Original Article

Mitigation of Delayed Sodium Hypochlorite-Induced Lung Injury by Phosphodiesterase Enzyme Inhibitors (PDEIs), Pentoxifylline and Theophylline, in Guinea Pigs

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A B S T R A C T

Sodium hypochlorite (NaOCl) is widely used as an industrial material as well as an ingredient in household cleaning products. Exposure to high concentrations of NaOCl, a powerful oxidant, results in acute lung injury that may proceed to delayed airway hyper responsiveness and remodeling. The present study aims at investigating the effects of two nonselective PDEIs, pentoxifylline 'PTX' and theophylline 'THEO', versus dexamethasone 'DEX' on delayed airway functional and histopathological injury induced by NaOCl-inhalation in guinea pigs. Forty-eight guinea pigs were classified into 8 groups; 2 groups (control, 4% NaOCl-inhalation for 20 min) and another 6 groups were exposed to NaOCl and administrated intraperitoneally vehicle, PTX (50 mg/kg/day), THEO (50 mg/kg/day), DEX (20 mg/kg/day), PTX+DEX or THEO+DEX for 3 weeks. Guinea pigs were assessed for airway functional, biochemical and histopathological dysfunctions. Treatment with PENT or THEO, as monotherapy or in combination with DEX, reduced airway resistance and bronchial reactivity to methacholine. Similar findings were noticed with inflammatory markers such as total cell count, neutrophil percentage and TNF-α in bronchoalveolar lavage and lung myeloperoxidase activity and neutrophil infiltration. These data were parallel to lung histopathology and Aschorft fibrosis score that were improved in treatment groups. PENT, but not THEO or DEX could ameliorate oxidative stress biomarkers, malondialdehyde and superoxide dismutase, in lungs. Co-administration of PENT or THEO with DEX improved the effect of DEX on NaOCl-induced airway injury. In conclusion, 'PENT and THEO' are effective in mitigation of delayed NaOCl-induced lung injury in guinea pigs and if these findings were to translate into actual clinical benefit, they might provide a suitable alternative to corticosteroids or at least, reduce its dose needed in management of NaOCl and chlorine-induced lung toxicity.

Key Words: NaOCl-inhalation, Guinea pigs, Pentoxyfylline, Theophylline, Lung injury, Remodeling

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1. INTRODUCTION

Sodium hypochlorite (NaOCl) is widely used as an industrial material and an ingredient in household cleaning products as well as water disinfectant. Mixing NaOCl with strong acids (e.g. HCl) or ammonia releases chlorine gas or chloramine gases, respectively that are irritating to the respiratory tract *(Temple, 1983)*. There is an increasing opportunity for humans to be exposed to NaOCl-containing substances because household products such as mold-removing sprays that generate a NaOCl aerosol are widely sold and used. Patients suffered from recurrent asthma-like attacks after frequent use of a mold-removing spray containing NaOCl over several years. NaOCl is known as a powerful oxidant *(Mizutani et al., 1993)*.

When NaOCl dissolves in water, two substances form, which play a role in oxidation and disinfection, namely hypochlorous acid and hypochlorite ion (OCl-). By adding hypochlorite to water, hypochlorous acid (HOCl) is formed which then rapidly decomposes into hydrochloric acid (HCl) and oxygen (O). The latter is a very strong oxidator. The resulting HCl may react with part of the dissolved sodium hypochlorite resulting in release of chlorine (Cl_2) gas. The toxic mechanism of action of Cl_2 on the living cells is believed to be due to production of reactive hypochlorite ions, hypochloric acid and the free chloride ion which interact as a powerful oxidizing agent with the sulfhydryl groups and sulfur bonds of the cellular proteins *(Winder, 2001)*. These toxic agents (hypochlorite ions, hypochloric acid) that induce tissue injury by Cl_2 gas are the same as those produced by NaOCl when dissolved in water. Therefore, both NaOCl and Cl_2 induce tissue injury by mechanisms that seem to be similar.

Cl_2 is a highly toxic and widely used industrial chemical agent. It is employed in purification of drinking water and in the production of plastics, solvents, pharmaceuticals,

cleaning agents and maintenance of pathogen-free swimming pools. In the last few years, the effects of chronic lung exposure to Cl_2 and its byproducts have been suggested to play a role in the rising numbers of children and adolescents with asthma *(Beretta et al., 2011)*. Chlorination is potentially harmful to the respiratory tract and the swimmer can breathe during swimming and Cl_2 is even absorbed from skin *(Drobnic et2000; Bernard, 2007; Bernard et al., 2008)*.

The acute effects of Cl_2 gas inhalation can range from mild respiratory mucus membrane irritation to marked denudation of the mucosa, pulmonary oedema, and even death. Recovery from Cl_2-induced lung injury requires repair and/ or regeneration of the epithelial layer. The repair process after Cl_2 exposure may not restore normal structure and function as cases of subepithelial fibrosis, mucous hyperplasia, non-specific airway hyperresponsiveness, pulmonary fibrosis and remodeling have been reported in persons after recovery from Cl_2 injury *(Bernard et al., 2007; Tuke et al., 2008)*.

Cl_2 is a highly reactive oxidant gas and a common inhalational irritant, encountered both occupationally and environmentally *(Winder, 2001)*. Presently, the pathophysiological sequelae associated with Cl_2-induced lung injury in conscious animals, as well as the cellular and biochemical involved mechanisms, and the long-term consequences of this injury are not clearly understood *(Matalon and Maull, 2010)*. The airway epithelium is the first target of inhaled Cl_2 gas. Oxidative injury is likely involved, but further damage to the epithelium may occur with migration of inflammatory cells such as neutrophils into the airway epithelium with release of inflammatory cytokines and finally lead to airway remodeling and hyperresponsiveness *(Martin et al., 2003)*.

Currently, management of both animals and people exposed to Cl_2 consists of administration of supplemental oxygen to alleviate hypoxemia, β2-agonists to alleviate bronchoconstriction and enhance alveolar fluid clearance, and corticosteroids to reduce the inflammatory response *(Wang et al., 2002; Wang et al., 2004; Wang et al., 2005; Bell, 2008)*. However, the effect of corticosteroids on Cl_2-induced lung injury is quite controversial. Very recently, it has been reported that corticosteroids have no beneficial effect at the alveolar level on acute lung injury *(Dylan and Meulenbelt., 2011)*. Presently, there are no effective treatments for Cl_2 toxicity *(Matalon and Maull, 2010)*. Moreover, most of the tested therapeutic agents were for alleviation of acute Cl_2-induced lung injury and not for the delayed lung injury, like pulmonary fibrosis and remodeling. Therefore, searching for therapeutic agents that can ameliorate late Cl_2-induced lung injury especially at the alveolar level and prevent fibrosis and remodeling is warranted.

Drugs that increase intracellular levels of the signaling molecule cyclic AMP (cAMP) may be useful for treatment of acute lung injury through effects on alveolar fluid clearance, inflammation, and airway reactivity *(Hoyle, 2010)*. Pentoxifylline (PTX), a methylxanthine acts as a nonspecific phosphodiesterase inhibitor (PDEI) which can increase intracellular levels of cAMP. It participates in immune modulation by preventing lymphocyte and neutrophil cytotoxicity *(Lazarczyk et al., 2003)*. PTX, a potent anti-inflammatory drug, decreases inflammatory cytokine release such as TNF-alpha *(Pardakhti et al., 2009)*; on the other hand, it decreases oxidative stress and lipid peroxidation *(Radfar et al., 2005)*. Moreover, recent research revealed that PTX has produced an inhibitory effect on airway remodeling as well as on airway hyperresponsiveness and airway inflammation *(Kim et al., 2009)*.

Theophylline (THEO), another nonselective PDEI, has been used in the management of bronchial asthma and chronic obstructive pulmonary disease for over 50 years. It has not only a bronchodilating effect, but also an anti-inflammatory one conducive to the inhibition of airway remodeling, including subepithelial fibrosis *(Barnes, 2003; Morfín and Castillo, 2010)*.

Therefore, it can be hypothesized that PTX and THEO might ameliorate the delayed NaOCl or Cl_2-induced lung injury through their pre-proved anti-inflammatory, anti-oxidant and anti-remodeling potentials. To our knowledge, there is no previous experimental study testing the effect of PDEIs on delayed NaOCl or Cl_2-induced lung injury such as pulmonary fibrosis and airway remodeling. Therefore, the present study sought to investigate the effects of two nonselective PDEIs, pentoxifylline and theophylline, on delayed airway functional and histopathological changes induced by NaOCl-inhalation (as a source to hypochlorous acid and hypochlorite ion, the main toxic agents that mediate Cl_2-induced tissue injury) in guinea pigs and to compare their effects with those of dexamethasone as monotherapy or in combination with each PDEI. A trial to find an effective, well tolerated and less adverse effects drug therapy to Cl_2 or NaOCl-induced lung injury.

2. MATERIALS AND METHODS

2.1. Animals:
Male guinea pigs weighing 400±50 g were purchased from the Medical Research Centre, Faculty of Medicine, Ain Shams University, Cairo, Egypt. The animals were maintained in ordinary animal cages in a constant 12-h light/dark cycle, temperature ≈ 25°C and 50–60% relative humidity. Food (Meladco for Animal Food, El-Obour, Egypt) and water were available ad libitum. Experimental procedures were conforming following ethical conduct of research on animals, Ethical committee, Faculty of Medicine, Ain Shams University, Egypt .

2.2. Drugs and chemicals:
Pentoxyphylline, theophylline, dexamethasone, sodium hypochlorite and acetyl-beta methylcholine bromide (Methacholine) powders were purchased from Sigma-Aldrich chemicals Co., Germany. All drugs were dissolved in distilled water except dexamethasone powder which was dissolved in ethanol (25 mg/ml) and then diluted with distilled water (vehicle).

2.3. Experimental procedures:
2.3.1. Study design:
Forty-eight male guinea pigs were allocated to 8 groups (n = 6 per group): a control group, a NaOCl-inhalation group and

the other 6 groups were exposed to NaOCl inhalation and after 30 minutes, animals received intraperitoneal (i.p.) injection of vehicle (ethanol in distilled water), pentoxyphylline 'PTX' 50 mg/kg/day *(Parra et al., 2002)*, theophylline 'THEO' 50 mg/kg/day *(Aoki et al., 2001)*, dexamethasone 'DEX' 20 mg/kg/day *(Toward and Broadley, 2004)*, PTX+DEX or THEO+DEX for three weeks. No statistically significant difference has been found between NaOCl inhalation-untreated group and NaOCl inhalation vehicle-treated group in all outcome measures (data not shown). All drug treatment groups were compared with vehicle-treated group.

2.3.2. Sodium hypochlorite inhalation:

Guinea pigs were exposed for 20 minutes to a nebulized 4% sodium hypochlorite or its vehicle (distilled water in control group) using a nebulizer (Norditalia Elettromedicali S.R.L. – 25010 S. Martino della Battaglia – Italy) to deliver the aerosol at a rate of 0.25 ml/min.

2.3.3. Measurement of airway function in conscious guinea pigs:

Before NaOCl inhalation (base line) and at the end of the third week, conscious guinea pigs were placed in a double flow plethysmogragh (Hugo Sachs Elektronik- Harvard Apparatus GmbH, D-79232 March-Hugstetten, Germany) for measurement of specific airway resistance (sRaw) in cm H_2O x sec and tidal volume (TV) in ml. The plethysmograph was connected to a non-invasive respiratory analyzer (Plugsys Module, Bus-Terminator Module Type 653-2) via a differential pressure transducer (Validyne DP45). For data acquisition (Pulmodyn Pennock W) was used. The method used for recording sRaw was previously described by *Pennock et al. (1979)*, which is based on measuring the time delay between thoracic and nasal air flow or respiration i.e. the increasing phase displacement (Plugsys Module calculated it by specific serial equations) accompanying the increased airway resistance. The sRaw was calculated as airway resistance multiplied by the thoracic gas volume (Cm H_2O x sec). sRaw provides an indirect index of obstruction in the lower airways *(Finney and Forsberg, 1994)*.

2.3.4. Measurement of airway reactivity to methacholine *(Duan et al., 2003)*:

After measuring sRaw and TV, animals were exposed to consecutive doubling concentrations of aerosolized methacholine [0.0156-1mg/ml or '65 μM- 4 mM'] until sRaw was approximately increased to 100% of its base line value for each individual animal. The provocation concentration causing a 100% increase of sRaw (PC_{100}) was derived by linear interpolation of the concentration-sRaw response curve and was used as an index for airway reactivity towards methacholine.

2.3.5. Bronchoalveolar lavage 'BAL' *(Toward and Broadley, 2000)*:

After 1 hour from assessing post-exposure airway reactivity to methacholine, the animals underwent a bronchoalveolar lavage (BAL). The guinea-pigs were anesthetized by urethane (1.2 g/Kg i.p.) and the trachea was cannulated. Guinea pigs lungs were lavaged by flushing with 5 ml of Phosphate buffer saline 'PBS' (PH 7.4) through the

cannula into the lungs, and recovered 3 min later. This was repeated 4 times (total 20 ml).

2.3.5.1. Total and differential cell count in BAL:

Total cell count of the pooled BAL fluid (BALF) was determined by a haemocytometer (cells/ml BALF). The lavage fluid was centrifuged at 4°C (1,000 r.p.m. for 7 minutes) and the precipitated cells were differentially stained (Leishman's: 1.5% in methanol, 6 min), and a minimum of 300 cells (macrophages, eosinophils and neutrophils) were counted and expressed as percentages of total cell count. The BAL supernatant was stored in -20°C until measurement of TNF-α *(Toward and Broadley, 2000)*.

2.3.5.2. TNF-α protein level in BAL:

This was taken as a marker of inflammation. TNF-α level was measured in BAL supernatant using commercially available ELISA kits (Genzyme Immunobiologicals, Cambridge, U.K.) following the protocol provided by the manufacturer. Results are expressed as nanograms per milliliter of bronchoalveolar lavage fluid (ng/ml BALF).

2.3.6. Biochemical analysis of lung tissue homogenate:
2.3.6.1. Evaluation of Myeloperoxidase Activity:

Myeloperoxidase (MPO) activity was used as a marker for leukocyte infiltration in tissues. MPO was determined as previously described in lung homogenates *(Goldblum and Jay, 1985)*. MPO activity was defined as the quantity of enzyme degrading 1 μMol of peroxide/min at 37°C and was expressed as units per gram tissue protein (U/g protein). Protein concentration was determined according to the method described by *Bradford (1976)*.

2.3.6.2. Determination of Malondialdehyde *(Sunderman et al., 1985)*:

Malondialdehyde (MDA), an end-product of peroxidation of cell membrane lipids caused by oxygen-derived free radicals, is considered a reliable marker of oxidative stress and was determined by measurement of the chromogen obtained from the reaction of malondialdehyde with 2-thiobarbituric acid, according to *Aruoma et al. (1989)*. The MDA values are expressed as nanomole per milligram of tissue protein (nmol/g protein).

2.3.6.3. Assessment of superoxide dismutase activity:

Superoxide dismutase (SOD) was also measured in lung homogenate as a reliable marker of oxidative stress. SOD activity of each lung homogenate was measured by colorimetric assay that relies on the ability of the enzyme to inhibit the phenazine methosulphate (PMS)-mediated reduction of nitoblue tetrazolium dye. SOD activity is expressed as U/g tissues = % inhibition x 3.75 /g tissue protein *(Peskin et al., 2000)*.

2.3.7. Histological examination:

After BAL technique, lung specimens were fixed in 10% formalin for preparation of paraffin blocks. Five micrometer thick sections were cut and stained with Hematoxylin-Eosin (H&E). Neutrophil sequestration was determined by counting the number of neutrophils using image analyzer (Leica Q 500 MC program, Heidelberg, Germany) in 5

different areas of the lung, each 200 x 200 μm area , and the numbers obtained from areas were averaged for each animal. Masson trichrome (MT) stain was used for collagen fibers demonstration in inter-alveolar septa. The severity of fibrosis was semi-quantitatively assessed according to *Ashcroft and co-workers (1988)*. Briefly, the grade of lung fibrosis was scored on a scale from 0 to 8 by examining randomly chosen fields of the left middle lobe. Criteria for grading lung fibrosis were as follows: grade 0, normal lung; grade 1, minimal fibrous thickening of alveolar or bronchiolar walls; grade 3, moderate thickening of walls without obvious damage to lung architecture; grade 5, increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous masses; grade 7, severe distortion of structure and large fibrous areas; grade 8, total fibrous obliteration of fields. Grades 2, 4 and 6 were used as intermediate pictures between the aforementioned criteria *(Failla et al., 2006)*.

3. Statistical analysis:

The statistical analyses were all carried out on the software program, Prism version 4.03, Graphpad software Inc., CA, USA. (2005). Results were expressed as mean ± S.E.M. and were analyzed using one-way analysis of variance (ANOVA) followed by post hoc (Tukey's test) to determine which of the groups were responsible for the observed significant difference. Differences were considered statistically significant at $P < 0.05$. For calculation of methacholine (MCh) PC_{100}, MCh concentrations were converted to Log Molar and the response to % change in sRaw from pre-MCh.

4. RESULTS

4.1. Delayed airway functional changes (airway resistance, tidal volume and bronchial reactivity to methacholine) induced by NaOCl inhalation.

As demonstrated in Table (1) and Figure (1) , after 21 days from exposure to NaOCl inhalation vehicle-treated guinea pigs exhibited significant ($P < 0.01$) increase in airway resistance (sRaw) and decrease in tidal volume (TV) in comparison to control group. Chronic treatment with pentoxifylline, theophylline or dexamethasone significantly ($P < 0.05$) decreased sRaw. Although, treatment with pentoxifylline, theophylline or dexamethasone could increase TV (1.21 ± 0.24, 1.30 ± 0.43 and 1.06 ± 0.27, respectively vs 0.63±0.07 ml), this monotherapy effect was not statistically significant. However, combination of dexamethasone with pentoxifylline or theophylline produced significant increase in TV ($P < 0.01$ and $P < 0.05$, respectively). Statistical analysis with unpaired t-test revealed that addition of pentoxifylline or theophylline to dexamethasone significantly increased the ameliorative effect of dexamethasone on sRaw ($P = 0.0249$, $P = 0.0160$; respectively).

Table (2) and Figure (2) show that exposure to NaOCl inhalation induced significantly ($P < 0.01$) reduced methacholine (MCh) PC100 from 0.521 ± 0.056 in control group to 0.088 ± 0.015 (mg/ml) indicating airway hyperresponsiveness. Chronic treatment with pentoxifylline, theophylline or dexamethasone reduced airway response to MCh as evident by significant ($P < 0.05$) increase in PC100

of MCh in comparison to NaOCl inhalation vehicle-treated. Unpaired *t*-test indicated that addition of pentoxifylline or theophylline to dexamethasone significantly potentiated dexamethasone effect on airway responsiveness to inhaled MCh ($P = 0.0021$, $P = 0.0095$; respectively).

4.2. Airway inflammatory markers in broncho-alveolar lavage fluid (BALF): Total and differential cell count in BALF and tumor necrosis factor-α (TNF-α) protein level in BALF of guinea pigs exposed to NaOCl inhalation.

After 21 days from exposure to NaOCl inhalation, there was a significant ($P < 0.001$) increase in total cell count and neutrophils % in BALF of vehicle-treated guinea pigs compared to control group indicating a state of airway inflammation. Macrophages % significantly decreased in NaOCl inhalation vehicle-treated group while % did not change. Chronic treatment with pentoxifylline, theophylline or dexamethasone significantly decreased total cell count and neutrophils % in comparison to vehicle-treated group ($P < 0.05$, $P < 0.05$ and $P < 0.01$, respectively). When pentoxifylline or theophylline were co-administrated with dexamethasone, they significantly ($P = 0.0140$, $P = 0.0063$; respectively) increased the dexamethasone effect on neutrophils % in BALF (Table 3).

TNF-α protein level was significantly ($P < 0.01$) increased in BALF after 21 days from NaOCl inhalation (44.28 ± 9.3 vs 6.13±1.6 ng/ml BALF) when compared to control group, (Figure 3). Pentoxifylline, and dexamethasone, but not theophylline could significantly ($P < 0.05$) reduced TNF-α level in BALF (18.32 ± 2.54, 23.10 ± 3.31 and 27.57 ± 8.18, respectively vs 44.28 ± 9.3 ng/ml BALF) compared to vehicle-treated group. Statistical analysis with unpaired *t*-test revealed that addition of pentoxifylline or theophylline to dexamethasone significantly ($P = 0.0425$, $P = 0.0223$; respectively) potentiated the effect of dexamethasone on TNF-α level in BALF (13.70 ± 2.33 and 12.78 ± 1.91 ng/ml BALF, respectively).

4.3. Airway inflammatory markers in lung tissue: Myeloperoxidase (MPO) activity in lung tissue homogenate and average neutrophil cell count /200 x 200 μm lung area of guinea pigs exposed to NaOCl inhalation.

As shown in Figure (4), after 21 days from exposure to NaOCl inhalation, vehicle-treated guinea pigs exhibited significant increase in MPO activity (30.62 ± 4.74 vs 4.80±1.28 Units/g protein), a marker for leukocyte accumulation in tissues, and neutrophil infiltration(380.8 ± 50.73 vs 27.67 ± 7.579 cell/200 x 200 μm lung area) in lung tissue ($P < 0.01$, $P < 0.001$, respectively), indicating lung inflammation compared to control group. Chronic treatment with pentoxifylline, theophylline or dexamethasone significantly ($P < 0.05$) reduced lung MPO activity (13.48 ± 3.52, 16.23 ± 2.51 and 18.10 ± 2.08 vs 30.62 ± 4.74 Units/g protein; respectively) and neutrophil count (169.3 ± 28.25, 211.5 ± 50.73 and 178.0 ± 51.58 vs 380.8 ± 50.73 cell/200 x 200 μm lung area; respectively) in comparison to NaOCl inhalation vehicle-treated group. Unpaired *t*-test indicated that combination of pentoxifylline or theophylline with dexamethasone significantly potentiated dexamethasone effect on MPO activity ($P = 0.0190$,

$P = 0.0492$; respectively) and neutrophil count ($P = 0.2722$, $P = 0.0308$; respectively).

4.4. Airway lipid peroxidation and oxidative stress markers in lung tissue: Malondialdehyde (MDA) level and superoxide dismutase (SOD) activity in lung tissue homogenate of guinea pigs exposed to NaOCl inhalation.

As shown in Figures (5) and (6), exposure of conscious guinea pigs to Na hypochlorite inhalation for 20 minutes caused significant ($P < 0.01$, $P < 0.001$, respectively) increase in lung lipid peroxidation product, MDA (957.7 ± 148.2 vs 329.3 ± 37.90 nmol/g protein) and depletion of SOD activity (214.8 ± 57.10 vs 817.0 ± 103.5 Units/g protein) in comparison to control group, indicating lung oxidative stress injury. Pentoxifylline displayed a significant antioxidant effect evidenced by reducing MDA and increasing SOD activity in lung tissue ($P < 0.01$, $P < 0.05$, respectively). Although, theophylline and dexamethasone monotherapy induced insignificant effect on MDA (804.5 ± 136.1 and 844.7 ± 208.5 vs 957.7 ± 148.2 nmol/g protein; respectively) and SOD (414.2 ± 240.2 and 350.0 ± 206.6 vs 214.8 ± 57.10 Units/g protein; respectively) when they were used in combination therapy, they induced significant reduction in lung MDA and increase in SOD activity ($P < 0.01$, $P < 0.05$, respectively). Addition of pentoxifylline to dexamethasone induced more significant antioxidant effect (significant reduction in lung MDA and increase in SOD activity) than using dexamethasone alone ($P = 0.0179$, $P = 0.0329$, respectively).

4.5. Delayed airway histopathological changes and remodeling induced by Na hypochlorite (NaOCl) inhalation (Figures 7, 8 and 9)

Light micrographs of guinea pigs lung sections stained with Hematoxylin and Eosin show that after 21 days from NaOCl inhalation, vehicle-treated group exhibited airway remodeling evidenced by marked thickening of the alveolar wall, rupture of the alveolar septa and interstitial hemorrhage. There were also dilation and thickening of vascular wall, increased smooth muscle thickness around bronchial passages and inflammatory cellular infiltration. This picture was ameliorated in treatment groups in variable degrees. Although, dexamethasone group was the least improved group, combination therapy groups showed the best improvement in histopathological changes induced by NaOCl (Figure 7).

Regarding airway fibrosis, Figures (8) and (9) show that exposure to NaOCl inhalation induced marked collagen interstitial deposition (green color with Masson trichrome stain) and significant ($P < 0.0001$) increase in Aschorft pulmonary fibrosis score (5.50 ± 0.428 vs 0.50 ± 0.223). Chronic treatment with pentoxifylline or dexamethasone, but not theophylline, significantly ($P < 0.05$) reduced Aschorft pulmonary fibrosis score, in comparison to NaOCl inhalation vehicle-treated group and decreased collagen deposition (green stain). Although, treatment with theophylline alone insignificantly reduced Aschorft fibrosis score (4.167 ± 0.703 vs 5.50 ± 0.428), applying Unpaired t-test revealed that addition of theophylline to dexamethasone could significantly ($P = 0.0219$) potentiate the anti-remodeling effect of dexamethasone (2.000 ± 0.365 vs 3.667 ± 0.494). Combination of pentoxifylline with dexamethasone also showed significant ($P = 0.0325$) improvement in Aschorft fibrosis score when compared to dexamethasone alone (2.333 ± 0.211 vs 3.667 ± 0.494).

Table 1: Effect of chronic treatment with pentoxifylline (PTX) '50 mg/kg/day i.p.' or theophylline (THEO) '50 mg/kg/day i.p.', alone or in combination with dexamethazone (DEX) '20 mg/kg/day i.p.', on specific air way resistance (sRaw) and tidal volume (TV) of guinea pigs 21 days after exposure to Sodium hypochlorite (NaOCl) inhalation.

Animal group	sRaw (Cm H_2O_{xs})	Tidal volume (ml)
Control	2.93 ± 0.34	1.86 ± 0.38
NaOCl inhalation vehicle-treated	$6.67^{**} \pm 0.35$	$0.63^{**} \pm 0.07$
NaOCl inhalation PTX-treated	$4.75^{\#} \pm 0.50$	1.21 ± 0.24
NaOCl inhalation THEO-treated	$5.14^{\#} \pm 0.39$	1.30 ± 0.43
NaOCl inhalation DEX-treated	$4.86^{\#} \pm 0.41$	1.06 ± 0.27
NaOCl inhalation DEX + PTX-treated	$3.31^{\#\# a} \pm 0.56$	$1.58^{\#\#} \pm 0.18$
NaOCl inhalation DEX + THEO-treated	$3.52^{\#\# b} \pm 0.20$	$1.39^{\#} \pm 0.27$

Data are mean ± SEM. (n=6 per group).
One way ANOVA followed by Dunnett's Multiple Comparison Test: $^{**}P < 0.01$ vs control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs NaOCl inhalation vehicle-treated group. Unpaired t-test: $^{a}P = 0.0249$, $^{b}P = 0.0160$ compared to NaOCl inhalation DEX-treated group.

Table 2: Effect of chronic treatment with pentoxifylline (PTX) '50mg/kg/day i.p.' or theophylline (THEO) '50 mg/kg/day i.p.', alone or in combination with dexamethazone (DEX) '20 mg/kg/day i.p.' on bronchial reactivity to methacholine of guinea pigs 21 days after exposure to NaOCl inhalation.

Animal group	PC$_{100}$ of methacholine (mg/ml)
Control	0.521 ± 0.056
NaOCl inhalation vehicle-treated	$0.088^{**} \pm 0.015$
NaOCl inhalation PTX-treated	$0.249^{\#} \pm 0.054$
NaOCl inhalation THEO-treated	$0.231^{\#} \pm 0.045$
NaOCl inhalation DEX-treated	$0.186^{\$} \pm 0.039$
NaOCl inhalation DEX + PTX-treated	$0.424^{\#\# a} \pm 0.043$
NaOCl inhalation DEX + THEO-treated	$0.388^{\#\# b} \pm 0.050$

Data are mean ± SEM. (n=6 per group). One way ANOVA followed by Dunnett's Multiple Comparison Test: $^{**}P <0.01$ vs control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs NaOCl inhalation vehicle-treated group.
Unpaired t test: ap $= 0.0021$, $^{b}P = 0.0095$ compared to NaOCl inhalation DEX-treated, $^{\$}P = 0.0403$ vs NaOCl inhalation vehicle-treated group.
PC100 = The provocation concentration of inhaled methacholine required to produce 100% increase of the airway resistance over the base line.

Table 3: Effect of chronic treatment with pentoxifylline (PTX) '50mg/kg/day i.p.' or theophylline (THEO) '50 mg/kg/day i.p.', alone or in combination with dexamethazone (DEX) '20 mg/kg/day i.p.' on total cell count (X106/ml BAL) and differential cell count (percentage from total cell count) in bronchoalveolar lavage fluid (BALF) of guinea pigs exposed to NaOCl inhalation

Animal group	Total cell count (X10⁴/ml BALF)	Differential cell count (% from total cell count)		
		Neutrophils	Macrophages	Lymphocytes
Control	2.04 ± 0.53	1.46 ± 0.81	74.7 ± 1.56	4.12 ± 0.091
NaOCl inhalation vehicle-treated	$21.25^{**} \pm 1.08$	$65.1^{***} \pm 8.31$	$10.2^{***} \pm 5.87$	3.92 ± 0.022
NaOCl inhalation PTX-treated	$11.83^{\#} \pm 0.78$	$41.0^{\#\#} \pm 5.12$	$32.5^{\#} \pm 8.113$	3.39 ± 0.036
NaOCl inhalation THEO-treated	$15.12^{\#} \pm 2.79$	$49.1^{\#} \pm 3.08$	$40.1^{\#} \pm 6.90$	3.97 ± 0.087
NaOCl inhalation DEX-treated	$10.16^{\#\#} \pm 1.66$	$35.7^{\#\#} \pm 6.97$	$51.5^{\#} \pm 9.66$	4.89 ± 0.282
NaOCl inhalation DEX + PTX-treated	$8.99^{\#\#} \pm 2.10$	$21.7^{\#\#\#a} \pm 2.89$	$61.3^{\#\#} \pm 5.28$	3.92 ± 0.053
NaOCl inhalation DEX + THEO-treated	$9.03^{\#\#} \pm 0.93$	$16.7^{\#\#\#b} \pm 1.09$	$70.5^{\#\#c} \pm 3.99$	4.20 ± 0.071

Data are mean±SEM. (n=6 per group). One way ANOVA followed by Dunnett›s Multiple Comparison Test: $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs NaOCl inhalation vehicle-treated group. Unpaired *t*-test: $^aP = 0.0140$, $^bP = 0.0063$, $^cP = 0.0056$ compared to NaOCl inhalation DEX-treated group.
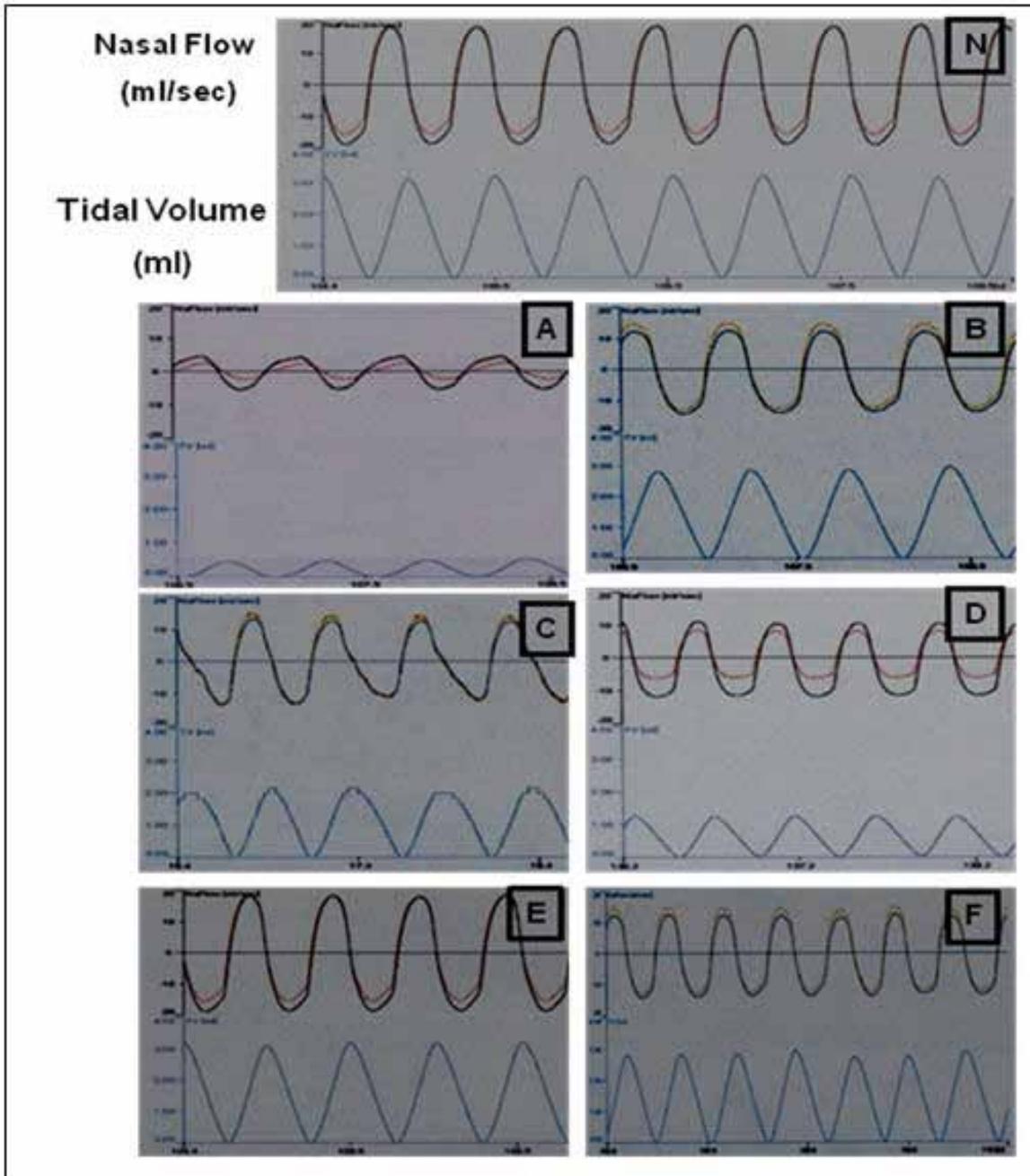

Figure 1: Tidal volume (ml) and Nasal flow (ml/sec) of normal guinea pig (N) and guinea pigs 21 days after exposure to NaOCl inhalation; vehicle-treated group (A), pentoxifylline 'PTX'- treated group (B), theophylline 'THEO'- treated group (C), dexamethazone 'DEX'- treated group (D), PTX+DEX- treated group (E) and THEO+DEX- treated group (F).
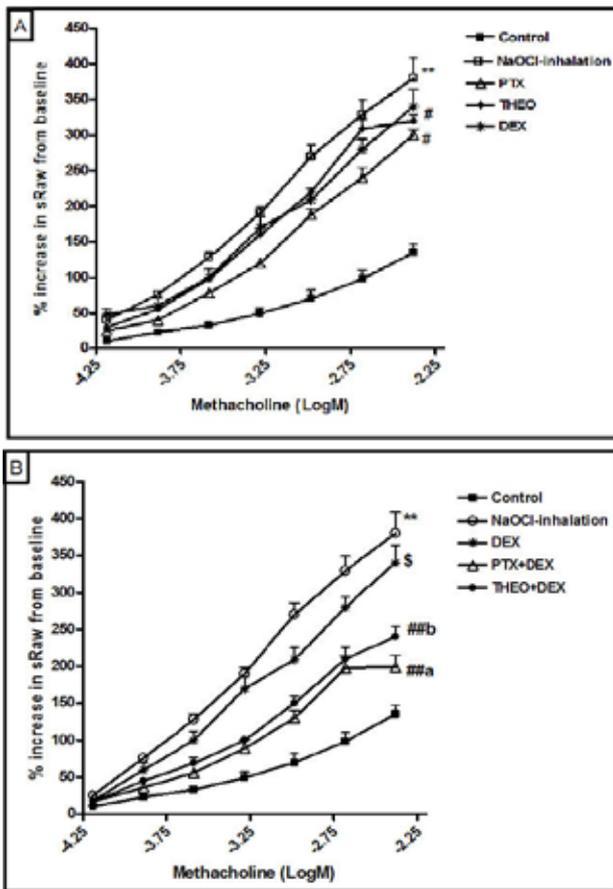
Figure 2: Effect of chronic treatment with pentoxifylline (PTX) '50mg/kg/day i.p.' or theophylline (THEO) '50 mg/kg/day i.p.', alone or in combination with dexamethazone (DEX) '20 mg/kg/day i.p.' on bronchial reactivity to methacholine in guinea pigs exposed to NaOCl inhalation. Data are mean±SEM. (n=6 per group). One way ANOVA followed by Dunnett's Multiple Comparison Test: $^{**}P < 0.01$ vs control group. $^{#}P < 0.05$, $^{##}P < 0.01$ vs NaOCl inhalation vehicle-treated group. Unpaired t test: $^{a}P = 0.0021$, $^{b}P = 0.0095$ vs NaOCl inhalation DEX-treated group, $^{$}P = 0.0403$ compared to NaOCl inhalation vehicle-treated group
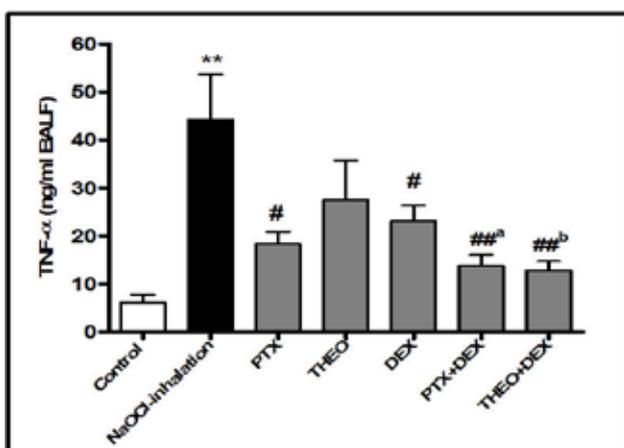
Figure 3: Effect of chronic treatment with pentoxifylline (PTX) ‹50mg/kg/day i.p.› or theophylline (THEO) ‹50mg/kg/day i.p.›, alone or in combination with dexamethazone (DEX) ‹20mg/kg/day i.p.› on tumor necrosis factor-α (TNF-α) protein level in bronchoalveolar lavage fluid ‹ BALF› of guinea pigs exposed to NaOCl inhalation. Data are mean±SEM. (n=6 per group). One way ANOVA followed by Dunnett›s Multiple Comparison Test: $^{**}P < 0.01$ vs control group. $^{#}P < 0.05$, $^{##}P < 0.01$ vs NaOCl inhalation vehicle-treated group. Unpaired t-test: $^{a}P = 0.0425$, $^{b}P = 0.0223$ compared to NaOCl inhalation DEX-treated group.
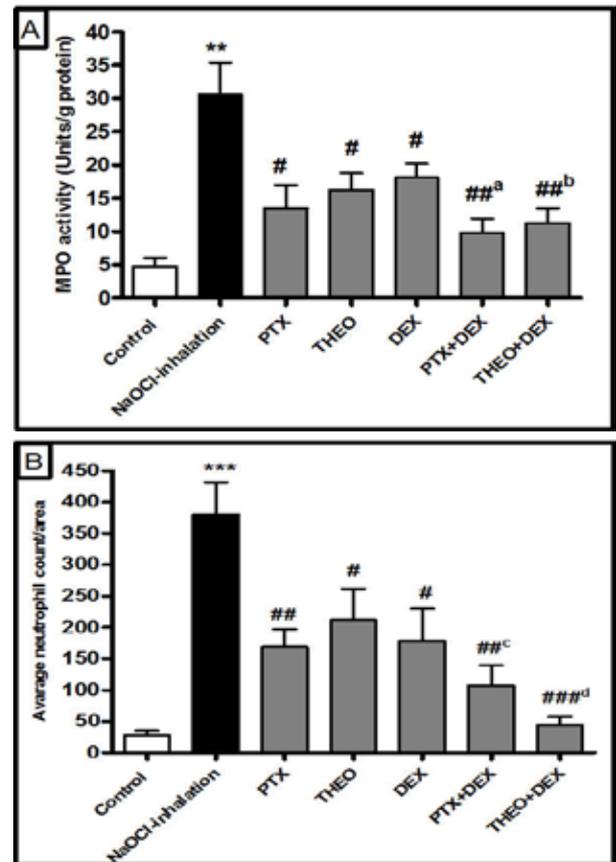

Figure 4: Effect of chronic treatment pentoxifylline (PTX) ‘50mg/kg/day i.p.’ or theophylline (THEO) ‘50mg/kg/day i.p.’, alone or in combination with dexamethazone (DEX) ‘20mg/kg/day i.p.’ on myeloperoxidase (MPO) Activity in lung tissue homogenate (A) and average neutrophil cell count/200 x 200 μm lung area (B) of guinea pigs exposed to NaOCl inhalation.

Data are mean±SEM. (n=6 per group). One way ANOVA followed by Dunnett's Multiple Comparison Test: $^{**}P < 0.01$, $^{***}P < 0.001$ vs control group. $^{#}P < 0.05$, $^{##}P < 0.01$ vs NaOCl inhalation vehicle-treated group. Unpaired t-test: ap=0.0190, $^{b}P = 0.0492$, $^{c}P = 0.2722$, $^{d}P = 0.0308$ compared to NaOCl inhalation DEX-treated group.
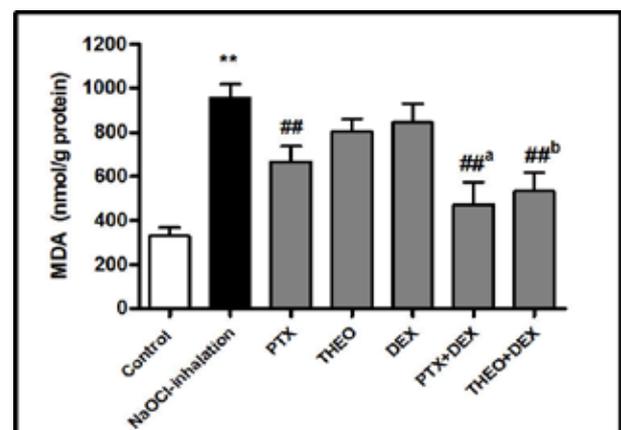

Figure 5: Effect of chronic treatment with pentoxifylline (PTX) ‘50mg/kg/day i.p.’ or theophylline (THEO) ‘50mg/kg/day i.p.’, alone or in combination with dexamethazone (DEX) ‘20mg/kg/day i.p.’on malondialdehyde (MDA) level in lung tissue homogenate of guinea pigs exposed to NaOCl inhalation. Data are mean ± SEM. (n=6 per group). One way ANOVA followed by Dunnett's Multiple Comparison Test: $^{**}P < 0.01$ vs control group. $^{#}P < 0.05$, $^{##}P < 0.01$ vs NaOCl inhalation vehicle-treated group. Unpaired t-test: $^{a}P = 0.0179$, $^{b}P = 0.0261$ compared to NaOCl inhalation DEX-treated group.
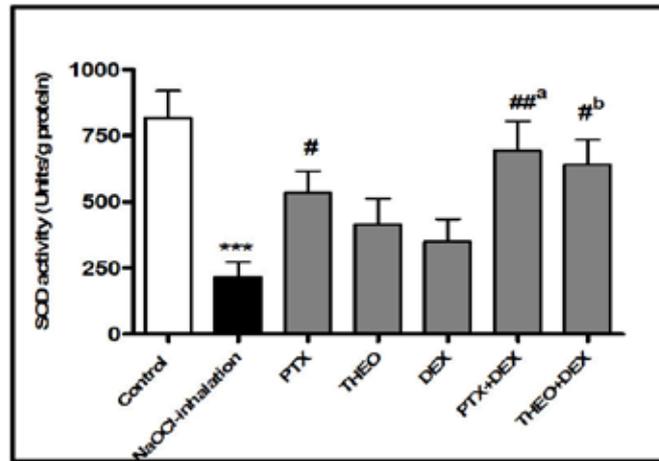
Figure 6: Effect of chronic treatment pentoxifylline (PTX) '50mg/kg/day i.p.' or theophylline (THEO) '50 mg/kg/day i.p.', alone or in combination with dexamethazone (DEX) '20 mg/kg/day i.p.'on superoxide dismutase (SOD) activity (Units/g protein) in lung tissue homogenate of guinea pigs exposed to NaOCl inhalation. Data are mean±SEM. (n=6 per group). One way ANOVA followed by Dunnett's Multiple Comparison Test: ***$P < 0.001$ vs control group. #$P < 0.05$, ##$P < 0.01$ vs NaOCl inhalation vehicle-treated group. Unpaired *t*-test: [a]$P = 0.0329$, [b]$P = 0.0436$ compared to NaOCl inhalation DEX-treated group.
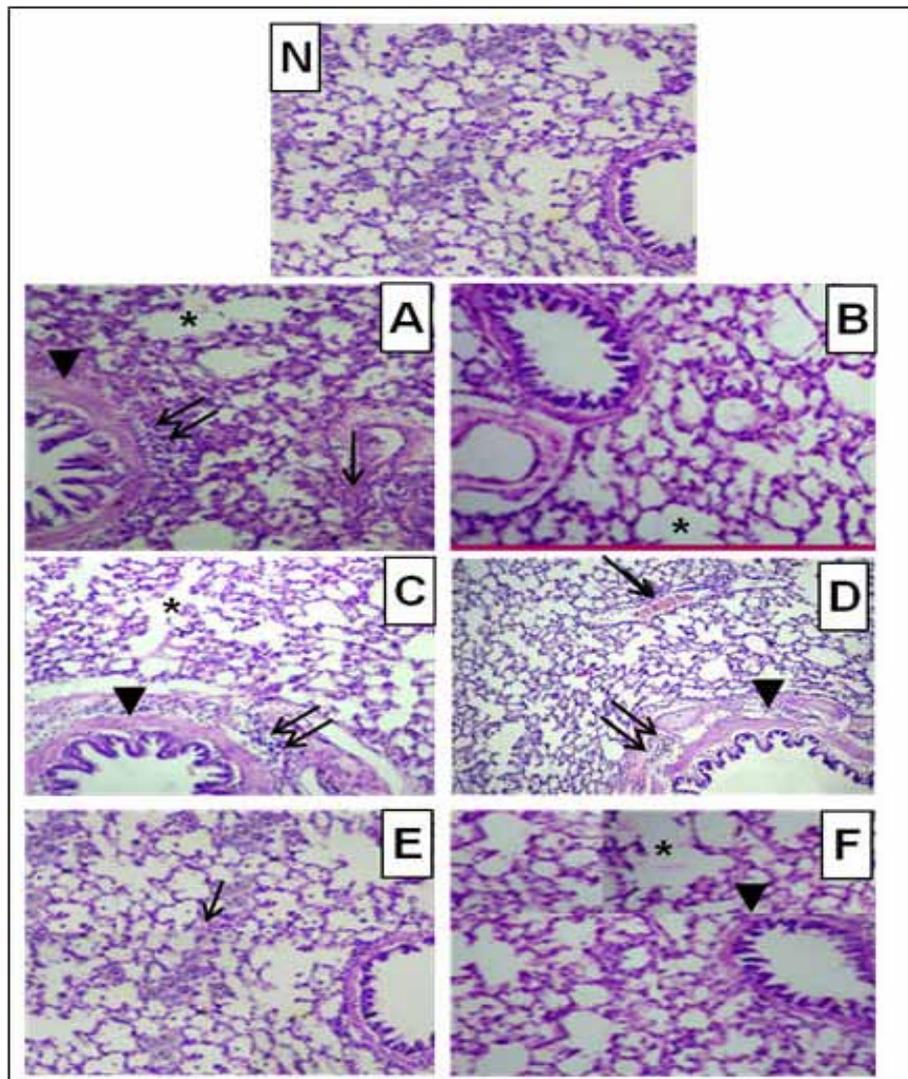

Figure 7: Light micrographs of guinea pigs lung sections stained with Hematoxylin-Eosin (H&E) (X200). N: normal unexposed group. A: NaOCl inhalation vehicle-treated group. B: NaOCl inhalation pentoxifylline 'PTX'- treated group. C: NaOCl inhalation theophylline ' THEO'- treated group. D: NaOCl inhalation dexamethazone 'DEX' treated group. E: NaOCl inhalation PTX+DEX- treated group. F: NaOCl inhalation THEO+DEX- treated group. After 21 days from NaOCl inhalation, the untreated group showed thickening of the alveolar wall, rupture of the alveolar septa (*), Interstitial hemorrhage (↓). There were also dilation and thickening of vascular wall, increased smooth muscle thickness around bronchial passages (►) and inflammatory cellular infiltration (↓↓). This picture was ameliorated in treatment groups in variable degrees.
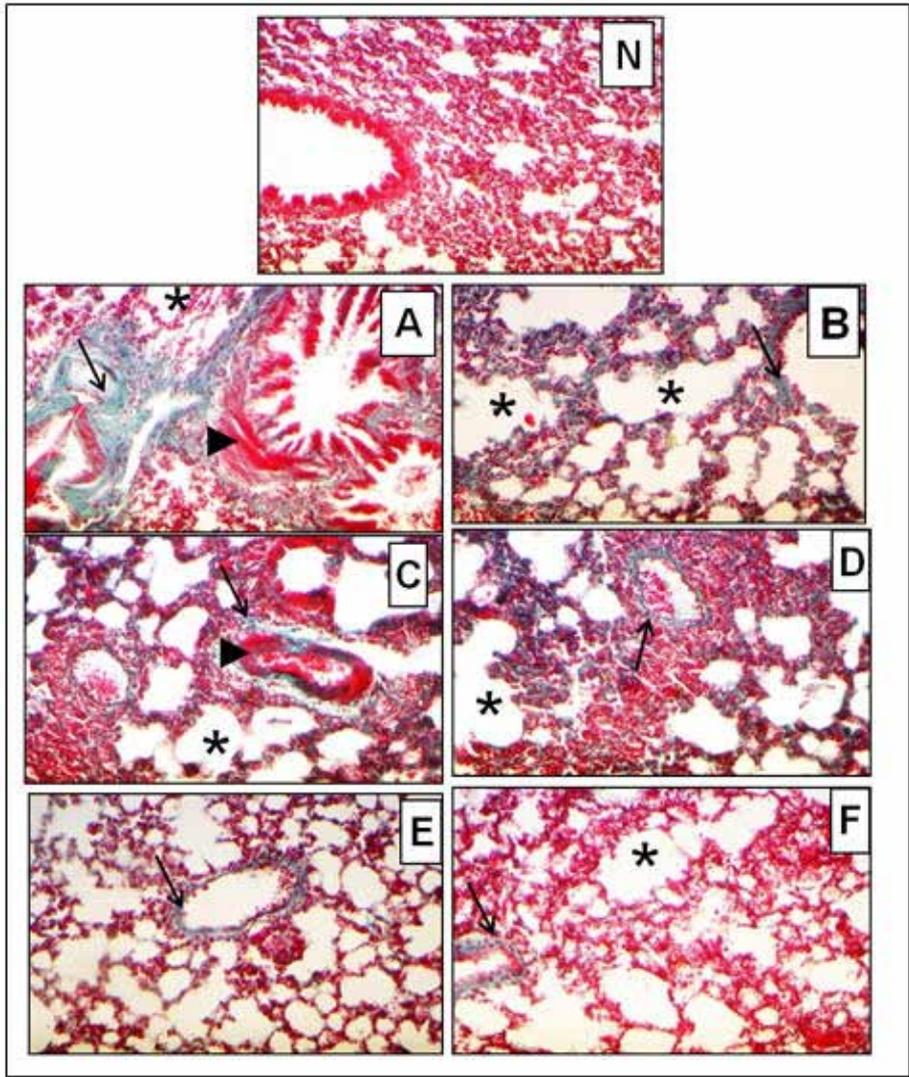
Figure 8: Light micrographs of guinea pigs lung sections stained with Masson trichrome stain (X250). The used stain shows collagen in green colour. N: normal unexposed group. A: NaOCl inhalation vehicle-treated group. B: NaOCl inhalation pentoxifylline 'PTX'- treated group. C: NaOCl inhalation theophylline ' THEO'- treated group. D: NaOCl inhalation dexamethazone 'DEX' treated group. E: NaOCl inhalation PTX+DEX- treated group. F: NaOCl inhalation THEO+DEX- treated group. After 21 days from NaOCl inhalation, the untreated group showed thickness of the alveolar wall, rupture of the alveolar septa (*), Interstitial fibrosis 'green colored areas' (↓). There were also dilation and thickening of vascular wall, increased smooth muscle thickness around bronchial passages (►). This picture was ameliorated in treatment groups in variable degrees.
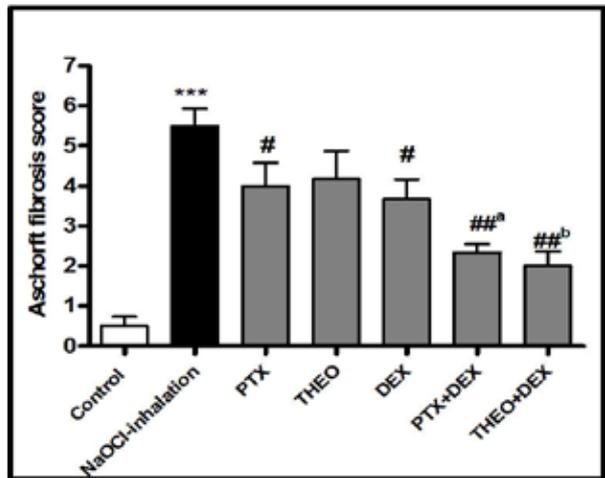

Figure 9: Effect of chronic treatment with pentoxifylline (PTX), theophylline (THEO) and dexamethazone (DEX) alone or in combination on Ashcroft score, a marker for pulmonary fibrosis, in lung sections of guinea pigs exposed to NaOCl inhalation.
Data are mean±SEM. (n=6 per group). One way ANOVA followed by Dunnett's Multiple Comparison Test: ***$P < 0.0001$ vs control group. #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ vs NaOCl inhalation vehicle-treated group. Unpaired *t*-test: [a]$P = 0.0325$, [b]$P = 0.0219$ compared to NaOCl inhalation DEX-treated group.

5. DISCUSSION

The present study was designed to investigate the effects of two nonselective PDEIs, pentoxifylline and theophylline, on delayed airway functional and histopathological changes induced by NaOCl-inhalation (as a source to hypochlorous acid and hypochlorite ion, the main toxic agents that mediate Cl_2-induced tissue injury) in guinea pigs and to compare their effects with those of dexamethasone as monotherapy or in combination with each PDEI. Results of the present study indicate that 21 days after exposure to 4% NaOCl inhalation for 20 min., conscious guinea pigs exhibited airway dysfunction, as evidenced by increased specific airway resistance (SRaw) and bronchial reactivity to MCh and reduced tidal volume (TV). These functional changes were associated with elevation in inflammatory injury markers such as BALF total cell count, neutrophil percentage and TNF-α level and lung myeloperoxidase (MPO) activity and neutrophil infiltration, in addition to alteration in oxidative stress injury markers as increase in lipid peroxidation product, malondialdehyde (MDA) and depletion of superoxide dismutase (SOD) activity in lung tissue homogenate. These findings were consistent with histopathological lung changes and Aschorft lung fibrosis score that revealed NaOCl-induced airway remodeling.

Very few experimental studies tested the effect of NaOCl-inhalation on airway function. Mizutani and his colleagues evaluated the effect of NaOCI (1.2-10%) inhalation on specific airway resistance and the possibility that NaOCl is involved in causing airway hyperreactivity in guinea pigs. In this study, inhalation of NaOCl caused an increase in SRaw in a concentration-dependent manner and inhalation of 3.6% NaOCl induced airway hyperreactivity.

On the other hand, many experimental studies investigated the effect of chlorine (Cl_2)-inhalation on airway function. The toxic agents, hypochlorite ions, hypochloric acid, that induce tissue injury by Cl_2 gas are the same produced by NaOCl when dissolved in water. Therefore, both NaOCl and Cl_2 may induce their tissue injury by mechanisms that seem to be similar.

Indeed, exposure of animals and humans to high concentrations of Cl_2 has resulted in acute and chronic lung injury, especially in people with asthma and chronic obstructive lung disease *(Evans, 2005; Yadav et al., 2010)*. Clinical symptoms of Cl_2 intoxication include dyspnea, airway obstruction, cough, pulmonary edema, pneumonitis, cyanosis, nausea, vomiting, and loss of consciousness. Common features of lung injury in such models are epithelial cell damage, vascular leakage, pulmonary edema, airway hyperreactivity, production of inflammatory mediators, and influx of neutrophils into lung tissue *(Holey, 2010)*. The airway epithelium is the first target of inhaled Cl_2 gas. Although the exact mechanism of epithelial damage is unknown, oxidative injury is likely involved as Cl_2 gas can combine with reactive oxygen species to form a variety of highly reactive oxidants *(Winder, 2001)*. It is scrubbed from the airstream with high direct oxidative injury to the epithelium may occur immediately with exposure to Cl_2, but further damage to the epithelium may occur with migration of inflammatory cells such as neutrophils into the airway epithelium and the subsequent release of inflammatory cytokines e.g. TNF-α, oxidants and proteolytic enzymes that finally lead to airway remodeling and airway hyperresponsiveness *(Martin et al., 2003)*.

Some animal studies tested systemic or inhaled corticosteroids administered immediately following high-level exposure to Cl_2 that were suggested to attenuate Cl_2-induced lung injury in rats and guinea pigs *(Wang et al., 2002; Wang et al., 2005)*. Other experimental studies showed possible benefits from nebulized sodium bicarbonate *(Bosse, 1994)*, cromoglycate *(Pipelzadeh et al., 2002)* and terbutaline *(Wang et al., 2004)*. Leustik et al. (2008) reported that Cl_2-induced alveolar damages were ameliorated by low-molecular-weight antioxidants (Ascorbic acid, deferoxamine, and N-acetyl-L-cysteine) and more recently, post-exposure administration of sodium nitrite mitigates airway and epithelial injury induced by Cl_2 inhalation in rats *(Yaday et al., 2011)*. However, till now, there are no effective treatments for Cl_2 toxicity *(Matalon and Maull, 2010)*.

In the present work, chronic treatment with PDE inhibitors, pentoxifylline and theophylline, and corticosteroid, dexamethasone as monotherapy significantly mitigated airway functional and histopathological changes induced by NaOCl-inhalation. Pentoxifylline could significantly ameliorate inflammatory and oxidative stress markers, indicating anti-inflammatory and antioxidant effects. On the other hand, theophylline and dexamethasone significantly reduced inflammatory markers while insignificantly affected oxidative stress markers. However, co-administration of dexamethasone with theophylline could significantly alleviate biomarkers of oxidant stress in lung tissue homogenates.

Pentoxifylline, a nonspecific PDE inhibitor that can increase intracellular levels of cAMP, has shown significant effects in ameliorating lung injury following exposure to acid aspiration in rats *(Kudoh et al., 1995, Pawlik et al., 2005)*. Pentoxifylline, a potent anti-inflammatory drug, decreases inflammatory cytokine release such as TNF-alpha *(Pardakhti et al., 2009)*; on the other hand, it decreases oxidative stress and lipid peroxidation *(Radfar et al., 2005; Vircheva et al., 2010)*. Moreover, recent research revealed that pentoxifylline has produced an inhibitory effect on airway remodeling as well as on airway hyperresponsiveness and airway inflammation in a mouse model of chronic asthma using house dust mite antigen *(Kim et al., 2009)*.

Theophylline, another nonselective PDE inhibitor, has an anti-inflammatory effect and inhibits of airway remodeling, including subepithelial fibrosis *(Barnes, 2003)*. It has been proposed that the observed anti-inflammatory and anti-fibrotic effects of theophylline could be attributed to PDE inhibition and at least partly, through the cAMP-PKA pathway *(Yano et al., 2006; Morfin and Castillo, 2010)*. To our knowledge, no previous study has investigated the effect of chronic treatment with pentoxifylline or theophylline on chlorine induced lung injury in experimental animals

including guinea pigs. However, *Hoyle (2010)* tested the effects of the PDE4 inhibitor, rolipram, on airway hyperreactivity induced by chlorine inhalation in mice and found that delivery of rolipram to the lungs via intranasal administration 1 hour and 10 hours after chlorine exposure inhibited chlorine-induced airway hyperreactivity.

Cyclic AMP (cAMP) is an intracellular signaling molecule that regulates normal cell function and responses to injury. Agents that raise cAMP levels are known to stimulate alveolar fluid transport, to inhibit inflammation, and to induce bronchodilation *(Hoyle, 2010)* and this agrees with ability of PDEIs, pentoxifylline and theophylline, to mitigate airway functional and histopathological injury induced by chlorine inhalation in the present work. Increased alveolar fluid transport following acute lung injury is beneficial because of the potential to speed the resolution of pulmonary edema. Increased cAMP level stimulates alveolar fluid transport through its effects on the expression and function of ion channels such as the epithelial sodium channel and the cystic fibrosis transmembrane conductance regulator chloride channel *(Matthay et al., 2005)*. Agents that raise cAMP levels produce widespread dampening of inflammatory processes, including decreased inflammatory mediator production and inhibition of macrophage, lymphocyte, eosinophil, and neutrophil function (adhesion, chemotaxis, and degranulation) in acute lung injury *(Souness et al., 2000)*. Treatments that increase cAMP levels also inhibit endothelial permeability *(Suttorp et al., 1993)*, and this may contribute to suppression of inflammatory cell influx from the circulation into the injured lung *(Hoyle, 2010)*. An additional beneficial effect of elevated cAMP levels in the lung is bronchodilation produced by smooth muscle relaxation.by stimulation of Gs-coupled G protein–coupled receptors by activating PKA that inhibit of calcium release, stimulate of myosin light chain phosphatase, and inhibit of Gq signaling *(Deshpande and Penn, 2006)*. Therefore, cAMP signaling appears to play a minor role in maintenance of basal airway smooth muscle tone under normal conditions, but agents that raise cAMP levels are effective inhibitors of bronchoconstriction and airway hyperreactivity in pathologic states *(Hoyle, 2010)*.

In the current study, benefit was obtained by treatment with dexamethasone after chlorine inhalation. This is in agreement with *Demnati et al. (1998)* and *Wang et al. (2002 and 2005)*, where their studies showed improved pulmonary and cardiovascular function by administration of corticosteroid following exposure to chlorine inhalation. However, the effect of corticosteroids on Cl_2-induced lung injury is quite controversial, as recently demonstrated, corticosteroids may not have beneficial effect at the alveolar level in acute lung injury, which is caused by severe exposure to water-soluble compounds (e.g. chlorine). In the recovery phase, corticosteroids may even be harmful, because corticosteroids hamper the division of type II alveolar cells and hamper the differentiation from type II into type I alveolar cells. The latter is important for the re-epithelialization of the alveolus and removal of excess of water in the alveolus *(Dylan and Meulenbelt, 2011)*.

Data obtained from the present experiment suggest that co-administration of pentoxifylline or theophylline with dexamethasone significantly improved the ameliorative effect of dexamethasone on airway functional, biochemical and histopathological changes induced by NaOCl inhalation. This improvement might be related to involvement of oxidative stress in pathogenesis of NaOCl or Cl_2-induced lung injury. This is in accordance with the theory suggested by *Marwick et al. (2008)* who reported that oxidative stress reduces the anti-inflammatory corticosteroid action and may therefore contribute to the relative corticosteroid insensitivity. Low concentrations of theophylline can restore the anti-inflammatory action of corticosteroids in oxidant exposed cells, however the mechanism remains unknown. Theophylline restores corticosteroid repression of pro-inflammatory mediator release and histone acetylation in oxidant exposed cells. On the other hand, the present results indicate that pentoxifylline improved dexamethasone effect too; this may be explained by its antioxidant effect and reducing oxidative stress that was evidenced by decreasing MDA level and increasing SOD activity in lung tissue homogenate of guinea pigs exposed to NaOCl- inhalation in the present study.

In conclusion, the present study demonstrate that chronic administration of PDEIs, pentoxifylline and theophylline, as monotherapy or in combination with dexamethasone, is effective in alleviation of delayed airway functional, biochemical and histopathological injury induced by NaOCl-inhalation in guinea pigs. If these findings were to translate into actual clinical benefit, then PDEIs might prove to be a suitable alternative to corticosteroids as therapeutic approach for management of NaOCl and Cl_2-toxicity or at least can be combined with corticosteroid to reduce its dose-dependent adverse effects and improve its efficacy.

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