Synergistic Effects of Erythromycin and N-Acetyl-cysteine on Amelioration of Cigarette Smoke-Induced Chronic Obstructive Pulmonary Disease in Adult Male Rats

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Abstract

Background: Chronic obstructive pulmonary disease (COPD) affects various structural and functional domains in the lungs. It is characterized by damage of small airways due to an inflammatory process, oxidative stress and imbalance of proteolytic and anti-proteolytic activities. The aim of this work is to investigate the mechanisms involved in COPD and the role of erythromycin and/or N-acetyl cysteine in amelioration of these mechanisms by their role in the extracellular matrix remodeling together with their anti-inflammatory and antioxidant effects.

Methods: In this study a total of 50 male albino rats were divided into five groups (10 rats/group); control group, COPD group, COPD group treated by erythromycin, COPD group treated by N-acetyl-cysteine and COPD group treated by both erythromycin and N-acetyl-cysteine. In lung tissue, levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured using ELISA techniques, glutathione (GSH), catalase, myeloperoxidase activity (MPO) and malondialdehyde (MDA) were measured by specific chemical methods, together with gene expression of Interleukin-8 (IL-8), matrix metalloproteinase-9 (MMP-9) and tissue inhibitors of matrix metalloproteinase-1 (TIMP-1) by RT-PCR, histo-pathological examination of lung tissues to detect the damage level was also done.

Results: When compared with the control group, COPD group showed significant increase of IL-8, IL-6, TNF-α, MPO activity, MDA and MMP-9 with significant decrease of GSH, catalase and TIMP-1 together with tissue damage shown by the histo-pathological examination. The use of erythromycin or N-acetyl-cysteine lead to significant decrease of IL-8, IL-6, TNF-α, MPO activity, MDA and MMP-9 with significant increase of GSH, catalase and TIMP-1 and improvement of histo-pathological damages, but the use of erythromycin affected significantly the inflammatory aspect of COPD (IL-8, IL-6, TNF-α, MPO activity, MDA and MMP-9 and TIMP-1 balance), while N-acetyl-cysteine affect significantly oxidative stress in COPD (TNF-α, MPO activity, MDA, GSH and catalase). However, treatment of COPD by both erythromycin and N-acetyl-cysteine lead to improvement of inflammation, alveolar structure destruction (emphysema) and oxidative stress in COPD.

Conclusion: Administration of erythromycin or N-acetyl-cysteine can ameliorate the changes that occur in COPD by their anti-inflammatory, antioxidant effects and their role in regain the balance between extracellular matrix degradation and deposition, while treatment of COPD using combination of the two drugs resulted in better improvement.


Introduction

CHRONIC obstructive pulmonary disease (COPD) is defined as a preventable and treatable disease with pulmonary component which is characterized by Inflammation of the lungs in the small and the large airways and in the pulmonary vessels [1]. In addition, systemic inflammation is present in many of these patients to the point that some authors have suggested that COPD is a part of a chronic systemic inflammatory syndrome [2]. Evidence of systemic inflammation in COPD suggests the possibility of a “spill-over” phenomenon from lung inflammation [3].

The association between systemic inflammation and COPD was due to activation of circulating inflammatory cells, increased levels of pro-inflammatory cytokines and acute-phase reactants as well as increased oxidative stress [4].

The lungs are exposed continuously to oxidants generated either from air pollutants or cigarette smoke. In addition, intracellular oxidants, such as those derived from mitochondrial electron transport, are involved in many cellular signaling pathways. Lung cells are protected against this oxidative...
challenge by well-developed enzymatic and non-
enzymatic antioxidant systems. When the balance
between oxidants and antioxidants shifts in favor
of oxidant form, by excess of oxidants and/or
depletion of antioxidants, oxidative stress occurs
[5].

In COPD, the extracellular matrix (ECM) deg-
radiation and deposition were imbalanced and ab-
normally activated. There was imbalance between
matrix metalloproteinase and tissue inhibitors of
matrix metalloproteinase of the lung tissue, which
may contribute to the pathogenesis of airflow
limitation through airway remodeling and alveolar
structure destruction (emphysema) [6].

Smoking is the main etiologic factor in COPD; cigarette smoke (CS) contains around 1017 oxidant
molecules per puff which lead to increase oxidative
stress in smokers with and without COPD [7]. Both
reactive oxidant species from inhaled cigarette
smoke and those endogenously formed by inflam-
matory cells constitute an increased intrapulmonary
oxidant burden. Structural changes to essential
components of the lung are caused by oxidative
stress, contributing to irreversible damage of both
parenchyma and airway walls. In addition, oxidative
stress results in alterations in the local immune
response, increasing the risk of infections and
exacerbations, which, in turn, may decrease the
lung function [8].

Suspension of the inflammatory response is a
logical approach to the treatment of COPD and
might improve symptoms, improve health status,
and reduce exacerbations. The long-term treatments
may reduce disease progression. However, no therapeu-
tic agent till now has been shown to reduce
the numbers of the important inflammatory cells,
macrophages, neutrophils, and CD8 + lymphocytes
present in the lung in COPD. Further, there is
currently no evidence that current therapies can
influence the systemic inflammation. For this
reason, attention has largely been focused on inhi-
bition of recruitment and activation of these cells,
and on antagonism of their products, also using of
antioxidant is essential in COPD to reduce the
airway remodeling and emphysema [9].

Increasing evidence shows that erythromycin
(ERY) ameliorates chronic inflammation via mech-
anisms independent of its antibacterial activity via
its anti-inflammatory and anti-apoptotic actions
[10]. N-acetyl-cysteine (NAC), a glutathione pre-
cursor, has been also applied in COPD patients in
order to reduce symptoms by its antioxidant effect

The aim of this work is to investigate the mech-
anisms involved in the genesis of chronic obstruc-
tive pulmonary disease and the role erythromycin
and/or N-acetyl-cysteine in amelioration of these
mechanisms by their role in the extracellular matrix
remodeling together with their anti-inflammatory
and antioxidant effects.

Material and Methods

Animals and experimental design:

In this study a total of 50 male albino rats,
weighing 150-200gm, 10-12 weeks old were used.
The rats were housed in wire mesh cages in a
constant temperature (22-24 °C) and light controlled
room on an alternating 12:12h light-dark cycle and
had free access to food and water. Veterinary care
was provided by laboratory animal house unit of
faculty of medicine, Cairo University during 2012.
The animals were divided into five groups (10 rats/
group) as follows:

Group I (Control): Administered saline orally and
exposed to ambient air.

Group II (COPD): In which COPD was induced
by cigarette smoking.

Group III (COPD treated by erythromycin): In
which erythromycin was administered in a dose
of 100 mg/kg orally/day, during the last month
of smoking period [10].

Group IV (COPD treated by N-acetyl-cysteine):
In which N-acetyl-cysteine was administered
in a dose of 600 mg/kg orally/day, during the
last month of smoking period [11].

Group V (COPD group treated by both erythromy-
cin and N-acetyl-cysteine): In which both drugs
were administered in the same above doses
daily, during the last month of smoking period.

Induction of COPD by cigarette smoking:

Cigarette smoke exposure was done according
to Hüseyin et al. [12] with some modifications in
the period of exposure. Cigarette smoke inhalation
was achieved in a wooden box (100 x 50 x 20cm)
with the help of an aquarium air pump, which had
been fixed inside the box. One end of a plastic
tube was implanted into the air pump and the other
end placed into a glass container in which 2 lit
cigarettes were placed. In each period of exposure
40 rats (in 4 cages, 10 rats/cage) were placed in
the exposure box for 10 minutes. Popular Egyptian
filter-tipped cigarette were used containing 25mg
tar and 1.8mg nicotine. The frequency of exposure
periods was increased gradually in the first five
days with a period of an hour and half interval
Biochemical analysis:

Detection of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) by ELISA methods.

Detection of malondialdehyde (MDA), glutathione (GSH), catalase, and myeloperoxidase (MPO) activity by colorimetric methods.

Gene expression of Interleukin-8 (IL-8), matrix metalloproteinase (MMP-9) and tissue inhibitors of matrix metalloproteinase (TIMP-1) in lung tissue by RT-PCR.

- Measurement of IL-6 and TNF-α in lung tissue:
  
  The lungs were homogenized for 30 s in lysis buffer containing 10mM HEPES, 150mM NaCl, 1 mM EDTA, 0.6% ipegal, 5mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, 1 mg/ml aprotinin, 10mg/ml soybean trypsin inhibitor, and 1mg/ml pepstatin. The homogenates were centrifuged at 10,000 rpm at 4°C for 10 min, and the supernatants were collected. IL-6 and TNF-α were quantitated with commercially available ELISA kits (R&D Systems, Minneapolis, MN, USA) [13,14].

- Measurement of GSH:

  Lung tissue homogenate was prepared using 10% (w/v) ice-cold 0.1M PBS (pH 7.4), the homogenate was centrifuged at 9000 rpm for 20min to obtain the supernatant. One milliliter of the supernatant was mixed with 1ml of 5% TCA (w/v), the mixture was allowed to stand for 30min and centrifuged at 2500 rpm for 15min. 0.5ml of the supernatant was taken and 2.5ml of 5'5’-Dithionitrobenzoic acid (DTNB) was added, mixed thoroughly, incubated for 1hour and absorbance was recorded at 412nm. Concentrations of GSH were calculated using the standard curve and the results were expressed as µmol/g tissue [15].

- Measurement of catalase activity:

  Lung tissue homogenate was prepared using 10% (w/v) ice-cold 0.1M PBS (pH 7.4), the homogenate was centrifuged at 12,000 rpm for 20min to obtain the supernatant. One milliliter of the phosphate buffer and 0.4ml water was added to 0.1ml of the supernatant. Reaction was started by adding 0.5ml H2O2 and the mixture was incubated at 37°C for 1min. Reaction was stopped by adding 2ml of dichromate: Acetic acid reagent and kept at a boiling water bath for 15min. The mixture was cooled, and absorbance was read at 570nm. Catalase activity was defined as the degradation of 1 µmol H2O2/min/mg protein, and the enzyme activity was expressed as U/mg protein [16].

- Determination of myeloperoxidase (MPO) activity:

  Assaying of MPO activity was described by Mizutani et al. 2003 [17]. Briefly, lung tissue (0.5gm) was homogenized in 10% (w/v) 0.05M phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and rehomogenized for 20 seconds. After centrifugation (4500g for 20 minutes at 4°C), 0.1mL of the supernatant was added to 0.55mL of 0.1 M phosphate buffer (pH 6.0) containing tetramethylbenzidine, as substrate (1.5 mmol/L) and H2O2 (0.5 mmol/L). After 5 minutes, changes in absorbance at 460nm were measured using a spectrophotometer. The activity of purified known human neutrophil MPO was used as the standard. Results are expressed as units of MPO activity per 100mg of tissue.

- Measurement of MDA:

  To measure the MDA concentration, 100mg of the lung tissue was homogenized in 1mL PBS, pH 7.0 with micro-pestle in micro-tube. 20% TCA was added to the homogenate to precipitate the protein, and centrifuged. Supernatants were collected and thiobarbituric acid (TBA) solution was added to the supernatants. After boiling for 10 minutes in water bath, the absorbance was measured. Concentrations of MDA were calculated using the standard curve. The concentration of MDA tissue was expressed in nanomole/milligram protein [18].

- Detection of IL-8, MMP-9 and TIMP-1 gene expression by reverse transcription -polymerase chain reaction (RT-PCR):

  - RNA extraction: RNA was extracted from lung tissue homogenate using RNeasy Purification
Reagent (Qiagen, Valencia, CA) according to the manufacturer’s instructions. The final RNA amount was determined by the spectrophotometer at 260/280nm.

- **RT-PCR:** Reaction mixture of RT reaction containing 1 µg total RNA, 0.5 µg random primer, 5xRT buffer, 2.5mmol/L dNTP, 20 U RNase inhibitor and 200 U MMLV reverse transcriptase in a total volume of 25 µl was incubated at 37°C for 60 minutes, then heated to 95°C for 5 minutes to inactivate MMLV. For PCR, 4 µl cDNA were incubated with 30.5 µl water, 4 µl 25mM MgCl2, 1 µl dNTPs (10mM), 5 µl 10X PCR buffer, 0.5 µl (2.5 U) Taq polymerase and 2.5 µl of each primer containing 10pmol. Primer sequences were shown in Table (1). The reaction mixture was subjected to 40 cycles of PCR amplification as follows: Denaturation at 95°C for 1 min, annealing at 67°C for 1min and extension at 72°C for 2min.

- **Agarose gel electrophoresis:** All PCR products were electrophoresed on 2% agarose stained with ethidium bromide and visualized by UV trans-illuminator.

- **Semi-quantitative determination of PCR products:** Semi-quantitation was performed using the gel documentation system (BioDO, Analyser) supplied by Biometra. According to the following amplification procedure, relative expression of each studied gene (R) was calculated following the formula:

\[ R = \frac{\text{Densitometrical Units of each studied gene}}{\text{Densitometrical Units of GAPDH}} \]

GAPDH was amplified with the same run of tested genes as a house keeping gene to detect RNA integrity.

| Table (1): Oligonucleotide primers sequence. |
|-----------------|-----------------|-----------------|
| **Gene** | **Primer sequence** | **PCR products sequence** |
| IL-8 | Forward: 5’ACGCTGGCTTCTGACAACACTAGT -3’  
Reverse: 5’-CTTCTCTGTCCTGAGAGAAGG -3’  
(according to Dinesh et al. 2008 [ ]) | 105 bp |
| TIMP-1 | Forward: 5’-CACAGACAGCCTTCTGCAAC -3’  
Reverse: 5’-CATTTCCCAAGCCTTGTAAT -3’  
(according to gene bank number NM 01044384) | 502 bp |
| MMP-9 | Forward: 5’-CATTCCGGTTGATAAGGAGT -3’  
Reverse: 5’-ACCTGTGTTACCTCAGTGTC -3’  
(according to gene bank number NM-013599) | 350 bp |
| GAPDH | Forward: 5’TGCTGGTGCTGAGTATGTCG 3’  
Reverse: 5’T TGAGAGCAATGCCAGCC 3’  
(according to gene bank number NM_017008) | 290 bp |

**Histological examination:**

Lungs were embedded in paraffin, and mid-sagittal sections were stained with hematoxylin and eosin (H&E), followed by examining under a light microscope with 100x magnification. Three non-consecutive lung sections from each animal and three non-overlapping random fields from each section were examined [19].

**Statistical analysis:**

Analysis of data was performed using SPSS 17 (Statistical Package for Scientific Studies) (Chicago, IL, USA). Data were presented as mean ± SD and evaluated by one-way ANOVA followed by post hoc, Kruskal-Wallis & Mann-Whitney tests. Differences of \( p \leq 0.05 \) were considered significant [20].

**Results**

- The Levels of IL-8 (arbitrary units), IL-6 (pg/mg ptn) and TNF-α (pg/mg ptn) in the Studied Groups:

There is a significant increase in the gene expression of IL-8 in lung tissue (Fig. 1) in COPD group (II) when compared to control group (I) (1.72±0.41 versus 0.35±0.17) \( (p<0.05) \). Treatment of COPD by erythromycin (group III) and by N-acetyl-cysteine (group IV), resulted in a significant decrease in the gene expression of IL-8 when compared to COPD group (II) (0.70±0.19 versus 1.72±0.41) and (0.92±0.22 versus 1.72±0.41) respectively \( (p<0.05) \), but the decrease is more in group III indicating the role of erythromycin in suppression of inflammation in COPD. Treatment with both drugs erythromycin and N-acetyl-cysteine (group V) resulted in a highly significant decrease
in the gene expression of IL-8 when compared to COPD group (II) (0.36 ± 0.19 versus 1.72 ± 0.41) (p < 0.001) and it is returned near to control levels.

As regard IL-6 (Fig. 2), a significant increase in its level in lung tissue in COPD group (II) was found when compared to control group (I) (133.67 ± 13.23 versus 25.37 ± 3.68) (p < 0.05). Treatment of COPD rats by erythromycin (group III) or by N-acetyl-cysteine (group IV), showed a significant decrease in the tissue levels of IL-6 when compared to COPD group (II) (99.92 ± 13.52 versus 133.67 ± 13.23) and (94.70 ± 4.99 versus 133.67 ± 13.23) respectively (p < 0.05). Treatment with both drugs (group V) resulted in a significant decrease in the level of IL-6 when compared to COPD group (II) (76.28 ± 12.52 versus 133.67 ± 13.23) (p < 0.05).

Value of TNF-α (Fig. 3) was significantly elevated in lung tissue in COPD group (II) compared to control group (I) (109.85 ± 8.40 versus 30.92 ± 6.47) (p < 0.05), treatment of COPD by erythromycin (group III) or by N-acetyl-cysteine (group IV), resulted in a significant decrease in the level of TNF-α when compared to COPD group (II) (88.20 ± 11.14 versus 109.85 ± 8.40) and (75.78 ± 13.97 versus 109.85 ± 8.40) respectively (p < 0.05).

- The levels of MPO activity (U/100mg tissue) in the studied groups:

As observed in (Fig. 4), the Levels of MPO activity was significantly increased in lung tissue in COPD group (II) compared to control group (I) (1.65 ± 0.42 versus 0.21 ± 0.4) (p < 0.05). In group III and group IV, a significant decrease in the levels of MPO activity was found when compared to COPD group (II) (0.51 ± 0.20 versus 1.65 ± 0.42) and (0.76 ± 0.18 versus 1.65 ± 0.42) respectively (p < 0.05), but erythromycin is still more effective in reduction of MPO activity than N-acetyl-cysteine. Combining both drugs (group V) resulted in a highly significant decrease in the level of MPO activity when compared to COPD group (II) (0.35 ± 0.12 versus 1.65 ± 0.42) (p < 0.001).

- The level of MDA (nmol/mg ptn) in the studied groups:

As shown in (Fig 5), the levels of MDA were highly significantly increased in lung tissue in COPD group (II) compared to control group (I) (9.55 ± 1.34 versus 1.26 ± 0.27) (p < 0.001), treatment with erythromycin (group III) and with N-acetyl-cysteine (group IV), resulted in a significant decrease in the levels of MDA when compared to COPD group (II) (6.99 ± 1.27 versus 9.55 ± 1.34) and (5.80 ± 1.31 versus 9.55 ± 1.34) respectively (p < 0.05).

Using both drugs (group V) resulted in a significant decrease in the levels of MPO when compared to COPD group (II) (4.58 ± 0.49 versus 9.55 ± 1.34) (p < 0.05).
**Fig. (3): Levels of TNF-α in all studied groups.**

* Denotes significant difference versus control subjects.
# Denotes significant difference versus COPD subjects.
$ Denotes significant difference versus COPD subjects treated with erythromycin.
@ Denotes significant difference versus COPD subjects treated with N-acetyl-cysteine.
% Denotes significant difference versus COPD subjects treated with both drugs.

**Fig. (5): Levels of MDA in all studied groups.**

* Denotes significant difference versus control subjects.
# Denotes significant difference versus COPD subjects.
$ Denotes significant difference versus COPD subjects treated with erythromycin.
@ Denotes significant difference versus COPD subjects treated with N-acetyl-cysteine.
% Denotes significant difference versus COPD subjects treated with both drugs.

**Fig. (4): Levels of MPO in all studied groups.**

* Denotes significant difference versus control subjects.
# Denotes significant difference versus COPD subjects.
$ Denotes significant difference versus COPD subjects treated with erythromycin.
@ Denotes significant difference versus COPD subjects treated with N-acetyl-cysteine.
% Denotes significant difference versus COPD subjects treated with both drugs.

**Fig. (6): Levels of GSH in all studied groups.**

* Denotes significant difference versus control subjects.
# Denotes significant difference versus COPD subjects.
$ Denotes significant difference versus COPD subjects treated with erythromycin.
@ Denotes significant difference versus COPD subjects treated with N-acetyl-cysteine.
% Denotes significant difference versus COPD subjects treated with both drugs.
The Levels of GSH (umol/gm ptn) and catalase (U/mg ptn) in the studied groups:

Cigarette smoke exposure triggered a significant decrease in the levels of GSH (Fig. 6) and catalase in lung tissue in COPD group (II) when compared to control group (I) (23.68±5.07 versus 54.72±7.95) and (59.53±7.01 versus 104.72±10.59) respectively (p<0.05), treatment of COPD with N-acetyl-cysteine (group IV) led to significant increase in the levels of GSH and catalase when compared with COPD group (II) (40.63 ±3.38 versus 23.68±5.07) and (81.88±13.42 versus 59.53±7.01) respectively (p<0.05), no significant change was observed in the levels of GSH and catalase of group III (erythromycin only) when compared to COPD group (II) (31.85±3.58 versus 23.68±5.07) and (71.10±7.59 versus 59.53±7.01) respectively (p>0.05), these results indicating the value of N-acetyl-cysteine as antioxidant and as a precursor of glutathione.

Treatment of COPD with both drugs (group V), resulted in a highly significant increase in the levels of GSH and catalase when compared with COPD group (II) (44.53 ±4.24 versus 23.68±5.07) and (95.58±11.07 versus 59.53±7.01) respectively (p<0.001), indicating the improvement of antioxidant defenses which counterbalance the effects of oxidants when both erythromycin and N-acetyl-cysteine were used.

The levels of MMP-9 (arbitrary units), TIMP-1 (arbitrary units) and MMP-9/TIMP-1 ratio in the studied groups:

Values of MMP-9 gene expression showed a high significant elevation in lung tissue in COPD group (II) compared to control group (I) (0.92 ±0.13 versus 0.13±0.02) (p<0.001), treatment of COPD by erythromycin (group III) and by N-acetyl-cysteine (group IV), resulted in a significant decrease in the values of MMP-9 gene expression when compared to COPD group (II) (0.55 ±0.15 versus 0.92±0.13) and (0.69±0.12 versus 0.92±0.13) respectively (p<0.05). Treatment of COPD with both drugs (group V), resulted in a high significant decrease in the values of MMP-9 gene expression when compared with COPD group (II) (0.32 ±0.07 versus 0.92±0.13) (p<0.001), these results indicated the value of combined erythromycin with N-acetyl-cysteine in reduction of extracellular matrix degradation.

As regard gene expression of TIMP-1, a significant decrease in its level in the lung was found in COPD group (II) compared to control group (I) (0.33±0.21 versus 1.33±0.31) (p<0.05), treatment of COPD by erythromycin (group III) and by N-acetyl-cysteine (group IV), resulted in a significant increase in gene expression of TIMP-1 when compared to COPD group (II) (0.76±0.15 versus 0.33±0.21) and (0.66±0.11 versus 0.33±0.21) respectively (p<0.05). Treatment with both drugs resulted in an insignificant difference when compared to COPD group (II) (0.34±0.09 versus 0.33±0.21) (p>0.05).

Fig. (7): Levels of MMP-9/TIMP-1 ratio in all studied groups.
* Denotes significant difference versus control subjects.
# Denotes significant difference versus COPD subjects.
$ Denotes significant difference versus COPD subjects treated with erythromycin.
@ Denotes significant difference versus COPD subjects treated with N-acetyl-cysteine.
% Denotes significant difference versus COPD subjects treated with both drugs.

Fig. (8): Agarose gel electrophoresis 2% stained by ethidium bromide showing gene amplification of: (A) IL-8 gene, (B) TIMP-1 gene, (C) MMP-9 gene, (D) GAPDH gene. Lane M: Ladder (100 bp for A, B,C and 50 bp for D), Lane 1: Control group, Lane 2: COPD group, Lane 3: COPD group treated with erythromycin, lane 4: COPD group treated with N-acetyl-cysteine and Lane 5: COPD group treated with both erythromycin & N-acetyl-cysteine.
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Fig. (9A): Histology of lung tissue of the control group.

Fig. (9B1, B2): Pulmonary morphological changes in cigarette smoke-induced COPD Group (II).

Fig. (9C): Pulmonary morphological changes in COPD group treated with erythromycin (Group III).

Fig. (9-D): Pulmonary morphological changes in COPD group treated with n-acetyl-cysteine (Group IV).

Fig. (9E): Pulmonary morphological changes in COPD group treated with erythromycin and n-acetyl-cysteine (Group V).
As observed in (Fig.7), MMP-9/TIMP-1 ratio was highly significantly elevated in lung tissue in COPD group (II) compared to control group (I) (4.30±2.88 versus 0.10±0.02) (p<0.001). Treatment of COPD by erythromycin (group III) and by N-acetyl-cysteine (group IV) resulted in a high significant decrease in MMP-9/TIMP-1 ratio when compared to COPD group (II) (0.78±0.33 versus 4.30±2.88) and (1.10±0.36 versus 4.30±2.88) respectively (p<0.001). Treatment with both drugs (group V), resulted in a high significant decrease in MMP-9/TIMP-1 ratio levels when compared to COPD group (II) (1.01±0.33 versus 4.30±2.88) (p<0.001).

From all these results we can suspect that erythromycin has more effective role than N-acetyl-cysteine in regain the balance between MMP-9 and TIMP-1 (protease/anti-protease balance) in lung tissues of COPD subjects.

- Histopathological analysis:

Histological examination of lung specimens of the normal control rats showed normal pulmonary architecture, the alveoli were patent and separated by average thickness of inter-alveolar septa, the walls of the alveoli were lined by flat type I pneumocytes and cuboidal type II pneumocytes (Fig. 9A).

Light microscopic examination of lung specimens from rats exposed to cigarette smoking (group II), showed obvious injury of the lung tissue with loss of the normal alveolar pattern. There was rupture of the inter-alveolar septa with the development of large emphysematous air spaces (Fig. 9B1). Some specimens showed marked cellular infiltration which led to thickening of the inter-alveolar septa and in massive consolidation which resulted in alveolar collapse. The pulmonary interstitium was invaded by many inflammatory cells, areas of hemorrhage which were sometimes associated with hemosiderin pigment deposition were seen, and also fluid exudate was seen in the interstitium (Fig. 9B2).

Light microscopic examination in COPD group treated with erythromycin showed mild damage in most specimens with mild affection of the normal alveolar pattern, where most of the alveoli were patent. Rupture of the inter-alveolar septa and formation of large air spaces were much less encountered in this group. Cellular infiltration and consolidation though were also much less than in the previous group (Fig. 9C).

Light microscopic examination revealed moderate damage with moderate affection of the normal alveolar pattern in COPD group treated with N-acetyl-cysteine (Fig. 9D).

As regard group of COPD treated with both erythromycin and N-acetyl-cysteine, light microscopic examination of lung tissues showed minimal damage and histological findings were comparable to control group (Fig. 9E).

Discussion

According to World Health Organization estimates, 80 million people have moderate to severe chronic obstructive pulmonary disease (COPD) [21]. COPD is an increasingly important cause of morbidity and mortality. It is now the fifth leading cause of death worldwide. In the developed world, cigarette smoking is by far the most important risk factor for COPD [22].

All measures for reducing smoking were failed due to its habitual and addictive nature. Also, the continuous scientific trials made to understand the mechanisms of COPD pathogenesis and the exacerbation of the stable disease placed a burden on health care systems. Moreover, results of many existing treatment interventions are still disappointing. All these factors necessitate the approach of a design of novel therapeutics.

COPD classically involves chronic bronchitis, emphysema, and small airway disease [23]; its pathogenesis involves several pathogenetic processes such as inflammation, alterations of cell growth, cellular apoptosis, abnormal cell repair, extracellular matrix destruction, and oxidative stress [24].

Inhaled cigarette smoke and other irritants activate epithelial cells and macrophages to release several chemotactic factors that attract inflammatory cells (e.g. monocytes, neutrophils, T helper cells, and cytotoxic T cells) from blood to the lungs. These inflammatory cells, together with tissue macrophages and epithelial cells, release proteases (e.g. matrix metalloproteinase (MMP) -9), which cause elastin degradation and emphysema. Neutrophil elastase also causes mucus hypersecretion. Epithelial cells and macrophages also release transforming growth factor (TGF)-β and fibroblast growth factors (FGFs), which stimulate fibroblast proliferation, resulting in fibrosis in the small airways, as well as the pro-inflammatory cytokines, TNF-α, IL-18, and IL-6, which amplify inflammation [25].
The current study demonstrated a significant increase in the levels of IL-6, TNF-α and IL-8 in lung tissue of COPD subjects when compared to controls. The study performed by Barnes et al. 2003 [26] supported these findings that, chronic inflammation in COPD is associated with an increase in the production of various mediators and pro-inflammatory proteins, including cytokines, chemokines, inflammatory enzymes and adhesion molecules, which are regulated by gene transcription factors. Among these mediators, those chemoattractant for inflammatory cells, in particular leukotriene B4 and IL-8, as well as pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6.

Foschino et al. [27] also found a high concentrations of IL-6, TNF-α and IL-4 either in plasma or in supernatant of induced sputum or in exhaled breath condensate of COPD subjects compared to healthy controls. Many other studies on humans and experimental models of COPD found that chronic exposure to cigarette smoke was associated with an increase in IL-6, IL-8, TNF-α and many other inflammatory markers in serum, sputum, bronchoalveolar lavage and lung tissue samples [28,29].

Myeloperoxidase (MPO) and elastase are elaborated from neutrophils during the inflammatory process. Since MPO acts as a parameter of neutrophil activity, the present study measured the levels of MPO in the lung tissue of COPD rats, and the results revealed that MPO activity was significantly increased in COPD group compared to control group. These results are supported by Fujimoto et al. 2005 [30] who found an increase in neutrophil numbers in COPD accompanied by an increase in level of MPO. Allegra et al. [31] also demonstrated that stimulated neutrophils generate superoxide, hydrogen peroxide, and release myeloperoxidase which has the unique property of converting chloride to hypochlorous acid (HOCI), which is considered to be the most powerful oxidant generated by neutrophils in association with endothelial dysfunction [32].

Oxidative stress plays a key role in the pathophysiology of COPD and amplifies the inflammatory and destructive process, as oxidants mediate inflammation, extracellular degradation, and failure of alveolar maintenance [24]. Reactive oxygen species from cigarette smoke or from inflammatory cells (particularly macrophages and neutrophils) result in several damaging effects in COPD, including decreased antiprotease defenses and activation of NF-kB, resulting in increased secretion of the cytokines IL-8 and TNF-α, which produce direct effects on airway function [33].

Increased levels of oxidants or reactive oxygen species (ROS) in the airways is reflected by increased markers of oxidative stress in the airspaces, sputum, exhaled breath, lungs and blood of the COPD patients [34]. In addition, the oxidant/antioxidant balance is deteriorated further by the depletion of antioxidant mechanisms with deficiencies in both enzymatic and non-enzymatic antioxidant systems [35,36].

MDA as a product of lipid peroxidation was measured in the present study, and a highly significant increase in its level in lung tissue of COPD group compared to controls was found. These results were in agreement with Doina et al. 2007 [37], who reported that the serum and lung MDA levels were significantly higher in cigarette smoke COPD group compared with control group. These findings were positively correlated with histopathological changes (squamous metaplasia and clear cell hyperplasia in the bronchium epithelium, emphysema) found in pulmonary tissue.

Another study done by Kluchová et al. 2007 [38], found that MDA levels correlate with disease severity, thus patients with severe COPD have the highest MDA levels.

Glutathione is abundant in the epithelial lining fluid of the respiratory tract, where more than 95% of it is in the reduced form. Glutathione is a key molecule in the antioxidant system not only because it is a critical substrate in the enzymatic machinery, but also because it is a major constituent of the non-enzymatic antioxidant defense [39]. GSH is considered a key adaptive response to protect the respiratory epithelium against cigarette smoke exposure [40].

Various cells of the respiratory tract, including macrophages and epithelial cells, produce antioxidant enzymes such as superoxide dismutase, and catalase [41].

In view of these data, we chose to assess the levels of GSH (as a non-enzymatic antioxidant), and catalase (as an enzymatic antioxidant) in lung tissue. In the current study cigarette smoke exposure triggered a significant decrease in the levels of GSH and catalase in lung tissue of COPD group when compared to controls. Similar results were obtained by Rahman et al. [42] and Marco et al. [43] who found that Acute exposure of cigarette smoke or its condensate to alveolar epithelial cells in culture or to lungs in vivo in rats and rabbits has been shown to deplete intracellular GSH. However, chronic inhalation of cigarette smoke in rats...
was associated with a dramatic depletion of GSH, with a significant increase in the levels of GSGS and protein S-thiolation in the lung.

Very recent studies confirmed the decrease in catalase levels through reporting that catalase was decreased at mRNA and protein levels in bronchial epithelium in smokers with COPD. They concluded that in mild COPD, the levels of catalase were elevated, as compared to healthy subjects due to an increase in the antioxidant activity in body in response to the mild oxidative stress. Greater the severity of the disease, higher will be the generation of the free radicals. Levels of catalase decreased from mild to very severe COPD [44-46].

On the contrary, Barnes [47] and Hanneke et al. [48] reported that catalase was markedly up-regulated in response to cigarette smoke and oxidative stress, and its activity was significantly higher in severe COPD patients compared with both controls and patients with mild COPD.

In COPD, the extracellular matrix (ECM) degradation and deposition were imbalanced and abnormally activated. There was imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinase (TIMPs) of the lung tissue, which may contribute to the pathogenesis of airflow limitation through airway remodeling and alveolar structure destruction (emphysema) [6]. The processes of inflammation and Oxidative stress may also impair the function of anti- proteases and thereby accelerates the breakdown of elastin in lung parenchyma [49].

In the current study, MMP-9 (as a documented protease biomarker related with pulmonary status), TIMP-1 (as a naturally occurring protein counter-acts MMPs) and MMP-9/TIMP-1 ratio (as the potential biomarker in diagnosis and treatment of emphysema) were chosen to study the extracellular matrix proteolysis or protease/anti-protease imbalance. The results showed high significant elevation of MMP-9 gene expression, significant decrease of TIMP-1 gene expression and a high significant elevation of MMP-9/TIMP-1 ratio in lung tissue in COPD group compared to controls.

Whereas data from other studies reported that patients with COPD caused by cigarette smoke showed higher expression levels of MMP-2, -9, and -12 transcripts in macrophages from broncho-alveolar lavage and from lung parenchyma when compared with controls [50,51]. Also, Segura-Valdez et al. 2000 [52] found increased MMP-9 expression by lung tissue samples of COPD patients.

Several studies found that MMP-8 and MMP-9 do not only act as secreted enzymes, but they are also bound to cells where they exert elastolytic activity. Thus, approximately 80% of the MMP-8 and MMP-9 synthesized by neutrophils remains associated with the surface and is not neutralized by TIMPs, so they may play a critical role in elastolysis [53,54].

In agreement with the present results, Abboud and Vimalanathan, 2008 [55], reported that COPD patients release less TIMP-1 in vitro than those from smokers without COPD and non-smokers. Several studies showed that cigarette smoke can promote MMP-9 gene expression and elevate the MMP-9/TIMP-1 ratio which may play a role in smoke-induced emphysema [56,57].

COPD is characterized by persistent pulmonary inflammation, which is thought to lead to progressive airflow limitation. Exacerbations of COPD are associated with increased lung inflammation and impair in the quality of life of patients. Thus anti-inflammatory antibacterial therapeutic interventions to reduce lung inflammation may lead to improved outcome in COPD patients [58].

There is also considerable evidence that an increased oxidative burden occurs in the lungs of patients with cigarette smoke-induced COPD results in an imbalance between oxidants/antioxidants or oxidative stress, leading to enhanced proteolytic activity, mucus hypersecretion and the enhanced inflammatory response. Most COPD patients are significantly deficient in antioxidants. Antioxidant therapy therefore would seem to be a logical therapeutic strategy for the prevention and treatment of COPD patients [59].

In the present study, treatment of COPD by erythromycin ERY (as an anti-inflammatory drug) (group III) or by N-acetyl-cysteine NAC (as an antioxidant drug) (group IV) resulted in a significant decrease in the gene expression of IL-8 and the levels of IL-6, TNF-α, MDA and MPO activity. Furthermore, a significant decrease in the value of MMP-9 gene expression and a highly significant decrease in MMP-9/TIMP-1 ratio with a significant increase in gene expression of TIMP-1. In addition, group IV showed significant increase in the levels of GSH and catalase, when compared to COPD group, indicating the value of NAC as antioxidant and as a precursor of glutathione.

Few studies have shown the protective role of ERY against the development of inflammation and pulmonary emphysema in COPD. But, until now, the involved mechanisms still remain to be explored.
In fact, the current study may be considered the first attempt to emphasize that the combination of ERY and NAC administration confers several protective mechanisms which may slow the progression of the COPD process.

Treatment of COPD subjects with both ERY and NAC (group V) resulted in a highly significant decrease in the gene expression of IL-6 and the levels of IL-6, TNF-\(\alpha\), MDA and MPO activity. Also, this combination of drugs resulted in a high significant decrease in the values of MMP-9 gene expression and MMP-9/TIMP-1 ratio. Furthermore, levels of GSH and catalase were highly significantly increased. Light microscopic examination of lung tissues showed minimal damage and histological findings were comparable to control group.

**Conclusion:**

According to the present results, it is obvious that administration of each of ERY or NAC alone in COPD patients can ameliorate the inflammatory changes, the oxidant insult, and the imbalance between extracellular matrix degradation and deposition in the lung tissue. While treatment of COPD with both ERY and NAC resulted in better improvement in pathogenesis of COPD due to their synergistic effects on decreasing inflammation, oxidative stress and the destruction of the alveolar walls.

**Recommendations:**

The current study may contribute to setting research priorities for clinical scientist as the results may spark future investigations to use a combination of ERY and NAC as a potential treatment of COPD patients.

**References**


Synergistic Effects of Erythromycin & N-Acetyl-cysteine


