Original Article

Evaluation of Propolis Supplementation on Lung Tissue Toxicity Induced by Aluminum chloride in Adult Male Albino Rats: A Histological and Immunohistochemical Study

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ABSTRACT

Introduction: Aluminum chloride is the most abundant metal present on the earth's crust. It is found in our daily life as in drinking water, soil and in commonly used cosmetics. The lung epithelium is the site for aluminum chloride accumulation. Propolis is wax-like bee product. It has a powerful anti-inflammatory antioxidant property.

Objectives: Evaluation of aluminum chloride toxicity on lung tissue and the possibility of Propolis to play a protective role.

Materials and Methods: Fifty adult male albino rats were divided into five equal groups received treatment for 6 weeks: (I) Control group received distilled water (II) Propolis group "50mg/kg ". (III) Aluminum chloride group "475mg/kg". (IV) Recovery group" kept untreated". (V) Propolis and aluminum chloride group. Finally, all animals were sacrificed. Lung samples were obtained for histological and immunohistochemical studies. Data was statistically analyzed.

Results: Groups (I& II) showed normal structure of lung tissues. Aluminum chloride group (III) revealed distorted lung tissues. Some alveoli were collapsed and the others showed compensatory dilatation. Lung tissue had numerous hemosiderin granules. Pneumocyte II showed condensed nucleus, cytoplasmic vacuolations, distended irregular mitochondria, dilated rough endoplasmic reticulum and degeneration of their lamellar bodies. There was thickening in the inter-alveolar septa, numerous inflammatory infiltrate and eosinophilic materials. Blood vessels were congested and surrounded by inflammatory cells. The bronchioles showed distortion. Marked collagen fibers and numerous accumulations of mast cells in connective tissue septae were detected. A significant decrease in BCL2 immune expression was noticed. These changes were moderately alleviated in the recovery group. Co-administration of Propolis with aluminum chloride restored lung tissue integrity.

Conclusion: Aluminum chloride induces marked lung tissue damage via, fibrosis and apoptosis. These changes can be alleviated moderately by stopping treatment for 2 weeks and markedly by combined treatment with Propolis. So we recommend Propolis supplementation for people exposed to aluminum chloride.

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Key Words: Aluminum chloride, BCL2, EM, propolis.

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INTRODUCTION

Aluminum chloride is the most common metal present on the earth's crust[1]. It is found in our daily life as in drinking water and soil. It is used in manufacture of cooking utensils and toothpaste[2]. Unfortunately, aluminum chloride entered in the newest industrial products like vaccines adjuvants, phosphate binders, dialysis, total parenteral nutrition solutions and foods, providing easy exposure to it[1]. Additionally, it is present in cans, foils, containers, baking powder, cake mixes, frozen dough, pancake mixes, self-rising flour, grains, and processed cheese[3]. Aluminum chloride has been proved to have toxic effects on many organs of the human body.

Exposure to aluminum through breathing is significantly affected by relevant practices, including industrial exposures[4] moreover, Presence of aluminum chloride as a component of many aerosol formulations of cosmetics particularly antiperspirants that are commonly used, will contribute significantly to exposure to aluminum through breathing. Topically applied cosmetics and related skin, hair and hygiene products are often significant sources of aluminum[5]. The highly dynamic nature of the lung epithelium means that it must be a site for the accumulation of aluminum chloride and a surface for the uptake of aluminum into lung tissues and access to the systemic circulation[6].

Aluminum chloride has been proved to have toxic effects on many organs of the human body[7]. It is capable of increasing the cellular oxidative stress by potentiation of pro-oxidant properties of transition metals such as iron and copper[8]. It leads to lipid peroxidation and oxidative degradation of cellular DNA and proteins. Thus it can induce tissue damage under chronic condition[9].

Propolis is a wax-like bee product, composed of more than 300 compounds as phenols aldehydes, sesquiterpene quinines, amino acids, steroids, polyphenols and coumarins[10]. Polyphenolic fractions that present in Propolis are responsible for its antibacterial, antiviral,

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antifungal, antimicrobial, analgesic, anti-inflammatory, antioxidant and anticancer effects[10]. Recently Propolis became the focus of a large number of research projects[11]. Moreover Propolis has been used as antioxidant effective against environmental pollutes like Lead[12].

Propolis can modulate cytokine secretion and inhibit reactive oxygen species (ROS)[13]. It also modulates the metabolism of blood lipid leads to a decrease in lipid peroxidation and scavenges the free radicals[14].

From forgoing, our study was designed to evaluate the effects of aluminum chloride on lung tissue and the possibility of Propolis to play a protective role, regarding to its demonstrated antioxidant and anti-inflammatory activity

MATERIAL AND METHODS

Chemicals

- Propolis was purchased as a sticky powder from Emtinan Company, Egypt.
- 2- Aluminum chloride powder was purchased from El Gomhoreya Company, Egypt. Both chemicals dissolved in distilled water (10 mg Propolis, and 95 mg Aluminum chloride). Continuous shaking was done for complete dissolving. The solution was prepared fresh every day
- Bcl-2 Antibody was purchased from Santa Cruz Biotechnology Company, USA

Animals

In this study, fifty adult male albino rats were used. They were weight 165-180 gm. strict care and hygiene was observed to keep them in healthy conditions. They were fed standard rat chow diet and allowed tap water ad-libitum. They were housed in stainless steel cages at room temperature (37°C) in the animal house at Faculty of Medicine, Menoufia University, Shebin el Kom, Menoufia, Egypt. Rats were treated according to the guidelines approved by the Animal Care and Ethical Committee of Faculty of Medicine, Menoufia, University (19719HIST25). They were distributed randomly into five equal groups (10 rats each)

Animalٰs groups

Group I (control): received 2ml distilled water by oral route and kept without any treatment for 6 weeks.

Group II (Propolis supplemented): rats received Propolis at a dose of 50mg/kg b.w/ day[15]. Each rat received 1 ml distilled water containing 10 mg Propolis by oral route for 6 weeks.

Group III (aluminum chloride treated): rats treated by aluminum chloride at dose of 475mg/kg B.W[16] once daily, by oral gavage for 6 weeks. Each rat received 1 ml distilled water containing 95 mg aluminum chloride.

Group IV (Recovery group): rats were treated with Aluminum chloride (475mg/kg B.W) for 6 weeks and then were kept untreated for another 2 weeks.

Group V (Propolis and aluminum chloride): rats received both aluminum chloride and Propolis once daily, by oral gavage for 6 weeks. The doses were similar to that of the previous experimental groups.

Methods

At the end of the experiment the animals were anesthetized by ether inhalation (2 ml) for about 2 min in a transparent acrylic jar[17]. Animal's body weights were recorded in all groups. Rats were sacrificed and a median incision was achieved to expose the lungs. The two lungs of each rat were carefully dissected out and were weighted.

For light microscopic study: Lung tissues were fixed in 10% formol saline and were processed for paraffin blocks. 5-6 µm thickness sections were prepared for Hematoxylin & Eosin (H &E) staining for general histological examination[18]; Mallory trichrome (M.T) for collagen fibers detection and toluidine blue (t.b) staining for analysis of mast cell[18].

For immunohistochemical study

Bcl2 (B-cell lymphoma 2) for detection of cells apoptosis. Immunoreactivity was performed on paraffin sections of 4 µm thick. The paraffin sections were deparaffinized and rehydrated. Endogenous peroxidase was blocked by incorporating the sections in 3% hydrogen peroxide (H_2O_2). The sections were incubated overnight at room temperature with primary anti Bcl2 antibody (rabbit polyclonal, Abcam). Dilution1:50 using IHC-TekTM antibody diluent (Cat# IW-1000 or IW-1001) was done to reduce background and unspecific staining. Then, the secondary antibody (biotinylated goat-polyvalent) was applied. The technique was completed using the streptavidin–biotin complex detection method. Controls slides were included in each run. Positive controls were normal human colon. Negative controls were obtained by excluding the primary antibody and replacing it with phosphate buffered saline (PBS). Finally, the slides were counterstained with hematoxylin stain[19].

For electron microscopic study

Lung tissues of each rat were excised rapidly and cut into pieces of 1x1 mm². These pieces were fixed in 3 % glutaraldehyde and 0.1 M phosphate buffer at pH 7.4, after that they were fixed in osmium tetraoxide, processed and embedded in epon. Semithin sections of 1 µm thick stained by toluidine blue. Ultrathin sections were contrasted with lead citrate and uranyl acetate. All sections of all groups were examined by using transmission electron microscope (Jeol) in Tanta E.M Unite in faculty of medicine, Tanta University[20].

Morphometric study

All measurements were applied on ten non overlapping sections of each group in the same magnification. The

morphometric study was carried out by using Image analyzer software (Image J 1.47v national institute of health, USA). It was performed on the following parameters:

1. The number of pneumocytes type II (× 400);
2. The thickness of inter-alveolar septa (× 200);
3. Percentage area of collagen deposition (× 200);
4. The number of mast cells (× 400);
5. The color intensity of Bcl2 immunostaining (× 400). The calculated data were used for comparison and statistical analysis.

Statistical study

Data were statistically analyzed by SPSS (Statistical Package for the Social Sciences) program, version 20 (SPSS Inc., Chicago, Illinois, USA). Data were expressed as mean and SD (standard deviation) and analyzed by using one-way analysis of variance (ANOVA) followed by post hoc Turkey test for comparison between the control and other groups. Differences were graded as significant if *P values* were < 0.05 and highly significant if *P value* was < 0.001[21].

RESULTS

Macroscopic Results: Lungs obtained from control and Propolis groups were homogeneous and glistening with bright red color (Figure 1a). Regarding the lung of aluminum chloride group, it appeared shrunken and collapsed with dark opaque red color with patches of hemorrhage (Figure 1b) while in recovery group it showed little hemorrhage. (Figure 1c). Lunges restored its bright red color in the Propolis and aluminum chloride group (Figure 1d).

Light microscopic results

H&E staining: Lung sections collected from the control and Propolis groups (Groups I and II) showed normal lung tissue with numerous patent alveoli. The alveoli are arranged into clusters called alveolar sac, that share acommon opening to the alveolar duct. Each alveolus was lined by 2 types of cells: pneumocytes I and pneumocytes II. Pneumocytes I appeared flattened with flat nucleus and scanty cytoplasm. Pneumocytes II appeared cubical with rounded nuclei and abundant cytoplasm. Moreover, alveolar macrophages appeared inside the alveoli. Thin inter-alveolar septa, blood vessels and normal bronchioles were noticed. The bronchioles were lined by ciliated cuboidal epithelium (Figure 2).

Aluminum chloride group (Group III) revealed distorted structure of lung tissues. Some alveoli were collapsed while the others showed compensatory dilatation with rupture of septa between the alveoli. Lung tissue had numerous depositions of hemosiderin granules that appeared as dark brown particles (Figure 3a). Pneumocytes type I & II showed dark pyknotic nuclei and vacuolated cytoplasm with discontinuity of the alveolar wall. There was thickening in the inter-alveolar septa, (Figure 3b). The thickened interstitium contained numerous inflammatory infiltrate and acidophilic homogenous materials (Figure 3c). The blood vessels were congested and surrounded by inflammatory cells (Figure 3a). There was an irregularity in the wall of the bronchioles with sloughing of the bronchial epithelial lining. Moreover, some hemorrhage was observed inside the bronchioles (Figure 3d).

Lung tissues showed moderate improvement after stopping aluminum chloride for 2 weeks in the recovery group (Group IV) where, some alveoli appeared collapsed with pyknosis of pnemocytes nuclei, congestion of the blood vessels and minimal cellular infiltrations were noticed. The inter-alveolar septa were still thickened (Figure 4).

Co-administration of Propolis with aluminum chloride in Group V showed marked enhancement in the lung tissues. Where, the integrity of lung alveoli, blood vessels and bronchioles were preserved to be more or less as in control group (Figure 5).

Mallory's trichrome staining: Mallory's trichrome stained section from the control and Propolis groups (Groups I and II) showed few collagen fibers in the inter-alveolar septa, around the bronchioles and around the blood (Figure 6). In the aluminum chloride group (Group III) massive deposition of collagen fibers was noticed mainly around the bronchioles and the congested blood vessels and in the inter-alveolar septa (Figure 7). While lung sections obtained after stopping aluminum chloride for 2 weeks (Group IV) revealed moderate deposition of collagen fibers (Figure 8). Propolis and aluminum chloride group (Group V) showed minimal collagen fibers in the inter-alveolar septa slightly more than the control group (Figure 9).

Toluidine blue staining: No mast cells could be detected in the control and Propolis groups (Figure 10). But there was dense accumulation of mast cell in the aluminum chloride group with rupture of some (Figure 11). There was little number of intact mast cells in the recovery group (Figure 12). While no mast cells were seen in the Propolis and aluminum chloride group (Figure 13).

BCL2 immunostaining: Control and Propolis groups showed positive brown cytoplasmic immune reaction for BCL2 in pneumocyte I and II (Figure 14) the reaction became very weak in aluminum chloride group (Figure 15). Sections collected two weeks after arrest of aluminum chloride showed moderate immune reaction to BCL2 (Figure 16). In Propolis and aluminum chloride group the reaction was intense as the control (Figure 17).

Electron microscopic results: Ultrathin sections of the lung in Control and Propolis groups (Group I&II) showed pneumocyte type I containing large oval nucleus with peripheral condensed chromatin and thin attenuated cytoplasm (Figure 18a). Pneumocyte type II appeared rounded with large central rounded vesicular

nucleus and prominent nucleolus. Its cytoplasm contained mitochondria, lamellar bodies, rough endoplasmic reticulum and lysosomes. Its apical surface exhibited numerous short apical microvilli (Figures 18 b,c). The alveolar macrophage was noticed with its characteristic nucleus and multiple pseudopodia on the surface. The inter-alveolar septum appeared clear (Figure 18c).

Aluminum chloride group (Group III) exhibited marked ultrastructure changes where, pneumocyte type I had flattened irregular nuclei, swollen mitochondria and multiple cytoplasmic vacuolations. Distorted tight junction between the pneumocytes I was noticed. The alveolar macrophages were filled with numerous residual and multivesicular bodies (Figure 19a). Pneumocyte type II showed irregular indented nucleus. Their cytoplasm contained multiple cytoplasmic vacuolations, distended irregular mitochondria and dilated rough endoplasmic reticulum (Figure 19b). The lamellar bodies revealed degenerative changes leaving irregular empty vacuoles. Few short microvilli appeared on the surface of pneumocyte II (Figure 19c). The inter-alveolar septum contained congested blood capillary (Fig 19 c), degenerated pneumocyte II, macrophages, red blood cells, eosinophil and marked collagen fiber (Figure 19d)

Stopping aluminum chloride treatment for2 weeks in the recovery group (Group IV) revealed no restoration of normal structure of the alveoli. Pneumocyte type II showed irregular nucleus, their cytoplasm contained enlarged mitochondria, dilated rough endoplasmic reticulum and cytoplasmic vacuolations. Distorted lamellar bodies and disturbed microvillous border were observed. The inter-alveolar septum still contained numerous collagen fibers and congested blood capillary (Figure 20).

Sections of Propolis and aluminum chloride group (Group V) showed great reduction in the structural changes demonstrated in aluminum chloride group. Pneumocyte type II restored its normal feature and appeared with large rounded vesicular nucleus and prominent nucleolus. Their cytoplasm demonstrated numerous rough endoplasmic reticulum and mitochondria and remarkable well defined lamellar bodies. Some cytoplasmic vacuolations were still appeared. Mild capillary congestion and few collagen fibers were noticed in the inter- alveolar septum (Figure 21).

Statistical and morphometric results

The animal body weight, lung weight and the ratio between them:

Aluminum chloride group revealed a high significant decrease of these parameters ($P< 0.001$) compared to control. Recovery group showed a significant decrease compared with control group ($P\leq 0.05$). There was non-significant relation in both Propolis supplemented & Propolis and aluminum chloride groups compared to control group (Table1).

The number of pneumocytes type II: There was a high significant increase in the number of pneumocytes type II ($P < 0.001$) in aluminum chloride group compared to control. In recovery group there was a significant increase in pneumocytes type II number compared with control group ($P< 0.05$) However, other groups showed a non-significant change ($P>0.05$) (Table 2 and Histogram1).

The thickness of inter-alveolar septa: The mean thickness of inter-alveolar septa showed a high significant increase ($P< 0.001$) in aluminum chloride group compared to control group. Recovery group exhibited a significant increase ($P< 0.05$) compared with control group. However, other groups including (Propolis group & Propolis and aluminum chloride group) showed a non-significant change ($P>0.05$) compared to control (Table 3 and Histogram 2)

The percentage area of collagen deposition: The mean percentage area of collagen fibers showed a high significant increase ($P< 0.001$) in aluminum chloride group and a significant increase ($P< 0.05$) in the recovery group compared to control. However, Propolis supplemented group & Propolis and aluminum chloride group showed non-significant change ($P>0.05$) compared to control (Table 4 and Histogram 3)

The number of mast cells: The mean number of mast cells revealed a high significant increase ($P< 0.001$) in aluminum chloride group and asignificant increase ($P< 0.05$) in the recovery group compared to control. There was a non-significant relation ($P>0.05$) in both Propolis supplemented group & Propolis and aluminum chloride group compared to control (Table 5 and Histogram 4)

The color intensity of Bcl2 immunostaining: The color intensity of Bcl2 showed a high significant decrease ($P< 0.001$) in aluminum chloride group and a significant decrease ($P< 0.05$) in the recovery group compared to control. However, Propolis supplemented group & Propolis and aluminum chloride group showed a non-significant change ($P>0.05$) compared to control (Table 6 and Histogram 5).

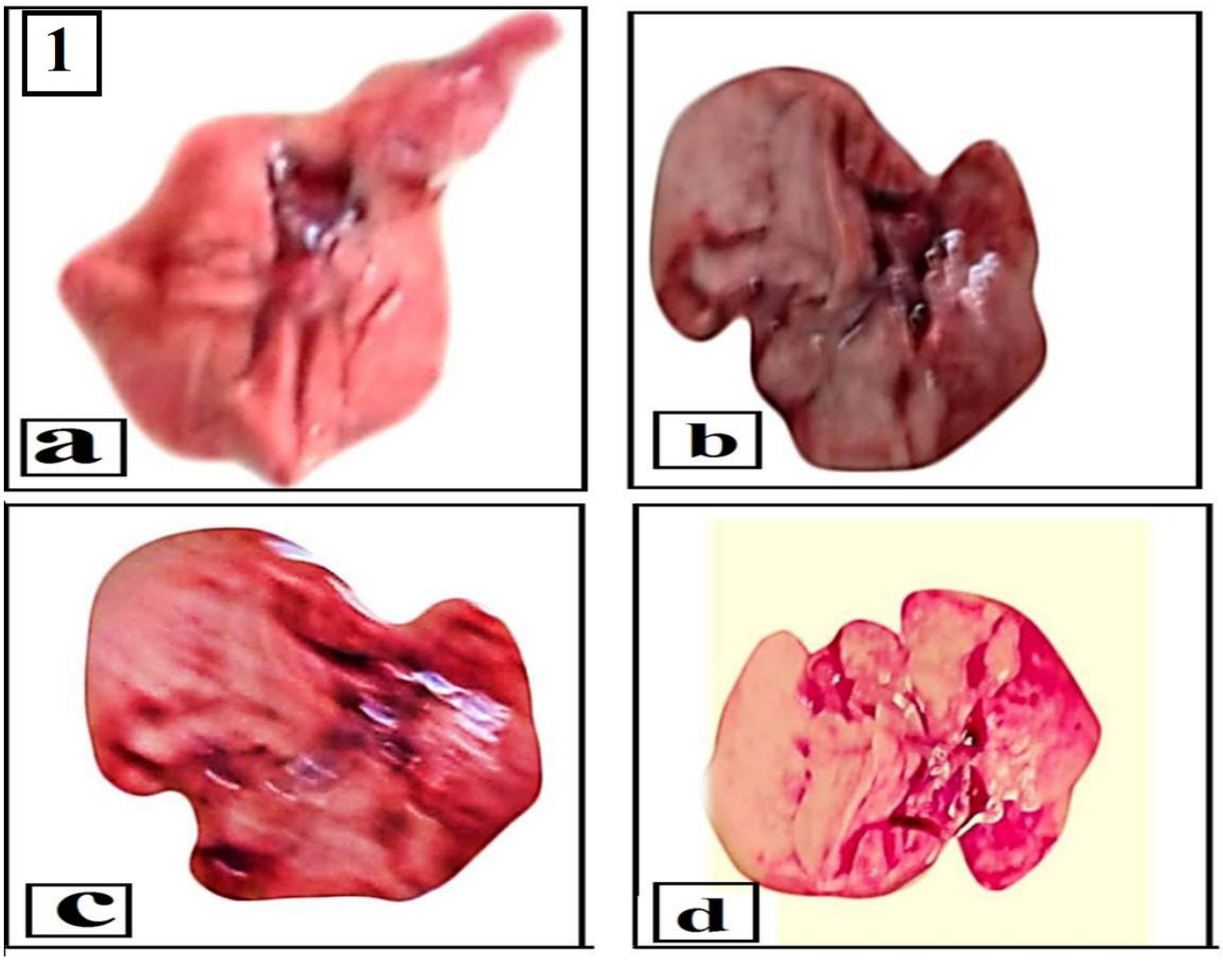


Fig. 1: Macroscopic picture of all expermintal groups
a) Control and propolis groups (Groups I and II) b) Aluminum chloride group (Group III) c) recovey group(Group IV) d) propolis and Aluminum chloride group(Group V).

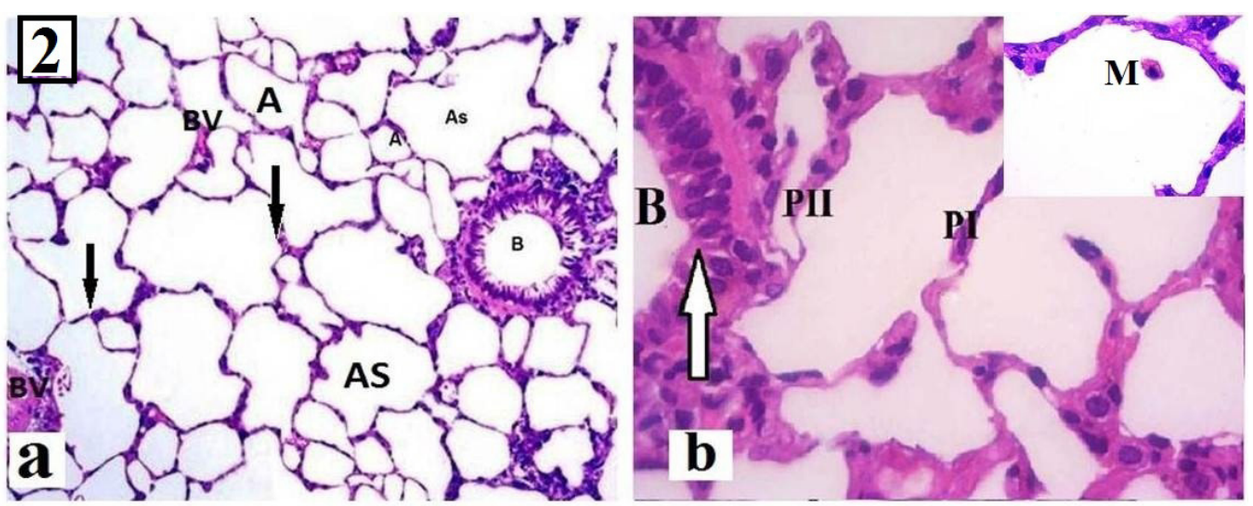


Fig. 2: Hx&E stained sections Control and propolis groups (Groups I and II) Showing a): normal lung tissue with thin inter-alveolar septa (arrow) and numerous patent alveoli (A). Notice: alveolar sacs (AS), blood vessels (BV) and bronchioles (B). (Hx&E x 100) b): alveoli are lined by pneumocytes type I (PI) with flattened nuclei and Pneumocytes type II(PII) with rounded nuclei. Bronchiole (B) is lined by ciliated cuboidal epithelium (arrow) ,Hx&E x 400 .inset: alveolar macrophage(M) inside the alveoli(Hx&E x 1000).

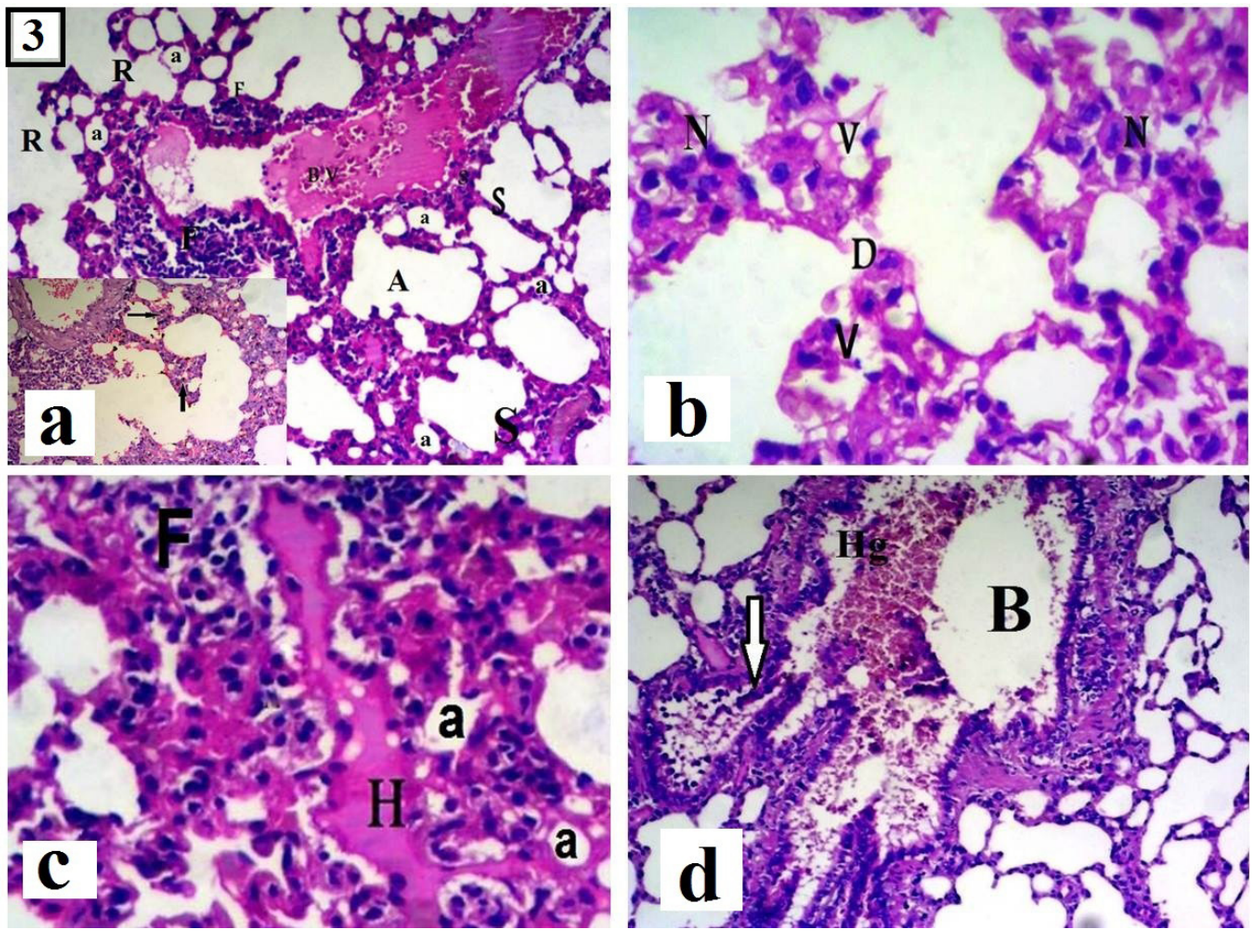


Fig. 3: Aluminum chloride group (Group III) showing a): Some alveoli were collapsed (a) and others shows compensatory dilatation (A) with rupture of septa between the alveoli(R). Notice: Thickened inter-alveolar septa (S) and cellar infiltration (F) around congested blood vessel (B.V). Hx&E x 100 inset: showing numerous depositions of hemosiderin granules (arrow) Hx&E x 200 b): degeneration of alveolar wall (D). Both type of pneumocyte shows cytoplasmic vacuolation (V) and pyknotic nuclei (N). Hx&E x 400 c): collapsed alveoli (a), thickening interstitium with inflammatory infiltrate (F) and hyaline degeneration (H) Hx&E x 400 d): irregularity in the wall of the bronchioles(B) with sloughing of the bronchial epithelial lining(arrow). Notice: hemorrhage (Hg) inside the bronchioles. Hx&E x 100

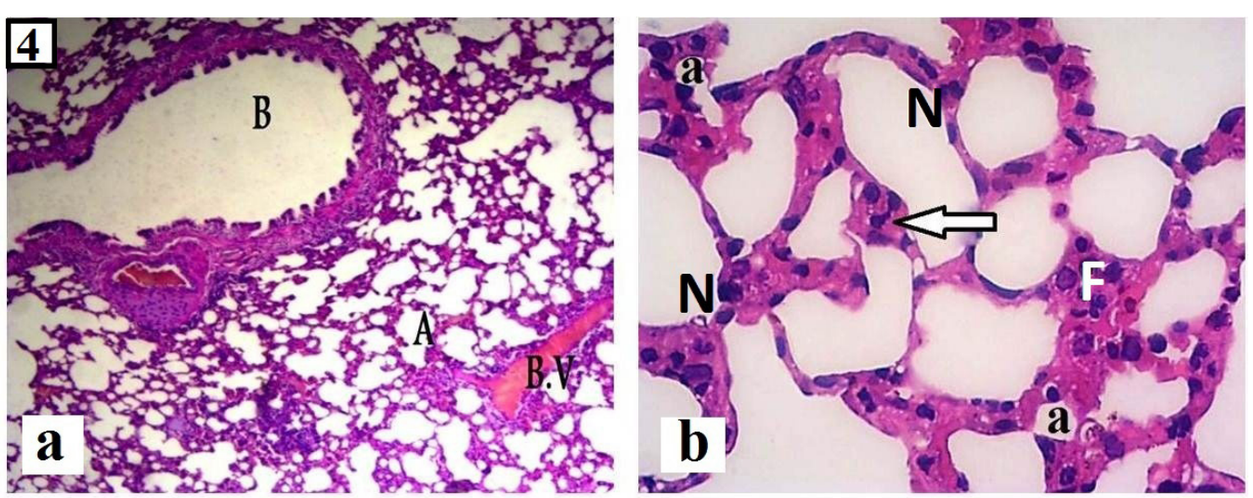


Fig. 4: Recovery group (Group IV) Showing a): lung tissue with patent alveoli (A) mild distortion of the bronchiole (B) and moderate congestion blood vessel (B.V). Hx&E x 100 b): some alveoli appear collapsed (a), pyknosis of some pneumocytes nuclei (N). Notice: minimal cellular infiltration (F) and thickened inter-alveolar septa (arrow). Hx&E x 400

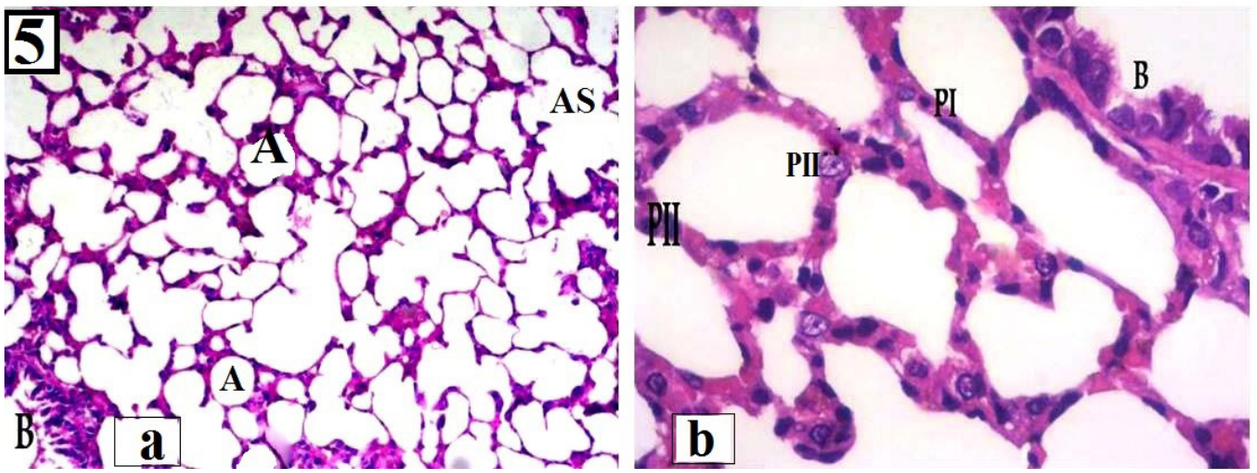


Fig. 5: Propolis and Aluminum chloride group (Group V) showing a): normal lung tissue with patent lung alveoli (A), alveolar sacs ((AS) and bronchiole (B). Hx&E x 100 b): Vesicular rounded nuclei of pneumocyte type II (PII) and flattened nuclei of pneumocyte type I (PI). Notice: Normal bronchiolar epithelium (B). Hx&E x 400

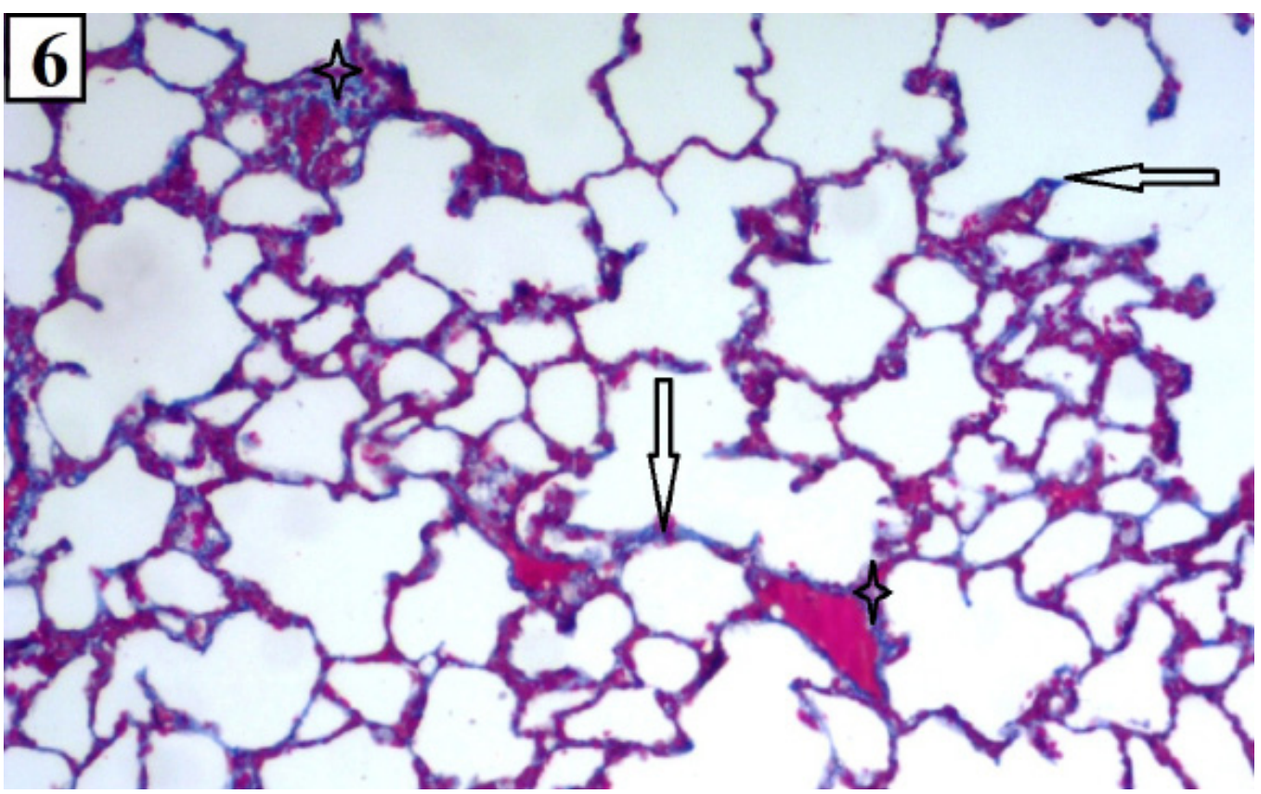


Fig. 6: Mallory Trichrome stained sections Control and propolis groups (Groups I and II) showing few collagen fibers in the inter-alveolar septa (arrow) and around the blood vessels (stars). (M.T. ×200)

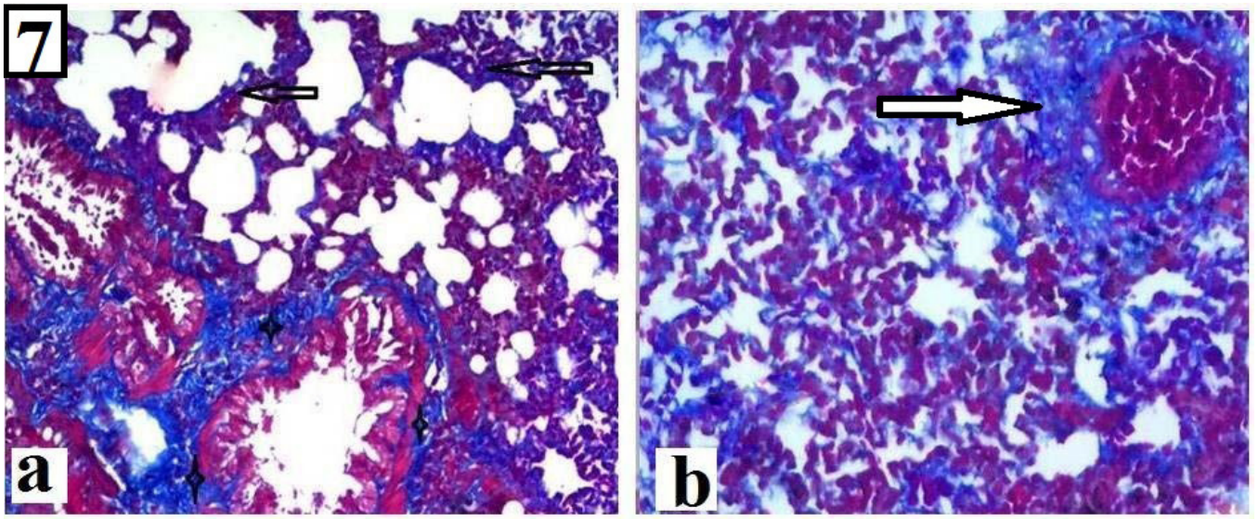


Fig. 7: Aluminum chloride group (Group III) showing a) massive collagen fibers deposition around the bronchioles and in the inter-alveolar septa (arrow). b) perivascular collagen fibers deposition (arrow) (M.T. ×200)

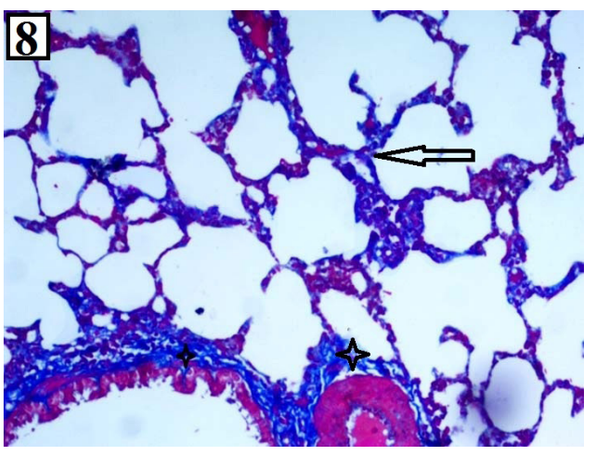


Fig. 8: Recovery group (Group IV) Showing moderate deposition of collagen fibers in the inter-alveolar septa (arrow), around bronchioles and blood vessels (stars (M.T. ×200)

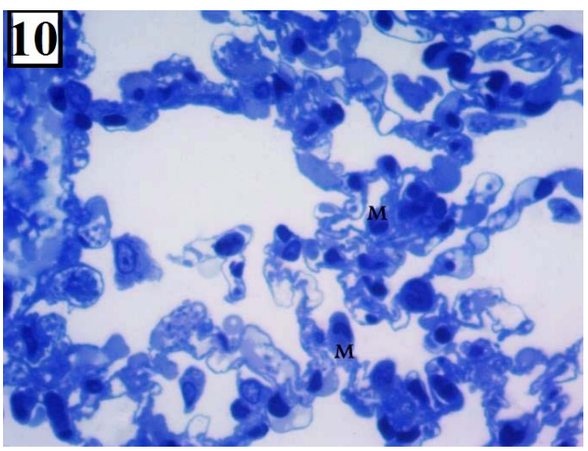


Fig. 10: Toluidine blue stained sections Control and propolis groups (Groups I and II) showing no mast cells could be detected Notice: Alveolar macrophages (M) with its characteristic eccentric kidney shaped nucleus and irregular outline. (T.B. × 400)

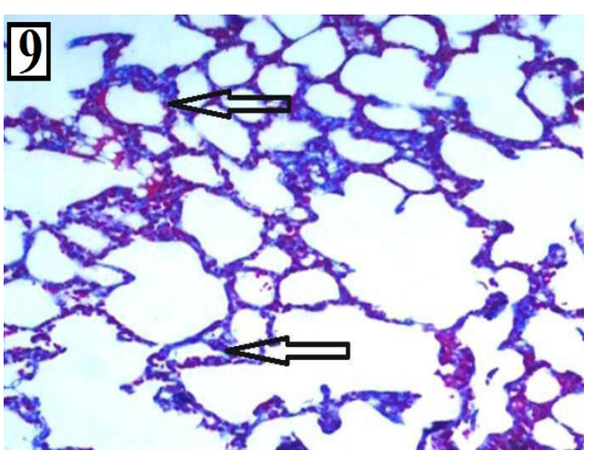


Fig. 9: Propolis and Aluminum chloride group (Group V) showing few collagen fibers in the inter-alveolar septa (arrow) slightly more than the control. (M.T. ×200)

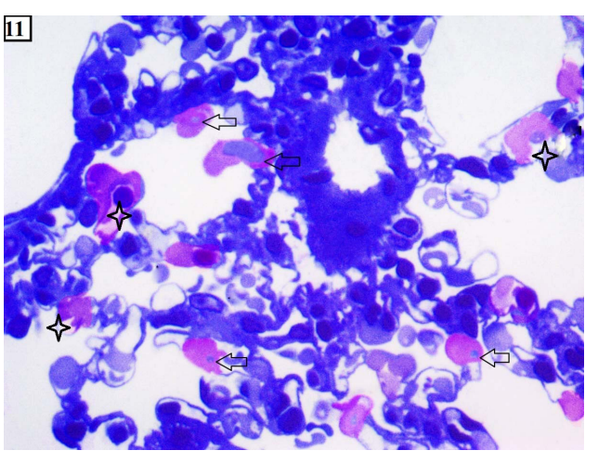


Fig. 11: A photo micrograph of Aluminum chloride group (Group III) showing dense accumulation of mast cells (arrow) in the Aluminum chloride group with rupture of some (stars) (T.B. × 400)

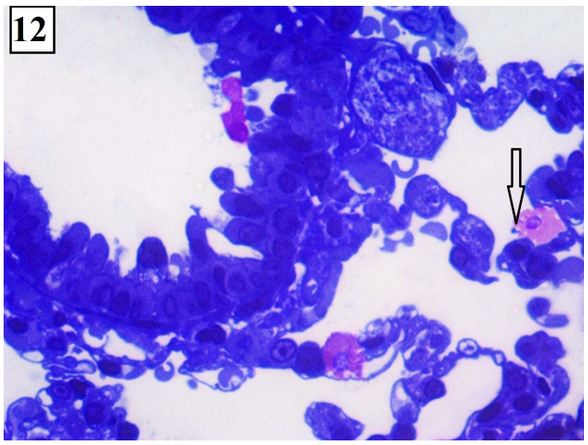


Fig. 12: A photomicrograph of recovery group (Group IV) Showing few number of intact mast cells (arrow) (T.B. × 400)

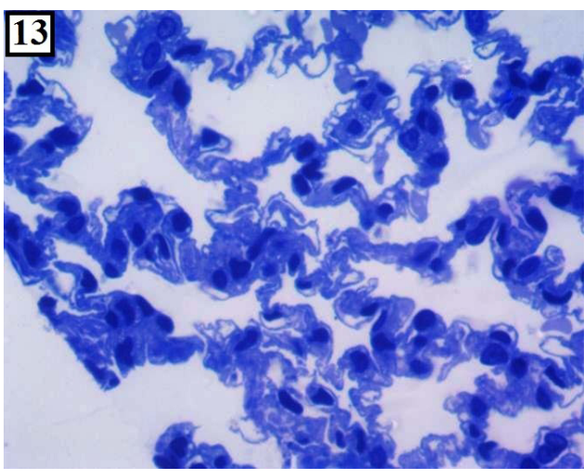


Fig. 13: A photomicrograph of Propolis and Aluminum chloride group (Group V) showing no mast cells (T.B. × 400)

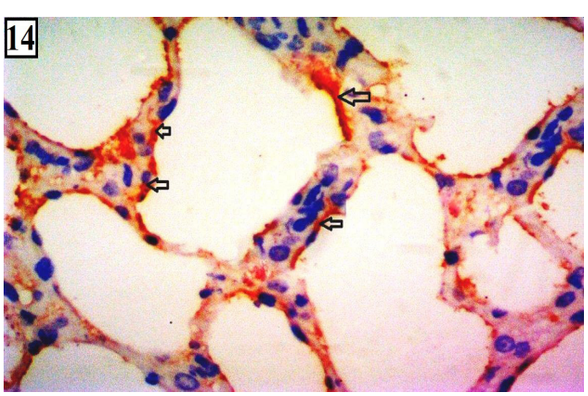


Fig. 14: BCL2 stained sections Control and propolis groups (Groups I and II) showing intense positive cytoplasmic immune reaction for BCL2 in the pneumocytes (arrow) (BCL2× 400)

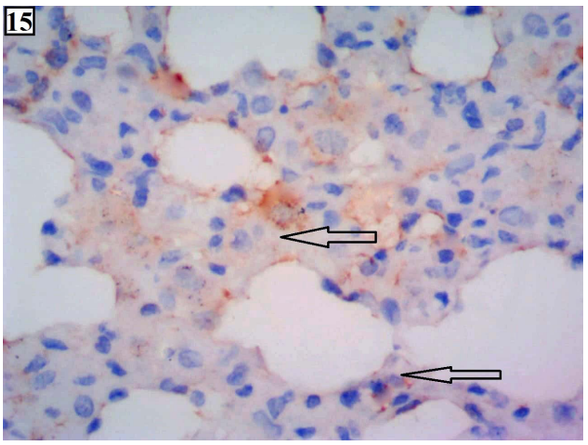


Fig. 15: Aluminum chloride group (Group III) showing very weak BCL2 immune reaction BCL2 (arrow) (BCL2× 400)

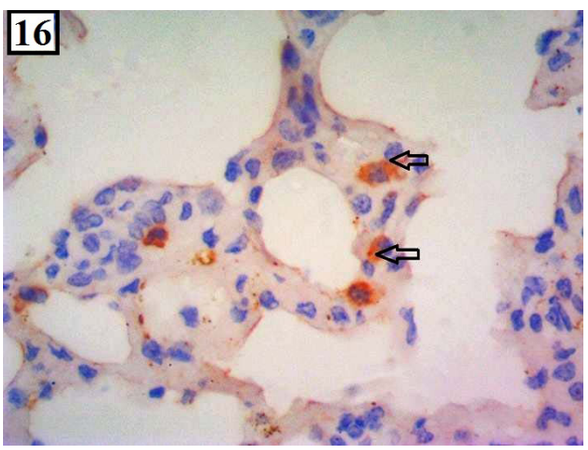


Fig. 16: Recovery group (Group IV) showing moderate BCL2 immune reaction to (arrow) (BCL2× 400)

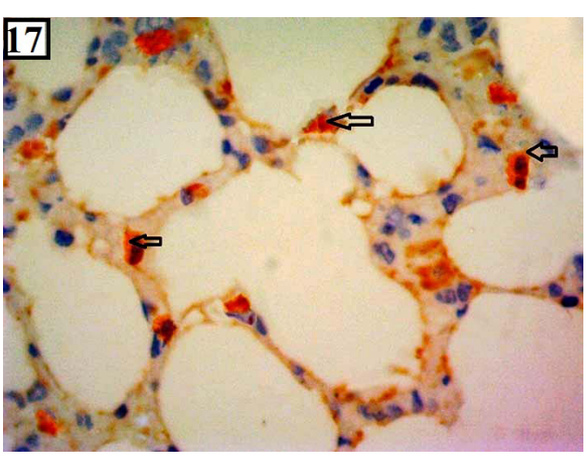


Fig. 17: Propolis and Aluminum chloride group (Group V) showing intense positive cytoplasmic immune reaction for BCL2 (arrow) (BCL2× 400)

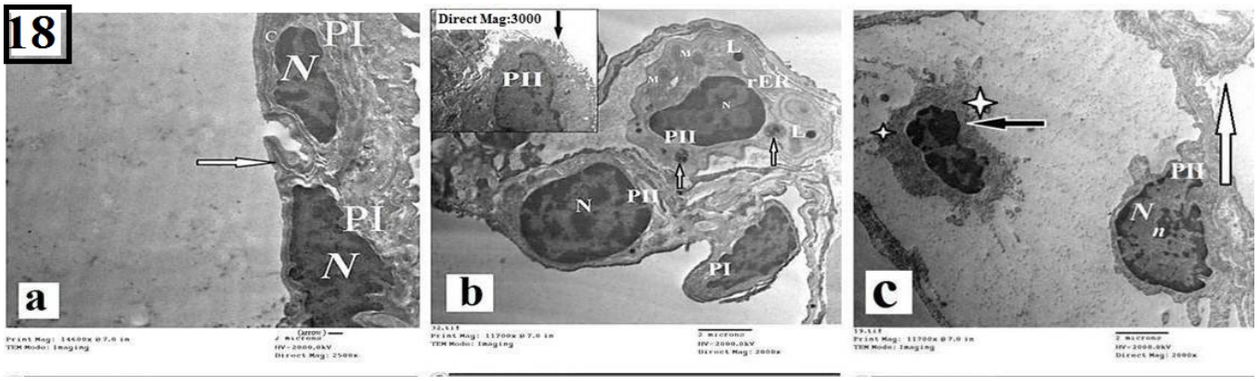


Fig. 18: Electron microscopic sections An electron micrograph of lung of Control and propolis groups (Groups I and II) showing a) two adjacent pneumocyte type I (PI) having large flattened condensed nucleus (N) and thin rim of cytoplasm(C). Notice: tight junction between the two cells (arrow). (x 2500) b) pneumocytes type II (PII) containing large central rounded vesicular nucleus (N) and abundant cytoplasm containing mitochondria (M), lysosome (L) , rough endoplasmic reticulum(rER) and remarkable well defined lamellar bodies (arrows). Notice: pneumocyte type I (PI) between pneumocytes type II (x 2000 inset: showing pneumocytes type II (PII) having regular short apical microvilli (arrow) .c) alveolar macrophages in the alveolus lumen (black arrow) with long pseudopodia (star). The inter-alveolar septum appears clear (white arrow). Notice: pneumocyte type II (PII) containing large central vesicular nucleus (N) with prominent nucleolus (n) and peripheral condensed chromatin. (x 2000

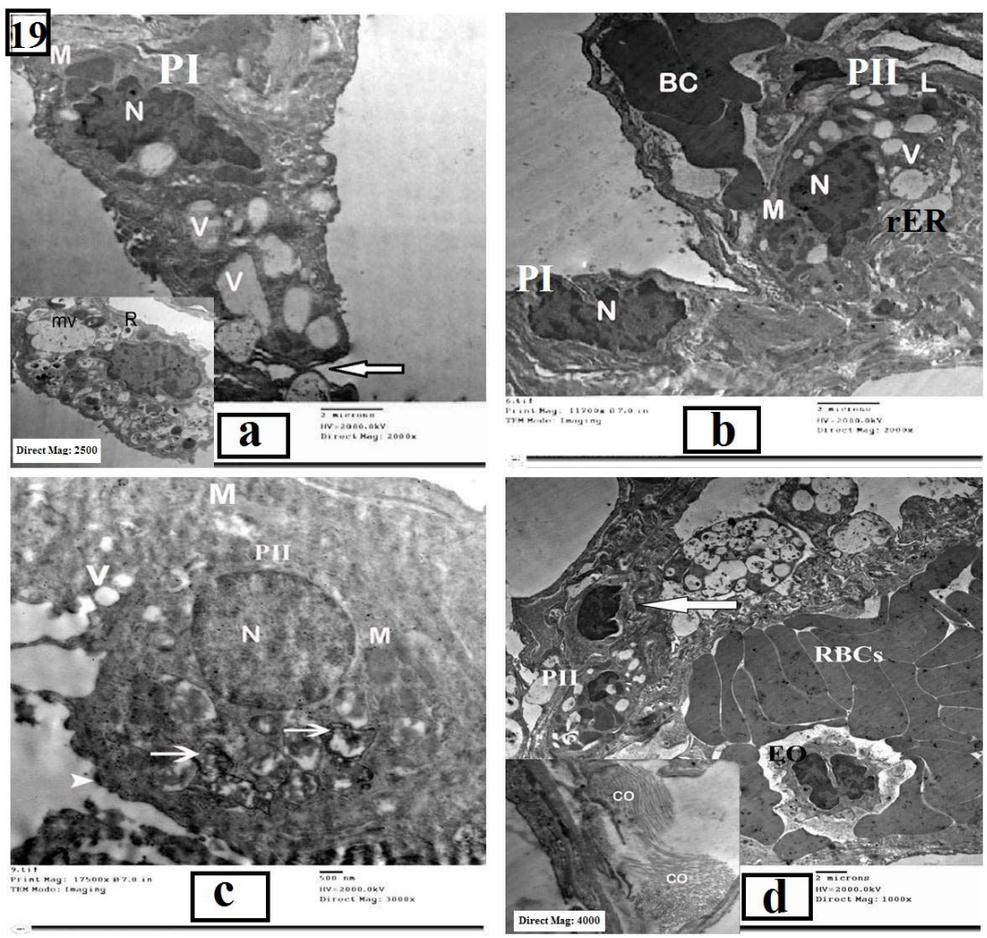


Fig. 19: Electron micrographs of Aluminum chloride group (Group III) showing a) pneumocyte type I(PI) with flattened irregular nuclei(N) ,swollen mitochondria(M) and multiple cytoplasmic vacuolation (V) . Notice: distorted tight junction between the two cells (arrow) x 2000) inset: showing alveolar macrophages containing multivesicular body (mv) and residual body (R) x2000) b) pneumocyte type II(PII) with irregular indented nucleus(N) containing multiple cytoplasmic vacuolation (V), enlarged irregular mitochondria(M) dilated rough endoplasmic reticulum (rER)and lysosomes(L). Pneumocyte type I(PI) appears with irregular flattened nucleus(N). Notice: congested blood capillary (BC).x 2000 c) pneumocyte type II (PII) is seen with rounded nucleus (N). The cytoplasm contains degenerative changes of the lamellar bodies leaving irregular empty vacuoles (arrow) and distorted mitochondria (M). Few short microvilli appear on the surface (arrow head). x 3000) d) inter-alveolar septum containing degenerated pneumocyte type II (PII), macrophages (arrow), numerous red blood cells (RBCs) and eosinophil (EO). x 1000) Inset: showing marked collagen fiber deposition in the inter-alveolar septum (CO).x 4000)

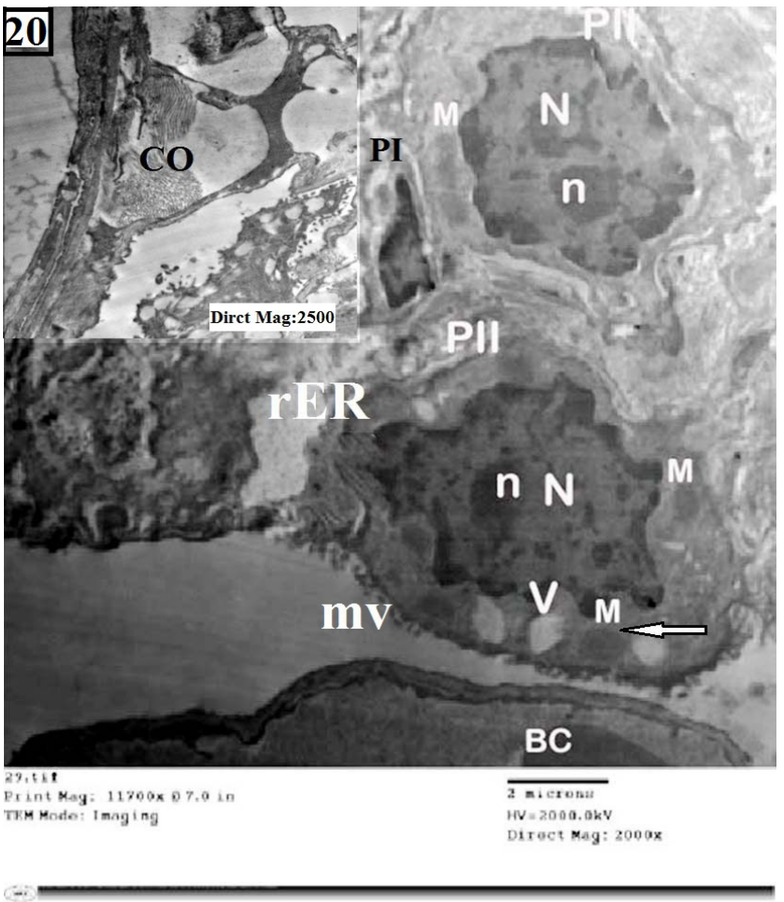


Fig. 20: Electron micrographs of recovery group (Group IV) showing two pneumocyte type II with slightly irregular nucleus (N) and prominent nucleolus (n), their cytoplasm contains mitochondria, dilated rough endoplasmic reticulum (rER). Distorted lamellar bodies (arrow) and cytoplasmic vacuolation (V). Disturbed microvillous border (mv) and congestion of blood capillary (BC) are noticed. Notice: pneumocyte type I between two pneumocyte type II x 2000) inset: collagen fiber deposition in the inter-alveolar septum (CO). x 2500)

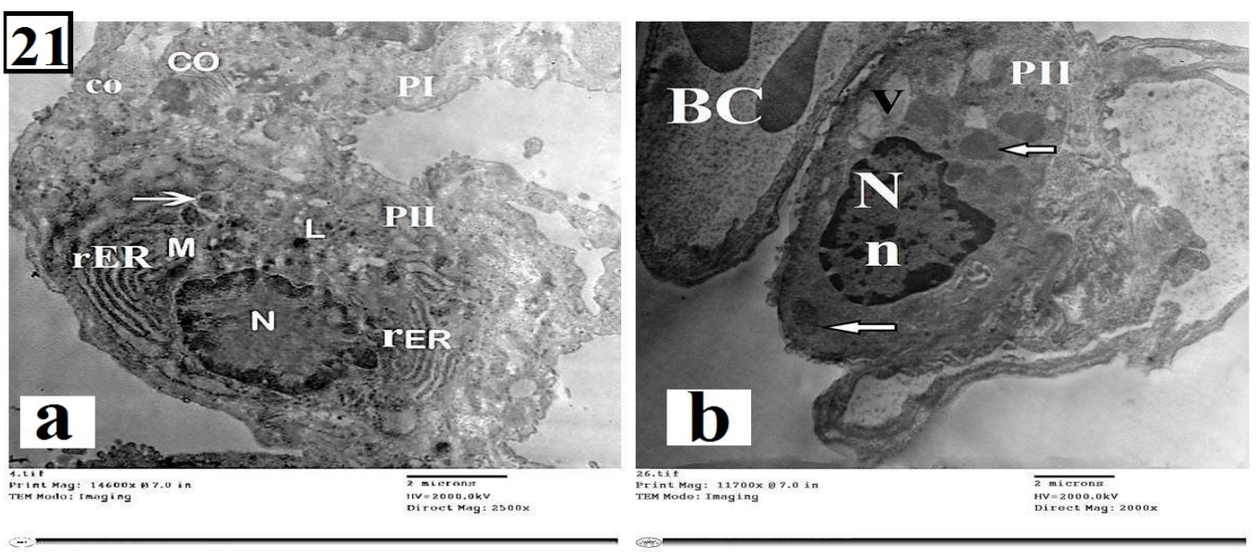


Fig. 21: Electron micrographs of propolis and Aluminum chloride group (Group V) showing a) pneumocytes type II (PII) with large rounded vesicular nucleus (N) and abundant cytoplasm containing mitochondria (M), lysosome (L), rough endoplasmic reticulum (rER), remarkable well defined lamellar bodies (arrow). Notice: part of pneumocytes type I and few collagen fibers in the inter- alveolar septum (CO). x 2500 b) pneumocytes type II (PII) with vesicular nucleus and prominent nucleolus (n). The cytoplasm contains lamellar bodies (arrows). Cytoplasmic vacuolation (V) and mild capillaries congestion (BC) are still appearing. x 2000)

Table 1: The animal body weight, lung weight and the ratio between them in all studied groups

	Group I (Control)	Group II (Propolis supplemented)	Group III (Aluminum chloride treated)	Group IV (Recovery)	Group V (propolis+ Aluminum chloride)
Body weight	193.4 ± 5.9 (185 - 200)	200.9 ± 6.87 (192 - 210)	160 ± 3.67* (155 - 164)	177.5 ± 8.33* (168 - 190)	191.8 ± 4.55 (187 - 198)
P value		0.1	<0.001**	0.008*	0.6
Lung weight	2 ± .2 (1.7 – 2.2)	2.1 ± .3 (1.8 – 2.4)	1.3 ± .2* (1.0 – 1.5)	1.6 ± .2* (1.4 – 1.8)	1.8 ± .2 (1.6 – 2.0)
P value		0.560	<0.001**	0.009*	0.145
Lung/body weight ratio %	1.03 ±. 11	1.04 ± .15	.81 ± .14*	.9 ± 0.08	.95 ± 0.092
P value		0.8	<0.001**	0.05*	0.2

* Significant vs control; ** high significant vs control

Table 2: Statistical means of pneumocytes type II number in all studied

Groups	Mean number of pneumocyte type II ±SD.	*P value*
Group I (Control)	25.3 ±2.48	----
Group II (Propolis supplemented)	25.8 ±2.52	----
Group III (Aluminum chloride treated)	87.7±2.43	0.000**
Group IV (Recovery)	41.2 ±4.9	0.032*
Group V (propolis+ Aluminum chloride)	28.3 ±3.2	0.498

* Significant vs control; ** high significant vs control

Table 3: Statistical means of thickness of inter-alveolar septa in all studied groups

Groups	Mean thickness of interalveolar septa ±SD	*P value*
Group I (control)	48.4 ±5.3 µm	---------
Group II (Propolis supplemented)	49.6 ± 6 µm	0.713
Group III (Aluminum chloride treated)	381.9 ± 124.6 µm	0.000**
Group IV (Recovery)	87.6 ±7.1 µm	0.043*
Group V ((propolis+ Aluminum chloride))	50.7 ± 7.7 µm	0.596

* Significant vs control; ** high significant vs control

Table 4: Statistical mean of percentage area of collagen fibers in all studied groups

Groups	% area of collagen fibers	*P Value*
Group I (Control)	15.2 ± 3.5	----------
Group II (Propolis supplemented)	15.5 ± 3	0.873
Group III (Aluminum chloride treated)	48.8 ± 7.2	0.00**
Group IV (Reccovery)	22.7 ± 6	0.041*
Group V (propolis+ Aluminum chloride)	17.6 ±3.1	0.281

* Significant vs control; ** high significant vs control

Table 5: Statistical means of mast cells in all studied groups

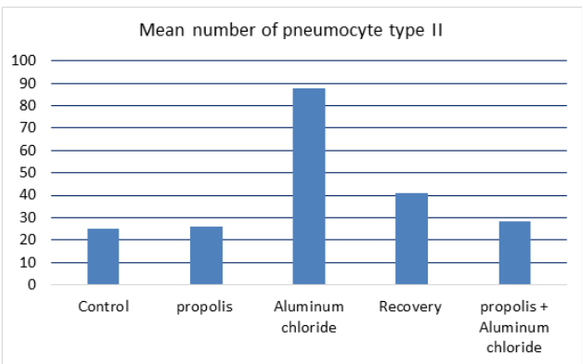
Groups	Mean number of mast cells ±SD.	*P Value*
Group I (Control)	--------	----------
Group II (Propolis supplemented)	---------	----------
Group III (Aluminum chloride treated)	6± 1	0.000**
Group IV (Recovery)	2 ± 1	0.034*
Group V (propolis+ Aluminum chloride)	---------	----------

* Significant vs control; ** high significant vs control

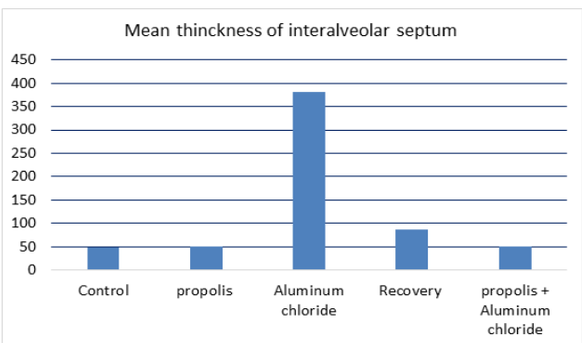
Table 6: Statistical means of Bcl2 color intensity in all studied groups

Groups	Bcl2 color intensity of ±SD.	*P Value*
Group I:(Control)	58.5± 8.2	---------
Group II:(Propolis supplemented)	56.4± 6.7	0.666
Group III:(Aluminum chloride treated)	20.4 ± 3.8	0.000**
Group IV:(Recovery)	31.2± 6.5	0.001*
Group V:(propolis+ Aluminum chloride)	53.2± 5.6	0.269

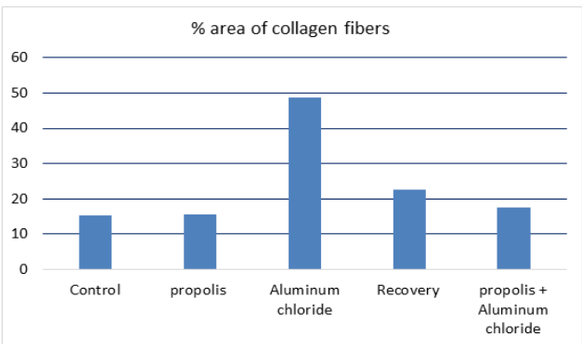
* Significant vs control; ** high significant vs control



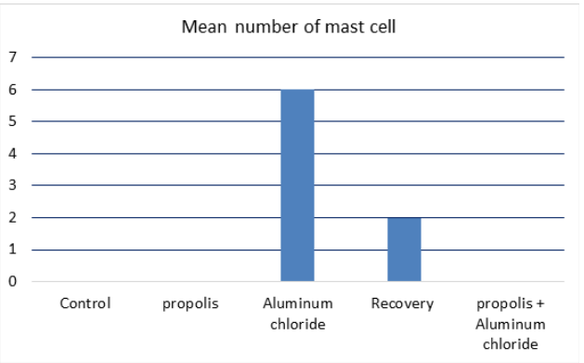
Histogram 1: Statistical means of pneumocyte type II number in all studied groups



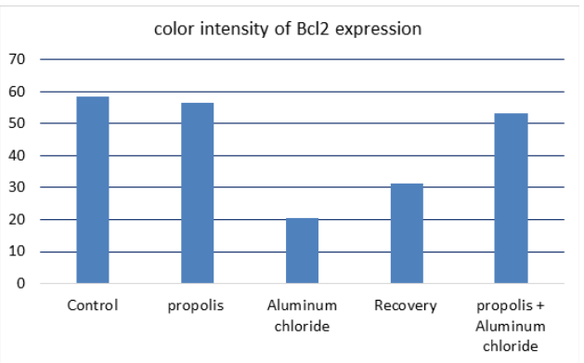
Histogram 2: Statistical means of the thickness of inter-alveolar septa in all studied groups



Histogram 3: Statistical means of percentage area of collagen fibers in all studied groups



Histogram 4: Statistical means of number of mast cell in all studied groups



Histogram 5: Statistical means of color intensity of Bcl2 expression in all studied groups

DISCUSSION

Lung lesions linked to aluminum exposure during the manufacturing of aluminum products are Granulomatous pneumonia, pulmonary granulomatosis, pulmonary alveolar proteinosis, desquamative interstitial pneumonia and pulmonary fibrosis[22].

During this study, a decrease in body weight was observed in Group III (aluminum chloride treated) when compared to the control group. Morphometric and statistical studies indicated that the weight of the rats, the weight of their lungs, and the ratio between them are significantly decreased when compared to the control.

These findings are with agreement with those of Zhu *et al*.,[23]. This reduction in the body weight was attributed to disorder absorption, consumption of nutrients and organ injury that led to diminished body protein synthesis.

The lung tissue of this group revealed distorted structure. This was in agreement with previous studies[6] which stated that lung epithelium may be a site for the accumulation of aluminum and a surface for its uptake into lung tissues and access to the systemic circulation. Mucus helps in removing aluminum from the lung and offers a substrate for the capture and dissolution of more labile forms of incipient aluminum. Sahar & Tarek[24] declared that variation in the alveolar shape between obliteration & dilatation, with thickened alveolar septa leading to severe airway injury,

fibrosis and chronic inflammatory lung diseases. John *et al*.[25] added that chronic obstructive air way disease is characterized by progressive and irreversible decrease of airway lumen diameters that develops as a result of varying perturbations in both airway and interstitial lung tissue. Moreover, these changes were in agreement with previous reports of pathological and functional changes associated with drug-induced pulmonary diseases, including chronic interstitial pneumonia which was previously reported by other investigators[26]. Gonzalez *et al*.,[27] added that aluminum chloride treatment caused decreasing activities of antioxidant enzymes like superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT).

At the same time, aluminum chloride administration increased the level of lipid peroxidation (LPO). These findings revealed that Al has a toxicity potential, which might be mitigated by changes in free radical production and antioxidant enzymes changes[28]. The thickened inter- alveolar septum is due to excess cellular infiltrate, inflammatory exudates, congestion of the capillaries, increased interstitial connective tissue and the related alveolar collapse.

Researchers,[29] recorded that aluminum chloride treatment is associated with diffuse alveolar damage and severe inflammatory reaction confirmed in autopsied cases, and added that, the recorded septal thickening due to aluminum chloride toxicity may result from infiltration with fibroblasts and inflammatory cells. The histological changes were corroborated by morphometric and statistical analysis, which revealed a highly significant increase in the mean thickness of inter-alveolar septa in group III compared to the control. Some researchers[30] reported collapse of some alveoli and subsequent compensatory overexpansion of neighboring alveoli with destroyed alveolar walls after exposure to Aluminum compounds. Aluminum chloride administration causes repeated lung inflammation, which damages and finally destroys the alveolar walls, resulting in huge air gaps, which is associated with emphysematous alterations. The alveolar septa are damaged first, removing a section of the capillary bed and increasing alveolar air volume. The pulmonary vascular congestion noticed in aluminum chloride treated rats may be attributed to vasodilator substances released by blood vessels into the blood stream[31].

Aluminum ions have been found to replace iron and magnesium ions, as well as impair Fe+2 binding to ferritin and disrupt hemoglobin production[32]. Another source of prior inhibition could be related to the heamooxygenase enzyme, which is required for hemoglobin production and is inhibited by aluminum toxicity, which accelerates the breakdown of RBCs, which then convert to bilirubin, resulting in hypoxia[33].

Hypoxia causes elevation of neurokinin-1 receptor expression in alveolar macrophages and epithelial cells. Activation of these receptors leads to inflammatory responses mediated by cytokines IL-1 (Interleukin-1), IL-6 (Interleukin-6) and TNFα (tumor necrosis factorα). Furthermore, alveolar macrophages have been implicated in the synergistic effects of hypoxia on pathogen-induced lung inflammation[34].

On the other hand, the current study found that proliferation of pneumocytes type II was consistent to others investigators[35], who stated that the chronic inflammatory process converts mesenchymal stem cells into type II pneumocytes, increasing their number The histological changes were corroborated by morphometric and statistical analysis, which revealed a highly significant increase in the mean number of pneumocytes type II in group III when compared to the control group. Furthermore, the epithelial lining of bronchioles showed desquamation and cellular debris accumulation in the lumen, as previously reported by other researchers[36].

The dark brown hemosiderin granules in the areas of extravasated blood in the pulmonary interstium and inside the alveoli could be explained by breakdown of red blood cells followed by phagocytosis of the released iron pigment by the pulmonary alveolar macrophages as had been explained previously by researchers[37].

Other researchers confirmed the apoptotic changes observed in the current study, demonstrating that the early pneumocyte apoptosis index was significantly higher in the Al-treated rats[38]. They reported that apoptosis was responsible for counterbalancing cell proliferation and eliminating damaged cells in tissues, and that it was one of the fundamental measures for maintaining the body's cell number in a dynamic balance. It has also been proposed that the toxic effects of Al were caused by the production of reactive oxygen species (ROS), which resulted in the oxidative degradation of cellular lipids, proteins, and DNA. Furthermore, excessive ROS production would disrupt the balance of the oxidative antioxidative system in the liver, resulting in pneumocyte apoptosis[39].

Massive deposition of collagen fibers was seen in Mallory's trichrome stained sections from Group III, primarily around the bronchioles, congested blood capillaries, and inter-alveolar septa, as determined by morphometric and statistical analyses.

The studies of others investigators[40,41], stated that aluminum chloride exposure causes an inflammatory response and that more fibroblasts are drawn to the irritated area, where they play a role by laying down more fibrous connective tissue. Recent researches have shown that fibroblasts can originate from the lung epithelium and contribute to fibrosis in experimental animals via epithelial-mesenchymal transition (EMT)[42,43].

Toluidine blue staining showed a significant increase in mast cells number in aluminum chloride treated group. Mast cells are specialized granulocytes which participate in wound-healing process. These cells have an important role in the early asthmatic reaction. Mast cell derived mediators induce bronchoconstriction, mucosal edema,

and mucus secretion in early asthmatic stage. There is growing evidence that these cells may also play a role in the pathogenesis of other airway diseases[44]. It has been shown that mast cells and the mast cell-specific chymase MCPT4 can mediate acute lung inflammation and damage in mice[45].

Immunohistochemical reaction showed a decrease in the Bcl2 (an anti-apoptotic marker) immunoreaction in aluminum chloride treated group, and confirmed by morphometric and statistical analysis. Earlier studies suggested that mitochondria played an important role in cell survival/death and that the opening of the mitochondrial permeability transition pore could be regulated by reducing free radicals and regulating Bcl2 expression[46]. They also stated that in aluminum toxicity, oxygen free radicals directly caused structural damage to cells or the opening of the mitochondrial permeability transition pore[47,48] postulated that free radical damage and its potential to induce apoptosis is regulated by the intracellular levels of BCL-2. BCL-2 has been found in mitochondria, nuclear membranes, and the endoplasmic reticulum, all of which are sites where free radicals are produced.

Ultrathin microscopic examination confirmed light microscopic results as there was degeneration of pneumocytes type I & type II, loss of characteristic lamellar pattern of their lamellar bodies leaving empty vacuoles and swollen ballooned mitochondria. Also, there were massive hemorrhage and congestion of alveolar blood capillaries which are filled with red blood cells & lined by irregular endothelial cells. These changes might result in decrease in the total pulmonary surfactant and more decrease in the free pulmonary surfactant. This was in harmony with Muller *et al*.,[49] who found that surfactant-like liposome recycling was impaired in type II pneumocytes from injured lungs.

The mitochondrial cristae in these cells exhibited obvious signs of attenuation and the lamellar bodies were nearly empty. This was documented by Majida & Sawsan[50] who investigated the effects of aluminum on various cellular organelles such as mitochondria, endoplasmic reticulum, lysosomes, and the cell membrane According to Saleh *et al*.[51], intraperitoneal injection of aluminum chloride caused cellular organelles vacuolization, which resulted in biochemical and structural changes at the cellular level. Anane & Creppy,[52] added that aluminum toxicity could cause calcium buildup in the mitochondria, resulting in irreversible damage to the membrane. Elmore[53] attributed previous changes to the formation of transition pore of the mitochondrial permeability, a high conductance channel in the mitochondrial membrane. The opening of this channel leads to the loss of mitochondrial membrane potential and pH changes, resulting in failure of oxidative phosphorylation and progressive depletion of energy production (ATP). ATP depletion leads to derangement of cell physiology, with subsequent dilatation of cytoplasmic membranous components and formation of cytoplasmic vacuolation that was encountered in our study as a result of loss of selective permeability of cell membrane.

Many alveolar macrophages were detected in aluminum chloride treated rats. This suggests their role in phagocytosis of degenerated pneumocytes type II with their deformed secretory material[54].

Propolis and aluminum chloride group showed improvement in light and electron microscopic results more or less like control group. According to studies, Propolis can counteract aluminum chloride toxicity[55]. This was described by other researchers[56] who reported that Propolis is an important antioxidant that antagonize aluminum free radical-mediated cytotoxicity.

This was further described by other investigators[57] who stated that Propolis administration reduced cellular membrane lipid peroxidation. Pervious research[58] stated that Propolis and its constituents have antioxidant properties as it can bind metal ions and scavenge singlet oxygen, superoxide anions, hydroxyl radicals, proxy radicals, and proxy nitrite. Propolis has the effect of preventing damage and preventing the enzymes from leaking through cellular membranes. These findings are consistent with the findings of numerous researches[59]. It has a powerful ROS (reactive oxygen species) scavenger in rats, as well as having antioxidant properties[60]. Macrophage production in response to Propolis treatment could imply a regeneration role against aluminum chloride toxicity as a mechanism for clearing tissue debris and apoptotic cells to restore lung tissue flexibility[61]. Because of its antioxidant properties, Propolis has been reported to help with fibrosis by some researchers[62]. Other researchers suggested that Propolis have a modulatory effect on cytokine TGF-1-induced fibrosis[63].

In the present study, following arrest of aluminum chloride treatment (for two weeks), lung sections showed persistence of histopathological changes including dilated congested pulmonary vessels, minimal inflammatory cellular infiltrate & minimal hemorrhage.

These results are contributed to very long half-life (approximately 2 weeks) of aluminum chloride[64]. This is in harmony with several clinical studies which suggested that spontaneous regression may occur up to two years[65].

CONCLUSION

It was demonstrated that aluminum chloride resulted in considerable histological and ultrastructural changes in lung tissue. Propolis minimizes this hazardous effect of aluminum chloride on lung and provides a good protective role. So, Propolis supplementation is highly recommended for people exposed to aluminum chloride. We recommend further studies with longer period of recovery to determine if the aluminum chloride toxicity is reversible or not.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

دراسة عن تأثير تناول عكبر النحل على سمية أنسجة الرئة التي يسببها كلوريد الألومنيوم في ذكور الجرذان البيضاء البالغة: دراسة نسيجية وكيميائية مناعية

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المقدمة:. كلوريد الألومنيوم هو أكثر المعادن انتشارا علي القشره الأرضيه . يوجد في حياتنا اليومية في مياه الشرب والتربة ومستحضرات التجميل شائعة الاستخدام. النسيج المبطن للرئة هو مكان تراكم كلوريد الألومنيوم. عكبر النحل هو منتج من النحل يشبه الشمع. له خاصية مضادات الأكسدة القوية المضادة للالتهابات.

الهدف من الدراسة : تقييم تأثير كلوريد الألومنيوم على أنسجة الرئة وإمكانية عكبر النحل في أن يقوم بالدور الوقائي

المواد المستخدمة وطريقة البحث: تم تقسيم خمسين من ذكور الجرذان البيضاء البالغة إلى خمس مجموعات متساوية تلقت العلاج لمدة ٦ أسابيع: (أولا) المجموعة الضابطة: تلقت الماء المقطر (ثانيا) مجموعة عكبر النحل "٥٠ مجم / كجم". (ثالثا) مجموعة كلوريد الألومنيوم "٤٧٥ ملجم / كجم". (رابعا) مجموعة الاستشفاء "تركت بدون علاج". (خامسا) مجموعة عكبر النحل وكلوريد الألومنيوم. في النهاية ، تم ذبح جميع الحيوانات و الحصول على عينات من الرئة للدراسات النسيجية والكيميائية المناعية. تم تحليل البيانات إحصائيا.

النتائج: ظهرت أنسجة الرئة طبيعية في كل من المجموعة الأولي والثانية. أما مجموعة كلوريد الألومنيوم (III) تشوه في أنسجة الرئة. انكمشت بعض الحويصلات الهوائية وأظهر البعض الآخر توسعًا تعويضيًا. امتلات أنسجة الرئة بالعديد من حبيبات الهيموسيديرين. أظهر النوع الثاني من الخلايا الرئوية نواة مكثفة غير منتظمة ، فجوات حشوية ، ميتوكوندريا منتفخة غير منتظمة ، تمدد في الشبكية الإندوبلازمية الخشنة وتلف في الأجسام الصفائحية. كانت هناك سماكة في الحاجز بين الحويصلات الهوائية ، العديد من الارتشاح الالتهابي وترسب مادة هياليئية حمراء. كانت الأوعية الدموية محتقنة ومحاطة بخلايا التهابية. أظهرت القصيبات الهوائيه تشوهًا. تم الكشف عن ترسب ملحوظ لألياف الكولاجين وتراكمات عديدة للخلايا الصاريه في حواجز النسيج الضام. لوحظ انخفاض كبير في التصبغ المناعي BCL٢ في أنسجة الرئة. تم تخفيف هذه التغييرات بشكل معتدل في مجموعة الاستشفاء. أعاد تناول عكبر النحل مع كلوريد الألومنيوم لأنسجة الرئة شكلها الطبيعي.

الاستنتاج: يسبب كلوريد الألومنيوم تلفًا ملحوظًا في أنسجة الرئة عن طريق التليف وموت الخلايا المبرمج. يمكن تخفيف هذه التغييرات الهيكلية بشكل متوسط عن طريق إيقاف العلاج لمدة أسبوعين وبشكل ملحوظ عن طريق تناول عكبر النحل مع العلاج .لذلك نوصي بتناول عكبر النحل للاشخاص الذين هم أكثر عرضة كلوريد الألومنيوم.