducted according to Tanta University Ethical Guidelines for Animal Care and Welfare (No. IACUC-Sci-TU-0086).

Preparation ofTyrode solution: The solution was freshly prepared by mixing NaCl; Glucose; $NaHCO_3$; $CaCl_2$; $MgCl_2$; KCl; NaH_2PO_4 weighing 8, 1, 1, 0.2, 0.1, 0.2, 0.05 (g) respectively, add distilled water till 1 litre (Freshney, 2005).

Experimental design: Unless otherwise mentioned, all chemicals were purchased from Sigma-Aldrich, (Saint Louis, MO, USA). Animals received a mixture of 1mg/kg ovalbumin (OVA)/100mg aluminium hydroxide ($Al(OH)_3$ suspended in 1ml of sterile normal saline (0.9% sodium chloride; purchased from Otsuka, Japan) I.P once on day 1 of the experiment (Careau et al., 2002). Three weeks after immunization, animals were treated with either oral salbutamol 2mg/kg (GlaxoSmithKline, UK), bambuterol 10mg/kg (AstraZeneca, Sweden), or prednisolone 3mg/kg (Sanofi, Egypt) (Guan et al., 2015; Hirano et al., 2011; Uzkeser et al., 2012). As well, different combinations were as described below. All drugs were administered in distilled water by oral gavage using 18-gauge stainless steel animal feeding needle for five consecutive days.

For the study design, the rats were equally and randomly divided into eight groups (n=8):

- Group 1: Control group received I.P 1ml normal saline.
- Group 2: OVA/$Al(OH)_3$- sensitized group.
- Group 3: OVA/$Al(OH)_3$-sensitized group treated with salbutamol.
- Group 4: OVA/$Al(OH)_3$-sensitized group treated with bambuterol.
- Group 5: OVA/$Al(OH)_3$-sensitized group with salbutamol and bambuterol.
- Group 6: OVA/$Al(OH)_3$-sensitized group treated with prednisolone.
- Group 7: OVA/$Al(OH)_3$-sensitized group treated with a combination of salbutamol and prednisolone.
- Group 8: OVA/$Al(OH)_3$-sensitized group treated with a combination of bambuterol and prednisolone.

Broncho-alveolar lavage (BAL): Animals were anaesthetized with pentobarbital (60 mg/kg/I.P) during 24h of the last drug treatment (Kips et al., 1992). Their thoracic cavities were carefully opened & tracheas were cannulated and lavaged with 4ml freshly prepared phosphate buffer saline (PBS). This process was repeated four times and the contents were pooled (Wu et al., 2017). BAL was then centrifuged at 1500 rpm for 10 minutes at 4°C. The cell sediments were suspended in Tyrode solution containing OVA (the challenge was performed by direct contact with OVA). BAL's cell count was adjusted to $2x10^6$ cells/ml. The cell suspension was gently wormed at 37°C for 20 min. Afterwards, the suspension was centrifuged at 1500 rpm for 10 minutes. The supernatant was aspirated for assays.

Tissue sampling and analysis: After washing with PBS, the lungs were carefully dissected, sliced, and kept at -80 °C. Later on, tissues were suspended in Tyrode containing OVA (challenge was performed by direct contact with OVA) (2ml/gm) (100µg/ml). Then the tissues are homogenized. Homogenates were incubated at 37°C for 20 minutes and then centrifuged at 13,000 rpm for 15 min. at 4°C. The supernatants were then frozen at -80°C for assessment of IL-4, TNFα, MIP1α, and PGE2 concentration by enzyme-linked immunosorbent kits (ELISA) (Alba-Loureiro et al., 2006; Holgate et al., 1997; Rai et al., 2015). The IL-4, MIP1α, PGE2 kits were purchased from (MyBioSource, San Diego, USA) whereas the TNFα kit was obtained from (RayBiotech, Norcross, Georgia, USA). The samples were assayed by sandwich ELISA (Rai et al., 2015). Malondialdehyde (MDA) was determined by Colorimetric assay (Ohkawa et al., 1979). NO concentration was determined by colorimetric assay using Griess reaction, according to the method of (Miranda et al., 2001).

Statistical analysis

All data were expressed as means ± SEM and analyzed by sigma plot (ver.12.5). Unless otherwise mentioned, data are considered to be significant at *(p<0.001*), *(p<0.05*) using one way RM ANOVA, followed by Tukey as a post-hoc test.

RESULTS

Assessment of mediators in BAL

The pellets of BAL cells were incubated with OVA. The resultant mediators were assayed in cell-free supernatants by ELISA and the following results were obtained: The concentrations of all tested mediators (IL-4, MIP1α, PGE2, and TNFα) were significantly *(p<0.001*) elevated in the OVA-sensitized rats compared with the untreated animals. While treatment with salbutamol or bambuterol and their combination significantly *(p<0.001*) reduced the BAL fluid concentrations of all tested mediators compared to levels seen in sensitized groups. There was no significant difference between salbutamol and bambuterol groups. Moreover, the application of prednisolone with salbutamol or bambuterol also significantly *(p<0.001*) reduced the levels of meditators compared to sensitized groups. In contrast, the levels of MIP1α, PGE2, and TNFα were normalized in all combined groups (Figure 1).

Assessment of mediators in lung homogenate

The lung tissue as a major supply for biological mediators, which could enhance or obstruct the pulmonary function, was also studied. Different samples of the different groups subjected to the present study were taken after lavaging the lung with PBS to examine the following parameters: The IL-4, MIP1α, PGE2, and TNFα levels were significantly *(p<0.001*) higher in the lung homogenate of sensitized rats compared with the control rats. The administration of salbutamol, bambuterol, and their combination revealed a significant *(p<0.001*) reduction in all mediators compared to the sensitized groups. Moreover, co-administration of prednisolone with salbutamol or bambuterol showed a remarkable *(p<0.001*) reduction in all mediators levels as compared with the sensitized groups. In contrast, this combination has shown a significant decrease in TNFα level as compared to the prednisolone group (Figure 2).

Assessment of Oxidative markers

The rats sensitized with OVA showed a significant *(p<0.001*) increase in MDA, NO levels

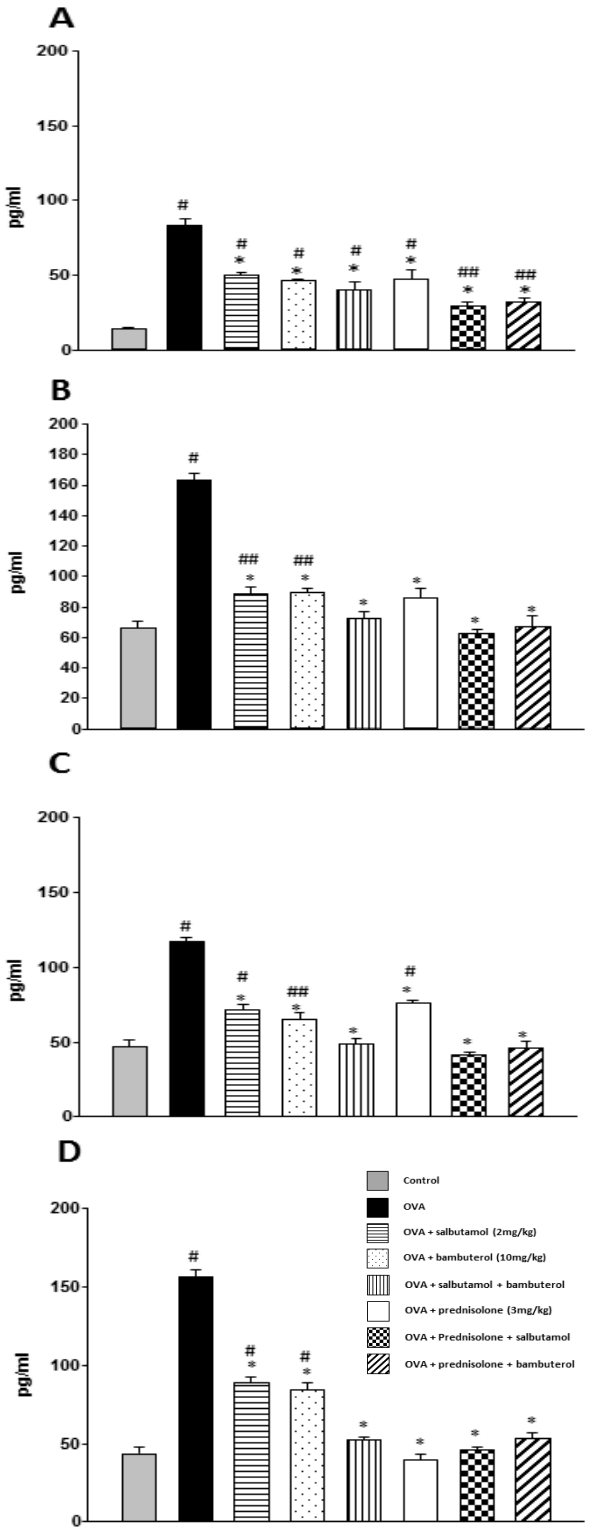

Figure 1. Effect of treatments on the levels of IL-4 (A), MIP1α (B), PGE2 (C), TNFα (D) in broncho-alveolar lavage of OVA sensitized animals. Data shown are mean ± SEM. (*) significant differences compared with sensitized groups ($p < 0.001$). (#) significant differences ($p < 0.001$), (##) significant differences ($p < 0.05$) compared with control group.

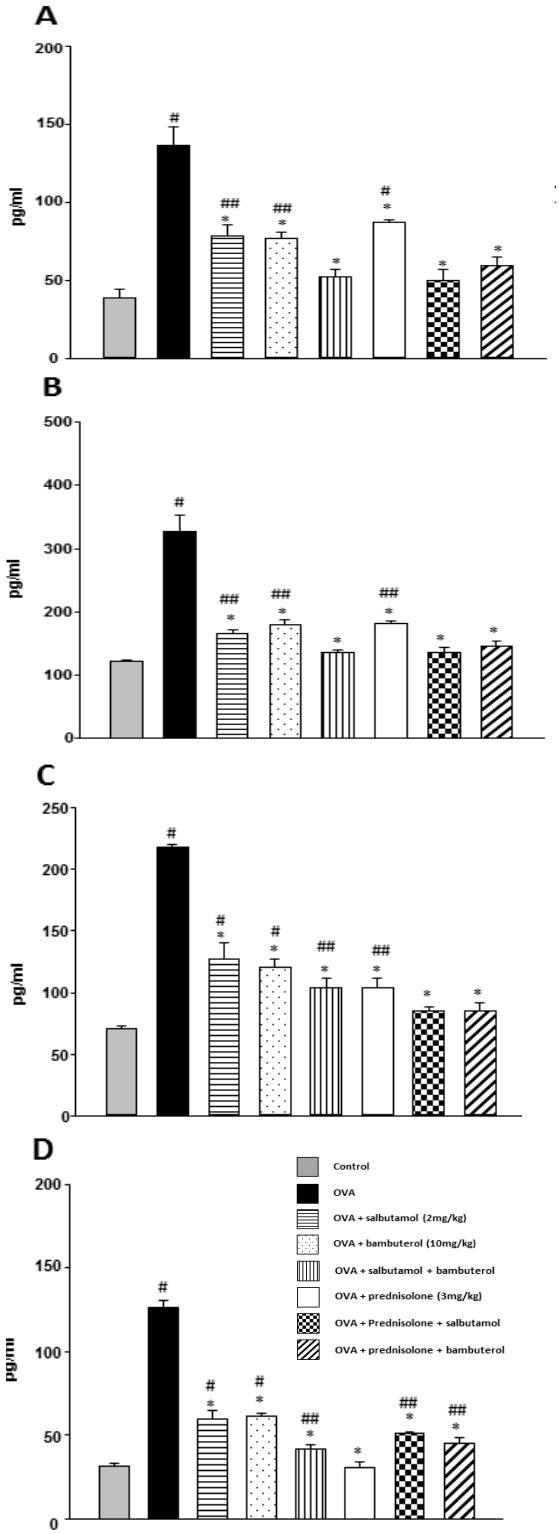

Figure 2. Effect of treatments on the levels of IL-4 (A), MIP1α (B), PGE2 (C), TNFα (D) in tissue homogenate of OVA sensitized animals. Data shown are mean ± SEM. (*) significant differences compared with sensitized groups ($p < 0.001$). (#) significant differences ($p < 0.001$), (##) significant differences ($p < 0.05$) compared with control group.

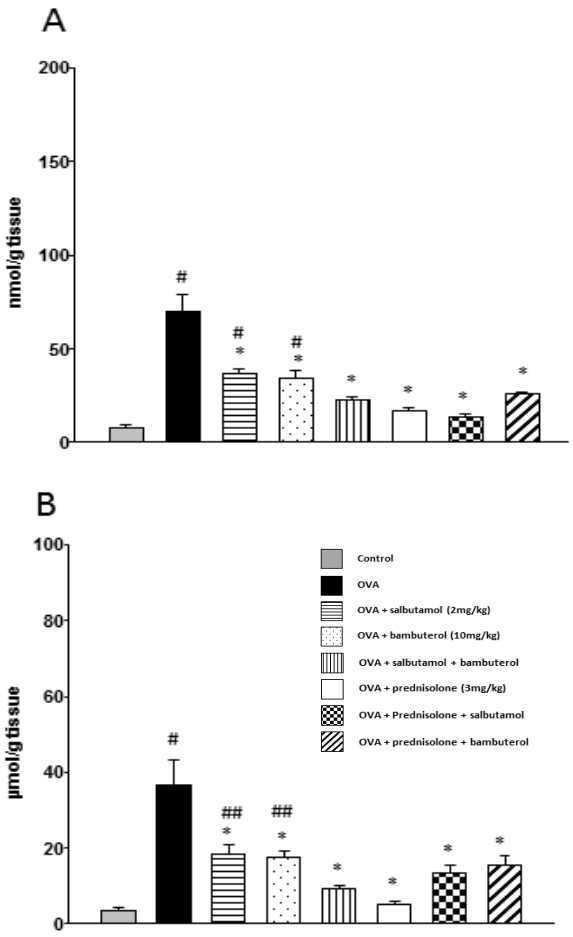

Figure 3. Effect of treatments on the levels of MDA (A), NO (B) in tissue homogenate of OVA sensitized animals. Data shown are mean ± SEM. (*) significant differences compared with sensitized groups ($p < 0.001$). (#) significant differences ($p < 0.001$), (##) significant differences ($p < 0.05$) compared with control group.

compared to the control groups. While compared with the asthmatic groups, the animals receiving salbutamol, bambuterol, and their combination significantly *(p<0.001*) reduced the MDA, NO levels. As well as, the combination of prednisolone with salbutamol or bambuterol significantly *(p<0.001*) inhibited the level of MDA, NO compared with the sensitized groups (Figure 3).

DISCUSSION

Bronchial asthma is a chronic inflammatory disorder of the airway characterized by infiltration and activation of many inflammatory cells which produce cell-signaling proteins called cytokines and inflammatory mediators. β_2 agonists are used as a bronchodilator in the treatment of asthma (Prentice et al., 2016). Moreover, they have been reported to possess anti-inflammatory properties as they inhibit the pro-inflammatory activity of different cells, such as macrophages, mast cells, eosinophils, neutrophils, and structural cells, such as smooth muscle, epithelial cells, and fibroblasts (Halwani et al., 2011; Lim et al., 2000; Maes et al., 2012).

However, the impact of the combination between short and long-acting β_2 agonists and their combination with glucocorticoids on the regulation of inflammatory mediators is still mysterious. The present study aimed to provide additional information on the effect of the orally administered combination of short-acting (salbutamol) and long-acting (bambuterol), and their combination with glucocorticoids (prednisolone) on the modulation of inflammatory mediators in both lung tissue and BAL fluid. Our study demonstrated that β_2 agonists salbutamol and bambuterol inhibited the production of inflammatory mediators IL-4, MIP1α, PGE2, TNFα in both lung tissues and alveolar cells of sensitized rats. It is known that cytokines and inflammatory mediators including IL-4, MIP1α, PGE2, TNFα play an important role in initiating and perpetuating asthma which makes them valuable treatment targets (Barnes, 2018). Interleukin-4 plays a unique role in allergic inflammation and the pathogenesis of asthma. It is responsible for the differentiation of TH0 into TH2 lymphocytes and also regulates IgE synthesis (Maes, Joos ; Brusselle, 2012). MIP1α is a pro-inflammatory chemokine responsible for leukocyte chemoattractant and airway remodeling (Halwani et al., 2011; Lim et al., 2000). PGE2 also plays an important role in asthma (Undem et al., 1988; Undem et al., 1990). Further, TNFα is an important multi-functional trans-membrane pro-inflammatory cytokine. Such cytokine plays a critical role in initiating and regulating that inflammatory reaction as it recruits many inflammatory cells and induces the production of many cytokines and inflammatory mediators (Brightling et al., 2008; Kips, 2001). Moreover, it induces mucus secretion and stimulates reactive oxygen species (ROS) generation. Interestingly, in the current asthma model, the increase of these inflammatory mediators was observed after OVA/$Al(OH)_3$ challenge in both lung tissue and alveolar cells.

The present study revealed that salbutamol and bambuterol significantly reduced IL-4 in both lung tissues and alveolar cells that were in consistence with the previously reported data (Sun et al., 2015) and In contradiction to previous reports which documented that β_2 agonists do not affect the expression of IL-4 (Barnes, 1999), such decrease in the IL-4 generation may be due to species variation, but also to variations in the sensitization conditions used.

As well, our results showed that β_2 agonists significantly reduced the MIP1α content in both lung tissues and alveolar cells. These data are in harmony with the previous study showed the efficacy of albuterol and formoterol on MIP1α in lipopolysaccharide (LPS)-sensitized mice (Bosmann et al., 2012). In addition, salbutamol and bambuterol decrease PGE2 in both lung tissues and alveolar cells. This result was in accordance with previous studies (Undem, Peachell ; Lichtenstein, 1988; Undem et al., 1990). Inconsistent with the present data, many studies have reported that short and long-acting β_2 agonists may play a role in the inhibition of TNFα production (Bissonnette andBefus, 1997; Gill et al., 2016; Keränen et al., 2016; Keränen et al., 2017).

The influence of β_2 agonists on inflammatory mediators could be explained by their ability to bind to β_2 receptors expressed on many cells, such as, leucocytes, macrophages, lymphocytes results in the stimulation of the receptor leading to increase cyclic adenosine monophosphate (cAMP) which may act as a direct inhibitor for many enzymes, such as phospholipase A2 resulting in inhibition of eicosanoids biosynthesis, such as PGE2 (Undem, Peachell ; Lichtenstein, 1988; Undem et al., 1990). Furthermore, cAMP activates cAMP-dependent protein kinase A (PKA) which is responsible for the activation of the nuclear transcription factor CAMP response element-binding protein (CREB) through translocation of its C-subunit to the nucleus and phosphorylate it. In turn, this binds to cAMP response element (CRE) on target DNA leading to regulating the expression of many genes (Keränen et al., 2016). Also, CREB may compete with the transcription factors, such as nuclear factor-kappa B (NF-KB) and activating protein-1 (AP-1) which responsible for the expression of many inflammatory proteins (Parry andMackman, 1997). In addition, CAMP inhibits cytosolic free Ca^{+2} concentrations which are a trigger for several enzymes (Johnson, 2001). Such a mechanism was also linked to the profound effect of β_2 agonists on promoting smooth muscle relaxation.

The current study has shown that there was no significant difference between short and long-acting β_2 agonists in immune modulation in the tissue and alveolar cells. The only difference between them is the duration of action and this could be attributed to the difference in their physical properties and pharmacokinetics as there is no difference in their mechanism of action (Bissonnette andBefus, 1997). Additionally, we noticed that the administration of a combination of both short and long β_2 agonists in the OVA/$Al(OH)_3$-sensitized rat asthma model demonstrates a greater decrease in the production of all tested inflammatory mediators than each drug alone in both BAL fluids and tissue samples. This could be explained by the augmentation effect of short and long β_2 agonists on each other.

In the present study, the effects of salbutamol, bambuterol, and their combination with prednisolone on the modulation of the immune system were compared with that of oral prednisolone in a rat asthma model. Both β_2 agonists and prednisolone exerted anti-inflammatory effects. Glucocorticoids including prednisolone are the most effective frequently prescribed anti-inflammatory drug that suppresses the level of many cytokines, such as IL-4 and TNFα, and chemokines such as MIP1α and prostaglandins like PGE2 (Barnes, 2011; Liu et al., 2001; Ramsahai andWark, 2018). Glucocorticoids and β_2 agonists are the mainstays in the treatment of asthma. In previous studies, the addition of long-acting β_2 agonists to glucocorticoids is recommended for patients not controlled with glucocorticoids alone instead of increase the dose of glucocorticoids (KIPS et al., 2000; Pauwels et al., 1997; Peters et al., 2016; Sun et al., 2015). These combinations are to achieve greater therapeutic benefits in the treatment of asthma as they improve the lungs' function and decrease exacerbation (Sin andMan, 2006).

The present study showed that the combination of prednisolone with short or long-acting β_2 agonists under current conditions caused a pronounced reduction in IL-4, MIP1α, and PGE2 in comparison with each drug alone in both lung tissue and alveolar cells. This synergistic effect could be explained by the action of prednisolone on β_2 receptors by enhancing their coupling to Gs protein. As well, it protects against the downregulation of beta2 receptors, moreover, increases the transcription of the receptors gene leading to increase receptors number. Furthermore, the β_2 agonists increase the translocation of activated glucocorticoid receptors (GR) from the cytoplasm to the nucleus (Adcock et al., 1996; Taylor andHancox, 2000).

On the other hand, the levels of TNFα, NO, and MDA were increased in both tissue and alveolar cells in sensitized rats when compared with the prednisolone-treated group that could be due to interaction between GR and CREB forming GR-CREB complex that leads to inhibition of binding of GR to glucocorticoid responsive elements(GRE). moreover, in some cases, pro-inflammatory cytokine as TNFα activates transcription factors e.g. AP-1, NFKB (Adcock, Stevens ; Barnes, 1996; Taylor andHancox, 2000).

Oxidative stress is considered a hallmark of asthma and increases the levels of oxidants which are markers of the inflammatory process (Ruprai, 2011). Oxidants are produced in high proportions in cases with asthma compared with healthy subjects (Kleniewska andPawliczak, 2017). Salbutamol and bambuterol also inhibit the transcription factors such as NFKB (Farmer andPugin, 2000; Parry andMackman, 1997) that regulate the expression of some inflammatory enzymes, like inducible nitric oxide synthase (iNOs) (Xie et al., 1994). iNOs are responsible for the production of NO which can react with superoxide forming peroxynitrite (Radi, 2018), a powerful oxidant that can initiate lipid peroxidation (Radi et al., 1991).

Also, the present experiment has shown that oral salbutamol and bambuterol significantly decreased the level of MDA produced due to lipid peroxidation such as inflammatory cells that produce free radicals which destroy cell membrane by attacking their lipids and producing peroxide radicals and aldehyde such as MDA (Sharma et al., 2003). The inhibition in MDA could be attributed to the decrease of TNFα and NO. These results were in agreement with those of previous investigators who studied the effect of oral salbutamol (2mg/kg) on the lipid peroxidation level in carrageenan injected to rat paws (Uzkeser et al., 2012).

CONCLUSION

In the present study, we provide evidence that combination of short and long-acting β_2 agonists (salbutamol and bambuterol) as well as the combination of salbutamol or bambuterol with prednisolone, have anti-inflammatory effects in addition to their antioxidant activities in both lung tissue and broncho-alveolar lavage on the experimentally induced asthma..

CONFLICTS OF INTEREST

All authors declared no conflict of interest.

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