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Protective Effects of N-acetylcysteine against 5-Fluorouracil-Induced Pulmonary Toxicity in Albino Rats

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Abstract

Background 5-fluorouracil (5-FU) is a potent chemotherapeutic drug widely used in the treatment of cancer and acts by blocking DNA synthesis. N-acetylcysteine (NAC) is a pharmaceutical drug and nutritional supplement represents the rapidly absorbed form of the amino acid L-cysteine and acts as antioxidant.

Objective The current study aims to investigate the protective role of N-acetylcysteine administration against 5-FU induced pulmonary toxicity in albino rats.

Methods The study was conducted on 18 healthy adult female and male Wistar albino rats which were randomly selected and equally distributed into three groups of 6 rats for each. Group I served as a control group. Group II received 5-FU (20 mg in 2ml normal saline per kg body weight) by intraperitoneal injection for 7 consecutive days. Group III received intraperitoneal injections of N-acetylcysteine (200 mg/kg) 24 hour prior to each intraperitoneal injections of 5-FU for 7 consecutive days. The specimens of lung tissue of the three groups were extracted and prepared for light microscopic examination. The tissue sections were stained with Harris Hematoxylin and Eosin (H&E) stain and with Masson's trichrome stain.

Results Structural changes were observed in Group II (5-FU recipient group compared to Group I (control group)including emphysematous dilatation of the alveoli, proliferation of BALT (bronchus associated lymphatic tissue), thickening of alveolar walls with mononuclear inflammatory cells infiltration, in addition to congestion and hemorrhage of pulmonary interstitium. Pretreatment with N-acetylcysteine effectively reduces the changes induced by 5-FUon the lung and reverts the abnormal pulmonary structure to become nearer to the norms.

Conclusion Treatment with N-acetylcysteine prior to 5-fluorouracil effectively attenuated 5-FU induced pulmonary damage and reverted the abnormal structural changes to near normal. Thus NAC has a protective potential in ameliorating 5-fluorouracil induced pulmonary toxicity.

Key words 5-Fluorouracil, N-acetylcysteine, rats, lung, emphysema.

List of abbreviation: 5-FU = 5-fluorouracil, TS = thymidylate synthase, ROS = reactive oxygen species, DPD = dihydropyrimidine dehydrogenase, NAC = N-acetylcysteine, LD_{50} = Lethal dose, BALT = bronchus associated lymphatic tissue, COAD = chronic obstructive airway disease.

Introduction

Chemotherapeutic drugs have been used worldwide for the treatment of a variety of human neoplasms given as a single or combined treatment protocol [1].

5-fluorouracil (5-FU) is pyrimidine analogue belongs to the family of antimetabolites. It is S-Phase specific drug which principally inhibits thymidylate synthase (TS) enzyme resulting in a decreased DNA synthesis. Moreover, it interferes with RNA processing and protein synthesis [2].

Cytotoxic effects of 5-FU may be exerted by generation of reactive oxygen species (ROS) resulting in apoptosis (programmed cell death)

or necrosis [3]. The metabolism of 5-fluorouracil occurs mainly in the liver and results in degradation products (e.g., carbon dioxide, urea, a-fluoro-*B* -alanine).It has a half-life of approximately 10 min, approximately 15-20% of the administered dose is excreted unchanged in the urine, the remaining 80-85% of the administered dose is excreted as carbon dioxide via expiration [4].

5-FU is degraded by the hepatic dihydropyrimidine dehydrogenase (DPD) which is the initial and rate limiting enzyme in 5-FU catabolism thus 5-FU.

Toxicity may be decreased if the catabolism is blocked by a genetic defect of DPD in the liver [5].

5-FU is used for the treatment of advanced colorectal cancer, breast cancer, carcinoma of the stomach, head and neck and pancreas [6], and topically (as a cream) for treating actinic keratoses and basal cell carcinoma [7] and in ophthalmic surgery [8].

Administration of 5-FU produces some adverse effects including stomatitis, mucositis and diarrhea in addition to leucopenia, hemolytic anemia and thrombocytopenia [9].

However, extensive investigations have been conducted on the toxicity of 5-FU including hepatotoxicity [10], cardiotoxicity and neurotoxicity of 5-FU [3,11] but there are limited information that concerned with the effects of 5-FU on the histology of the lung tissue.

5-FU causes excessive generation of ROS and induces a decrease in the antioxidant defense mechanism against oxidative damage resulting in cellular damage either as apoptosis (programmed cell death) or necrosis. Thus oxidative stress is an essential mechanism by which chemotherapy and radiotherapy work to kill cancer cells [12].

N-acetylcysteine (NAC) is the N-acetyl derivative of the amino acid L-cysteine. It exhibits direct antioxidant effect through its free sulphydryl (thiol) group which can reduce the free radicals [13].

In addition, NAC exerts an indirect antioxidant effect related to its role as a precursor of Glutathione which serves as an essential factor to overcome the harmful effects of internal and external toxic agents [14].

NAC is the drug of choice in acetaminophen overdose which is used frequently in self-poising [15]. Moreover, it is considered as mucus dissolving agent in the chronic obstructive pulmonary diseases such as bronchitis and cystic fibrosis [16].

NAC increases the resistance to influenza virus [17], it reduces the symptoms of schizophrenia, depression and bipolar disorder [18].

Additionally, NAC protects the body from toxic effects of alcohol and tobacco smoke. Recently, it has been used successfully to treat arsenic and mercury poisoning [19].

The aim of the present work is to evaluate the protective role of NAC against toxicity induced by 5-FU in the lungs of albino rats.

Methods

Eighteen adult healthy female and male Wistar albino rats of the same age group (2.5-3) months and weight (200-250 g) were obtained from the Animal House of the Experimental Research Unit, College of Medicine, and University of Mosul.

The animals were housed in a standard condition at a room temperature of about 25^{o}C and all animals were allowed for free access to laboratory pellet foods and tap water drink.

The experiment was conducted in the accordance of the ethical guidelines and internationally accepted principles for laboratory use and care in animal research. Lethal dose (LD_{50}), Pilot studies and related literature were taken into account and the accurate doses of 5-FU [20] and NAC [21] were calculated.

The body weight of each rat was recorded at the beginning of the experiment and recorded again at the end of the experiment just before killing of the animals.

The animals were randomly and equally divided into 3 groups of 6 animals each:

Group I: Each animal of this group was given2 ml/kg body weight/day of normal saline by

intraperitoneal injection for 7 consecutive days and served as a control group.

Group II: each animal of this group was given 5-FUin a dose of 20 mg in 2ml normal saline /kg /day by Intraperitoneal injection for 7 consecutive days.

Group III: first received NAC eats a dose of (200 mg/kg) by intraperitoneal injections and subsequently after 24 hour received 5-FU by intraperitoneal injection20 mg/kg/day for 7 consecutive days.

One day after the last injection of the three groups, all the animals were scarified and dissected under light ether to collect the two lungs from each animal, then the extracted lungs were fixed in 10% neutral buffered formalin solution for about 24 hours.

The histological sections were prepared according to Bancroft *et al* [22] in which small pieces of about 4-5 mm in thickness were cut from each lung and dehydrated in ascending grades of ethanol (70%, 90%, 100%). Clearing was done in xylene and embedded in paraffin wax. Serial ribbons of 4-7 sections of about (4-5) microns in thickness were collected from each paraffin block using Reichert's Rotatory Microtome.

The sections were spread in a hot water bath with 40-45^{o}C temperature then loaded on clean, labeled glass slides after making light scan of egg-albumin and put in oven at 60^{o}C for 30 minutes then left to dry at laboratory room temperature.

The sections were stained with Harris Haematoxylin and Eosin (H & E) stain and Masson's Trichrome stain according to Kim *et al* [23]. Then the stained sections were examined microscopically to detect any structural changes using (Olympus-BX51) light microscope using objective lenses X10, X40, X60 and eye piece lens X10. Micrographs from some sections were taken using Digital Camera (SONY-Cybershot 14.1 Mega Pixels) at X100, X400, X600 magnifications.

Morphometric Measurements

Morphometric estimation of the alveolar wall thickness was done by using a highly optimized microscope (Visopan projection microscope) at X400 magnification from 6 randomly chosen non-overlapping fields from the lung sections for each group then alveolar wall thickness was measured using perpendicular lines drawn across the section of the alveolar walls.

Statistical analysis

Statistical analysis for the animal's mean body weight and alveolar wall thickness was performed by SPSS version 20 for windows software. Data were presented as mean±SD and were analyzed using one-way Analysis of Variance followed by Bonferroni multiple comparisons for post-hoc analysis to compare the animals' mean body weight before and after injection of the drugs.

The cutoff point for statistical significance was set at 0.05. P value ≤ 0.05 were considered to be significant whereas P value > 0.05 were considered to be non significant.

Results

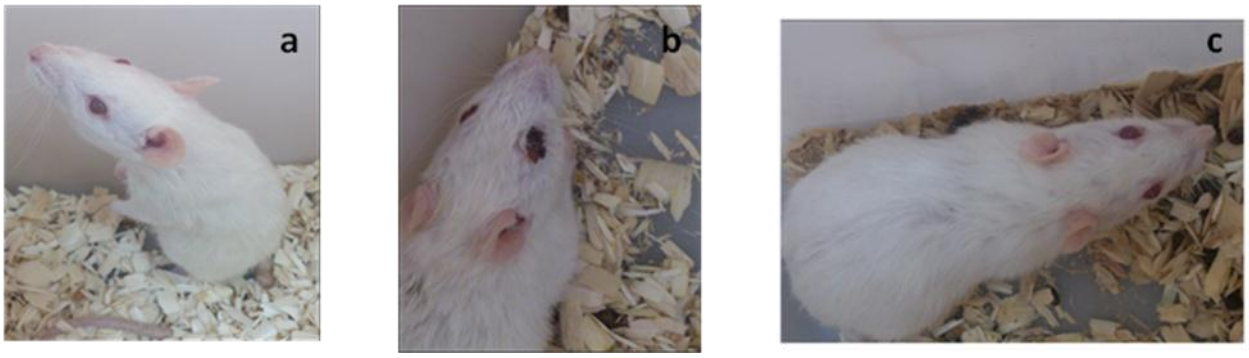
Physical Observations

The animals of the control group (group I) stayed alive till the end of the experiment. They were active, responded very quickly to stimuli, and they had a good appetite.

The sites of injections showed no swelling or inflammation **(Photo. 1a)** whereas the animals of group II became less active and gathered themselves at one corner of the cage on the 3rd day of the experiment and onward, their appetite was greatly reduced. Some rats had frequent diarrhea with ulcerations around the eyes and mouth and loss of furring of the skin which was recorded on the 7th day of the experiment **(Photo. 1b).** The animals of group III remained alert until the end of the experiment, their response to stimuli and food intake were normal **(Photo. 1c)**.

Histopathological results

(1) The lungs of the control group appeared pinkish, soft; with spongy like appearance, the left lung consisted of one large lobe while the right lung consisted of four lobes. The lung section of the control group showed:



Photograph showing normal appearance of rat from group I looks healthy and active (a); general appearance of rat from group II looks inactive with ulcerations around the eyes (b); general appearance of rat from group II showed return of activity (c).

1. Normal alveolar duct, alveolar spaces and terminal bronchioles with regular size and shape of the alveoli (Fig. 1).

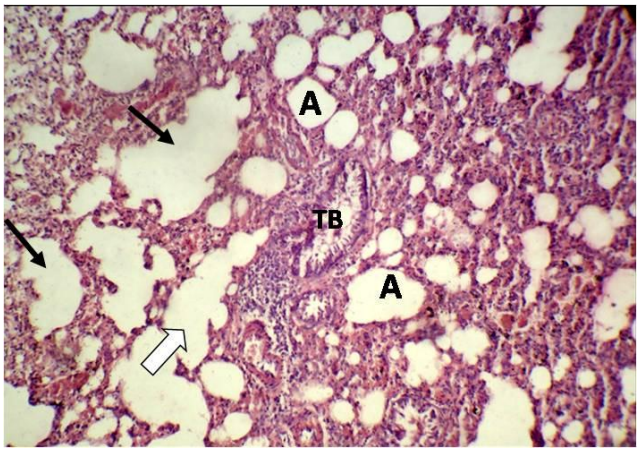


Fig. 1. Rat's lung of group I showing alveoli (A), alveolar duct (white arrow) alveolar sacs (black arrows) and terminal bronchiole (TB) (H&E X100).

2. Alveolar walls are of normal thickness and are lined by spindle shaped pneumocytes type I and rounded shaped pneumocytes type II (Fig. 2).
3. Few collagen fibers in the alveolar walls, in the wall of the pulmonary vessel and in the wall of the terminal bronchiole (Fig. 3).

(2) The lung tissue of group II showed focal areas of congestion and hemorrhage while the lung sections showed:

1. Abnormal alveolar spaces with destruction of the alveolar walls and fusion of some adjacent alveoli causing emphysematous dilatation with hyperplasia of bronchus associated lymphatic tissue (BALT) forming aggregates of lymphoid follicles (Fig. 4).

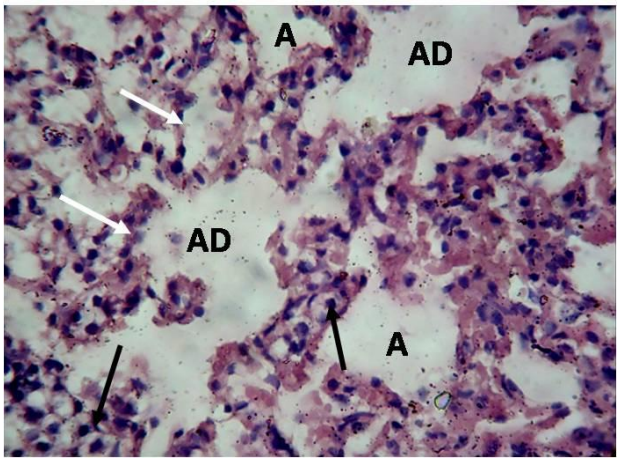


Fig. 2. Rat's lung of group I showing alveolar duct (AD), alveoli (A) with normal thickness of their wallslined by pneumocyte type I (white arrows) and pneumocyte type II (black arrows) (H&E X 400).

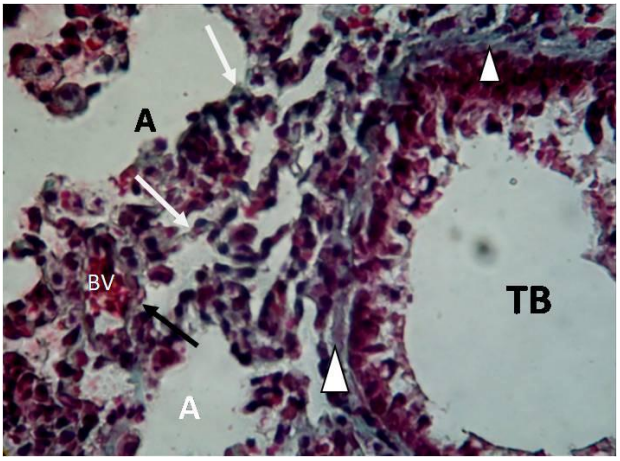


Fig. 3. Rat's lung of group I showing few collagen fibers stained with green color (white arrows) in the walls of the alveoli (A), in the wall of the blood vessel (BV)(black arrow) and in the wall of terminal bronchiole(TB)(arrow heads)(Masson's trichrome X 400).

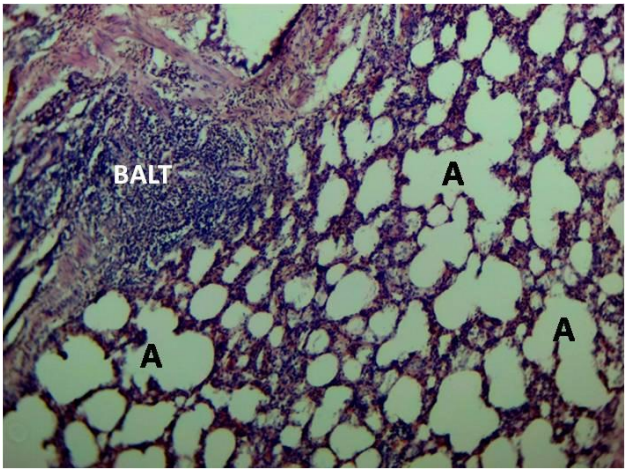


Fig. 4. Rat's lung of group II showing emphysematous dilatation of the alveoli (A) with hyperplasia of bronchus associated lymphatic tissue (BALT) (H&E X 100).

1. Less emphysematous dilatation of alveoli than that observed in group II with normal epithelial lining of the terminal bronchiole (Fig. 9).
2. Normal thickness of alveolar walls with no congestion of capillary bed (Fig. 10).
3. Thickening of alveolar walls with hemorrhage and congestion of the capillary bed and mononuclear inflammatory cells infiltration in the wall of the alveoli (Fig. 5).
4. Deposition of collagen fibers in the alveolar walls (interstitial fibrosis) (Fig.6).
5. Pulmonary vascular congestion and perivascular fibrosis with mononuclear inflammatory cells infiltration and adipocytes in the perivascular tissue (Fig. 7) and around the terminal bronchioles (Fig.8).

(3) The lung of group III appeared soft, pinkish, with few congested areas while the lung sections of group III showed:

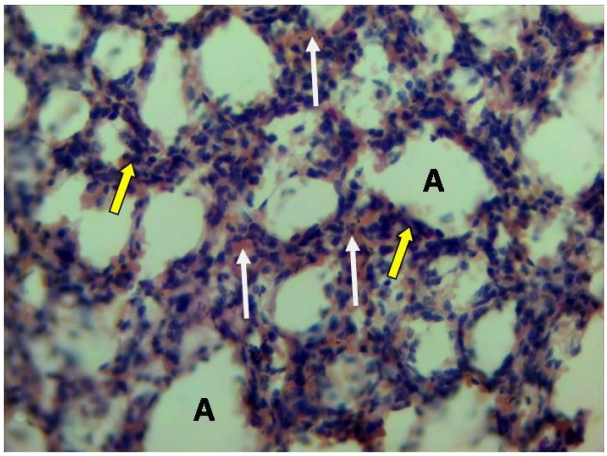


Fig. 5. Rat's lung of group II showing thickening in the walls of alveoli (A) with hemorrhage and congestion of the capillary bed (white arrows) and mononuclear inflammatory cells infiltration (yellow arrows) (H&E X 400).

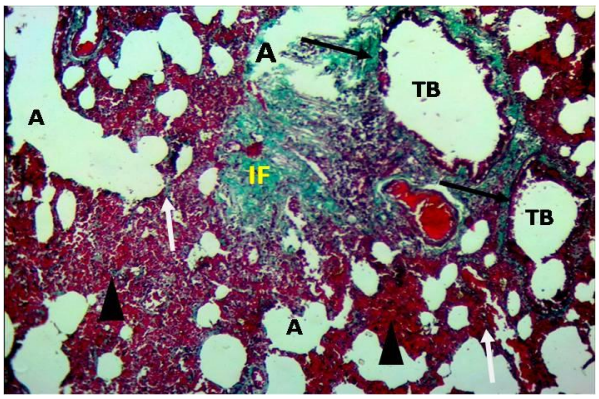


Fig. 6. Rat's lung of group II showing deposition of collagen fibers (interstitial fibrosis) (IF) between the alveoli (A) and around the terminal bronchioles (TB) (black arrows), thickening of alveolar walls with capillary congestion and fibrosis stained green (arrow heads) (Masson's trichrome X 100).

6. Few collagen fibers around the pulmonary vessels and around the terminal bronchiole (Fig.11) as well as in the alveolar walls (Fig.12).

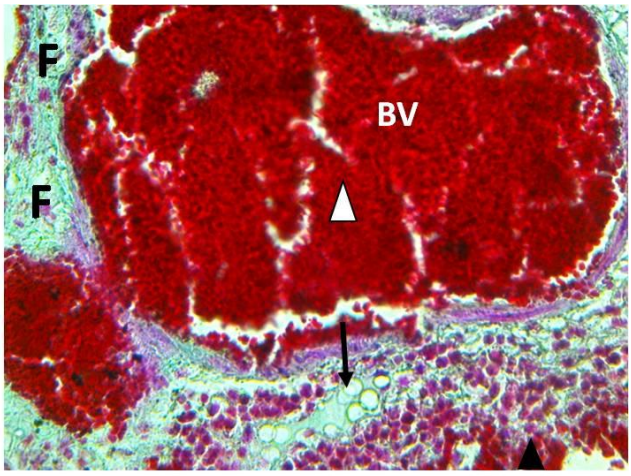


Fig. 7. Rat's lung of group II showing pulmonary vascular congestion (white arrow head) and perivascular fibrosis green in color (F) with mononuclear inflammatory cells infiltration (black arrow head) and adipocytes (black arrow) in the perivascular area (Masson's trichrome X 600).

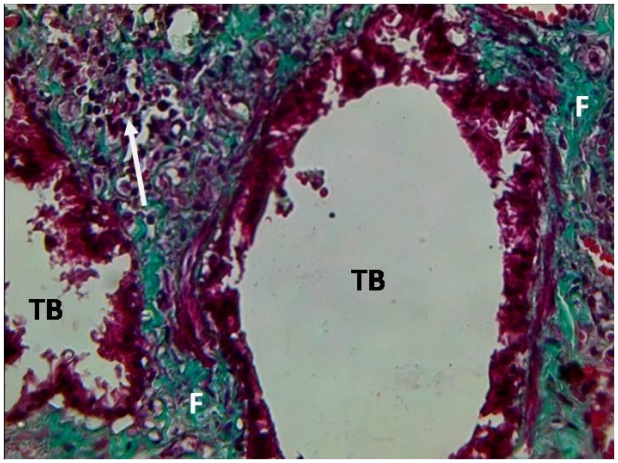


Fig. 8. Rat's lung of group II showing damage to the epithelial lining of the terminal bronchiole (TB) and fibrosis green in color (F) with mononuclear inflammatory cells infiltration (white arrow) around the terminal bronchioles (TB) (Masson's trichrome stain X 400).

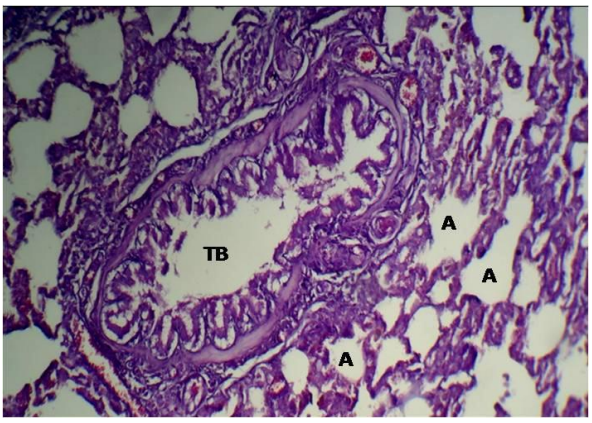


Fig. 9. Rat's lung of group III showing less emphysematous dilatation of the alveoli (A) with normal epithelial lining of the terminal bronchiole (TB) (H&E stain X 100).

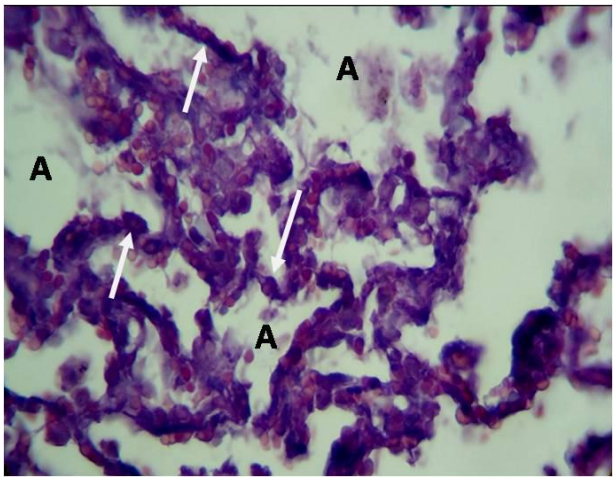


Fig. 10. Rat's lung of group III showing normal thickness of the alveolar walls (white arrows) between the alveoli (A) and mild emphysematous changes with no congestion of capillary bed (H&E stain X 400).

Body weight results

Results were expressed as mean±SD, very high significant reduction (P = 0.001) of the animals' mean body weight was observed in group II compared with the control group. Furthermore, group III showed no significant differences in the body weight compared to the control group (P = 0.1) but showed significant differences in their body weight compared to group II (P = 0.02) (Table 1).

Morphometric results

Results were expressed as mean±SD, very high significant increase (*P* = 0.001) in the alveolar wall thickness (14 ± 5.1) was observed in group II compared with the control group (4.3 ± 2.2).

Furthermore, group III showed no significant differences in the alveolar wall thickness (5.2 ± 2) compared to the control group (*P* = 0.2) but showed significant differences when compared with group II (*P* = 0.01) (Table 2).

Discussion

5-FU is a widely used chemotherapeutic drug which acts by blocking DNA synthesis. However, the clinical use of 5-FU is limited by its toxicity which interfere with its therapeutic efficacy [24] and several studies had been performed to prove the protective effects of certain agents against 5-FU induced toxicity [25]. However, there is very limited information on the protective role of NAC against 5-FU induced pulmonary toxicity.

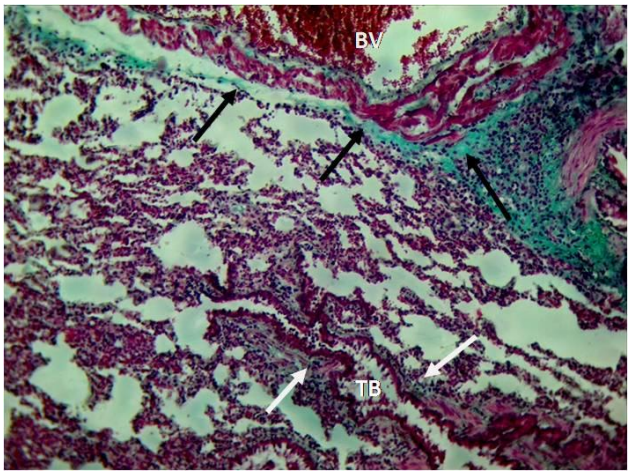


Fig. 11. Rat's lung of group III showing few collagen fibers around the pulmonary vessels green color (black arrows) and around the terminal bronchiole (TB) (white arrows)(Masson's trichrome stain X 100).

In the present study, the animals were injected by intraperitoneal 5-FU since this approach allows very high concentrations of 5-FU to deliver into the peritoneal cavity without increasing the risk of systemic toxicity [26]. Normal saline solution (0.9% sodium chloride) was used as a carrier for 5-FU in order to get a quicker absorption from the peritoneum and to achieve a higher level of toxicity [27].

The animals of group II showed very high significant reduction in their body weight .These results are in agreement with those reported by Cheah et al [28] who mentioned that 5-FU induced weight loss might be due to oral mucositis which is a painful condition associated with inflammation and ulceration affecting the mucosa of the mouth and causing a difficulty in eating and drinking and reduced food intake. The animals of group III showed no significant differences in their body weight compared to the control group. This observation was attributed to the ameliorative effect of NAC on the 5-FU induced oral mucositis.

In this study, the structural changes in the lung tissue induced by 5-FUinclude emphysema, mononuclear inflammatory cells infiltration and interstitial fibrosis are in agreement with that reported by Zidan [29] who stated that prolonged administration of amiodarone (an effective anti-arrhythmic drug indicated for cardiac arrhythmia) in rats may induce severe pulmonary changes such as.

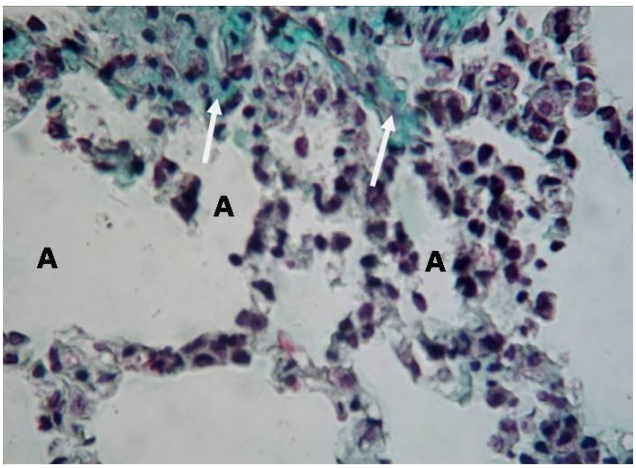


Fig. 12. Rat's lung of group III showing normal amount of collagen fibers (green color) (white arrows) in the alveolar walls (Masson's trichrome stain X 400).

Moreover, the results of the present work are nearly similar to what has been noted by Ahmed [30] who investigated the effects of Rifampicin and Isoniazide on the rat's lung tissue and found that combined rifampicin and isoniazid administration showed more intense emphysema and

inflammatory cells infiltration than when each drug was given separately.

The alveolar emphysematous changes observed in the rats treated with5-FU might be attributed to inadequate production of surfactant by pneumocytes type II due to direct cellular damage caused by the drug, thus most of the alveolar walls are destructed and the alveolar spaces are communicated with each other leading to emphysema.

Table 1. Body weight of the control group and experimental rats

Group	No.	Body weight	
		Before injection (Mean ± SD)	After injection (Mean ± SD)
Group I (control)	6	155 ± 16.43	158.5 ± 17.78**
Group II (5-FU)	6	160.5 ± 69.72	106.6 ± 39.39
Group III (5-FU+NAC)	6	185 ± 21.67	159.2 ± 28.17*

**: $P = 0.001$ (I versus II), $P = 0.1$ (I versus III), *: $P = 0.02$ (II versus III)

Similar changes were previously described by Ganesan *et al* [31] who referred these finding to the oxidative stress created by some oxidizing substances released from the alveolar macrophages and neutrophiles. In addition, some proteolytic enzymes such as metalloproteinases released by macrophages might cause alveolar collapse and emphysema thus alveolar macrophages play an important role in the pathogenesis of emphysema [32].

Table 2. Morphometric measurements of the alveolar wall thickness for the control group and experimental rats

Group	No.	Alveolar wall thickness (µm)
Group I (control)	6	4.3 ± 2.2**
Group II (5-FU)	6	14 ± 5.1
Group III (5-FU+NAC)	6	5.2 ± 2*

**: $P = 0.001$ (I versus II), $P = 0.2$ (I versus III), *: $P = 0.01$ (II versus III)

The presence of adipocytes in the perivascular tissue of pulmonary vessels in the present study was observed by previous workers [33,34] and may be explained by abnormal metabolism of phospholipids promoting accumulation of large quantities of adipocytes particularly in the perivascular areas.

This study showed thickening of alveolar walls due to congestion of capillaries, marked mononuclear inflammatory cell infiltration and edema.

Congested capillaries resulted from direct toxic effect of 5-FU on the wall of capillaries causing ischemia and necrosis followed by vasodilatation and escape of blood through their necrotic wall to the interalveolar septa and lumen of alveoli. This modifications in the vascular bed resulted in inflammatory cells infiltration and edema which is regarded as a defense mechanism against the toxic effects because the infiltrated cells assist in the rapid removal of tissue debris and red blood cells to facilitate regeneration [35]. The present finding agrees with previous studies which concluded that body defense reaction takes place against invading pathogenic bacteria or irritating agents due to the activity of alveolar macrophages which might release many mediators that augment the inflammatory response of the alveoli [36]. In addition, lymphoid hyperplasia of bronchus associated lymphatic tissue (BALT) might be provoked by some inflammatory chemotactic mediators released by the bronchial epithelium which stimulate lymphocytic proliferation around the terminal bronchioles. Similar activation of BALT had been previously observed in the rat's lung [37].

The pulmonary vascular congestion noticed in group II could be due to release of some vasodilator substances into the blood stream then the stagnant blood in the dilated vessels

will cause tissue hypoxia of the lung resulting in more pulmonary congestion. Similar finding had been noticed by [38] who observed congested pulmonary vessels in the rat's lung after exposure to aluminum chloride.

The lungs of group II rats showed interstitial fibrosis and deposition of collagen fibers in the perivascular and peribronchial area. This finding might be due to destruction of the lung tissue induced by the drug with subsequent inflammatory reaction thus, more fibroblasts might be brought to the irritated area leading to more collagen fiber deposition [39]. In addition, some previous studies revealed that in normal lung, pneumocytes type II are able to secrete prostaglandin E2, which acts to suppress fibroblast activity and proliferation and 5-FU might reduce prostaglandin E2 secretion which in turn leads to overproduction of fibroblasts with consequent interstitial fibrosis[40].

Danic *et al* [41] reported that one of the main events in the pathogenesis of broncho-pulmonary dysplasia and lung fibrosis following the administration of 5-FU is the formation of ROS. Pulmonary toxicity of 5-FU mediated by reactive oxygen species are nearly similar to the alterations observed in the chronic obstructive airway diseases such as desquamation of alveolar epithelium with increased vascular permeability, stimulation of mucous secretion, activation of fibroblast and mast cells with increased elastic and collagen fibers synthesis. All these changes can be attenuated by NAC which is a safe drug and easy to use in clinical practice [42].

The lung tissue of group III showed few congested areas with near normal appearance and this might reflect the improvement in the histological changes induced by 5-FU. Concomitant administration of NAC with 5-FU showed a considerable protection of the lung tissue thus the pulmonary architecture was preserved due to restoration of the oxidative imbalance by NAC directly by free radical scavenging and indirectly by glutathione synthesis thus, it increase pulmonary defense mechanisms. Supporting this finding, previous studies on a rat model revealed that administration of NAC together with cigarette smoke through the trachea might increase pulmonary glutathione, prevent thickening of the alveolar walls and improved phagocytic activity of the alveolar macrophages.

The lungs of group III rats showed less emphysematous dilatation of the alveoli with less fibrosis than that observed in group II and no congestion of the capillary beds. Such protective antioxidant role of NAC was previously demonstrated by Zhang *et al* [43] who reported that NAC can reduce ROS content, inhibit the mitochondrial apoptotic pathway and thus it can alleviate pulmonary fibrosis in rats exposed to intrapulmonary injection of silica suspension. Mononuclear inflammatory cells infiltration was alleviated by pretreatment with NAC due to reduction in the cytokins released from mast cells and alveolar macrophages [44].

Such protective antioxidant role of NAC was previously demonstrated in the liver tissue by Wang*et al* [45] who reported that acute liver damage induced by ethanol in mice was markedly alleviated by intraperitoneal injection of NAC. Additionally, Kilciksiz *et al* [46] noticed that the prophylactic use of NAC effectively reduce tissue damage caused by oxidative stress induced by radiation and give a clue about the probability of radioprotective effect of NAC.

In conclusion, the use of 5-FU for the treatment of some tumors seriously affects the structure of the lung causing emphysema and lung fibrosis. Using NAC before the injection of 5-FU protects the lung tissue against the toxic effects by increasing pulmonary defense mechanisms through its direct antioxidant action and its indirect role as a precursor in the glutathione synthesis. So that NAC may be considered as a useful dietary supplement for patients taking antineoplastic drugs like 5-FU.

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Author Contribution

The injection of the animals, histological preparation and staining of the sections, microscopical examination and photographing were done by Al-Hamdany. The reading, evaluation and writing of the article were done by Al-Hubaity.

Conflict of Interest

The authors declare of interest.

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