Original Research Article

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Ameliorating potential of clove oil on histological tissue damage induced by TCDD in lung of Wister albino Rats

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ABSTRACT: Dioxins are one of constant, bio-accumulative and toxic chemicals (PBTs) which accumulate in the environment with harmful level to ecological health either animals or human, also may travel long distances in air, can move readily from land to air and water (Rathoure 2018). The aim of the current research was to determine the potential for protective interventionsof clove oil in controlling tetra-chloro-dibenzodioxin (TCDD) toxicity in the lung tissue of Wister albino rats. Thirty female and male Wister rats were divided into three isolated groups (n=10). Group 1 (G1) served as normal control, group 2(G2), rats were received single intraperitoneal dose of TCDD (2µg/kg body wight dissolved in corn oil), while group 3 (G3), rats were treated with oral clove oil (CLO) (200mg/kg body weight day after day for 8 weeks) after TCDD treatment (same as group II). Lung tissue samples were collected for histopathological examination, Ultra-structural assessment and immunohistochemical examination for Proliferating Cell Nuclear Antigen (PCNA). Histopathological investigation from TCDD group revealed prominent broncho-interstitial pneumonia with thickened interalveolar tissues. Bronchial epithelium showed hyperplasia and desquamation and, in some cases, showed bronchiolitis obliterans. On the other hand, clove oil (CLO) co-administration ameliorated almost histopathological alterations in lung tissues. Clove oil administration could reduce the PCNA expression caused by TCDD in the immunohistochemical examination. In conclusion, exposure to 2, 3, 7, 8-TCDD caused oxidative stress damage and led to serious toxic effects in bronchioles and lung tissues and treatment with clove oil could ameliorate these toxic changes to an improved extend.

KEYWORDS: TCDD; Lung; TCDD; Clove oil; histopathology.

1. Introduction

Tetrachlorodibenzo-p-dioxin (TCDD) is considered as one of the most well-known environmental toxicants that build up either in the environment or the human body [1]. Dioxin is the conventional name for 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD), but it can also refer to the coplanar polychlorinated biphenyls (PCB), dibenzofurans (PCDF), and structurally and chemically similar polychlorinated dibenzo-para-dioxins (PCDD) [2]. According to [3] , dioxin is created as an inadvertent byproduct of numerous industrial processes, including industrial chlorination, the manufacture of PVC plastics, bleaching pulp and paper, the production of specific pesticides, waste-burning incinerators of various kinds, and backyard burn barrels [3] . Environmental spread of dioxins is worldwide, despite their local formation [4]). While plants, water, and air have extremely low amounts of these chemicals, some soils, sediments, and foods—particularly dairy products, meat, fish, and shellfish-have the largest concentrations of these substances [4]. The Aryl-hydrocarbon Receptor (AhR) is responsible for the toxicity of dioxins. It mediates the cellular metabolic adaptation to these planar aromatic xenobiotics through the classical transcriptional regulation pathway, which includes the heterodimeric transcription factor binding to dioxin-responsive elements that control the expression of genes involved in xenobiotic metabolism, AhR binding of ligand in the cytosol, receptor translocation to the nucleus, and dimerization with the AhR

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nuclear translocator [5]. In addition to being the most extensively researched AhR agonist over the past three decades, TCDD is thought to be the most hazardous of the dioxins and has the highest binding affinity for the AhR receptor [5, 6]. Dioxins cause many health problems to both animal and human including lung damage. [7] explained that exposure to dioxins, especially TCDD, can lead to oxidative DNA damage and lung tumor formation [7]. AhR activation can induce gene expression in lung and bronchial epithelial cells (Wang et al. 2011). Furthermore, dioxin exposure can damage mitochondrial function in lung cells that lead to a decrease in oxidative phosphorylation capacity [8]. Herbal products have been recognized as a significant source for detecting new medicinal compound which have been used to treat severe diseases [9]. The dried, unopened inflorescence of the clove tree, which is a member of the Myrtaceaceae family, is known as clove (Syzygium aromaticum). It is frequently used as a preservative in a variety of dishes and pickles, as well as to improve the flavor and aroma of the food. It is regarded as one of the most essential components and the second most beneficial and practical spice in Indian cooking. Ayurveda has been using cloves for medicinal purposes for over two thousand years. They are chewed to relieve toothache pain, and they are also commonly used as an oral anesthetic and to disinfect root canals in temporary fillings. Numerous studies have demonstrated the cloves' numerous significant and positive effects on human health. [10, 11, 12] Clove has a lot of qualities that can help with treating health related problems as it showed antimicrobial, antiviral, insecticidal, larvicidal, anti-inflammatory, cytotoxic, anesthetic, anti-oxidative (flavonoids) activities. It is among the most abundant sources of phenolic chemicals, including gallic acid, β-caryophyllene, α-humulene, eugenol, and eugenol acetate, which have significant applications in the food, cosmetic, pharmaceutical, and agricultural industries [11, 12, 13]. Eugenol is considered as the main component of clove oil which has antioxidant and anti-inflammatory properties, and prevents lipid peroxidation. Its anticancer properties were examined via several pathways as cell cycle arrest and inhibition of cell proliferation, migration, apoptosis, angiogenesis, and metastasis on multiple cell line models. Furthermore, eugenol is used as an adjuvant treatment in chemotherapy as this combinationhas an enhanced efficacy without major side effects or toxicity [14]. So, the aim of the work was to evaluate the possible protective role of administering clove essential oil on pulmonary injuries provokedby TCDD exposure in Wister albino rats.

2. Material and Methods

2.1. Ethical Considerations

All experimental procedures of the study were conducted according to the Declaration of the ethics committee's rules "Institutional Review Board" at Assiut University in Assiut, Egypt (approval number 04-2023-100073).

2.2. Materials

2.2.1. Drugs and Chemicals

TCDD was purchased from Chem-Impex International, Inc. Clove oil was purchased from Harraz company for food industry and natural products, Egypt.

2.2.2. Animals

Thirty male and female Wister strain albino rats, weighing 180–220 g, were acquired from Sohag University's experimental animal home. Four rats each were kept in sterile polypropylene cages in a temperature-controlled environment (27 ± 2 C) with a 12-hour light and 12-hour dark cycle. During the eight-week experiment, rats were given a regular pellet meal and unlimited access to water (8 weeks).

2.2.3. Experimental design

During the week of acclimatization, rats were closely monitored, and fecal samples from each group were analyzed using the concentration floatation method. Following centrifugation, the sediment and supernatant fluid

were analyzed independently for parasitic eggs and larvae, demonstrating that our test animals were parasite-free. Three groups of thirty rats, ten in each, were created and given the following treatment:

Rats in Group 1 (G1):

Normal control (n=10) were given drinking water and standard rat food.

Rats in Group 2 (G2)

Intraperitoneal (I/P) injection (n=10) of a single dosage of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (2µg/kg body weight) dissolved in maize oil was given to rats (Mai et al. 2020).

Rats in Group 3 (G3):

TCDD + Clove oil (CLO) (n=10) were given 2,3,7,8-TCDD (as in G2) and were gavaged with clove oil daily for 8 weeks at a dose of 200 mg/kg body weight [15].

2.3. Methods

Finally after 8 weeks, all experimental animals were decapitated and lung samples were taken from each group for assessment of histological examination, Ultrastructural assessment and immunohistochemical examination for Proliferating Cell Nuclear Antigen (PCNA).

2.4. Histopathological assessment

Following the experiment, the animals were sacrificed, and lung tissue samples were taken, dissected, and promptly fixed in 10% formalin for 24 hours. They were then dehydrated in a series of progressively stronger alcohols, cleared in xylene, and lastly wrapped in paraffin (Suvarna and Layton 2013). For histological assessment, tissue sections were cut to a thickness of 3 µm and stained with hematoxylin and eosin (H&E) (Bancroft et al. 1996). The OLYMPUS CX43 microscope and a microscope-adapted OLYMPUS SC52 camera were used to examine and take pictures of every tissue section (Department of Pathology and Clinical Pathology, Sohag University).

2.5. Morphometric analysis

Analysis of lung histopathology was performed by specifying a score, depend on the damage degree which recorded in different groups for semiquantitative measurements: 0 = no lesions; 1 = mild (1 to 25%); 2 = moderate, (26 to 45%); 3 = severe (> 45%) as described in previous literatures (O'Brien et al. 1996; Gibson-Corley et al. 2013; Hamdin et al. 2019). Lung tissue specimens were evaluated blindly. According to these documented histopathological classification, alveolar tissue lesions, interstitial tissue lesions, bronchial and vascular lesions were graded and scoring were assigned according to previous studies [16]. The morphometric was calculated at the unit of morphometric analysis at Pathology and Clinical Pathology Department, Faculty of Veterinary Medicine, Sohag University. Analysis of variance, or ANOVA, was used to compare the means of several groups using Prism 5 on the Microsoft Windows operating system. To determine whether there were mean differences, Prism 5 was utilized to do multiple comparison tests, such as One Way ANOVA with Newman Keuls test. Data accessed as the mean ± SD.

2.6. Semithin assessment

Lung samples were extracted and immediately preserved for two hours at 4°C in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4). The tissue samples were dried in ethyl alcohol, cleaned in propylene oxide, post-fixed in 1% osmium tetroxide at 4°C for two hours, and then embedded in epoxy resin [17]. Semi-thin slices (1µm thick) were cut with an ultramicrotome (Leica Ultracut; Leica, Berlin, Germany), stained with toluidine blue, and then seen under a light microscope (Electron microscope unit, Assiut University) to check for overall tissue arrangement.

2.7. Immunohistochemical assessment:

Recognition of proliferating cell nuclear antigen (PCNA) antibodies were carried outfor the assessment of proliferation of lung cells(1:200 dilution) by using Mouse monoclonal proliferating cell nuclear antigen (PCNA) antibody

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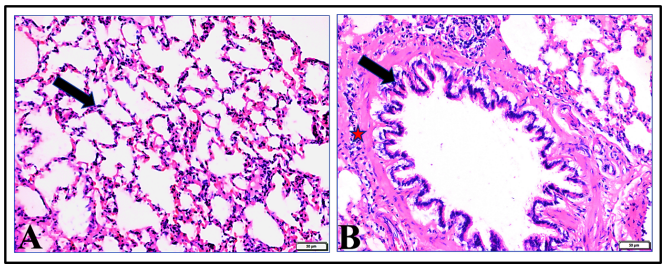

Figure 1: Photomicrograph of lung tissue section from control group showing: (A): alveoli of normal epithelium, size, and interalveolar tissue (arrows). (B): bronchial epitheliumshowed distinctive cellular structures (arrow)with slight normal thickening peribronchial lymphoid aggregation (star). HE. The bar size is (A, B=50 µm).

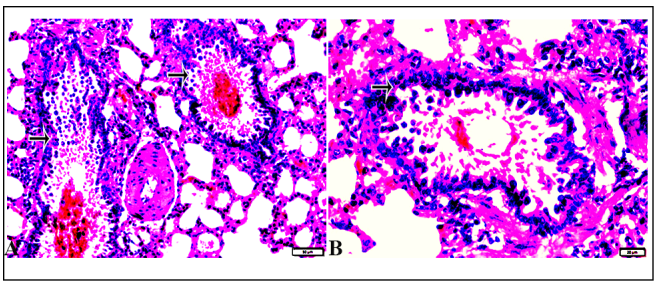

Figure 2: Photomicrograph of lung tissue section from TCDD group showing: (A): bronchiolitis with prominent hyperplasia and desquamation forming bronchiolitis obliterans (Blood + Inflammatory cells) (arrows). (B): prominent hyperplasia and desquamation of bronchiolar epithelium (arrows). HE. The bar size is (A=50µg, B=20 µg).

(PC10)(Invitrogen, Neomarks, Fremont, USA, Catalog # 13-3900). Deparaffinization in xylene and rehydration of sections were done. Antigen retrieval (5-10 min)was carried out in citrate buffer solution (at PH 6.0) boiling in the microwave. The endogenous peroxidase was blocked by 0.3% hydrogen peroxide for 5 min. Incubation of the sections was done overnight at 4°C with the primary antibody then the avidin-biotin technique was applied. Sections were counterstained with Mayer's Hematoxylin, dehydrated, and cleared. Positivity appeared as brown coloration [18]. Negative control was done by the omission of primary antibodies. The distinctive labeling patterns of each cell cycle stage were used to identify the cell cycle stage of the PCNA-stained cells [19]. The absence of nuclear or cytoplasmic staining was used to identify cells in G0 phase. Light-red, patchy to uniform nuclear staining without cytoplasmic staining was used to identify G1 cells. S phase cells lacked cytoplasmic staining and displayed a dense, homogeneous brown to red nuclear staining. While cells in M phase displayed a diffuse cytoplasmic staining along with mitotic structures, G2 cells displayed a diffuse, speckled nuclear and cytoplasmic staining. Cells in the G1, S, G2, and M phases of the cell cycle were those that had reached the growth fraction [20]. Using light microscopy Leica ICC50 Wetzlar (Germany), ten non-overlapped high-power fields (x400) were taken for each section at the Histology Department, Faculty of Medicine, Sohag University. Each field was analyzed using Image J1.51n software (National institutes of health USA Java 1.8.0_66 (32-bit).

3. Results

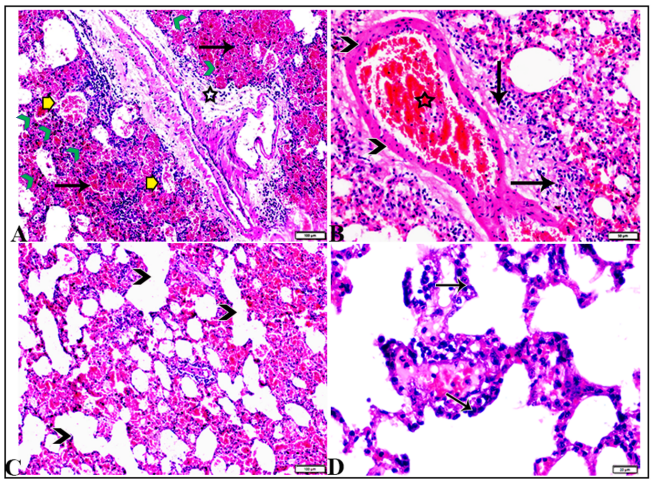

Figure 3: Photomicrograph of lung tissue section from TCDD group showing: (A): interstitial (black arrow) and interalveolar hemorrhage (yellow arrow) with prominent hemosiderosis (green arrowhead) and perivasculitis (star). (B): Blood vessels were severely dilated and engorged with blood mixed with inflammatory cells (star) with thickened and hyalinized wall (arrowhead), perivascular edema and mononuclear cells infiltration. (arrows). (C): Prominent alveolar emphysema (arrowhead) with congestion of interalveolar capillaries, perivasculitis (arrowhead). (D): Hyperplasia of pneumocyte typeπ cells lining alveolar wall. HE. The bar size is (A& C =100µm, B=50 µm, D =20 µm.

3.1. Histopathological assessment

Histopathological examination of lung tissue from experimental groups revealed normal histological structures

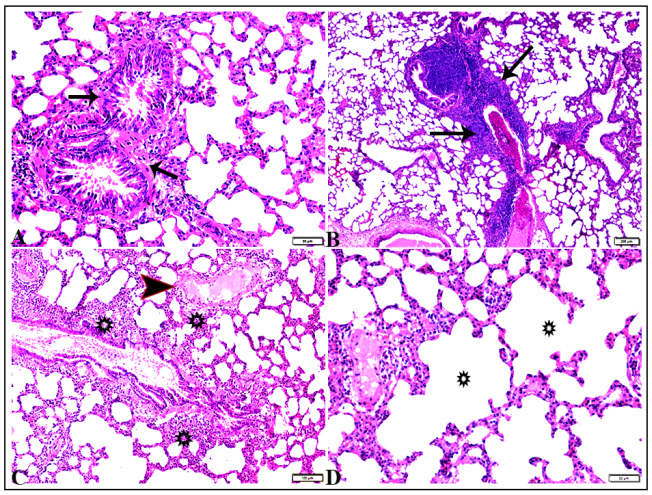

Figure 4: Photomicrograph of lung tissue section from TCDD group treated with Clove oil showing: (A): bronchiolitis with mild bronchial epithelium hyperplasia (arrows). (B): prominent hyperplasia of parabronchial lymphoid aggregation (arrows). (C): mild degree of interalveolar thickening (stars), perivasculitis (arrowhead). (D): mild alveolar emphysema. HE. The bar size is (A&D=50µg, B=200 µg, C=100 µg)

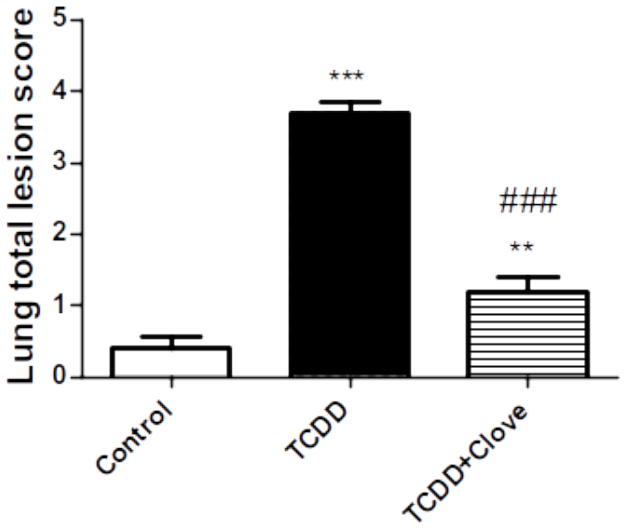

Figure 5: Histomorphometry graph showing semiquantitative measurements of total lesion scores recorded in lung tissue sections among the experimental groups:Data are expressed as means ± standard deviations. Significant differences versus the control group are marked by different asterisks while significant differences versusTCDD group are marked by different #, all through one-way ANOVA with Newman Keuls multiple comparison test: ** p ≤ 0.01, ###*** p

in sections obtained from control negative group (G1), whereas, bronchial epithelium showed distinctive cellular structures with slight normal thickening peribronchial lymphoid aggregation. alveoli showed normal epithelium, size, and interalveolar tissue (Fig. 1A), with normal vascular structure (Fig. 1B). Histopathological examination of lung from TCDD group revealed bronchointerstitial pneumonia with thickened interalveolar tissues. Bronchial epithelium showed hyperplasia and desquamation with hyperplasia of peribronchial lymphoid aggregation and in some cases showed bronchiolitis obliterans (Blood + Inflammatory cells) (Fig. 2A, Fig. 2B). There wereinterstitial and interalveolar hemorrhage with prominent hemosiderosis (Fig. 3A)).Blood vessels were severely dilated and engorged with blood mixed with inflammatory cells with thickened and hyalinized wall, perivascular edema and mononuclear cells infiltration (Fig. 3B)). Severe lymphoid tissue hyperplasia developed in lung tissue with prominent angiogenesis. Alveolar emphysema (Destruction of alveolar wall which lead to abnormal enlargement of air spaces distal to terminal bronchioles) was prominent with congestion of interalveolar capillaries (Fig. 3C). Hyperplasia of pneumocyte type π cells lining alveolar wall was also reported (Fig. 3D). Lung tissue examination in clove oil (CLO) group exhibited more or less normal histological structure of alveoli while little changes were reported in bronchial epithelium. Bronchial epithelium showed mild bronchiolitis in its lumen (Fig.4A). There was prominent hyperplasia of parabronchial lymphoid aggregation and perivasculitis (Fig. 4B). Thickening of interalveolar tissue was of mild degree (Fig. 4C) .Alveolar emphysema was present in mild degree (Fig. 4D) .Morphometric analysis of total lung lesions showed high significant increase (P≤0.001) in lesions in TCCD group in compared to control group. Whereas in clove group, lesions were highly significant decrease (P≤0.001) in compared to TCCD and to control group respectively Fig. 5.

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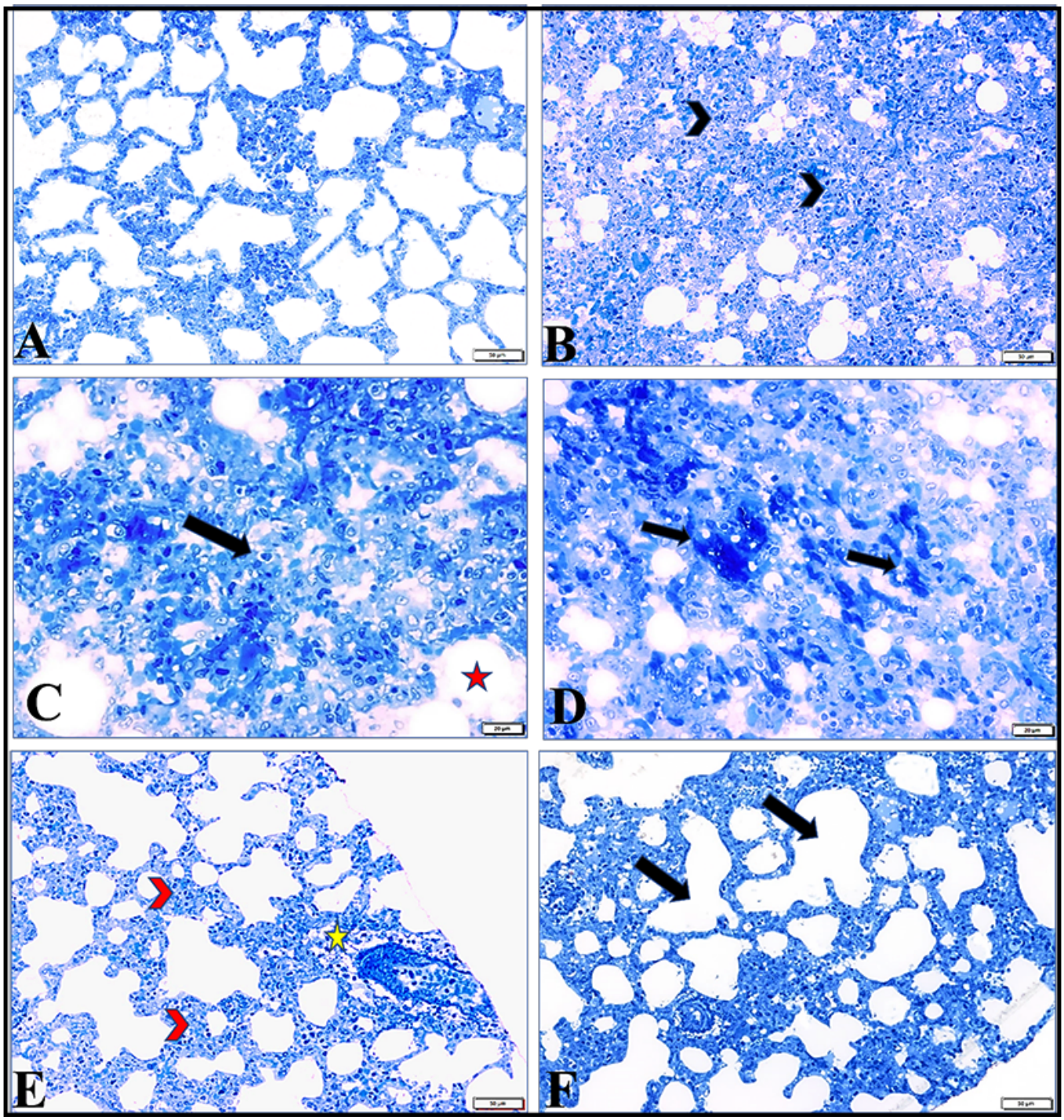

Figure 6: Photomicrograph of lung tissue section from: (A): control group showing normal tissue architecture with regular sized alveolar spaces, normal interalveolar septa.(B): TCDD group showingmarked thickening of interalveolar tissue (arrowhead).(C): TCDD group showing hyperplasia of pneumocyte type II (arrow), prominent alveolar emphysema (red star).(D):TCDD group showingcongestion and hemorrhage of interalveolar capillaries (arrow).(E):Clove group showing mild degree of interalveolar tissue thickening (arrowhead), perivasculitis (star). (F):Clove group showingmild alveolar emphysema.Toluidine blue stain. The bar size is (A, B,E&F=50µg,C&D=20 µg).

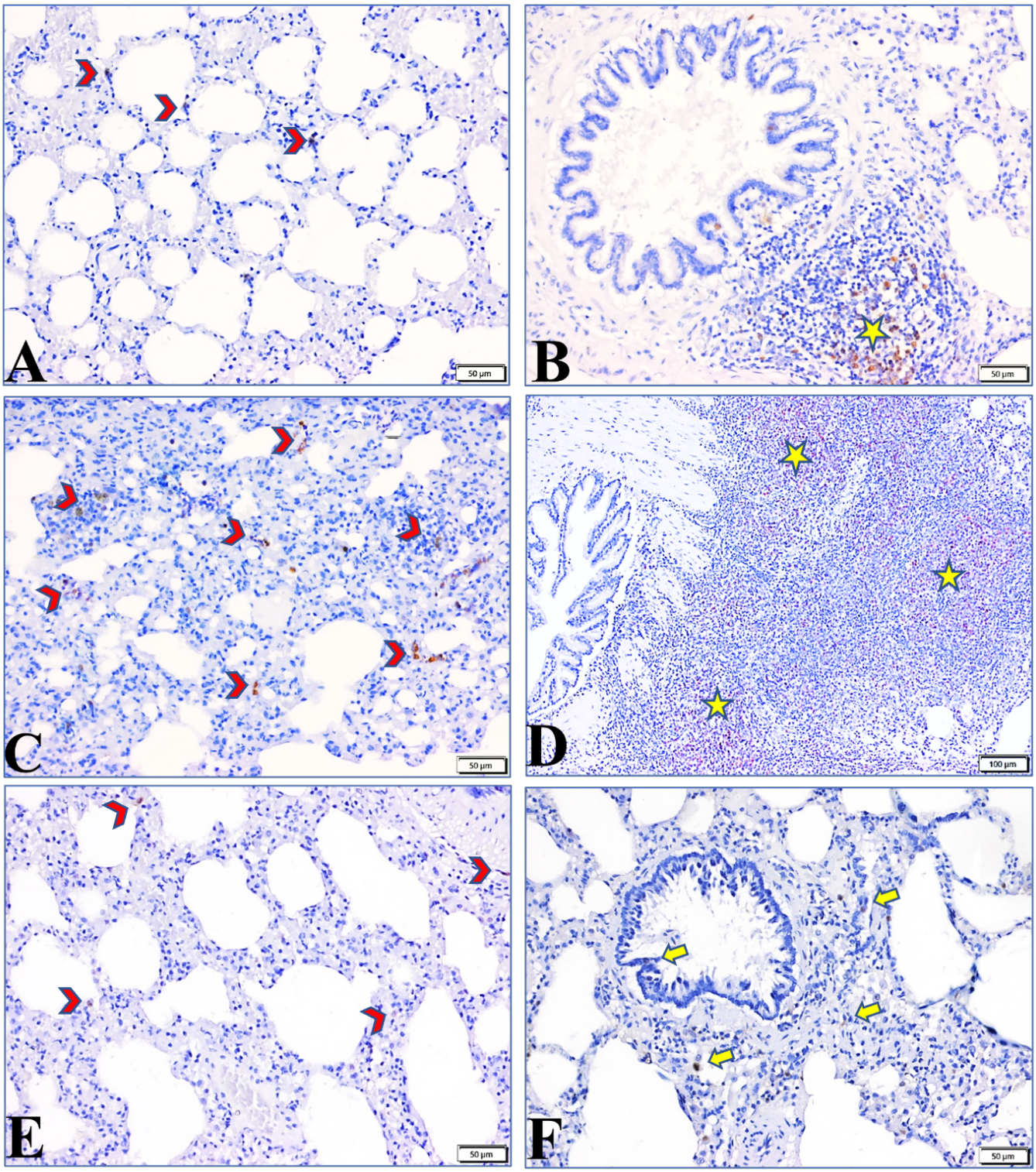

Figure 7: Immunohistochemical staining of lung tissue section from: (A): control group showing weak positive immunoreaction in the nuclei offew alveolar cells(arrowhead)(B): control group showing weak positive immunoreaction in bronchiolar epithelium and peribronchial lymphoid aggregation (star). (C): TCDD group showingstrongpositive immunoreaction in the nuclei ofalveolar epithelial cells (arrowhead). (D): TCDD group showing strongpositive immunoreaction in the nuclei of bronchiolar epithelium and peribronchial lymphoid aggregation (star). (E): Clove group showing weak positive immunoreaction in the nuclei of few alveolar cells(arrowhead). (F): Clove group showing weak positive immunoreaction in bronchiolar epithelium and peribronchial lymphoid aggregation (arrow). PCNA. The bar size is (A, B, C,E &F=50µg, D=100 µg).

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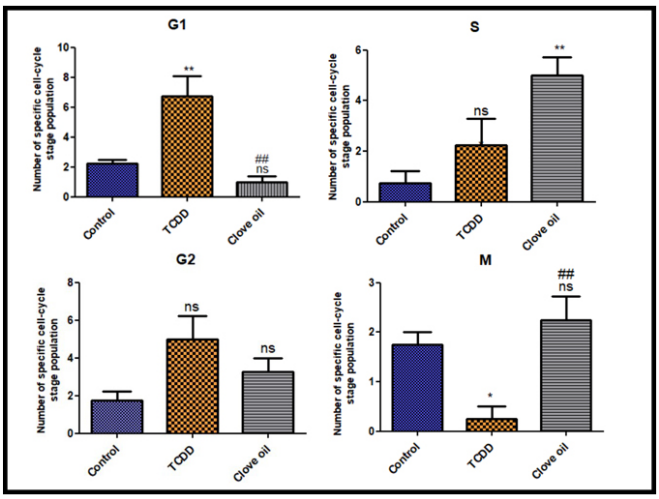

Figure 8: Histomorphometry graph showing quantitative measurements of specific Cell cycle distribution of total cellpopulation in lung tissue. G1, S phase, G2, and mitosis M phase. Control, Dioxin and Clove oil treated groups are as described. Designation of the specific cell-cycle stages was made using differential, PCNA immunohistochemical analysis. Data are expressed as means ± standard Error. Significant differences vs. the control group are marked by different asterisks, Dioxin significant differences vs. other groups are marked by different # through one-way ANOVA with Newman Keuls multiple comparison test: * #p ≤ 0.05, ** # #p ≤ 0.01, *** p ≤ 0.001).

3.2. Semithin assessment

In control group, semithin section examination of lung showed a normal tissue architecture with regular sized alveolar spaces, normal interalveolar septa and bronchioles with intact bronchial epithelium (Fig. 6A). In TCDD group, lung tissue showed marked thickening of interalveolar tissue (Fig. 6B) and hyperplasia of pneumocyte type II (Fig. 6C). There was prominent alveolar emphysema (Fig. 6C), congestion and hemorrhage of interalveolar capillaries with increased alveolar macrophage(Fig. 6D). Lung tissue in clove group showed mild thickening of interalveolar tissue (Fig. 6E), marked perivascular mononuclear cell infiltration. There was mild degree of alveolar emphysema (Fig. 6F)

3.3. Immunohistochemical assessment:

Immunohistochemical examination of lung tissue from control group showed weak positive nuclear immunoreaction for PCNA in the nuclei of few alveolar cells (Fig. 7A), bronchial epithelium and peribronchial lymphoid tissue (Fig. 7B). While in TCDD group, strongpositive immunoreaction for PCNA was noticedin thenuclei of alveolar epithelium (Fig. 7C), bronchial epithelium and peribronchial lymphoid tissue (Fig. 7D).Mostly of expressed positive cells were recorded in proliferated lymphoid tissues. Statistical analysis of different stages of positively expressed cells in TCDD group showed that no of G1cells increased significantly (P<0.01) in contrast to control group (Fig. 8). Also, no of S & G2 cells were insignificantly increased in contrast to control group. On the otherwise, M stage exhibited significant decreased (P<0.5) in compared to control group. Group III (Clove group)exhibited weak positive immunoreaction for PCNA in the nuclei of some alveolar epithelial lining (Fig. 7E), bronchial epithelium and peribronchial lymphoid aggregation (Fig. 7F) in compared to TCDD group. Whereas, G1 cells were decreased significantly (P<0.01) in contrast to TCDD group. M cells showed significant increase (P<0.01) in contrast to TCDD group. No of S cells was increased significantly (P<0.01) in contrast to control group (Fig. 8).

Discussion

Exposure to air pollutants as dioxins is associated with increased risk of lung diseases. These pollutants exert their harmful effects on lung by causing pulmonary and systemic inflammation, oxidative stress and endothelial dysfunction [21]. Histopathological findings of lung in TCDD group exhibited several proliferative changes were detected in the lung including alveolar epithelial hyperplasia and bronchiolar epithelial hyperplasia which represented as broncho-interstitial pneumonia with thickened interalveolar tissues (Pneumocyte type π cells hyperplasia) which agreed with [22] and in some cases showed bronchiolitis obliterans (Blood + Inflammatory cells) which obstruct pulmonary pathways and lead to prominent alveolar emphysema and may lead to chronic

obstructive pulmonary disease as discussed in [23]. Aryl-hydrocarbon Receptor (AhR) mediates TCDD toxicity. After being exposed to TCDD, activated AhR would travel to the nucleus and combine with Ah Receptor Nuclear Translocator (ARNT) to produce a dimer complex that would bind certain DNA elements. According to [24], this bonding will raise the expression of P450 cytochromes, particularly CYPIAI and CYPIBI [24]. Involved in the sequence of free radical generation that causes oxidative stress, cytochrome P450 can harm various vital biological components, including DNA, proteins, and lipids [25]. There was hyperplasia of peribronchial lymphoid aggregation and congestion of interalveolar capillaries as showed in [26, 27]. There were interstitial and interalveolar hemorrhage with prominent hemosiderosis as manifested in lamina propria of fundic mucosa in stomach of adult rat stomach exposed to TCDD [28]. On the other side, administration of clove oil in the present study showed marked improvement of lung tissue architecture with very mild alveolar emphysema, little degree of interalveolar thickening, and bronchiolitis with mild bronchial epithelial hyperplasia in compared to TCDD group. These results are agreed with [27] study which stated that clove oil administration offered protection against BP induced-lung toxicity, as clove can be efficient in protecting role through antagonizing free radicals; enhancing the antioxidant defense mechanisms and pollutant decontamination. All these properties of clove oil are related to its biochemical structure and its role in cell membrane stabilization via diminishing membrane fluidity employing antioxidant and anti-inflammatory properties [29]. PCNA immunohistochemical analysis can distinguish cells in the various cell-cycle stages (G1, S, G2, and M). Immunohistochemical examination for PCNA after TCDD toxicity revealed significant increase in proliferation of the positive immunoreactive cells especially G1 stage and to a little extent stage S & G2 in compared with control group. Also, it resulted in significant decrease of M phase cells in compared to control group and these results discussed at inhibitory effects of TCDD on hepatocyte proliferation induced by 2/3 partial hepatectomy in rat which proved that TCDD therapy caused a decrease in the S, G2, and M phases and an increase in the G1 phase. These statistics imply that TCDD might be contributing to a cell cycle delay or halt. The number of cells in the G1 phase of the cell cycle increased as a result of TCDD's apparent action at a restriction point in G1. The eventual production of lung cancers by persistent TCDD exposure may be significantly impacted by a suggested restrictive growth environment [20]. Immunoreactivity for PCNA was enhanced in clove treated group in contrast to the TCDD group. G1 cells were considerably reduced ($P<0.01$) in contrast to TCDD group. M cells were significantly increased ($P<0.01$) in contrast to TCDD group. No of S cells exhibited significant increase ($P<0.01$) in contrast to control group. Clove oil co-administrationoffered promising chemopreventioncandidate against TCDD induced-lung toxicity and possibility of lung cancer, as clove can beefficient in inhibition ofinflammation and abnormal cell proliferation as discussed in [27, 30]

Conclusion

Researchers and physicians consider the environmental pollution as a serious and an emerging factor in the development of lung diseases.Rat intoxication with TCDD causes changes in both lung tissue and its function. Clove oil showed higher radical scavenging activity which able to mitigate the deleterious effect of oxidative stress induced by TCDD. Dioxins extensively affect the histological lung tissue structure, which negatively affects its function. It has been observed that the administration of clove oil to TCDD-treated rats resulted in a reduction in these changes and assumed that it might potentially be used as a defensive agent against lung injury.

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Conflict of interest

There are no conflicting interests, according to the authors.

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