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Research Article

Histological effects of water-soluble fraction of diesel (WSFD) on liver, gill, and kidney of common carp (*Cyprinus carpio*)

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Abstract

This research aimed to study the histological effects of the acute water-soluble fraction of diesel (WSFD) on the liver, gill, and kidney of common carp (*Cyprinus carpio*). The fish were divided into four experimental groups; control (group1) (G1) without WSFD, and three groups of WSFD with densities of 4% (G2), 8% (G3), and 16% (G4). After 48 hours of exposure, sampling of liver, gill, and kidney was performed from all experimental groups, simultaneously. The results showed that the main alterations observed in the gill included goblet cell increasing, epithelial lifting, complete and incomplete fusion, filamentous edema, blood congestion, aneurysm, and infiltration of inflammatory cells causing the rank of gill tissue lesions in the G4 (3.30) and G3 (2.70) significantly differed from the G1(0.00) (p<0.005). Sever sinusoid dilation, sever blood congestion, hypertrophy a of nucleus, nucleus in lateral position, cytoplasmic vacuolization and pyknotic nucleus were observed in the liver. The rank of liver tissue lesions in the G4 (3.30) and G3 (2.70) significantly differed from the G1(0.00, p<0.005). Tubular disorganization, shrinkage and necrosis of tubule, and melanomacrophage aggregation were observed in the kidney. The rank of kidney tissue lesions in the G4 (3.30) significantly differed compared to the G1 and G2 (0.00) (p<0.005). Total lesion rank of whole aforementioned tissues in G4 (9.70) significantly differed from the G1 (0.00) and G2 (3.70) (p<0.05). The results showed that WSFD causes pathological lesions incidence in the fish liver, gill, and kidney and increase in WSFD level causes more severe tissue damages.

Keywords: Water-soluble fraction of diesel (WSFD), Histopathology, Gill, Liver, Kidney, Common Carp.

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Introduction

Seventy-five percent of the earth is water, and many creatures live in this aquatic environment. Different pollutions such as diesel constantly affect the aquatic environment and its living creatures, so it is essential to pay attention to contaminant factors that can risk aquatic animals (Carvalho *et al*., 2020). Diesel with the chemical formula (C12H24) is a crude oil refining product used as fuel in diesel engines. A large volume of this fuel is consumed daily in the world, therefore at the time of transportation, the entrance of diesel into aquatic environments is possible and causes pollution to aquatic animals (Ismail *et al*., 2009; Ekanem *et al*., 2018). When diesel leaks into an aquatic environment, some of it evaporates due to sunlight, some remains insoluble on the surface, a percentage of it deposits on the bottom of the aquatic environment, and the rest dissolves in water (Simonato *et al*., 2006). There are aromatic hydrocarbon compounds in WSFD, such as BTEX (Toluene, Ethylene, and Xylene) and PAHs (Naphthalene). Also, the soluble diesel phase contains some heavy metals like iron, nickel, and copper (Vanzella *et al*., 2007; Nogueira *et al*., 2011; Moreira *et al*., 2014; Delunardo *et al*., 2020). The PAHs have a great tendency to accumulate in various tissues of the fish body because of their lipophilicity. Gill, the digestive system, and body surfaces (skin) can absorb these materials (Teh *et al*., 2004; Ebonwu and Ugwu, 2016; Freire *et al*., 2020).

On the other hand, the aggregation of these toxic materials (PAHs) causes a loss of physical and chemical balance by disrupting cell membrane lipids and proteins that cause fish tissue damage (Rodrigues *et al*., 2010; Olyaei *et al*., 2014). Also, as a result of PAHs metabolism free radicals are produced that have high toxicity for body tissues (McCallum *et al*., 2017). The polycyclic aromatic hydrocarbons (PAHs) toxicity in the WSFD causes histopathological changes in fish's vital organs such as the liver, kidney, and gill (Akaishi *et al*., 2004; Simonato *et al*., 2008).

The liver is one of the vital organs of the fish body, which has an essential role in food digestion and body metabolism. Also, it is known as the most significant gland in the body. The liver has a detoxification role in the fish body (Haque *et al*., 2017). If it suffers a problem, toxins accumulate, and the general system of the body will get into trouble (Bin-Dohaish, 2012). Because of the high sensitivity in contact and detoxification of the liver contaminants, this tissue organ can be used for histological evaluation to detect contamination of fish with soluble diesel phase (El-ghazaly *et al*., 2006; Biuki *et al*., 2012). WSFD can cause damage to gill tissue and disrupt gas interchange, and fish suffer from hypoxia, eventually (Afifi *et al*., 2014). Gill has other responsibilities such as regulating the osmotic pressure and excreting some waste compounds such PAHs cause gill mechanism disorders (Evans *et al*., 2005; Prakash *et al*., 2019). The kidney has a vital role in the osmotic regulation of water and ions through the excretion of waste products in the fish body.

Therefore, it is one of the first organs to be affected at the time of exposure to oil pollution and its products (Hadi and Alwan, 2012).

Cyprinidae is the most prominent family of freshwater fish. This has been cultured worldwide except in South America, Madagascar, and Australia in terms of natural distribution. There are 386 genus and 3162 species in this Family (Hameed and Al-azami, 2016; Mohammed and Hameed, 2018). Many farmers select different kinds of this family for rearing, and they allocate a significant part of aquatic products, so in European countries, common carp forms 80% of aquaculture products volume (Maktabi *et al*., 2015). Common carp (*Cyprinus carpio*) is one of the most crucial culture species of this family. It is one of the most consequential economic species in the Caspian Sea and Iranian fish farms (Ardakani and Jafari, 2015). WSFD can cause poisoning in fish specifically common carp resulting in various effects on the body of carp (Rodrigues *et al*., 2010; Hamed and Al-azami., 2016; Ekanem *et al.*, 2018; Mohammed and Hameed., 2018; Khatun *et al*., 2021).

Based on aforementioned references, this study aims to investigate the effect of toxic compounds in the solution phase of diesel on the tissues of vital organs of the body such as; the liver, gill, and kidney while acute exposure in common carp is occurring.

Materials and methods

Research design

Two aquariums with a volume of five hundred liters and dimensions of 60x70x120 cm were used to quarantine (adapt) fish and twelve aquariums with two hundred L capacity and dimensions of 40x50x100 cm to expose fish to the soluble phase of diesel to conduct this research. Three six hundred L fiberglass tubs with dimensions of 70x77x125 cm were accommodated to prepare water contaminated with diesel solution phase. Diesel preparation tubs were installed away from the laboratory not to pollute the laboratory air with the gas emitted by mixing water with diesel. Tap water that was kept for Ninety-six hours to remove chlorine was used to supply water for quarantine tanks and mix with diesel. During the research, oxygen was provided to all quarantined and experimental aquariums with an air pump (2hp, Hila-China) to keep the oxygen level at 7 mg/L. The heater was placed in the laboratory to keep the environment temperature at 22^{o}C.

Fish

Ninety-six common carp weighing 50±0.5 g and 16±0.5 cm were bought from the carp aquaculture center located in Sangar, Gilan province. Double-walled plastic boxes filled with oxygen were used to transport the fish to the research laboratory. After the acclimation process in transfer boxes water and quarantine aquarium water, the fish were brought out of the box one by one. They were examined in terms of the existence of clinical signs, including

disease, wounds on the body surface, scaling, presence of erosion in fins and tails, existence of parasites or superficial fungi, and congenital disabilities. Before transferring fish to quarantine tanks, they were bathed in 100-liter aquariums containing 2% sea salt (iodine-free) to ensure eliminating the parasites. Then the fish were divided into two groups and stored in quarantine aquariums to be quarantined (adapted) for two weeks. The supply water was adjusted at 22°C, pH of 6.7, and oxygen of 7mg/L. All the physical and chemical parameters of water were monitored and recorded daily. Light and darkness duration was considered 12:12 h darkness. Feeding was done two times a day in the morning and the evening based on 12% biomass of a commercial feed (carp growing stage EX-CG Beyza 21 Feed Mill Company).

Preparation of diesel solution phase

The diesel was supplied from petroleum products distribution centers. Two hundred liter of trade diesel was prepared in a particular chemical carrying container and was transferred to the laboratory. A volume of 500 cc of diesel was sent to the chemical assay test for diesel compounds assessment that was used in this research (Table 1).

The presence of aromatic hydrocarbons was then tested, and the number of different types of aromatic compounds in the diesel was adjusted flowing the measurement of Rodriguez *et al*. (2010) (Table 2).

Table 1: Percentage of hydrocarbons compounds in diesel (%).

Group	Level
Alkanes	40.0556
Cycloalkanes	14.8795
Bicycloalkanes	7.6154
Alkylbenzene	16.1719
Indene and tetra lines	9.1537
Naphthalene's	8.6773
Tricycloalkane	1.5647
Diaromatic	1.2240
phenanthrene	0.6577

Table 2: Percentage of the presence of aromatic hydrocarbons in diesel.

Hydrocarbons	Level
Benzene	289.06
Toluene	754.28
Ethylbenzene	289.92
Xylene	1771.82
Total BTEX	2815.16
Naphthalene	31.33
Acenaphthelene	nd
Fluorene	3.46
Phenanthrene	3.89
Anthracene	nd
Fluoranthene	nd
Pyrene	nd
Benz[a]anthracene	nd
Chrysene	nd
Benzo[b]fluoranthene	nd
Benzo[k]fluoranthene	nd
Hydrocarbons	nd
Benzo[a]pyrene	nd
Indeno[1.2.3-C.D]pyrene	nd
Dibenzo[a,h]anthracene	nd
Benzo[ghi]perylene	nd
Total PAHs	38.61
ΣHPAs and BTEX	2853.77

The method of Anderson *et al*. (1974) was used to prepare the soluble diesel phase. The diesel was mixed with water to obtain proportionally selected density and placed in a dark place for 23 h. Then it was gently mixed with an electric mixer during this time. The mixture of diesel and water was then allowed to

remain for 1 h. The mix of diesel and water was then exposed to direct sunlight for 5 h and was mixed with an electric mixer. Finally, the water and diesel mixture was allowed to remain for 1 h at which the soluble phase was separated from the insoluble one. The whole solution phase was carefully separated from the bottom of the mixture container by siphoning and transferred to the specific aquariums. The leftover insoluble diesel phase was returned to the particular chemical containers and sent to the relevant centers to eliminate environmental pollution.

Exposure to the diesel solution-phase test

To study acute toxicity of soluble diesel phase considering the amount of LC_{50} of WSFD being 640±30 mg/L (Rodriguez *et al*., 2010; Hedayati *et al*., 2017). The fish were divided into four groups in triplicates: one control (group1, G1) excluded WSFD and three groups included WSFD with densities 4% (G2), 8% (G3), and 16% (G4) Every individual replicate with 8 fish was transferred to each 200-liter aquarium filled with WSFD according to the selected density and were the same as quarantine tank in physical and chemical conditions. Samples were exposed to WSFD for 48 hours to create an acute phase. Lightening and darkness duration were considered 12 and 12 h. Feeding was done two times a day in the morning and evening like quarantine time (adaptation).

Histology

After 48 hours of acute exposure to WSFD, six samples were selected randomly from each replication, and sampling of liver, gill, and kidney was performed. The samples were fixed in 50cc sterile falcons containing formalin 10% (Merck formalin, Germany). Formalin was renewed after 6 h to complete the stabilization process. Samples were then packed in suitable boxes next to the ice pack and were sent to the laboratory. Samples were eventually removed from formalin and 1 cm^2 of each sample was cut and given a unique code, then placed in tissue tags. In the next step, a routine histology procedure was done and sections of 5µ were prepared using a rotary microtome. Then the slides were stained with the hematoxylin-eosin staining technique (Herrera *et al*., 2009; Rheubert *et al*., 2017). The stained slides were then examined under a light microscope (Olympus BH-2), and their pictures were recorded with a digital camera. Lesions observed in tissues were evaluated and graded according to the designed tables, so the score of zero to a tissue indicates no tissue lesion, and the score of five was considered for the most severe tissue damage. More description of each score for grading pathological changes was given in Table 3.

Table 3: Histopathological grading for gill, liver and kidney tissue examinations

	0	1	2	3	4	5
Gill	No lesion	Aneurysm (very low) Hyperplasia and hypertrophy of the gill epithelium Epithelial lifting of lamellae (very low) Goblet cell increasing(low) Incomplete fusion(Low-Moderate) Complete fusion (very low) Filamentous edema (very low) Edema (very low)	Aneurysm(low) Hyperplasia and hypertrophy of the gill epithelium Epithelial lifting of lamellae(low) Goblet cell increasing(Low-Moderate) Incomplete fusion(Moderate) Complete fusion(low) Filamentous edema(low) Edema(low) WBC infiltration as Acute (very low) Disorganization & detachment (very low) Clubbing(low)	Aneurysm(Low-Moderate) Hyperplasia and hypertrophy of the gill epithelium Epithelial lifting of lamellae(Moderate) Goblet cell increasing(Moderate) Incomplete fusion(Moderate-High) Complete fusion(Low-Moderate) Filamentous edema(Low-Moderate) Edema(Low-Moderate) WBC infiltration as Acute(low) Disorganization & detachment(low) Blood congestion(low) Clubbing(Moderate)	Aneurysm(Moderate) Hyperplasia and hypertrophy of the gill epithelium Epithelial lifting of lamellae(Moderate-High) Goblet cell increasing(Moderate-High) Incomplete fusion(High) Complete fusion(Moderate) Filamentous edema(Moderate) Edema(Moderate) WBC infiltration as Acute(Low-Moderate) Disorganization & detachment(Low-Moderate) Blood congestion(Moderate) Tissue necrosis(low) Clubbing(High)	Aneurysm(High) Hyperplasia and Hypertrophy of the gill epithelium Epithelial lifting of lamellae(High) Goblet cell increasing(High) Incomplete fusion (very High) Gomplete fusion(High) Filamentous edema(High) Edema(High) WBC infiltration as Acute(Moderate) Disorganization & detachment(Moderate) Blood congestion(High) Hemorrhage(High) Tissue necrosis(Moderate) Clubbing(very High)
Liver	No lesion	Hypertrophy of nucleus and cytoplasm (very low) The nucleus in lateral position(low)	Hypertrophy of nucleus and cytoplasm(low) The nucleus in lateral position(Low-Moderate) Blood congestion(low) Sinusoid dilation(low) Pyknotic nucleus (very low) Hypertrophy of nucleus(low)	Hypertrophy of nucleus and cytoplasm Low-Moderate) Blood congestion(Low-Moderate) Sinusoid dilation(Moderate) Piknotic nucleus(Low-Moderate) Hypertrophy of nucleus(Low-Moderate) Cytoplasmic vacuolation (low) Macrophage cells increase(Moderate) Melanoma macrophage aggregation(low)	Hypertrophy of nucleus and cytoplasm(Moderate) Blood congestion(Moderate) Sinusoid dilation(Moderate-High) Pyknotic nucleus(Moderate) Hypertrophy of nucleus(Moderate) Cytoplasmic vacuolation (Moderate) Macrophage cells increase(High) Melanoma macrophage(Low-Moderate) Hepatocyte necrosis(low)	Hypertrophy of nucleus and cytoplasm(High) Blood congestion(High) Sinusoid dilation(High) Hypertrophy of nucleus(Moderate-High) Cytoplasmic vacuolation (High) Macrophage cells increase (very High) Melanoma macrophage(Moderate) Hepatocyte necrosis(Low-Moderate) Tissue and vascular rupture with bleeding(Low-Moderate)
Kidney	No lesion	Glomerular hypertrophy (very low) Tubular cell cytoplasm hypertrophy (very low) Tubular edema (very low)	Glomerular hypertrophy (low) Tubular cell cytoplasm hypertrophy(low) Disorganization tubular and glomerular(low) Increase of bowman's space(low) Tubular edema(low)	Disorganization tubular and glomerular(Moderate) Melanoma macrophage(low) Hyaline droplet(low) Increase of bowman's space(Moderate) Tubular edema(Low-Moderate)	Disorganization tubular and glomerular(Moderate-High) Melanoma macrophage(Moderate) Hyaline droplet(Low-Moderate) Increase of bowman's space(Moderate-High) Blood congestion(low) Tubular and glomerular necrosis(low) Tubular edema(Moderate)	Disorganization tubular and glomerular(High) Melanoma macrophage(High) Hyaline droplet(Moderate) Increase of bowman's space(High) Hemorrhage in Bowman's space and tubular(High) Blood congestion(Moderate) Tubular and glomerular necrosis(Moderate) Tubular edema(High)

Statistical method

To compare the difference between the average rankings of tissue damage in exposed groups, Kruskal-Wallis Test was used. Mann-Whitney U test was followed to compare group mean in pairs and two by two using SPSS, 26.

Results

Gill, liver, and kidney tissue structures, exposed to 4%, 8%, and 16% density of soluble diesel phase, differed from the group that was not affected by water pollution. According to the results reported in Table 4 and the results of the

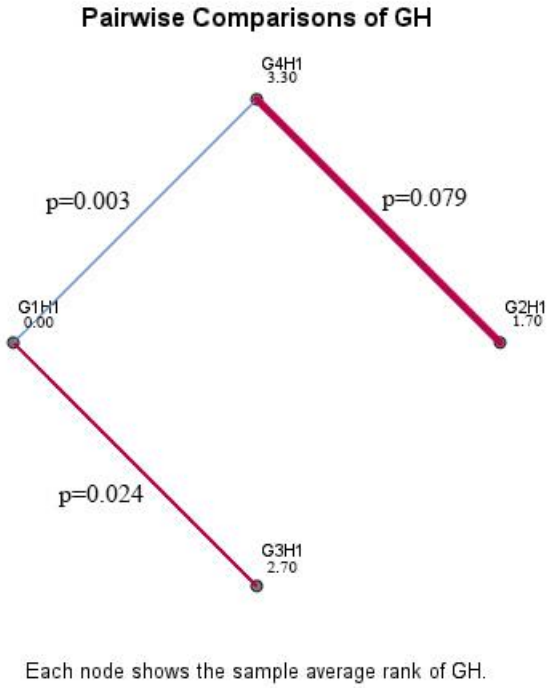
Kruskal-Wallis test, the probability of a difference between the four groups is as follows:

According to Graph 1, the average rank of gill tissue lesions in the G1 (0.00) showed a significant difference from the mean rank of gill lesions in the G3 (2.70) (p=0.024). Also, the average rank of gill lesions in the G1 (0.00) had a significant difference from the mean rank of gill lesions in the G4 (3.30) (p=0.003). The average rank of gill lesions in the exposed groups did not demonstrate a difference (p>0.05).

Table 4: Pathologic statistical assessment results.

Index	*P*-value
Gill	*0.021
Kidney	*0.012
Liver	*0.035
Total	*0.014

*is a significant difference at 0.05 level among groups.

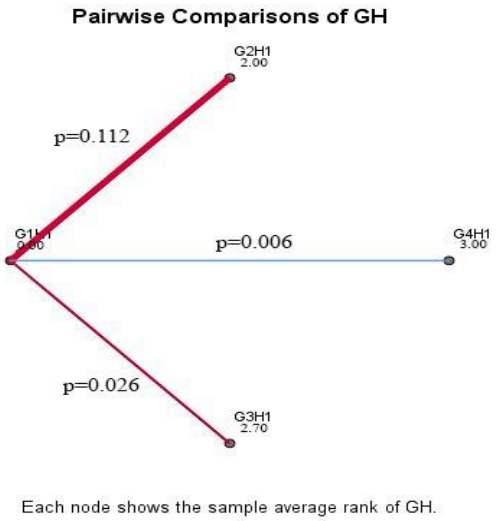


G1=control G2=%4 diesel G3=%8 diesel G4=%16 diesel H1=48 hours
Graph 1: Quantitative comparison of lesions formed in gill tissue in different groups.

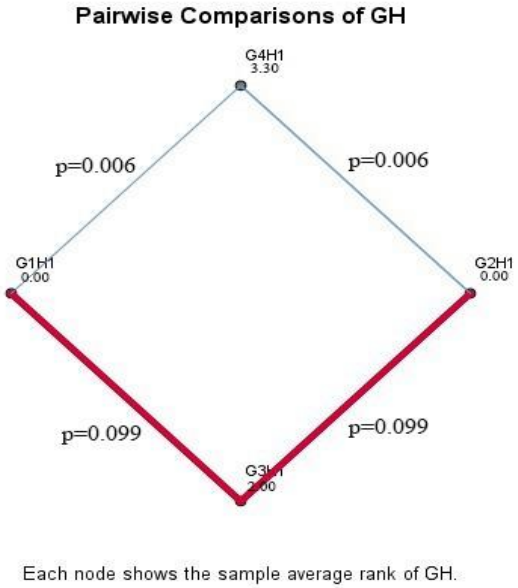
According to Graph 2, the average rank of liver tissue lesions in the G1 (0.00) showed a significant difference from the mean rank of liver lesions in the G3 (2.70) (p=0.026). Also, the average rank of liver lesions in the G1 (0.00) had a significant difference from the mean rank of liver lesions in the G4 (3.30) (p=0.006). The average rank of liver lesions in the exposed groups did not demonstrate a difference (p>0.05).

According to Graph 3, the average rank of kidney tissue lesions in the G1 (0.00) showed a significant difference from the mean rank of kidney lesions in the G4 (3.30) (P=0.006). Also, the average rank

of kidney lesions in the G2 (0.00) had a significant difference from the mean rank of kidney lesions in the G4 (3.30) (p=0.006).



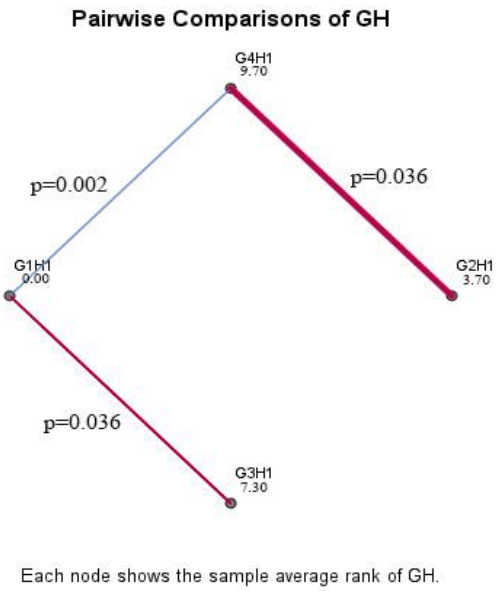
G1=control G2=%4 diesel G3=%8 diesel G4=%16 diesel H1=48 hours
Graph 2: Quantitative comparison of lesions formed in liver tissue in different groups.



G1=control G2=%4 diesel G3=%8 diesel G4=%16 diesel H1=48 hours
Graph 3: Quantitative comparison of lesions formed in kidney tissue in different groups.

According to Graph 4, the average overall ranking of tissue damage in the G1 (0.00) showed a significant difference from the average overall ranking of tissue damage in the G3 (7.30) (p=0.039). Also the average overall ranking of tissue damage in the G1 (0.00) had a significant difference from the

average overall ranking of tissue damage in the G4 (9.70) (p=0.002). The average overall ranking of tissue damage in the G2 (3.70) had a significant difference with the average overall ranking of tissue damage in the G4 (9.70) (p=0.039).



G1=control G2=%4 diesel G3=%8 diesel G4=%16 diesel H1=48 hours
Graph 4: Quantitative comparison of the overall ranking of tissue damage in different groups.

Gill tissue damage evaluation results in Table 5 showed that with the increase of WSFD, the destruction of gill tissue increased. As in the G1, change was not observed in the structure of gill tissue, and the average rank was 0.00. The average rank of gill tissue lesions in the G2 was 1.70, which was increased in comparison to the G1, but it was less in comparison to the G3 with the average rank of 2.70 in tissue lesions and the G4 (3.30) in tissue lesions (Table 5). In the G1, gill lamellas are regular, and the number of mucous cells and epithelial cells status were normal (Fig. 1a). In the G2, irregularity, hyperplasia, epithelial cells hypertrophy, and mucosal cell increase with complete and incomplete fusion in the posterior region of lamella (Fig. 1b). Incidence of tissue lesions was more severe in the G3 compared to the G1 and G2 so that the percentage of mucosal cells and complete fusion was increased compared to the incomplete fusion in secondary gill lamellas, and the expansion of the lesion was extended from the posterior part of gill lamella to the middle part (Fig. 2a). Disruption of gill lamella tissue, mucosal cells proliferation, increased complete fusion of secondary lamella compared to incomplete fusion included lesions expansion to more sections of gill lamellas and also blood congestion along with aneurysm, clubbing of lamella tips, and infiltration of inflammatory cells in G4 happened more intensely compared to other experimental groups (Fig. 2b).

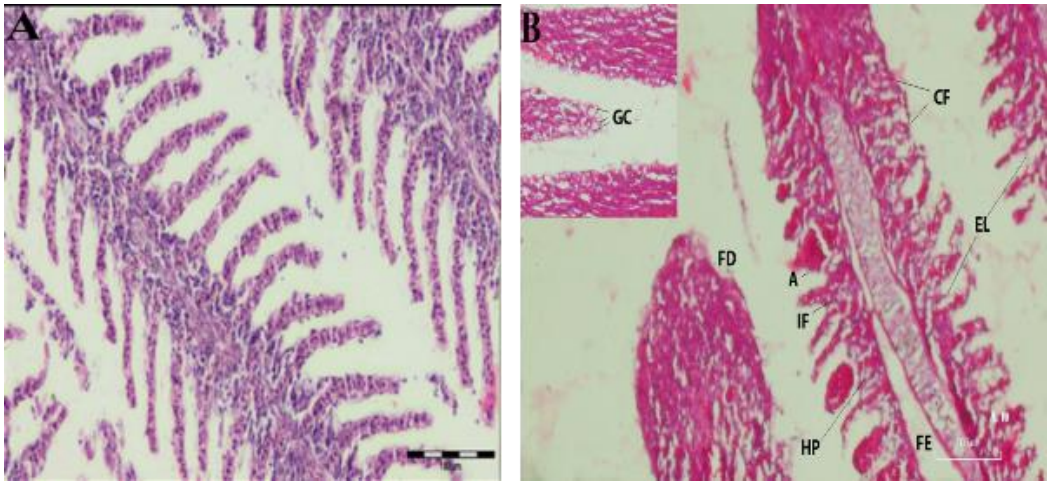


Figure 1. Gill histopathology of common carp. (A) G1: the normal structure of gill tissue (H&E, Bar: 100 µm) (B) Common carp exposed to %4 of WSFD (G2) for 48 h: GC: increased goblet cells (low-moderate), CF: complete fusion (low) in the posterior region, FD: complete fusion in distal lamellae (low), IF: incomplete fusion (low-moderate), A: aneurysm (low), HP: hyperplasia (moderate), EL: epithelial lifting (low), FE: filamentous edema (low), (H&E, Bar: 100).

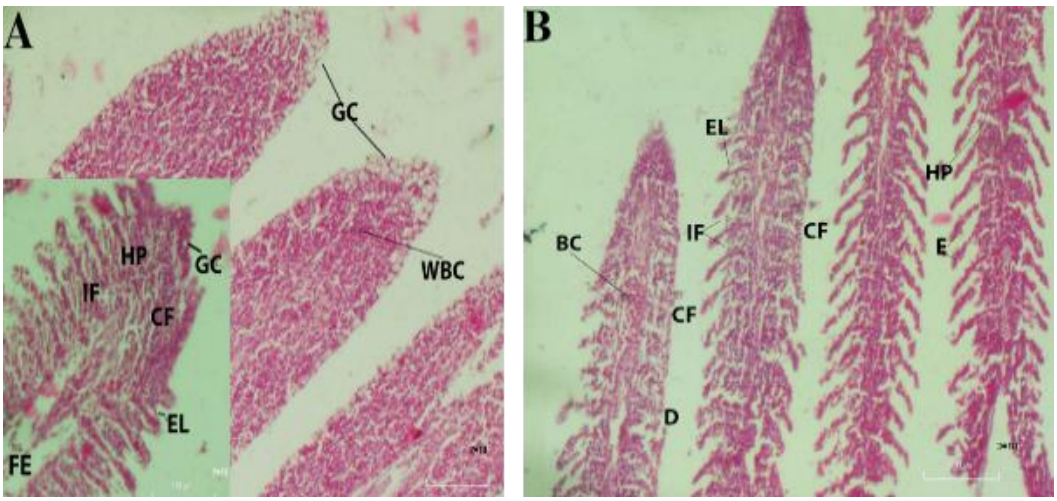


Figure 2. Gill histopathology of common carp. (A) Common carp exposed to %8 of WSFD (G3) for 48 h: GC: increase in goblet cell (moderate), CF: complete fusion in the middle and posterior region (low-moderate), IF: incomplete fusion (moderate), EL: epithelial lifting (low-moderate), HP: hyperplasia (moderate-high), FE: filamentous edema (low-moderate), WBC: infiltration of inflammatory cells (low), (H&E, Bar: 100 µm). (B) Common carp exposed to %16 of WSFD (G4) for 48 h: BC: blood congestion (low), CF: complete fusion (moderate) in the middle and posterior part of the lamella, IF: incomplete fusion (moderate-high), HP: hyperplasia (high), E: edema (moderate), D: disruption of gill tissue structures (moderate), EL: epithelial lifting (moderate), H&E, Bar: 100 .

Liver tissue damage evaluation results in Table 5 showed that with the increase of WSFD, the destruction of liver tissue increases. As in the G1, change was not observed in the structure of liver tissue, and the average rank was 0.00. The average rank of liver tissue lesions in the G2 was 2.00, which was increased compared to the G1, but it was less compared to the G3 with the average rank of 2.70 in tissue lesions (Table 5) and to the G4 with the average rank of

3.00 in tissue lesions. In the G1, lesions were not seen (Fig. 3a). In the G2, these lesions were reported: hypertrophy of nucleus, sinusoid dilation, blood congestion, cytoplasmic vacuolation, and pyknotic nucleus (Fig. 3b) and with increase of G3, the Incidence of lesions was more than G2 and G1 (Fig. 4a). The most severe liver pathological changes was related to G4 comparing to other experimental groups (Fig. 4b). This result indicates that the increase of WSFD causes increase in severity of liver tissue lesions.

Table 5: Histopathologic damage evaluation results in 4%, 8% and 16% groups of WSED after 48 hours (Mean±SD).

Tissue	Gill*	Liver	Kidney	Total
Control Group	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
4%	1.70 ± 0.58^{b}	2.00 ± 1.00^{b}	0.00 ± 0.58^{a}	3.70 ± 1.15^{b}
8%	2.70 ± 0.58^{c}	2.7 ± 0.58^{c}	2.00 ± 0.58^{b}	7.30 ± 0.58^{c}
16%	3.30 ± 0.58^{d}	3.00 ± 1.00^{c}	3.30 ± 0.58^{c}	9.70 ± 0.58^{d}

*Different superscripts show a significant difference (p<0.05) among groups of each column.

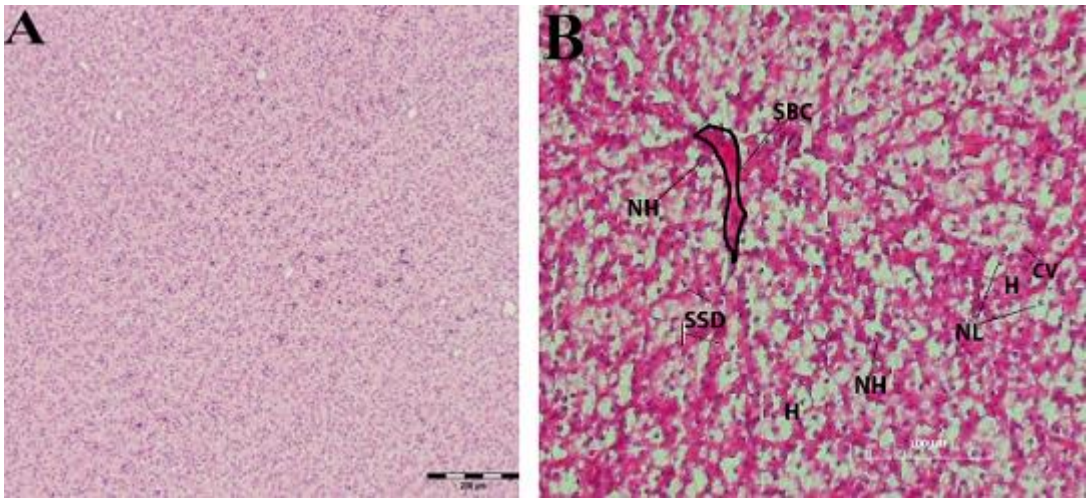


Figure 3: Liver histopathology of common carp (A) G1: the normal structure of liver tissue (H&E, Bar: 200 µm). (B) Common carp exposed to %4 of WSFD (G2) for 48 h: H: cytoplasm hypertrophy of hepatocytes (low), SSD: sinusoids dilation (low), NH: hypertrophy of nucleus (low), SBC: sever blood congestion (low), NL: hepatocytes nucleus lateral position (moderate), CV: cytoplasmic vacuolation (low), (H&E, Bar: 100.

Kidney tissue in G2 did not show any identifiable lesion, and its tissue structure was the same as the of G1 (Fig. 5b). The average rank of created lesions in kidney tissue was 0.00 in G1 and G2 (Table 3). Kidney tissue lesions in the G3 mainly contain melanomacrophage center formation (very low), glomerular hypertrophy (low), tubular necrosis (very low), tubular and glomerular disorganization (low), tubular hypertrophy (low) (Fig. 6a). The average damage rank in this group was 2.00, indicating an increase in the severity of tissue damage compared to G2 and G1. The average rank of tissue lesions in the G4 was reported to be 3.30, which shows the incidence of lesions was significantly higher than the average rank of tissue lesions in the G1 (0.00), G2 (0.0), and G3

(2.00). So that the presence of melanomacrophage centers (low), tubular necrosis (low), tubular and glomerular disorganization (moderate), increase of bowman's space (low-moderate), glomerular hypertrophy (low), indicates further destruction of kidney tissue in the exposure of G4 (Fig. 6b).

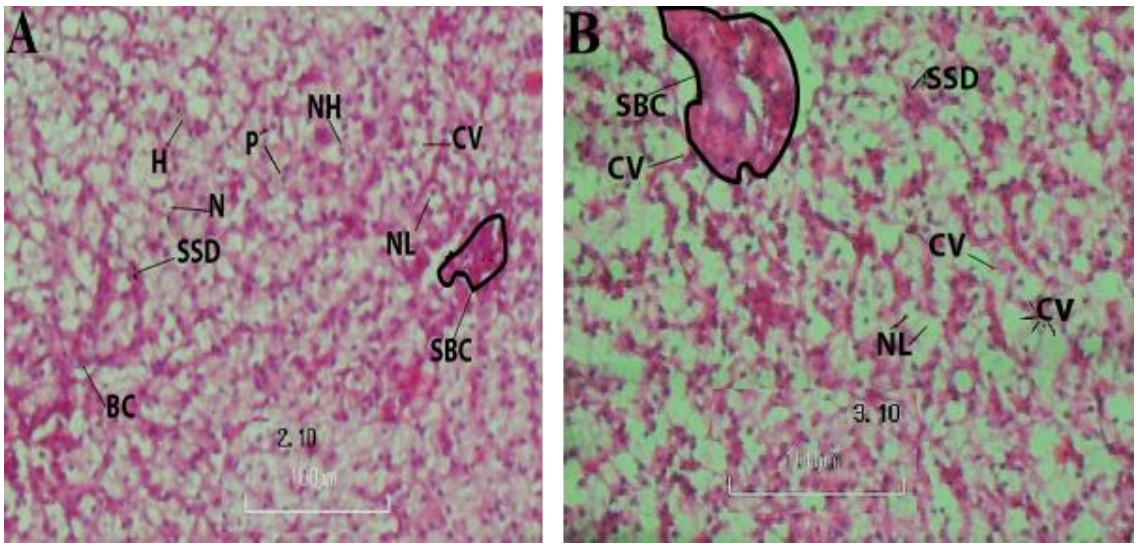


Figure 4: Liver histopathology of common carp (A) Common carp exposed to %8 of WSFD (G3) for 48 h: P: pyknotic nucleus (very low), SSD: sinusoids dilation (low-moderate), SBC: sever blood congestion (moderate), N: necrosis (very low), NH: hypertrophy of nucleus (low-moderate), BC: blood congestion (low) NL: hepatocytes nucleus lateral position (moderate), H: cytoplasm hypertrophy of hepatocytes (low-moderate), CV: cytoplasmic vacuolation (low-moderate), (H&E, Bar: 100 µm). (B) Common carp exposed to %16 of WSFD (G4) for 48 h: SSD: sinusoids dilation (moderate), SBC: sever blood congestion (moderate-high), NL: hepatocytes nucleus lateral position (moderate), CV: cytoplasmic vacuolation (moderate), H&E, Bar: 100.

Based on the results of Table 5, the mean grade of tissue lesions were increased within WSED-increase dependent manner. No significant difference was observed between G3 and G4 for liver lesions. Kidney lesions showed that 4% WSED could not be effective on kidneys to damage it after 48h.

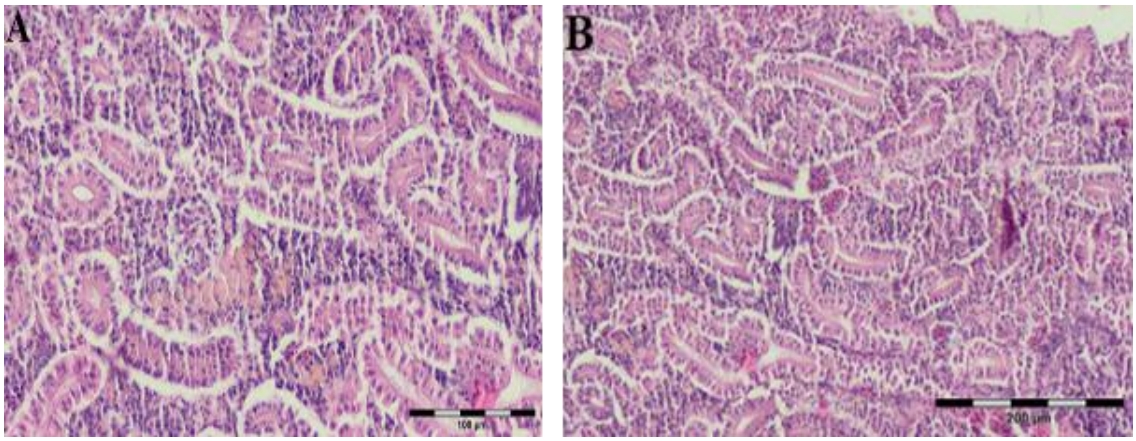


Figure 5: Kidney histopathology of common carp. (A-B) G1 and Common carp exposed to %4 of WSFD (G2) for 48 h tissue damage were not observed, (H&E, Bar: 100 µm and Bar: 200 µm).

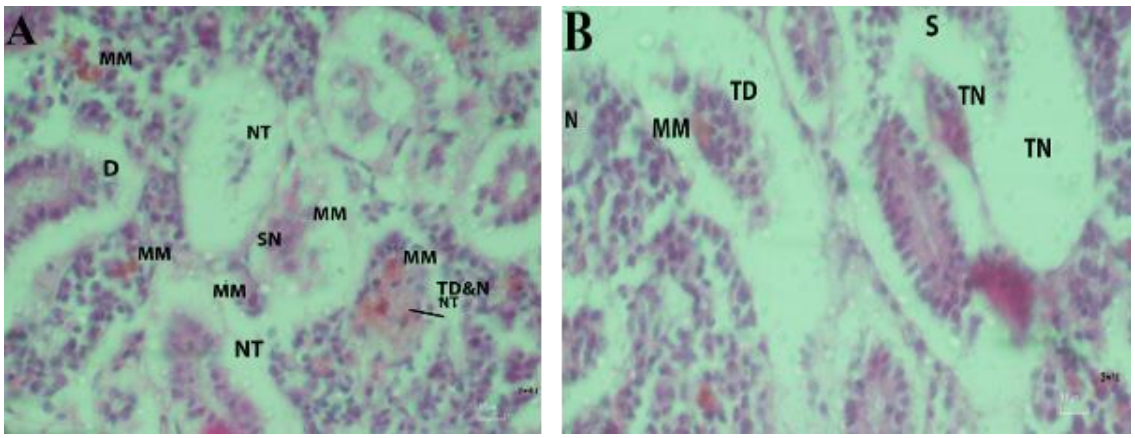


Figure 6: Kidney histopathology of common carp. (A) Common carp exposed to %8 of WSFD (G3) for 48 h: MM: melanoma macrophage centers (very low), N: necrosis (very low), SN: Shrinkage and necrosis of tubule (very low), NT: tubular necrosis (very low), D: disorganization (low), TD: tubular structures disorganization (low), (H&E, Bar: 10 µm). (B) Common carp exposed to %16 of WSFD (G4) for 48 h: MM: melanoma macrophage centers (low), NT: tubular necrosis (low), N: necrosis, S: Shrinkage of tubule, TD: tubular structures disorganization (low-moderate), (H&E, Bar: 10).

Discussion

Oil and its products have been introduced as one of the most common harmful factors to the environment and aquatic animals. Compounds like aromatic hydrocarbons and heavy metals (iron, copper, and nickel), the main components of oil and its products, have high toxicity for aquatic animals. Diesel is a derivative of oil that is consumed in large quantities daily in the world. Therefore, the contamination of aquatic environments while transferring this substance is possible (Anderson *et al*., 1974; Pacheco and Santos., 2002; Rodrigues *et al*., 2010). In the present study, common carp fish were exposed to the sub-lethal density of a water-soluble fraction of diesel for 48 hours. There was a significant difference between exposed and unexposed groups to WSFD after tissue damage classification and statistical analysis of data (p<0.05). Also, a significant difference was reported in

the severity of the lesions among the exposed groups (p<0.05).

Gill is one of the vital organs of the fish body that performs many vital activities such as gas exchange (respiration), osmotic pressure adjustment, acid and base balance, and disposal of nitrogenous waste (Dessouki *et al*., 2013). The first part of the gill that was exposed to WSFD was the external region of the gill lamellae. At the time of the WSFD exposure to the gill's outer region, the increase of mucosal cells was reported, which is a kind of defense against pollutants and causes the increase of mucous on the gill space and lamellae. This condition occurs to dilute and neutralize toxins in the gill (Olyaei *et al*., 2014). The lowest amount of mucosal cells in this study was seen in 4% density (p>0.05), and the highest amount of them excited in 16% density (p<0.05). Also, the increase of mucosal cells in flatfish exposed to polluted water with aromatic hydrocarbon compounds due to oil refinery effluent was reported, and subsequently, an accumulation of mucous on gill lamellae's surface and gill's space was reported (Khan, 2003). Hyperplasia and hypertrophy were seen in 4%, 8%, and 16% densities exposed groups (p<0.05). Sharifpour *et al*. (2011) announced that the cause of hyperplasia on the epithelial cells' surface was the cells' inability to separate due to increased mitotic division that was because of the gill exposure to petroleum hydrocarbons and heavy metals. Edema and epithelial lifting in secondary lamellae and edema in first lamellae were other tissue lesions in the WSFD exposed groups. As the contaminants density increased, the incidence of edema and epithelial lifting in the secondary lamellae and edema in the first lamellae increased compared to the control group which was 4%<8%<16%. In the study by Ismail *et al*. (2009) on tilapia, it was observed that soluble diesel phase density increase in water from 10 ppm to 500 ppm caused increasing the severity of edema and detachment of epithelial tissue. The results of their study were consistent with the results of the present study. Research observations showed that the increase of soluble diesel density in the water increased the incidence of fusion between secondary lamellas, and the highest fusion was reported in the 8% and 16% groups compared to the control group (p<0.05). As the soluble diesel phase density increased from 4% to 16%, the fusion rate progressed from incomplete fusion to complete fusion, and also, the conflict area extended from the external surface of lamellae to the middle surface. Similarly, gill histopathological lesions increased from day 8 in yellowfin sea bream (Kazempour *et al*., 2020). In a study that was performed on the effect of different densities of soluble diesel phase on *Huso huso* gill, it was found that the 40 ppm soluble diesel phase density caused more fusion in the lamellas than the 0, 5, 10, and 20 ppm (Jahanbakhshi and Hedayati, 2013). Aneurysm and clubbing of the end of the secondary lamellas, and blood congestion in the capillaries were indicative of the high density of WSFD so these lesions were not significantly different from the control group to the

4% density (p>0.05). Nevertheless, a significant difference was reported in 8% and 16% densities compared to the control group (p<0.05). Aneurysm in the gill of milkfish exposed to water polluted with diesel was one of the lesions reported by Hesni *et al*. (2011) similar to this study.

The liver is another vital organ that plays a significant role in fish metabolism. Also, it plays a role as one of the essential glands in the fish body (Authman *et al*., 2013). It plays a vital role in detoxification, and a large volume of blood is entered into this organ daily. As a result, in the case of water pollution, many toxins are entered the fish body and are absorbed by the liver through the blood, causing structural and functional changes in this organ (Barja-Fernandes *et al*., 2013). Sinusoid edema, hepatocytes nucleus and cytoplasm hypertrophy, and blood congestion were tissue damages observed in the larvae of *Odontesthes argentinensis* liver, which were affected by water polluted with soluble diesel phase. Also, with the increase of the WSFD, the occurrence of pathological changes became more severe, so most liver tissue damages were reported at more than 16% densities (Rodrigues *et al*., 2010). The results of the study by Rodrigues *et al*. (2010) were similar to the results of the present study so that hepatocytes nucleus and cytoplasm hypertrophy, sinusoid edema, and blood congestion were the significant pathological changes in liver tissue in the WSFD exposed groups which caused a considerable difference between the exposed groups compared to the control group (p<0.05). Also, the 8% and the 16% groups showed the highest severity of tissue damage compared to the control group (p<0.05). A study was performed on the effect of feeding common carp with food contaminated by heavy oil. It was found that the accumulation of aromatic hydrocarbons in hepatocytes led to the enzyme CPY1 secretion, which caused the production of a large number of secondary metabolites such as free radicals that led to nucleus formation with irregular shapes, hepatocytes nucleus in a lateral position, and hepatocytes cytoplasm vacuolation (Pal *et al*., 2011). These findings were similar to this research results and showed the effect of diesel compounds on pathological changes in liver tissue. Also, in the present study, there was a significant difference between the non-exposed and exposed groups in the liver tissue damage (p<0.05). Increased activity of hepatocytes for detoxification caused the production of a large amount of ATP, which upset the balance between energy consumption and production. Also, it caused the loss of glycogen and fat stores, which was the factor of hepatocytes nucleus and cytoplasm vacuolation (Olyaei *et al*., 2014). In the 4% and 8% of soluble diesel phase densities, most of the lesions included hepatocytes hypertrophy, cytoplasmic vacuolation, and nucleus position change, but with the increase of soluble diesel phase density in water, the intensity of hepatocyte activities to eliminate the cause of poisoning increased. As a result, more severe tissue damages occur due to loss of energy

stores so that in 16% density tissue lesions included cell degeneration and necrosis. Hague *et al*. (2017) studied the effect of aromatic hydrocarbons on liver tissue, sinusoid edema, endothelial cell destruction, nucleus, and cytoplasm hypertrophy, hepatocyte cells vacuolation, and early sign of necrosis were observed, which were the most apparent liver pathological lesions in the exposure of polluted water by the aromatic hydrocarbon compounds. The results of this study were matched with the results of the Hague *et al*. (2017) study that increasing soluble crude oil phase density in water increased the incidence of pathological lesions up to 33%. Also, in another research, melano macrophage centers formation and increased necrosis areas on the liver tissue surface were reported in the group exposed to 50% soluble oil phase density (Akaishi *et al*., 2004) that these results showed that increasing the soluble diesel phase density, the severity of tissue lesions increased.

The kidney in fish is divided into anterior and posterior parts, and each part plays a vital role in fish life. The frontal part hematopoietic area is responsible for producing blood cells and phagocytes. The posterior part that is consisted of glomeruli and urethra is responsible for the disposal of the waste and maintenance of the chemical balance (Basir and Peyghan *et al*., 2020). The kidneys are sensitive to various pollutants, especially diesel. Time of exposure to oil pollution and its products is one of the first organs to be affected (Hadi and Alwen, 2012). The study was performed by Ebonwu and Ugwu (2016) on tilapia larvae exposed to the density of 0, 0.25%, 0.55%, 0.85%, and 1.25% mg/l of the soluble crude oil phase. The kidney tissue damages included tubular edema, epithelial cells vacuolation, hyaline droplets, and glomerular and tubular disorganization. They also reported more severe tissue damage with increasing pollution density in water (Ebonvou and Ugwu, 2016). In the present study, after 48 hours, common carp exposed to 4%, 8%, and 16% WSFD, significant pathological changes included melanomacrophage aggregation, tubular necrosis, glomerular and tubular disorganization, increase of bowman's space, and glomerular edema. The severity of the lesions increased with increasing the water-soluble fraction of diesel density so that there was a significant difference between the 16% group with the control and 4% groups (p<0.05). The results of Olyaei *et al*. (2014) research showed that tissue lesions in the kidney increased with the increase of water-soluble hydrocarbon compounds. In this study, common carp were exposed to the density of 10, 50, 100 micrograms per liter of pyrene for 35 days. It was observed that tissue lesions in the ten micrograms per liter of pyrene group were macrophages containing hemosiderin dispersion, renal tubular degeneration, kidney interstitial area hyperemia, and the presence of urinary casts in some parts. However, in 100 micrograms per liter of pyrene group, more severe pathological damages were reported, including renal tubules degeneration, increase of bowman's

space, melanoma macrophages containing hemosiderin, interstitial tissue necrosis, and focal necrosis. These results were equivalent to the present study results that the increase of water-soluble fraction of diesel density caused the increase in severity of tissue lesions in the 16% group compared to control and 4% groups (p<0.05).

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