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(RESEARCH ARTICLE)

Histological investigation for the effect of non-steroidal anti-inflammatory drug [diclofenac] on myocardium and lung of local breed Rabbit

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Abstract

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used as analgesics, anti-inflammatory agents, and antipyretics. The goal of this study was to perform a histological assessment of therapeutic and overdose dosages of diclofenac on the heart and lung tissues and serum Troponin I, Lactate Dehydrogenase, and Creatine Kinase. Thirty local breed rabbits weighing 1000 and 1400 grams were used, divided into four groups: therapeutic group I, overdose group II, overdose group III, and control group. Each group was injected intraperitoneum with different doses of diclofenac (2, 20, 50 mg/kg) once daily for 20 days, while the control group was injected with normal saline. The results of heart sections in the experimental groups showed numerous histological alterations. In group I, muscle fibers were degenerated, lipid droplets within hemolyzed, and fibrinoid deposition in congested blood vessels. Group II showed a woven appearance of muscle fibers, atrophied muscle fibers, and an increased presence of fibroblasts between blood vessels and adjacent muscle fibers. In group III, colloid appearances, pavement of inflammatory cells on the tunica intima of a congested blood vessel, muscle fiber necrosis, and hemosiderin deposits were observed between muscle fibers. The lung results referred to that group I had thickened the interstitial connective tissue, hyperplasia in connective cells, and cytoplasmic vacuolation of type I alveolus cells. In group II, the results revealed folded alveolar walls, droplets in hemolyzed congested blood vessel, and aggregation of desquamated cells within the lumen of alveoli. Group III showed engorged pulmonary vessel, folded alveolar walls surrounded with inflammatory cells infiltration. The results of serum Troponin I, creatine kinase and Lactate dehydrogenase showed a significant increase in between the experimental group when compared with control group, otherwise, the results of Troponin I in group III showed a significant decrease when compared with the control group on level 0.05%. Finally, the results of serum cholesterol a significant increase in between experimental when compared with control group on level 0.05%.

Keywords: Nonsteroidal anti-inflammatory; Diclofenac; Troponin I; Creatine kinase and heart muscle fibers

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most common drugs used as inflammatory, analgesics, and antipyretics[1]. The mechanism action of NSAIDs blocks the activity of cyclooxygenase (COX2) enzyme in the process of converting arachidonic acid as a substrate into prostaglandins [2]. COX-1 is mainly considered to be expressed constitutively in most tissues; otherwise, COX-2 triggers the inflammation mediator [4]. NSAIDs were classified as nonselective COX1 inhibitors and specific COX-2 inhibitors [3]. COX-1 and COX -2 enzymes activate to produce prostaglandin H2, D2, E2, and F2, which participate in immune response, cardiovascular, elementary canal, renal, pulmonary, central nervous system, and reproductive function [5]. In 2005, the FDA revealed all data about selective and nonselective COX inhibitors' role in cardiovascular risks [6]. Studies revealed the adverse effects of NSAIDs in the elementary canal, Cardiovascular system[7], hepatocytes and renal toxicity [8], male and female reproductive system [9, 10], and central nervous system[11] . Studies have shown the adverse effect of NSAIDs on skeletal muscle satellite cells. Satellite cells differentiate into myoblasts after muscle fibers atrophy [12]. The inhibition activities of NSAIDs in

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prostaglandin (PGE2) production induced muscle inflammation and muscle fibers atrophy [13]. Troponins act as regulatory sarcomeric proteins in the myocardium, which are responsible for regulating sarcomeric Ca2+ and participating in myocardium contraction [14]. Troponins are considered to be specific markers of heart muscle fiber damage[15]. Creatine Kinase-MB (CK-MB) enzyme participates in cellular energy transport[16]. And act as a specific marker for determining heart damage [17]. Lactate Dehydrogenase (LDH) levels are also secondary markers in determining short half-life heart damage [18]. Respiratory diseases are common and serious health problems induced by NSAIDs [19]. Regular administration of acetaminophen may, induce depletion of antioxidants, which increases the risk of lung tissue damage

1.1. The aim of study

The aim of this study was designed to evaluate the role of therapeutic and overdoses of diclofenac on heart , lung tissue and estimate the levels of heart serum factors Troponin I, Lactate Dehydrogenase and Creatine Kinase.

2. Material and method

A study designed to assess the histological and serum Troponin I, Lactate Dehydrogenase, Creatine Kinase, and Triglyceride parameters in local breed rabbits The study was done in the biology lab. / department of biology/ Education college for women/ university of Kirkuk. Thirty 30 adult local breed rabbit mature (11 and 13 months), weighing (100- 1400 grams) obtained from the College of Veterinary/ Tikrit University. They were maintained on 12:12 light: dark bases and 24 ±2°C. Animals had free access to food and tap water.

2.1. Experimental protocol

30 rabbits were divided into four groups: I, II, III, and control group, each group housed in steel cages, each measuring 1.250 * 0.5 * 0.5 meters, with wood shavings as bedding material. Animal left 7 days for preparation before the intraperitoneal injection day. Saline was injected into the control group for 20 days. The experimental/therapeutic I groups received 2 mg, group II received 5mg, and group III received 20 mg of diclofenac. On the 20th day, blood samples were collected from the heart cardiac puncture on the last day, and then the heart and lung tissue were immediately removed. The samples were fixed in 10% formalin for 24 hours.

2.2. Measurement of lipid profile

The amount of cardiac troponin I (cTn-I), Creatine kinase (CK), and lactate dehydrogenase (LDH), was measured in serum according to standard kits[20] The number of total triglyceride (TG), was measured in serum [21].

2.3. Histological investigation

Fixed tissue samples of the heart and lungs were prepared for histological studies. The tissues were paraffinized into paraffin blocks and cut into 7- 7.5µ ribbons. Hematoxylin and eosin (H&E) staining was performed, and sections were visualized by light microscope [22].

3. Results

The results of histological study of control group of heart muscle revealed intact texture of muscle fiber those fibers were invested by loose connective tissue which represent the endomysium which have the branches of coronary blood vessels and greater blood vessels were present in the perimysium with fat cells (Fig1). The results of group I revealed degeneration of muscle fibers. Other spots showed zonal degeneration around the nucleus, blood hemolyzed from congested blood vessels and muscle fibers atrophy(figs 2, 3). Other sections showed lipid droplets vacuolated in a colloid appearance, congested blood vessels infiltrated with inflammatory cells, and fibrinoid deposition within them (fig 4). The results of group II showed that the histological alterations increased with a given dosage, such as the woven appearance in muscle fibers. Spread out of fibroblasts in between blood vessels and muscle fibers, infiltration of inflammatory cells in between muscle fibers. Focal degeneration in sarcoplasm of muscle cells , and lipid droplets aggregate within blood vessels (fig 5). Other sections showed muscle fiber necrosis adjacent blood vessels demonstrated, vacuolation in the blood vessel milieu, and zonal degeneration sarcoplasm of muscle fibers; in different spots, revealed karyolysis of muscle fibers and pyknosis of some muscle cell nuclei (fig 5,6).

The results of group III showed a colloid appearance of congested blood vessels with a pavement of inflammatory cells with endothelial layer (Fig 7). Other sections showed detachment of endothelium (squamous epithelium layer) and degeneration of His bundles (fig 8); another section showed atrophied muscle bundles and congested Blood vessels.

And fibrinoid deposit in others (fig 9); other section showed hemorrhage was seen in between muscle fibers with hemosiderin deposit, necrosis in muscle fibers, and infiltration of inflammatory cells (fig10). Other sections showed atrophy of muscle fibers, infiltration of inflammatory cells in many regions of tissue, and degeneration in muscle fibers. Spread out of fibroblasts in between blood vessels and muscle fibers, partial hemolysis of RBCs within congested blood vessels, and pyknosis of some muscle cell nuclei (fig 11, 12). The lung tissue sections revealed normal lung texture with a segmental air sac and air sac (alveolus). Numerous patent alveoli were arranged into clusters called alveoli that contribute a standard opening to the alveolar duct (Fig 13).

The results of group I showed extensive thickness in the interstitial of the connective tissue, particularly collagen bundle hemolyzed blood associated with WBCs and Hemosiderin deposition, the alveoli had small narrow (Fig 14). The other section showed the alveolar walls of most alveoli containing alveolar cells type I with cytoplasm vacuolation. Also there was pycnotic nuclei and dust cells in the interstitial connective tissue (Fig 15). Group II showed the presence of inflammatory cells, folded alveolar wall, congestion of hemolyzed blood and associated with fat droplets (fig 16); other section showed infiltration of inflammation within interstitial connective tissue, cytoplasmic vacuolation of dust cells, and aggregated desquamated cells within lumen of alveoli (fig 17) and severed Interstitial pneumonia was evidently associated with multiple diffusion of WBCs and dust cells, the alveolar walls were thickened with narrowing of alveolar spaces (fig 18) . The results in Group III demonstrated folded the alveolar walls air sacs, which had infiltration with inflammatory cells. The pulmonary vein was engorged with blood masses, surrounded by delicate connective tissue associated with inflammatory cells (Figure 19). Other sections of lung tissue contained profuse blood hemorrhage engorged with white inflammatory cells and dust cells. Other spots revealed blood hemolysis with minute fat droplets within blood capillaries in between alveoli (Figure 20). The current study's results (table 1) revealed a significant increase in Troponin I, Creatine kinase, Lactate dehydrogenase, and Triglyceride in experimental groups I, II, and III. Otherwise the results of Troponin I in group III showed a significant decrease when compared with control groups on level 0.05%

Table 1 The levels of Troponin I, Creatine kinase, Lactate dehydrogenase, and Triglyceride among experimental groups I, II and III

Parameters	Control	Group I	Group II	Group III
Troponin I	0.0514 * 0.0107 C	0.8114* 0.3346 B	3.1800* 0.3191 A	0.0300* 0.0129 D
Creatin kinase	151.29* 13.74 C	190.14* 13.33 B	224.43* 22.74 A	151.00* 10.55 C
LDH	682.1*219.0 C	1435.4*541.8B	1581.9* 170.3 A	266.4* 17.3 D
Triglyceride	39.86* 13.17 C	71.14* 9.15 B	95.43* 15.00 A	86.71* 7.54 A

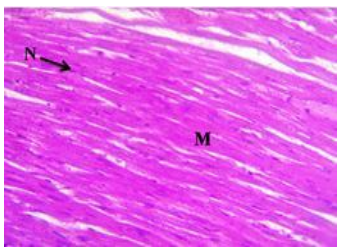


Figure 1 Heart section of the control group shows cardiac muscle bundles (M) and the nuclei of muscle cells (Nu).

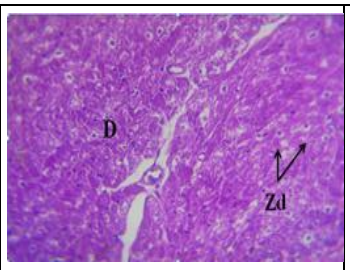


Figure 2 Heart section of group I shows degenerating muscle cells (D) and zonal degeneration around nucleus of muscle cells (Zd). (10x H. &E.)

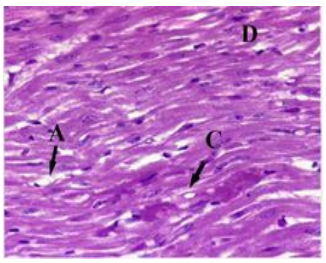


Figure 3 Heart section of group I revealed increased spaces between muscle bundles (A), complete blood hemolysis of congested blood vessels (C), and degeneration muscle cells (D). (40x H. &E.)

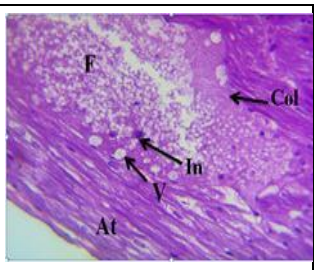


Figure 4 Heart section of group I shows atrophy of muscle fibers (A), inflammatory cells in (B).(V), with colloid of RBCs (Col).in congested blood vessels (C)., vacuollation of lipid droplets (V) and fibriniod F. (40x H. &E.)

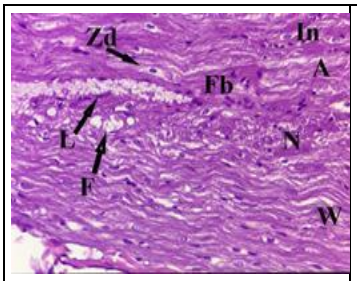


Figure 5 Group II Heart section of rabbits shows a woven appearance in muscle fibers (W), necrosis of entire muscle fibers (N), atrophy of muscle fibers (A), spread out of fibroblasts in between blood vessels and muscle fibers (Fb), infiltration of inflammatory cells among muscle fibers (In), focal cytoplasmic degeneration in muscle fibers (F), zonal degeneration around nucleus of muscle cells (Zd) and aggregate of lipids inside blood vessels (L). (40x H. &E.)

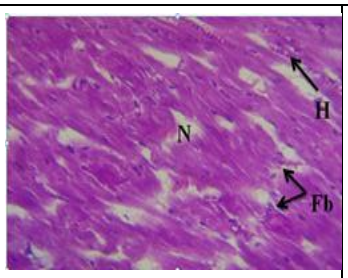


Figure 6 Group II: Heart section of rabbits shows necrosis throughout section (N), and spread of fibroblast among necrotic muscle cells (Fb). (40x H. &E.)

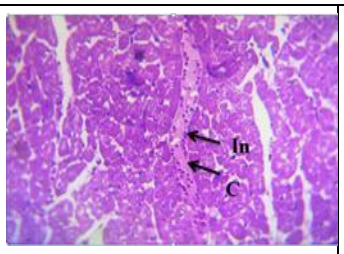


Figure 7 Group III: Heart section of rabbits shows inflammatory cells in (B.V), with colloid of RBCs Col.in congested blood vessels ©. (10x H. &E.)

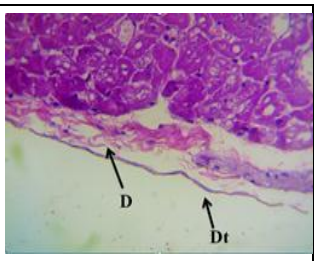


Figure 8 Group III: Heart section of rabbits shows detachment of the endometrium squamous layer (Dt) and degeneration of His bundles (D). (40x H. &E.)

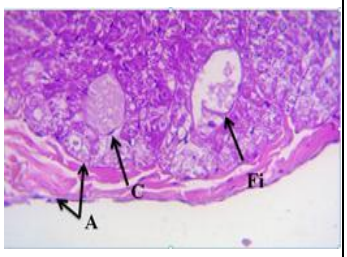


Figure 9 Group III, Heart section of rabbits shows atrophied muscular bundles (A)., congested Blood vessels C, and fibreniod in blood vessel (Fi) (40x H. &E.)

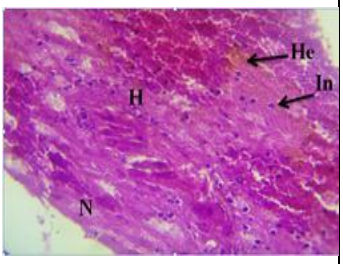


Figure 10 Group III, Heart section of rabbits shows hemorrhage among muscle fibers (H), hemosiderin deposition (He), and necrosis in muscular bundles section (N), and infiltration of inflammatory cells throughout the section (In). (40x H. &E.)

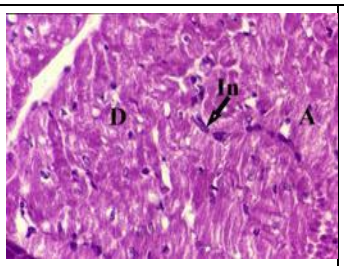


Figure 11 Heart section of group III rabbit shows atrophy of muscle fibers (A), infiltration of inflammatory cells between muscles bundles (In)., degeneration in cytoplasmic in muscle fibers (D)

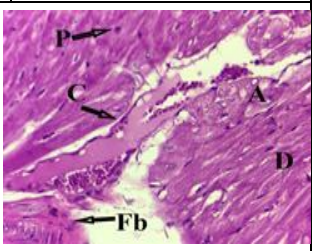
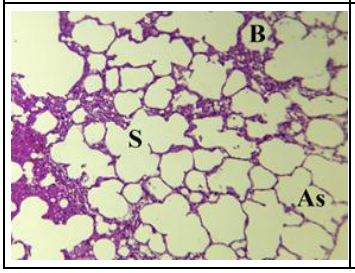
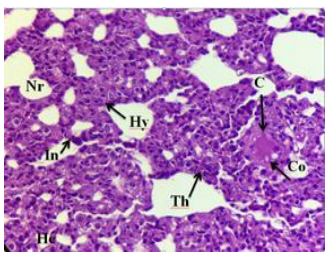
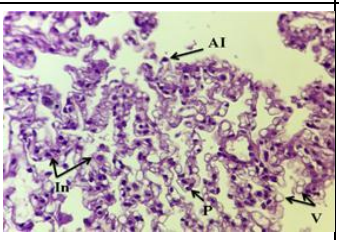


Figure 12 Heart section of group III rabbits shows atrophy of muscle fibers (A). adjacent blood vessel, spread of fibroblasts between blood vessel and muscle fibers (Fb), partial hemolysis of RBCs within congested blood vessels (C), degeneration in cytoplasmic muscle fibers (D), and pyknosis of some muscle cells nucleus (P)







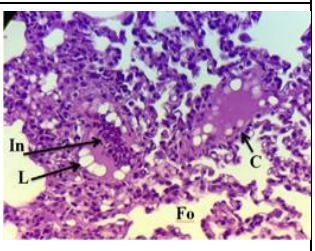


Figure 13 Control group: Lung section of rabbit shows segmental air sacs (S), and (alveolus) As	**Figure 14** Group I: Lung section of rabbit shows extensive thickness of the interstitial connective tissue, particularly collagen bundle. There is also hyperplasia of the connective tissue cells (Hy) , and in between , there is leaked hemolyzed blood associated with WBCs (He). The alveoli contain small, arrow-shaped cavities (Nr)	**Figure 15** Group I: Lung section of rabbit shows that the alveolar walls of the most alveoli contain type I alveolar cells (AI) with cytoplasm vacuolation (V). There are also alsopycnotic nuclei (P) and dust cells in the interstitial C.T. (In)	**Figure 16** Lung section of group II of rabbits shows the presence of inflammatory white blood cells (In), folded alveolar walls (Fo), congested hemolyzed blood (C) and associated fat droplets (L)

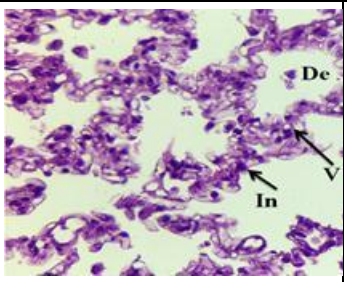
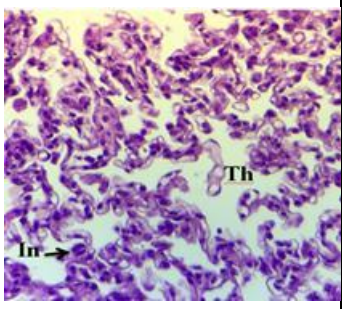
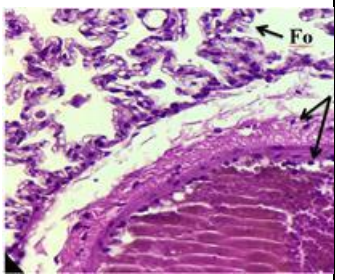
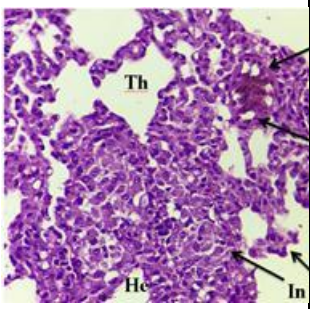

			

Figure 17 Lung section of group II rabbits shows the interstitial connective tissue contains white blood cells and dust cells (In).The alveolar cells exhibited cytoplasm vacuolation (V), and the lumen of alveoli contains certain desquamated cells (De)	**Figure 18** Group II of rabbit lung shows sever Interstitial pneumonia, evident associated with multiple diffusion of WBCs and dust cells. The alveolar walls are thickened,with narrowing of the alveolar cavities (Nr)	**Figure 19** Group III of rabbit lung shows that lung tissue demonstrates folded alveolar walls (Fo), which have infiltration of WBCs (In). The pulmonary vein is engorged with blood masses ©, surrounded by delicate connective tissue associated with inflammatory WBCs around the wall of blood vessel (B.V)	**Figure 20** Group III of rabbit lung shows that the lung tissue contains profuse blood hemorrhage, engorged with white blood cells and dust cells. Blood capillaries between the alveoli contain hemolyzed blood with minute fat droplets

4. Discussion

Diclofenac a member of NSAIDs group act as anti-inflammatory non-steroidal drugs. The current study's results are designed to reveal the effect of different dosages of diclofenac drugs on heart, lung tissues, and serum parameters (Troponin I, CK, LDH, and triglyceride) in albino rabbits in experimental groups. The microscopical examination of myocardium in experimental group I in the present study showed numerous histological alterations in muscle fibers, such as degeneration, zonal degeneration around the nucleus of muscle fibers, atrophy of muscle fibers, and aggregation of inflammatory cells. These findings agreed with Nakhaee et al., who showed tramadol toxicity induced enormous histological alteration in cardiac muscle fibers, including cell morphological changes, inflammatory cell infiltrates, and cellular apoptosis [23]. The fundamental reason for the effect of the NSAIDs (COX 2 inhibitor) on muscles enhanced inflammatory response and muscle atrophy is the inhibition production of Prostaglandin in skeletal muscles [8]. Abdelatty et. al. showed the effect of 10mg/ day doxorubicin on rat cardiac muscle fibers induced cytoplasmic eosinophilia, loss of striation, cytoplasmic vacuolation, and coagulative necrosis were observed along with sarcoplasmolysis, cardio muscle fibers atrophy, and occasional beginning of focal fibroplasia [24] Group II showed histological alteration increased with a given dosage, such as, woven appearance in muscle fibers, indicated as cells atrophy, focal degeneration in muscle cells, other spots showed necrosis in muscle fibers, karyolysis of muscle cells, and pyknosis of some muscle cells nucleus, these alterations reflect the toxic effect of diclofenac on cardiac muscle fibers. These finding agreed with Botelho et. al. revealed drug-induced heart toxicity through the production of reactive oxygen species (ROS), progress to modulation of sodium and potassium pump on cytoplasmic membrane that leads to cellular alterations in heart muscles fibers, they showed the effect of digoxin, Ouabain, and oleandrin on rats cardiac muscle which likes focal loss of muscle striation, mild and moderate necrosis, [25]. Indomethacin affects the cellular environment of various organs through increased production of reactive oxygen species (ROS) and decreasing the

endogenous antioxidant pathway like SOD, CAT, and GSH [26]. Diclofenac is an NSAID that blocks prostaglandin production by acting as both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitors which converted prostaniod acid to Prostaglandin D2 in the skeletal muscle fibers [13]. The results of group III in the present study revealed numerous defects in heart muscle fibers, such as atrophy of muscle fibers and necrosis in muscle bundles. These findings were in agreement with several studies that declared that NSAIDs acted to inhibit the production of PGE2 through inhibition of the activity of cyclooxygenase-2, which is involved in muscle inflammation, muscular cell atrophy [8], and macrophage activation as an inflammatory response [27]. At the same time the results of experimental groups I, II, and III in the current study showed lipid droplets within engorged blood vessels with aggregate inflammatory cells within it in blood vessel which nourish myocardium, this finding was in agreement with Rasheed et. al. they pointed the hyperlipidemia in rats may induced many cardiomyocytes alteration, such as atrophied the degenerated muscle fibers, cytoplasmic vacuolation, engorged blood vessels and marked increase in collagen deposition adjacent it. [28]. Hyperlipidemia may, progress the cardiac muscle cells alteration mechanisms due to extensive mitochondrial overload [29]. Researchers recorded the progress of lipotoxicity and oxidative stress in myocardium fiber alteration [30]. Other investigators declared lipid metabolism, which induced numerous cellular changes, such as apoptosis, interstitial cardiomyocyte fibrosis, and muscle fiber hypertrophy [31]. The lung tissue results revealed an iron deposition in the lung as hemosiderin due to decreased RBCs circulating throughout congested pulmonary vessels.Studies pointed out that iron deposition induced ROS production by various stimuli. Therefore, ROS-induced endothelial cell damage and hyperpermeability may lead to iron deposition. [32].

The lung tissue of group I demonstrates vacuolation in alveolar cells type I, the thickness of the interstitial tissue. the outcome of our results

Popper *et al*. noticed that chronic obstructive airway disease is distinguished by a gradual and irreversible reduction in the diameters of the airway lumen due to varying disorders in both bronchioles and interstitial lung tissue [33]. Our results agreed with El_Roghy; they showed the thickened inter alveolar septum is due to the infiltration of inflammatory cells, inflammatory exudates, congestion of pulmonary blood vessels, overproduction of collagen fibers within interstitial connective tissue, and alveolar collapse. [34]

In group II, histological examination revealed folded and thickened alveolar sacs with decreased alveolar sac lumen. Our findings agreed with Steffen et al., revealing congestion of pulmonary blood vessels and aggregation of neutrophils and macrophages as a part of the inflammatory response. Collapsed alveolar sac due to degeneration of type II alveolar cell, which is responsible to surfactant protein [35] Dust cells (macrophages) can assist in lung injury through the secretion of pro-inflammatory cytokines-induced neutrophil neutrophil diapedesis from the blood vessels to injured tissue [36]. The current group II results revealed congested blood vessels with fat droplets. These results agreed with Saadat et al.'s declaration that hyperlipidemia induces an inflammatory response and inflammatory cell infiltration within intestinal tissue between adjacent air sac blood vessels [37]. The COX-2 inhibitor drug affected prostaglandin E2 production and activated myofibroblasts, which induced pulmonary blood vessel fibrosis. [38].

The results of serum troponin, which acts as a heart infraction indicator, the level of troponin in present study showed a significant increase between experimental groups; on the other hand, results in group III showed a significant decrease when compared with the control group. Troponin is considered to be the primary marker of drugs-inducedthe primary marker of drugs-induced cardiac cell misfortune in humans and animals [39]. The level of serum cardiac troponin I increased in patients with type 2 myocardial injury [40]. The doxorubicin drug's effect on cardiac muscle cells is accompanied by cytoplasmic membrane alterations, which lead to leakage of a high level of serum troponin [41]. The increased myocardial protein-mediated serum levels indicate myocardial apoptosis [42].

Digoxin drugs induced to elevate the level of serum troponin level may be due to the myocardial toxicity that increases with a given dosage. There is a strong relationship between cardiomyocyte alteration and the level of troponin in serum. [43]. Our finding, in agreement with Pan et al. 2016 revealed that decreased TroponinI expression would cause a pure total TroponinI level decrease in the heart in mice. [44]. The results of the present study showed a significant elevation of Creatine Kinase in the experimental group when compared with the control group. Our finding in this research agreed with the increasing administration of diclofenac (a COX-2 inhibitor) being related to an elevated Creatine Kinase enzyme level, which represents myocardial necrosis [45].

The drug may affect the heart, induce a significant elevation of CK–MB and cTnT in serum, and gradually elevate these enzymes due to sarcoplasmolysis and cardiomuscle fiber atrophy [24].

These findings in the present study agreed with Okwakpam et al. (2023), who showed the toxic effects of diclofenac on albino rats. These effects were significantly increased in the levels of both Troponin I and LDH, specific markers of heart

damage. These elevations are due to lipid peroxidation, decreased glutathione levels, and increased ROS in the heart muscle fibers [46]. The results obtained from an experimental group of the current study showed a significant increase in serum triglyceride compared with the control group. Several experimental studies showed the effect of NSAIDs on organs, which induced a cellular imbalance mechanism, elevating the lipid profile level [47]. A high level of serum triglycerides in systemic circulation is due to an imbalance between the synthesis of triglycerides and the rate of release [84]. A high level of LDL-C and VLDL-C decreases the level of HDL-C, increasing the risk of cardiovascular disease [26]. Since the diclofenac in the present study showed aggregation of lipid droplets, inflammatory response, and atherosclerosis within blood vessels which nourish the myocardium, these histological findings agreed with (Zhao et. al., 2020, showed a high level of cholesterol and LDL lipoprotein-induced inflammatory cells infiltration to subendothelial layer, the LDL- C aggregation, which caused the transformation of atherosclerotic plaques [46],

5. Conclusion

In conclusion, the long-term administration of diclofenac leads to the impairment of myocardial function, mainly the muscle fibers' function. Troponin I, Creatine Kinase, Lactate Dehydrogenase, and triglyceride levels increase. The histological findings in heart and lung tissue point to local tissue damage. Moreover, the obtained data depict that high-dose intraperitoneal administration affects the cardiovascular system

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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