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Original Article

Antiinflammatory Effect of Montelukast versus Dexamethasone on a COPD Model induced by Chronic Exposure to Lipopolysaccharide in Guinea Pigs

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A B S T R A C T

Background: Although corticosteroids are currently drugs of choice for anti-inflammatory therapy, the inflammatory process in chronic obstructive pulmonary disease (COPD) is essentially steroid-resistant.

Aim: The aim of the present study was to assess the effects of oral treatment with montelukast (10 and 30mg/kg) or dexamethasone (20 mg/kg) for 20 days on COPD model induced by chronic exposure to lipopolysaccharide (LPS) in guinea pigs.

Methods: Six groups of six male guinea pigs were studied. Group 1: naïve group, group 2: exposed to saline nebulization. Groups 3, 4, 5 & 6: exposed to 9 nebulizations of LPS (30 μg/ml) for 1 h, 48 h apart with or without treatment with montelukast or dexamethasone. Airway hyperreactivity (AHR) to methacholine (MCh), histopathological study and bronchoalveolar lavage fluid (BALF) as well as lung tissues analyses were performed 48 hours after the final exposure to LPS(day20).

Results: LPS-induced pulmonary dysfunction was associated with increase neutrophil count, leukotriene (LT) B4 and tumor necrosis factor (TNF)-α in BALF. Besides, there was a decreases in malondialdehyde (MDA) level and histone deacetylases(HDAC) activity in lung tissue. Inflammatory cells in lung parenchyma were also detected. Both montelukast (10 or 30 mg /kg) and dexamethasone reduced significantly neutrophils count in BALF and inflammatory cells in lung parenchyma as well as TNF-α, and MDA levels. However, dexamethasone was more effective ($p<0.05$). Montelukast, at a dose of 30 mg /kg, was effective in ameliorating the pulmonary dysfunction. This was evidenced by reducing specific airway resistance after the 9th LPS exposure, attenuating AHR to MCh, decreasing LTB_4 and increasing HDAC activity.

Conclusions: These results suggest that relieving inflammation with montelukast (30 mg /kg) can be useful as a therapeutic approach in chronic airway inflammatory diseases including COPD in a model of pulmonary neutrophilic inflammation poorly responsive to glucocorticoids.

Key Words: Airway hyperreactivity, dexamethasone, lipopolysaccharide, montelukast, histone deacetylases, chronic obstructive pulmonary disease

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1. INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory airway disease with progressive and irreversible airflow limitation. Because airway inflammation in COPD is refractory to corticosteroids, COPD treatment is hindered and insufficient **(Barnes and Adcock, 2009).** New investigations into the mechanisms of glucocorticoid action have broadened and deepened our understanding of glucocorticoid resistance. Cellular and molecular factors, receptors, and complex signaling pathways have all been implicated in glucocorticoid resistance. Indeed, based on molecular biological studies, excessive activation of intracellular transcription factors, impaired histone deacetylase activity, and epigenetic factors (such as miR-18 and miR-124a) may result in glucocorticoid resistance **(Wang et al., 2010).**

The major anti-inflammatory effects of glucocorticoids appear to be largely due to interaction between the activated glucocorticoid receptor and transcription factors, notably nuclear factor-kappa B (NF-kappa B) that mediates the expression of

inflammatory genes. NF-kappa B switches on inflammatory genes via a process involving recruitment of transcriptional coactivator proteins and changes in chromatin modifications such as histone acetylation. The interactions between NF-kappa B and the activated glucocorticoid receptor result in differing effects on histone acetylation and deacetylation processes **(Milara et al., 2011)**.

Acetylation of histones by histone acetyltransferases activates inflammatory genes, whereas histone deacetylation results in inflammatory gene repression. Glucocorticoids exert their anti-inflammatory effects partly by inducing acetylation of anti-inflammatory genes , but mainly by recruiting histone deacetylase-2 (HDAC2) to activated inflammatory genes. HDAC2 deacetylates acetylated glucocorticoid receptors so that they can suppress activated inflammatory genes in asthma. In COPD, there is resistance to the antiinflammatory actions of glucocorticoids, which is explained by reduced activity and expression of HDAC2 **(Barnes, 2009)**.

Neutrophils provide a defense against infections that cause exacerbations of COPD, but persistent neutrophilia in the absence of infection in COPD reflects the chronic inflammatory state of the airways in this condition **(Thompson et al., 1989).** Leukotriene (LT) B_4 is a potent chemoattractant and activator of neutrophils and eosinophils **(Claesson et al., 1992).** Neutrophils rapidly release large amounts of LTB_4 in response to activating stimuli and have a high density of cell-surface LTB_4 receptors **(Chaney et al.,1992).** This suggests a potential involvement of LTB_4 in the induction of neutrophil survival by lipopolysaccharide (LPS) activation **(Conklyn et al., 1997).**

Montelukast antagonizes effectively the proasthmatic/proinflammatory/priming activities of cysteinyl leukotrienes (CysLTs) and forms part of numerous international guidelines for asthma therapy **(Bateman et al., 2008).** Interestingly, recent evidence suggests that montelukast possesses a range of secondary anti-inflammatory activities, apparently unrelated to antagonism of cysLTs receptors **(Tintinger et al., 2010).** These CysLT receptor - independent, anti-inflammatory mechanisms of action of montelukast may be particularly effective in controlling the glucocorticoid-insensitive inflammation and are the major focus of the current study.

The aim of the present study was to determine the influence of therapeutic (10mg/kg, **Tsuchida et al., 2008**) and high (30mg/kg, **Wu et al., 2006**) doses of montelukast in comparison to dexamethasone (20 mg/kg) on the functional and histopathological effects of chronic pulmonary inflammation induced by repeated exposure of guinea pigs to LPS as a model for the progressive inflammatory processes of COPD (neutrophilic inflammation) **(Toward and Broadley, 2001)**. The mediators measured in this study were LTB_4 and tumor necrosis factor (TNF)-α released in bronchoaveolar lavage fluid (BALF). The effects of montelukast on malondialdehyde (MDA) level and HDAC activity in lung tissue in comparison to dexamethasone were also assessed.

2. MATERIALS AND METHODS

2.1. Animals

Male guinea pigs, weighing 300 to 400 g, were used in this study. Animals were kept under standard laboratory conditions (12/12 h light/dark cycle, 22 ± 2ºC room temperature, 50-60% humidity) for at least 1 week before starting the experiments. All animal procedures were approved by the Institutional Animal Ethics Committee for Ain Shams University, Faculty of Medicine.

2.2. Drugs and chemicals

Lipopolysaccharides from Escherichia coli serotype 055-85, methacholine chloride, carboxymethylcellulose sodium and dexamethasone sodium phosphate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Montelukast sodium was generous gift from Merk, Sharp and Dohme, USA. LPS and methacholine were dissolved in phosphate-buffered saline (PBS) with pH 7.4. Dexamethasone and montelukast were suspended in a 0.5% solution of carboxymethylcellulose sodium as a vehicle **(Kaneko et al., 2007).**

2.3. Experimental design

Guinea pigs were randomly allocated into six groups (each consisted of 6 animals) as follows:

- N: naive group
- C: control; saline exposed and administered orally 1 ml solution of carboxy methylcellulose sodium (0.5%) daily for 20 consecutive days.
- LPS: LPS exposed and administered orally 1 ml solution of carboxy methyl cellulose (0.5%) daily for 20 consecutive days.
- LPS/Mont (10): LPS- exposed and orally treated with montelukast (10 mg/kg/day) for 20 consecutive days.
- LPS/Mont (30): LPS- exposed and orally treated with montelukast (30 mg/kg/day) for 20 consecutive days.
- LPS/Dex: LPS- exposed and pretreated with dexamethasone (20mg/kg/day) for 20 consecutive days.

All groups (except naïve group) were exposed for 1 h to an aerosolized solution of LPS (30 μg/ml) or saline. The aerosol was generated by PARI Jet nebulizer (HSE, Germany), at a rate of 0.5 ml/min. Animals were exposed to the aerosolized solution 9 times, 48 h apart **(Toward and Broadley, 2001).** The average of two specific airway resistance (sGaw) measurements was obtained prior to exposure (baseline) and 48 h after the first and final exposure. Treatment with the test drugs and vehicle were started one day before first LPS exposure and continued for 2 days after the last LPS exposure i.e. for 20 days. Methacholine (MCh)-induced airway hyperreactivity (AHR) was measured 48 h after the last exposure to LPS, and then BALF was collected and the lungs were isolated. BALF was used for estimation of LTB_4 and TNF α and the isolated lungs were used for histological studies and for estimation of MDA level as well as HDAC activity.

2.3.1. Measurement of respiratory function

Respiratory function was measured according to **Pennock et al. (1979).** Conscious guinea pigs were placed in cylindrical double box body plethysmograph. Respiratory flow in the body chamber of plethysmograph was measured indirectly by changes in the thoracic gas volume (body box) during respiration. These primary flow signals (nasal and thoracic) were transmitted via a differential pressure transducer (Validyne D P 45-14) and amplifier in the form of PLUGSYS with CFBA module to a data acquisition/analysis system (Pulmodyn software) from HSE (Hugo Sachs Electronic). Specific airway resistance (sGaw) is determined from the phase displacement between the nasal and thoracic flow. A minimum of five breaths were analyzed for each animal at each time point. Before each experiment, the animals were handled and familiarized with the equipment to reduce stress. sGaw was expressed as mmHg/s and increases in sGaw were expressed as percentage of baseline values before drug treatment and were compared with control values. Furthermore, changes in sGaw were expressed as percentage of value of drug-treated group against that of LPS control.

2.3.2. Measurement of airway hyperreactivity

MCh-induced AHR were determined by modification of the method of **Tulić et al. (2000) and Kasahara et al. (2005).** Increasing concentrations of MCh (0.125, 0.25, 0.5, 1, 2, and 4 mg/ml) were delivered by a PARI Jet nebulizer (HSE; Germany), at a rate of 0.5 ml/ min. Each concentration of MCh was delivered for 1 min at 5 min interval. Peak values of sGaw after each concentration of MCh were recorded. The AHR of each group was expressed as PC100 MCh (concentration of MCh that causes an increase of specific airway resistance by 100% over the baseline value). The PC100 was calculated by linear interpolation of graphed data.

2.3.3. Bronchoalveolar lavage fluid analysis

Bronchoalveolar lavage was performed as follows: Guinea pigs were sacrificed with an overdose of pentobarbital sodium (400 mg/ kg; i.p.). The lungs were lavaged via tracheal cannula with 50 ml of PBS (5 × 10 ml), which were aspirated after a gentle chest massage. The pooled BALF was centrifuged at 500g, at 4°C for 10 min, and the pellet was resuspended in 0.25% NaCl to lyse residual erythrocytes; after centrifugation, the pellet was resuspended again in 1 ml of 0.9% NaCl. A total cell count (cells/ml) of the pooled BALF was determined using a hemocytometer (Neubauer). A Cytospin smear (Shandon Centrifuge: 1000 rpm – 7 min) of the BALF samples was differentially stained (Leishman's: 1.5% in methanol, 6 min), and a minimum of 200 cells were counted. The results were expressed as a percentage of total cells and as the actual number of each cell type **(Underwood et al., 2000).**

2.3.4. Biochemical parameters

2.3.4.1. Bronchoalveolar lavage fluid

A. Measurement of Tumor necrosis factor-α

The levels of TNF-α in BALF supernatants were assessed by commercially available ELISA using Rat Biosource International (California USA) micro titer strips diagnostic kit. The assay was performed according to the instructions of the manufacturers. The detection limit was 4.5 pg/ml.

B. Measurement of Leukotriene B_4

LTB_4 was measured by enzyme immunoassay (Cayman Chemicals, Ann Arbor, MI, USA). The assay was performed according to the instructions of the manufacturers. The detection limit was 7 pg/ml**.**

2.3.4.2. Lung tissue homogenates

After measurement of AHR, the lungs were removed. Care was taken to remove adhering tissues such as bronchia and vessels. The right lung lobe and the trachea of each lung were cut for histological studies. The other parts of the lung were then homogenized in 10 ml PBS. An aliquot of 0.5 mL of homogenate was hydrolyzed by 0.5 mL of 12N HCl at 120°C for 24 h and used for the measurement of malodialdehyde and histone deacetylase.

A. Measurement of lung malondialdehyde

Lipid peroxides (LP) levels were determined as thiobarbituric acid reactive substances (TBARS) according to the method of **Buege and Aust (1978)**. Results were expressed as nanomoles of malondialdehyde (MDA), / mg wet tissue weight.

B. Measurement of lung histone deacetylase

Histone deacetylase (HDAC) activity was measured using a fluorometric assay kit (Sigma-Aldrich) based on a two-step enzymatic reaction. The measured fluorescence is directly proportional to the deacetylation activity of the sample **(Wegener et al., 2003).** HDAC activity was monitored with excitation at 490 nm and emission at 525 nm.

2.3.5. Lung histopathology

Histological examination of the lungs was undertaken to determine whether the inflammatory cell profile obtained from BALF was representative of tissue inflammation. After lavage, the lungs were removed from the thoracic cavity, the right lung lobe and the trachea of each lung were cut as mentioned above and immersed in neutral-buffered formalin for at least 72 h. After fixation, samples were cut, dehydrated in 70 to 100% ethanol/xylene, and embedded in paraffin wax. Sections were cut (6 μm), deparaffinized, and stained with hematoxylin and eosin. Inflammatory cells were identified by standard morphometry. Numbers of cells in tissue and air spaces were counted in 10 random, nonoverlapping parenchymal fields at × 100 magnifications under bright field illumination **(Tulić et al., 2000).**

2.3.6. Statistical Analysis

Changes in sGaw from the baseline values (taken before the procedure) are presented as a percentage of the mean baseline value preceding the first LPS challenge. Changes in airway function between groups were compared using analysis of variance, followed by Newman-Keuls Multiple Comparison Test. Differences were considered statistically significant when $p < 0.05$. The airway hyper-reactivity of each group is expressed as PC100 methacholine. The PC100 was calculated by linear interpolation. BAL fluid cell counts , MDA, TNF-α, LTB_4 and HDAC activity were compared using analysis of variance, followed by Newman-Keuls Multiple Comparison Test. Differences were considered statistically significant when $p < 0.05$.

3. RESULTS

3.1. Airway function studies

3.1.1. Effects of montelukast and dexamethasone on respiratory function

First exposure to LPS caused an immediate bronchoconstriction (+15.24% increase from baseline sGaw values) lasting 30 min; however, this effect was not significantly different ($p > 0.05$) from the response to saline (Figure 1). All subsequent LPS exposures caused nearly the same response. The 9th exposure to LPS caused a significant elevation in sGaw of +31.1% and 27.22% from baseline and saline control group values, respectively, this outcome persisted for more than 2 h.

Again, montelukast (10 and 30 mg/kg) and dexamethasone (20 mg/kg) treatment did not significantly affect ($p>0.05$) the initial LPS-induced bronchoconstriction after the first exposure. Moreover, montelukast (10mg/kg) did not significantly affect ($p>0.05$) the LPS-induced bronchoconstriction after the ninth exposure. On the other hand, dexamethasone tended to exaggerate the bronchoconstrictor response to LPS after the 9th ninth LPS exposure, (+12.33% increase from LPS control group sGaw value). In contrast, guinea pigs treated with montelukast (30 mg/kg) developed significant ($p < 0.05$) bronchodilation (-15.58% decrease from LPS control group sGaw values) after 9th exposure to LPS (Table 1 and Figure 1).

3.1.2 Effect of montelukast and dexamethasone on airway hyperreactivity

Airway hyperreactivity was determined 48 h after the 9th exposure to LPS. Responses of each group to increasing concentration of MCh are shown in Figure 2 (A and B). Inhalation of various concentration of MCh (0.125-4.0 mg/ml) caused an increase in the sGaw over the baseline in saline - exposed guinea pigs. As anticipated, the control LPS group showed the highest response to MCh among all groups studied. The PC100 value of the LPS control group was significantly reduced, compared with that of the saline control group with percent reduction of 64.19% ($P<0.05$).

Treatment with montelukast (10 and 30 mg/kg) and dexamethasone (20 mg/kg) caused an elevation of 19.0%,70.13% and 37.66% in the mean PC100 of inhaled MCh compared to that of LPS control group, respectively. The increase in the mean PC100 of inhaled MCh in montelukast (10 mg/kg) or dexamethasone- treated groups was insignificant compared to that of LPS control group. Moreover, there was no significant between these two groups ($P>0.05$). The PC100 value of montelukast(30 mg/kg) treated group was raised significantly compared to that of LPS control group.

3.2. Bronchoalveolar lavage fluid

The total leukocyte count in BALF in the control LPS group was significantly ($p < 0.05$) amplified ($31.78\pm1.17\times 10^6$ cells, $p < 0.05$), after the 9th exposure to LPS, compared with cells in the saline exposed group. Differential cell counts revealed that the number of macrophages ($16.62\pm0.12\times 10^6$ cells, $p < 0.05$), eosinophils ($2.8\pm 0.1\times 10^6$ cells, $p < 0.05$), and neutrophils ($12.30\pm1.3\times 10^6$ cells, $p < 0.05$) were significantly more in the control LPS group compared with those in the saline- exposed group (Figure 3 and Table2).

Treatment with montelukast (10 and 30 mg/kg) and dexamethasone significantly reduced the number of macrophages (41.03%, 49.16%, and 58.1%, respectively), eosinophils (65%, 78.93%, and 87.5%, respectively), and neutrophils (25.85%, 33.58% and 45.69%, respectively) in the BALF, at 48 h after the 9th LPS exposure. The resultant airway neutrophilia was significantly reduced in a dose-related fashion by administration of montelukast ($P < 0.05$)

3.3. Biochemical Parameters

3. 3.1. Tumor necrosis factor- α

Levels of TNF-α were significantly ($p < 0.05$) higher in LPS-exposed guinea pigs as compared to saline - exposed guinea pigs (Figure 4A). Pretreatment with montelukast (10 and 30 mg/kg) and dexamethasone (20 mg/kg) significantly ($p < 0.05$) reduced the TNF-α levels in the BALF as compared to LPS- exposed guinea pigs .

3.3. 2. Leukotriene B_4

Levels of LTB_4 were significantly ($p < 0.05$) higher in LPS- exposed guinea pigs as compared to saline exposed guinea pigs (Figure 4B) . Pretreatment with montelukast (10 mg/kg) did not significantly alter LTB_4 levels in the BALF. In contrast, montelukast (30 mg/kg) and dexamethasone (20 mg/kg) significantly ($p < 0.05$) reduced the LTB_4 levels compared to LPS exposed guinea pigs. Montelukast (30 mg/kg) was however more effective as compared with dexamethasone ($p < 0.05$).

3.3.3 Malondialdehyde

Lung MDA contents in the control LPS group were significantly increased ($p < 0.05$), compared with those in the control saline group. Lung MDA contents in the montelukast (10 and 30 mg/kg) and dexamethasone (20 mg/kg) treated groups were found to be significantly reduced compared with those in the LPS exposed group. There was a significant difference between montelukast (10mg/kg) and dexamethasone (20 mg/kg) treated groups but the difference between montelukast (30mg/kg) and dexamethasone were not significant ($p > 0.05$) (Figure 5A).

3. 3.4. Histone deacetylase

Lung HDAC activity was significantly decreased in LPS group. Pretreatment with montelukast (30 mg/kg) significantly ($p < 0.05$) increased HDAC activity. On the other hand, montelukast (10 mg/kg) and dexamethasone (20 mg/kg) had insignificant ($p > 0.05$) effect on the decrease in HDAC activity induced by LPS (Figure 5B).

3.4. Lung histopathology

The average number of inflammatory cells in 10 randomly chosen fields (500 μm squares) counted with light microscopy is shown in Figure 6. The number of cells counted in the control LPS group was significantly increased (47.1 ± 5.17 cells/field, $p < 0.01$), compared with the cells in the saline ~~control~~ group (14.1 ± 0.60 cells/field). The increase in the number of cells induced by LPS was significantly inhibited by montelukast (10 mg/kg group, 34.7 ± 2.76 cells/field; 30 mg/kg group, 23.1 ± 3.05 cells/field; $p < 0.05$) and dexamethasone (29.1 ± 1.07 cells/field; $p < 0.05$).

Table (1): The Effect of oral treatment with montelukast and dexamethasone on airway function of conscious guinea pigs exposed to chronic lipopolysaccharide (LPS) inhalation

Groups	Naïve	Saline control	LPS control	Montelukast (10 mg/kg) /LPS	Montelukast (30 mg/kg) /LPS	Dexamethasone (20 mg/kg) / LPS
Measurements						
sGaw(cm H2O×s)						
Baseline	2.98±0.09	3.1±0.08	3.28±0.09	3.12±0.16	3.17±0.12	3.17±0.16
First exposure	3.05±0.06	3.2±0.19	3.78±1.7	3.45±0.19	3.32±0.07	3.71±0.12
%change from LPS control				-8.73%	-12.17%	-3.35%
%change from dexamethasone				-7.0%	-10.51%	
Ninth exposure	3.2±0.11	3.38±0.15	4.3±0.24	4.1±0.11	3.63±0.13	4.83±0.26
%change from LPS control				-4.65%	-15.58% #	+12.33%
%change from dexamethasone				-15.11%*	-24.84%*	

Chronic exposure means 9 exposures to either nebulized LPS (30 mg/ml) or vehicle (saline), for 1 h, 48 h apart. Drugs treatment started 1 day before LPS exposure and continued daily for 20 days. Airway function was expressed as specific airway conductance (sGaw). Each value represents the mean ± S.E.M. (n = 6) Positive values represent bronchoconstriction. Negative values represent bronchodilation. Significance of differences from LPS exposures (#, p< 0.05) and Significance of differences from dexamethasone treatment (*, p < 0.05) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

Table (2): Effect of oral treatment with montelukast or dexamethasone on airway influx of inflammatory cells (leukocytes count) after chronic exposure to LPS or saline

Cells per Bronchoalveolar Lavage Sample (×10^6)						
Groups	**Naïve**	**Chronic saline**	**Chronic LPS**	**Chronic LPS and montelukast (10mg/kg)**	**Chronic LPS and montelukast (30mg/kg)**	**Chronic LPS and dexamethasone**
Measurements						
Total Cell Count	7.06 ±0.09	4.42±0.1	31.78±1.17	19.25±0.28	17.68±0.3	14.28±0.2
%change from LPS control				-39.43%#	-44.37%#	-55.07%#
%change from dexamethasone				+25.82%*	+19.23%	
Macrophages	6.48±0.11	3.58±0.05	16.62±0.12	9. 8±0.07	8.45±0.1	6.82±0.12
%change from LPS control				-41.03%#	-49.16%#	-58.1%#
%change from dexamethasone				+43.7%*	+23.9%*	
Eosinophils	0.3±0.02	0.62±0.05	2.8± 0.1	0.98± 0.03	0.59±0.02	0.35± 0.01
%change from LPS control				-65%#	-78.93%#	-87.5%#
%change from dexamethasone				+64.29%*	+39.8%*	
Neutrophils	0.0±0.0	0.07±0.01	12.30±1.3	9.12±0.05	8.17±0.12	6.68±0.08
%change from LPS control				-25.85%#	-33.58%#	-45.69%#
%change from dexamethasone				+36.53%*	+16.34%	

Total and differential cell counts (macrophages, eosinophils, and neutrophils) were made in bronchoalveolar lavage fluid removed from guinea pigs 48 h after chronic (nine) exposures (1 h, 48 h apart) to LPS (30 mg/ml) or vehicle (saline) by inhalation . Values represent the mean ± S.E.M. (n = 6) of the cells per sample (×10^6). Significance of differences from LPS exposures (#, p< 0.05) and significance of differences from dexamethasone treatment (*, p < 0.05) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

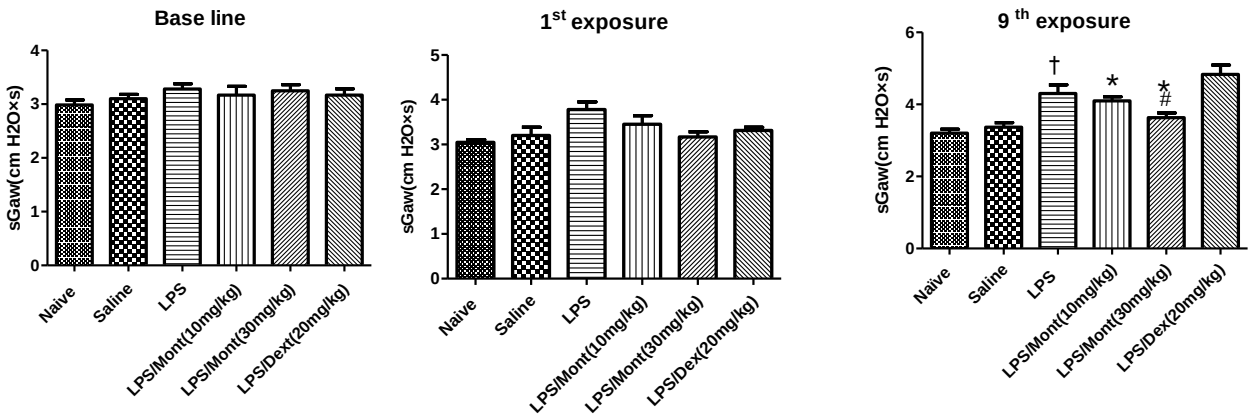

Fig. 1: Effect of oral treatment with montelukast (Mont) (10 and 30mg/kg) and dexamethasone (Dex) on airway function of conscious guinea pigs during chronic exposure (9 times) to LPS (30 mg/ml) or vehicle (saline) inhalation, for 1 h, 48 h apart. Drug treatment started 1 day before LPS exposure and continued daily for 20 days. Airway function was expressed as specific airway conductance (sGaw). Each point represents the mean ± S.E.M. (n = 6). sGaw (cm of H_2O/s). Significance of differences from saline exposure (†, p< 0.05), significance of differences from LPS exposures (#, p< 0.05) and significance of differences from dexamethasone treatment (*, p < 0.05) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

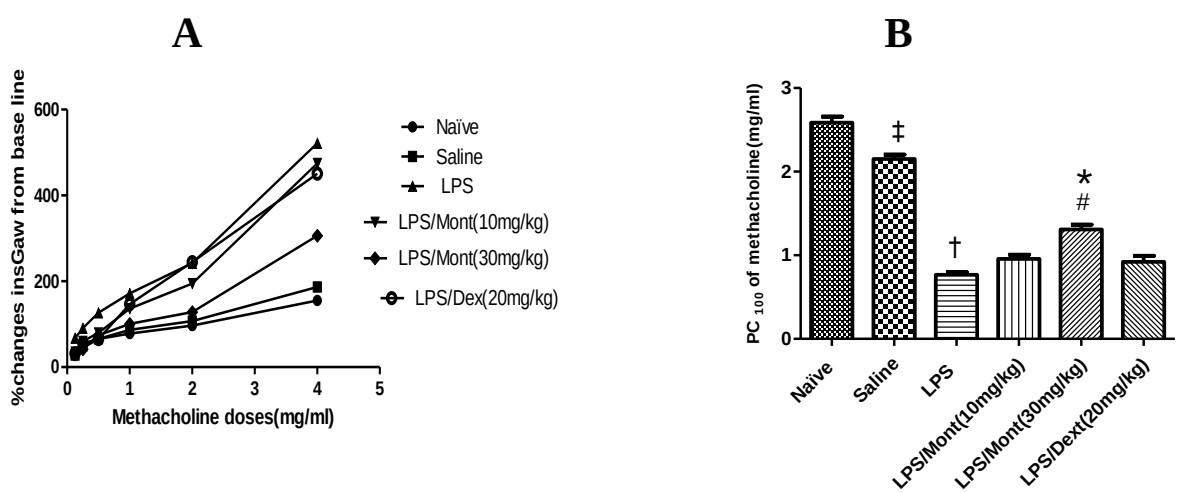

Fig. 2: Effect of oral treatment with montelukast (Mont) (10 and 30mg/kg) and dexamethasone (Dex) on airway hyperreactivity (AHR)

AHR was examined 48 h after the 9th exposure to LPS with increasing concentration of methacholine.
(**A**) Airway response to gradual increasing doses of inhaled methacholine (mg/ml) is expressed as relative bronchoconstriction (percent increase in specific airway resistance from base line).
(**B**) Airway hyperreactivity is expressed as PC100 (the methacholine dose, in mg/kg, at which 100% bronchoconstriction was induced. Values given are means ± S.E.M. (n=6). Mont: montelukast, Dex: dexamethasone. Significance of differences from naïve group (‡, p < 0.05), significance of differences from saline exposure (†, p< 0.05), significance of differences from LPS exposure (#, p< 0.05) and significance of differences from dexamethasone treatment (*, p < 0.05) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

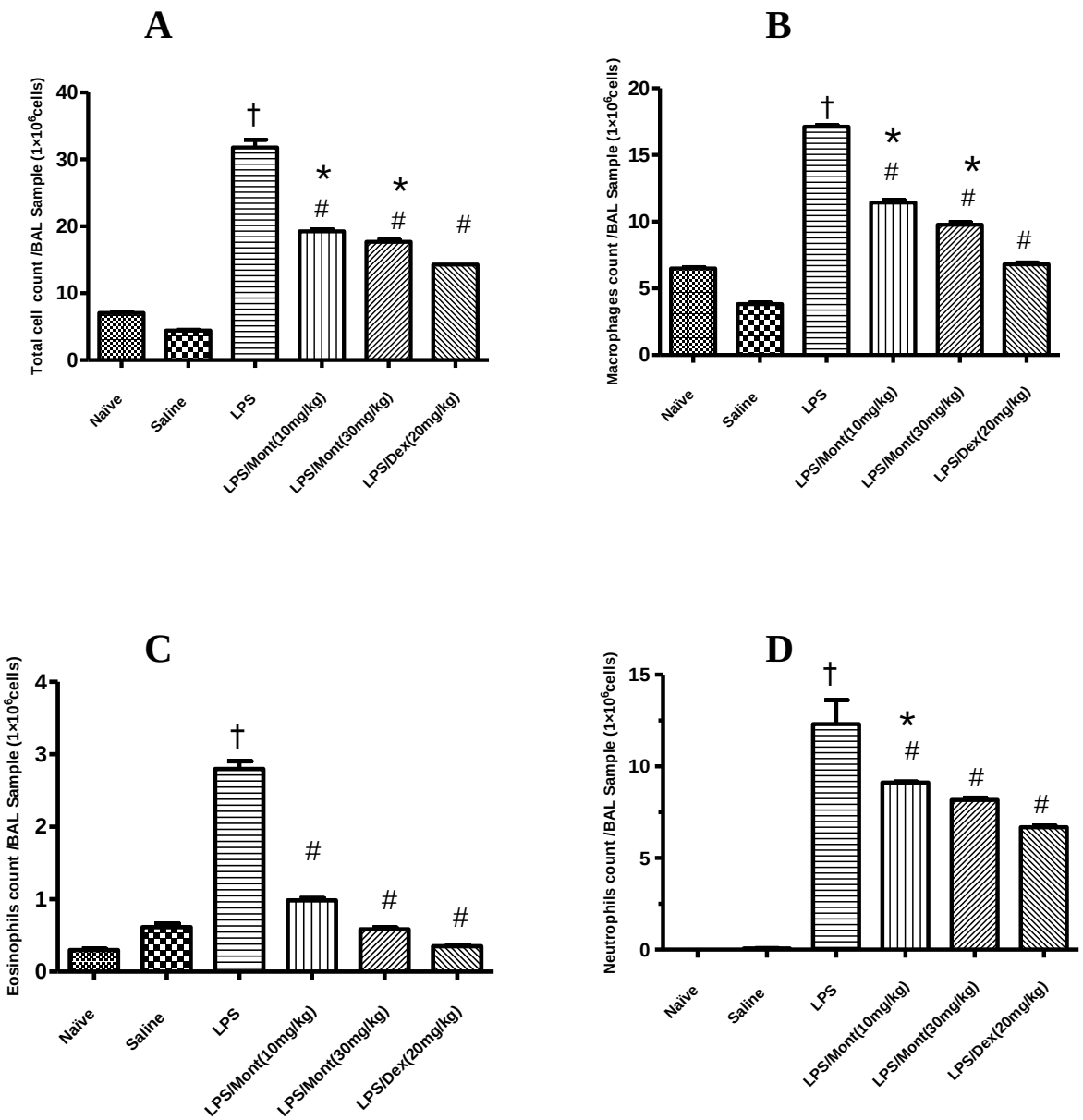

Fig. 3: Effect of oral treatment with montelukast (Mont) (10 and 30mg/kg) and dexamethasone (Dex) on the total number of cells (A), macrophages (B), eosinophils (C) and neutrophils (D) in samples from the bronchoalveolar lavage (BAL).

Cells were counted 48 h after the 9th exposure to LPS. Total cell counts and differential cell counts were carried out with light microscopy after staining with Leishman's. Mont: montelukast, Dex: dexamethasone. Each column represents means ± S.E.M. (n=6). Significance of differences from saline exposures (†, $p< 0.05$), significance of differences from LPS exposures (#, $p< 0.05$) and significance of differences from dexamethasone treatment (*, $p < 0.05$) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

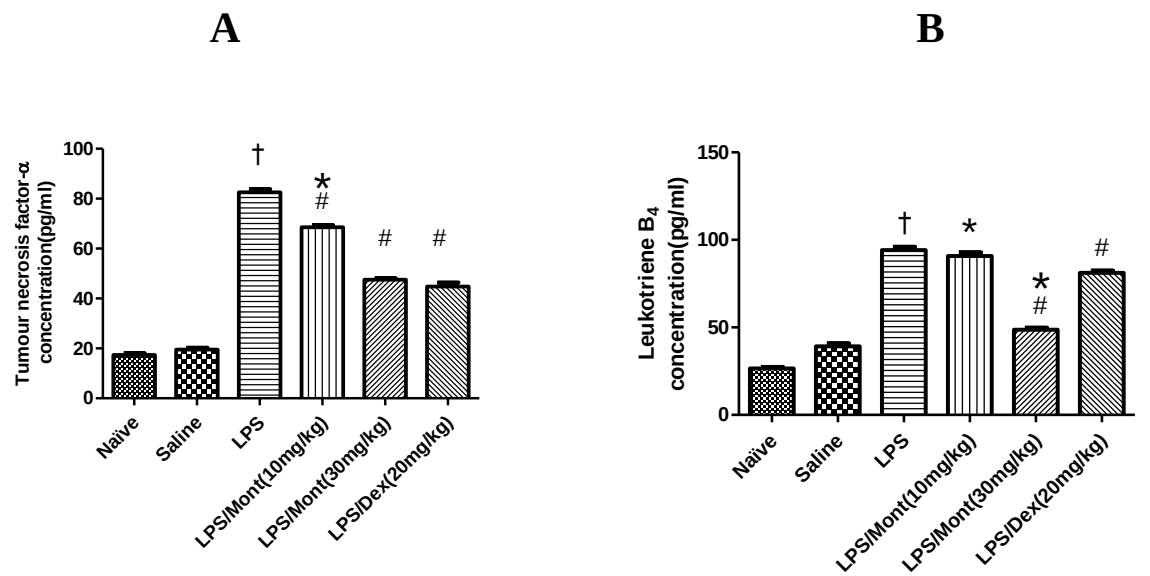

Fig. 4: Effect of oral treatment with montelukast (Mont) (10 and 30mg/kg) and dexamethasone(Dex) on release of **A:** Tumor necrosis factor-α . **B:** Leukotriene B_4 in BALF 48 h after the 9th exposure to LPS in guinea pigs. Each column shows means ± S.E.M. (*n*=6). Significance of differences from saline exposures († $p< 0.05$), significance of differences from LPS exposures (# $p< 0.05$) and significance of differences from dexamethasone treatment (*$p < 0.05$) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

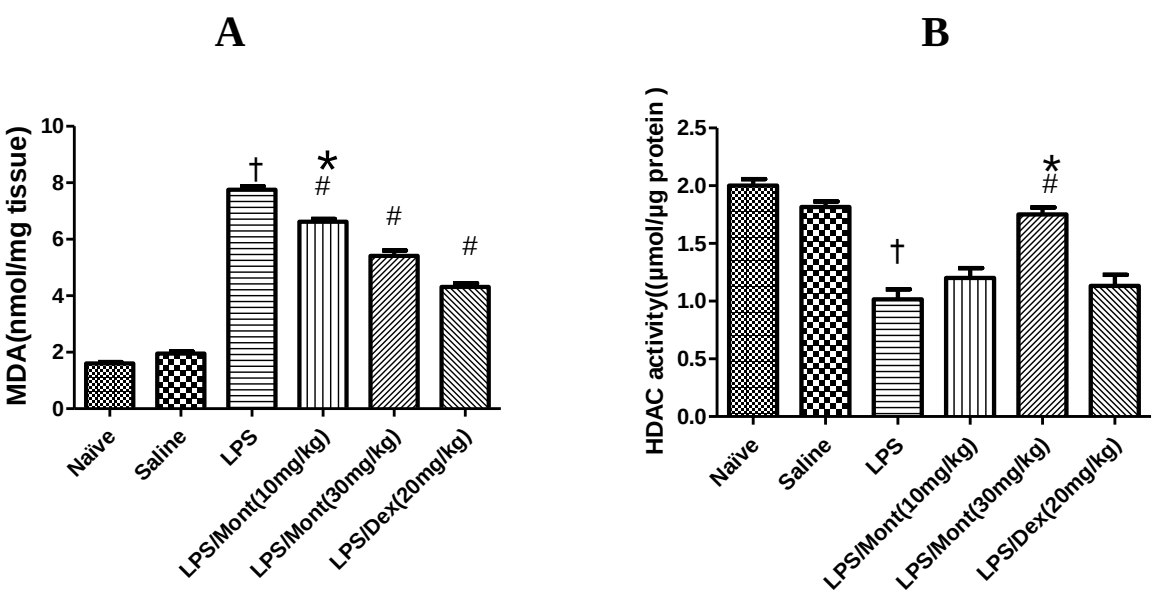

Fig. 5: Effect of oral treatment with montelukast (Mont) (10 and 30mg/kg) and dexamethasone (Dex) on A: malondialdehyde (MDA) level and B: Histone deacetylase (HDAC) activity in lung tissue homogenate 48 h after the 9th exposure to LPS in guinea pigs. Each column shows means ± S.E.M. (*n*=6). Significance of differences from saline exposures (†, $p< 0.05$), significance of differences from LPS exposures (#, $p< 0.05$) and significance of differences from dexamethasone treatment (*, $p < 0.05$) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

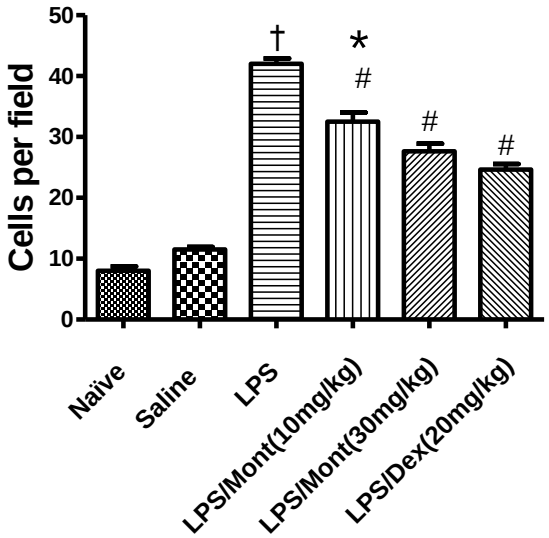

Fig. 6: Effect of oral treatment with montelukast (Mont) (10 and 30mg/kg) and dexamethasone(Dex) on average number of inflammatory cells in ten randomly chosen fields (500 μm squares) measured from 10 randomly selected lines passing through the lung in H&E stained sections taken 48 h after the 9th exposure to LPS. Mont: montelukast, Dex: dexamethasone. Each column represents means ± S.E.M. (n=6). Significance of differences from saline exposures (†, $p< 0.05$), significance of differences from LPS exposures (#, $p< 0.05$) and significance of differences from dexamethasone treatment (*, $p < 0.05$) were determined by analysis of variance (single factor), followed by Newman-Keuls Multiple Comparison Test.

4. DISCUSSION

In the present study bacterial inflammation by exposure to aerosolized LPS, exhibited COPD-like pathophysiological pulmonary features similar to those reported for airway obstruction (the increase in specific airway resistance), airway hyperresponsiveness (AHR) to inhaled MCh and increased total cell count in the BALF, predominantly as a result of neutrophil influx. Many studies have documented the prominence of neutrophils after administration of LPS, either via systemic or inhaled routes **(Lefort et al., 1998).** Inflammatory response is evident in the lung parenchyma after exposure to LPS in the present study and this was demonstrated previously by **Pauwels and coworkers (1990).** In addition, LPS exposure in the present study upregulated the production of TNF-α and LTB_4 in the BALF, as well as elevated MDA level and lowered HDAC activity in the lung tissue.

Dexamethasone (20 mg/kg), amplified the bronchoconstrictor response to LPS after the 9th LPS exposure but not to any significant extent. In fact, Toward and Broadler (2001) demonstrated that dexamethasone (20 mg/ kg, i.p.) exacerbated bronchoconstrictions in guinea pigs chronically exposed to LPS. In contrast, montelukast (30mg/kg)-treated guinea pigs developed significant bronchodilation after the 9th exposure to LPS.

In the present study, AHR was examined as a functional endpoint and it was found that the control LPS group showed the highest response to MCh among all studied groups. Low dose of montelukast did not significantly inhibit the airway response to gradual increasing doses of MCh. This is in agreement with Tsuchida **et al (2008)** who found that montelukast (10 mg/kg, p.o.), inhibited airway hyperresponsiveness to MCh in ovalbumin-induced asthma models, a milder form of asthma with low airway hyperreactivity. An important finding is that AHR was resistant to dexamethasone therapy. Pretreatment with high dose montelukast (30mg/kg), however, inhibited the development of LPS–induced AHR, indicating that montelukast played a central role in the inhibition of steroid-resistant AHR.

Results of the present study revealed that, pretreatment with montelukast (10 and 30 mg/kg) and dexamethasone (20 mg/kg) significantly reduced the number of macrophages, eosinophils, and neutrophils in the BALF, 48 h after the 9th LPS exposure. The resultant airway neutrophilia was significantly reduced in a dose-related fashion by administration of montelukast. In addition, the accumulation of inflammatory cells in the lung parenchyma after the 9th exposure to LPS was inhibited by treatment with either montelukast or dexamethasone.

Our results support the findings of Toward and Broadler (2001) who reported that dexamethasone reduced neutrophil influx at a dose of 20 mg/kg p.o. in a guinea pig model of pulmonary edema induced by chronic LPS exposure. Besides, Whelan (1996) reported inhibition of neutrophilia in guinea pigs after single exposure to LPS with treatment of 5 mg/kg dexamethasone**.** However, Kaneko et al. (2007) found that dexamethasone treatment (1mg/kg i.p.) did not

affect any increase in the total number of cells, neutrophils, and macrophages in BALF induced by chronic LPS exposure.

Belvisi (2004) reported that neutrophils are less sensitive to glucocorticoids than are eosinophils and T cells, and that macrophages from patients with COPD are less sensitive to steroid treatment under certain circumstances. **Whelan et al. (1995)** demonstrated that higher doses of dexamethasone were required to inhibit LPS-induced neutrophilia (ED50 10.8 mg/kg i.p.) in guinea-pig lungs. These differences in the responsiveness of activated inflammatory cells may help to explain why glucocorticoid treatment has been more beneficial for patients with asthma than for patients with COPD **(Belvisi , 2004)**.

Zhu et al. (2005) reported that although neutrophils do not produce CysLTs, they do possess receptors for LTC4 and LTD4, activation of which triggers relatively modest pro-inflammatory responses in these cells. Interference with neutrophil activation by CysLTs released from other cell types, such as monocytes/macrophages, mast cells or eosinophils, may therefore underlie the neutrophil-directed therapeutic efficacy of montelukast.

In the present study, montelukast significantly reduced the LTB_4 levels compared to LPS exposed guinea pigs. This is in agreement with **Anderson et al. (2009)** who demonstrated that montelukast markedly attenuated LTB4 production by platelets activating factor-activated neutrophils with maximal inhibition (89%) observed at concentrations of 2 μM.

TNF-α is one of the important inflammatory mediators in COPD and its level is overstressed in the sputum of patients with COPD **(Keatings et al., 1996).** In this study, guinea pigs exposed to LPS, showed significantly higher levels of TNF-α in BALF than saline- exposed guinea pigs. Pretreatment with montelukast (10 and 30mg/kg) and dexamethasone (20 mg/kg), significantly reduced TNF-α levels in the BALF. Both montelukast (30 mg/kg) and dexamethasone were more effective than montelukast (10 mg/kg); but there was no significant difference between montelukast (30 mg/kg) and dexamethasone treated groups.

Basyigit et al. (2010) evaluated the effects of montelukast (0.1 mg/kg i.p) in smoke-induced COPD in Wistar albino rats. They found that montelukast significantly decreased serum TNF-α levels and total histopathological damage score of the lung. There was no statistically significant difference between the montelukast group and healthy controls. The authors concluded that montelukast might have a protective effect on smoke-induced lung injury in rats both from a histopathological and inflammatory point of view.

Moreover, **Maeba et al. (2005)** examined the inhibitory effect of montelukast on LPS-induced TNF-α production in peripheral blood mononuclear cells. Montelukast ($10^{-5)}$ M) significantly inhibited LPS - induced TNF-α production in the peripheral blood mononuclear cells of controls and patients with asthma. In addition the study conducted by **Can et al (2006)** showed that montelukast improves clinical parameters and shows anti-inflammatory response by decreasing serum TNF-α level. Dexamethasone was less effective in reducing TNF-α release in guinea pigs exposed to LPS than montelukast (30mg/kg). This confirms previous data showing that neither inhaled nor oral glucocorticoids had any suppressive effect on TNFα regulation in COPD patients **(Barnes, 2003).**

The present results showed that lung MDA contents in the montelukast (10 and 30 mg/kg) and dexamethasone (20mg/kg) pretreated groups were significantly inhibited compared with those in the LPS- exposed group. There was a significant difference between montelukast (10 mg /kg) and dexamethasone pretreated groups, but the difference between montelukast (30 mg /kg) and dexamethasone were not significant. Similarly, the studies conducted by **Cuciureanu et al. (2009)** and **Sener et al. (2006)** indicate that montelukast can protect against experimental organ damage through its marked antioxidant effect by reducing MDA besides its anti-inflammatory effects by reducing TNF-α. Furthermore, the study conducted by **Du et al. (1998)** showed that MDA in the serum of asthmatic patients and in the serum and lung tissue of asthma models of guinea pigs were significantly decreased by dexamethasone inhalation.

In the present study HDAC activity was significantly less in lung homogenate from guinea pigs exposed to LPS as compared with saline - exposed group. Lung homogenate from guinea pigs exposed to LPS pretreated with montelukast (30 mg/kg) showed a significant increase in HDAC activity. In contrast, montelukast (10 mg/kg) and dexamethasone did not improve to any significant extent the decrease in HDAC activity induced by LPS. Reduction of HDAC activity by high dose montelukast may enhance inflammatory gene transcription that it may "unlock" the glucocorticoid resistance of COPD and potentiate its suppressive effects. This suggests that high dose montelukast may restore the antiinflammatory effect of glucocorticoids and thus controls the underlying disease process in COPD.

Tahan et al. (2008) reported that montelukast at concentrations of 0.01–10 μM resulted in substantial suppression of histone acetyl transferase (HAT) activity following activation of a monocyte/macrophage cell line with TNF-α. The precise molecular mechanism underlying these inhibitory effects of montelukast on HAT was not

established, but may involve interference with the activation of transcriptional coactivator proteins, a prerequisite for histone acetylation, chromatin unwinding, and gene transcription. Suppression of HAT activity may explain the increase in HDAC in the present study.

5. CONCLUSION

Montelukast improved some aspects of the pathological changes induced by chronic exposure to LPS. The HDAC-targeted anti-inflammatory activity of this agent demonstrated in the current study may contribute to the beneficial effects of this agent. In addition, montelukast attenuated the production of LTB4 representing an additional therapeutic activity that may contribute to the control of corticosteroid-insensitive neutrophil-mediated inflammation**.** Montelukast high dose might provide a way of managing this common and globally increasing disease. Nevertheless, this needs to be confirmed in clinical studies in COPD patients.

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