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Effects of acute crude oil exposure on basic physiological functions of Persian sturgeon, *Acipenser persicus*

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ABSTRACT

Hematological parameters are suitable biomarkers for evaluating the potential risk of the chemicals. The aim of this study was to investigate of acute crude oil exposure on basic physiological functions of Persian sturgeon, *Acipenser persicus*. 210 juvenile Persian sturgeon (9.4 ± 1g) were supplied by the Rajaei fish farm in Mazandaran Province, Iran. Juveniles were exposed to the crude oil (15, 16, 17, 18 and 19 ppm). The 96h-LC_{50} were detected under static condition by SPSS software. Hematological and biochemical parameters were compared between control group and treatment exposed to 96h-LC_{50}. The median lethal concentration was 16.5 ppm in 96 h toxicity test. WBC, RBC, Hb and MCHC decreased, while MCV, MCH and PCV were significantly higher in the treatment which exposed to LC_{50} concentration ($P<0.01$). Results of differential leukocyte count showed that after treatment with LC_{50} concentration, neutrophils and monocytes increased, while lymphocytes and eosinophils decreased ($P<0.01$). Biochemical parameters showed an increase in serum glucose (p<001). Other parameters including total protein, ALT, AST, ALP and LDH enzymes decreased in treatment group significantly ($p<0.01$). Our results provides evidences that crude oil may have disruptive action on the erythropoietic tissue which may be due to its influence on the viability of the cells. Crude oil also inhibited all of the enzymes activities leading to hyperglycemia due to stress inoculation.

Key words: Acute exposure, Biochemical parameters, Crude oil, Hematology, Persian Sturgeon.

INTRODUCTION

The Caspian Sea is the biggest land-locked body of water bordered by five countries: Azerbaijan, Iran, the Russian Federation, Kazakhstan and Turkmenistan (Kosarev & Yablonskaya 1994). It has five major inlet rivers but no outlets and acts as a watershed reservoir for the region. The key biological issues in recent years relate to the decline of fisheries and caviar harvesting, the massive mortality among seal populations and the introduction of invasive species in the Caspian Sea. There are several important fisheries in the Caspian Sea, but the greatest effect and hazard has always been placed on the sturgeons. Several anthropogenic factors, including both land-based and offshore pollution, threaten the survival of all fisheries, but especially sturgeon populations in the Caspian Sea (De Mora & Turner 2004). Among these, chemical contamination seems to be one of the most significant factors influencing the population of sturgeons in the Caspian Sea (Ivanov 2000; Pourkazemi 2006). Many potentially toxic contaminants released into the Caspian Sea, are lipophilic and insoluble in water. These properties increase their availability for uptake and accumulation by aquatic organisms. Previous investigations have demonstrated the occurrence of polycyclic aromatic hydrocarbons (PAHs) in fishes, water and sediments of the Caspian Sea (Tolosa *et al.* 2004; Khoshbavar Rostami *et al.* 2012; Eghtesadi Araghi *et al.* 2014; Mashroofeh *et al.* 2015).

European Union and U.S Environmental Protection Agency (US EPA) put these compounds in the priority pollutant list because of their mutagenic, carcinogenic, tetratogenic and toxic properties, environmental persistence, bioaccumulation and trophic transfer of PAHs in aquatic ecosystem (ATSDR, 1995; European Commission, 2011), and for this reason, the increase in levels of PAHs contaminations that has occurred in recent decades in the Caspian Sea, a landlocked system where the PAHs cannot be flushed out, is a cause for concern (Mashroofeh *et al.* 2015).

Furthermore, the life history of sturgeons may leave them particularly sensitive to effects of these pollutants. As an opportunistic bottom feeders, these fish frequently be in contact with sediments that may contain sediment adsorbed hydrophobic pollutants (Billard & Lecointre 2001; Kajiwara *et al*. 2003). Also, sturgeons are particularly long lived animals (up to 100 years in the wild) that take 5-30 years to reach sexual maturity (Mashroofeh *et al.* 2013; Billard & Lecointre 2001). These characteristics put the sturgeons at a high potential risk for accumulating persistent organic and inorganic contaminants in their tissues.

Previous studies reported induction of hepatic lesions, physiological and biochemical disorders in these fish (Ribeiro *et al.* 2005); and high levels of tumors or abnormalities in gonad development and gametogenesis and disturbances in the morphogenesis of organs have been noticed in the Caspian sturgeons since the late 1980s (Kajiwara *et al.* 2003). Acute toxicity data can help to determine the mode of toxic mechanism of a substance and may provide information on doses associated with target organ toxicity and lethality that can be used in setting dose levels for repeated dose studies.

This information may also be extrapolated for diagnosis and treatment of toxic reactions in humans.

The results of acute toxicity tests can provide information for comparison of toxicity and dose-response among numbers of chemical classes and application in selection of candidate material for future work (Hedayati *et al.* 2010).

However the environmental pollutions, such as spills of oil distillate products into the coastal waters are growing in the recent years in Caspian Sea, There are only few papers concerning the effects of crude oil exposure on physiological indices in sturgeons and there is a real need for information about the effects of this fuel oil on these fish species. Fish hematological indices are very sensitive to water contaminants, and its alternation in the hematological and immunological parameters can be used as toxicity indices of xenobiotics (Sancho *et al.* 2000). The aim of the present study was to investigate hematological and biochemical indices of the Persian sturgeon, *Acipenser persicus*, exposed to crude oil, as potential biomarkers, in order to assess pollution through these petroleum products and getting information on the threat imposed by these spills to this valuable fish species.

MATERIAL AND METHODS

The experiment was conducted on the juvenile specimens of Persian sturgeon (N = 210) with average weight of 9.4 ± 1g, supplied by Rajai fish farm in Mazandaran Province, Iran. Prior to the toxicity tests, fish were acclimated to laboratory conditions for at least two weeks in 160-L fiberglass tanks.

During acclimatization, fish were fed with commercial trout pellet (protein 36%, lipid 14%, ash 11%, fiber 3.5%, phosphorous 1%, wet 11%, carbohydrate 22.5%) and fish meal 50% twice a day. During the tests period, water was continuously monitored for temperature, dissolved oxygen, pH, and conductivity (Hedayati *et al.* 2010). Average temperature, dissolved oxygen, pH and total hardness were measured 22 ± 1°C, 8.2 ± 0.8 $mg.L^{-1}$, 7.5 ± 0.1 & 145 ± 5 $mg.L^{-1}$ respectively. Other water quality parameters were ammonia < 0.02 $mg.L^{-1}$, nitrite < 0.1 $mg.L^{-1}$, nitrate < 0.503 $mg.L^{-1}$ and phosphate < 0.285 $mg.L^{-1}$. Also fish were maintained under natural photoperiod (L: D=14:10). Range Finding Test values indicated that there is no mortality up to 15 ppm crude oil exposure while 100% mortality in 19 ppm. Then fish were transferred into the treatments with the different crude oil concentrations (15, 16, 17, 18 and 19 ppm) as a static exposure test (test medium was not renewed during the assay and no food was provided for animals), in tanks of 160 L, each containing nine fish. Three replicates were performed for each dose. Acute toxicity tests were done in order to calculate the 96-h LC_{50} for crude diesel oil, based on OCED (1984). Mortality was recorded after 24, 48, 72 and 96 h. Dead fish were immediately removed with special plastic forceps to avoid possible deterioration of the water quality. Value of LC_{50} was calculated from the data obtained in acute toxicity bioassays by SPSS statistical software.

After acute toxicity bioassay, two groups including ten specimens exposed to 96-h LC_{50} and one control group, exposed only to water (the same as that used for acclimation) were selected to determine the effect of acute crude oil exposure on hematological and biochemical indices of the Persian sturgeon.

Immediately after removing the fish from the tank, they were anesthetized with clove powder (200 ppm), and blood samples were taken from the caudal vein by means of heparinized plastic syringes (Hedayati & Safahieh, 2011). Subsequently, fish were killed by struck on head.

Red blood cells (RBCs) and white blood cell (WBCs) were measured immediately on fresh blood by diluting heparinized blood with Merck Giemsa stain at 1:30 dilution and cells were counted using a hemocytometer Neubauer under the light microscope (Banaee *et al.* 2008).

Blood smears were prepared, and leukocytes were categorized into lymphocytes, monocytes, neutrophils and eosinophils (Banaee *et al.* 2008). Hematocrit (Ht) was immediately determined after sampling by placing fresh blood in glass capillary tubes and centrifugation for 5 min at 10,000 rpm in a microhematocrit centrifuge (Hettich, Germany) then measured the packed cell volume (PCV) (Goldenfarb *et al.* 1971). Hemoglobin (Hb) levels were determined colorimetrically by measuring the formation of cyanomethemoglobin according to Lee *et al.* (1998). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were calculated from RBC, PCV and Hb according to Lee *et al.* (1998). Ultrapure water was used for all serum dilutions and standard preparations and duplicate readings were recorded for standards and serum samples. The quantitative determination of serum glucose was carried out using commercially available diagnostic Experimental Protocols kits (Pars Azmoon, Iran), at 546 nm and 37 °C by the glucose oxidase method (Hedayati & Safahieh 2011). Serum total protein level was determined using kits provided by Pars Azmoon Co., Iran, with bovine serum albumin serving as standard by the method of Canli (1996) at 546 nm and 37°C. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined with Pars-Azmoon Diagnostics Infinity AST reagent kit and Sigma Diagnostics Infinity ALT reagent kit, respectively, by enzymatic methods with a Technicon Ra1000 auto-analyzer. Alkaline phosphatase (ALP) was determined by enzymatic method with the automate apparatus auto-analyzer, a Technicon Ra1000, with Darman Kave kit at 37°C and 410 nm. Lactate dehydrogenase (LDH) was determined with Pars-Azmoon Diagnostics Infinity kits with a Technicon Ra1000 auto-analyzer.

For each index, the data were tested for normality and homogeneity of variances by Kolmogorov-Smirnov test. T test was used to determine significant differences to evaluate the effect of crude oil on blood/serum parameters. The differences between means were analyzed at the 5% significance level. Data are reported as means ± standard deviation. The software SPSS, version 17 (SPSS, Richmond, VA, USA) was used as described by Dytham (1999).

RESULTS

All controls resulted in low mortalities fewer than 5%, which indicated the acceptability of the experiments. The mortality of Persian sturgeon due to crude oil was examined during the exposure times at 24, 48, 72 and 96 h in Table 1.

Fish exposed during the period of 24-96h had significantly increased number of dead individual with increasing concentration ($P<0.05$).

There were significant differences in number of dead fish between the duration of 24 and 96h. Considering the crude oil bioassay, LC_5 and LC_{50} of 24, 48, 72 and 96h were 0.0, 15.6, 14.9 and 14.3 ppm and also 0.0, 22.1, 18.2 and 16.5 ppm respectively. Fish exposed to crude oil for 96h showed a significant change in most hematological indices ($P<0.01$).

Results showed that PCV and MCV increased, while Hb, RBC, WBC and MCHC decreased significantly after crude oil exposure.

Only Mean corpuscular hemoglobin (MCH) had no significant difference ($P>0.05$) compared to control group (Fig. 1A-B).

Table 1. Cumulative mortality of Persian sturgeon during acute exposure to crude oil (n = 10, each concentration).

Concentration (ppm)	Mortality (%)			
	24 h	48 h	72 h	96 h
Control	0	0	0	0
15	0	3	13	17
16	0	7	20	33
17	0	10	23	43
18	0	17	33	57
19	0	23	77	100

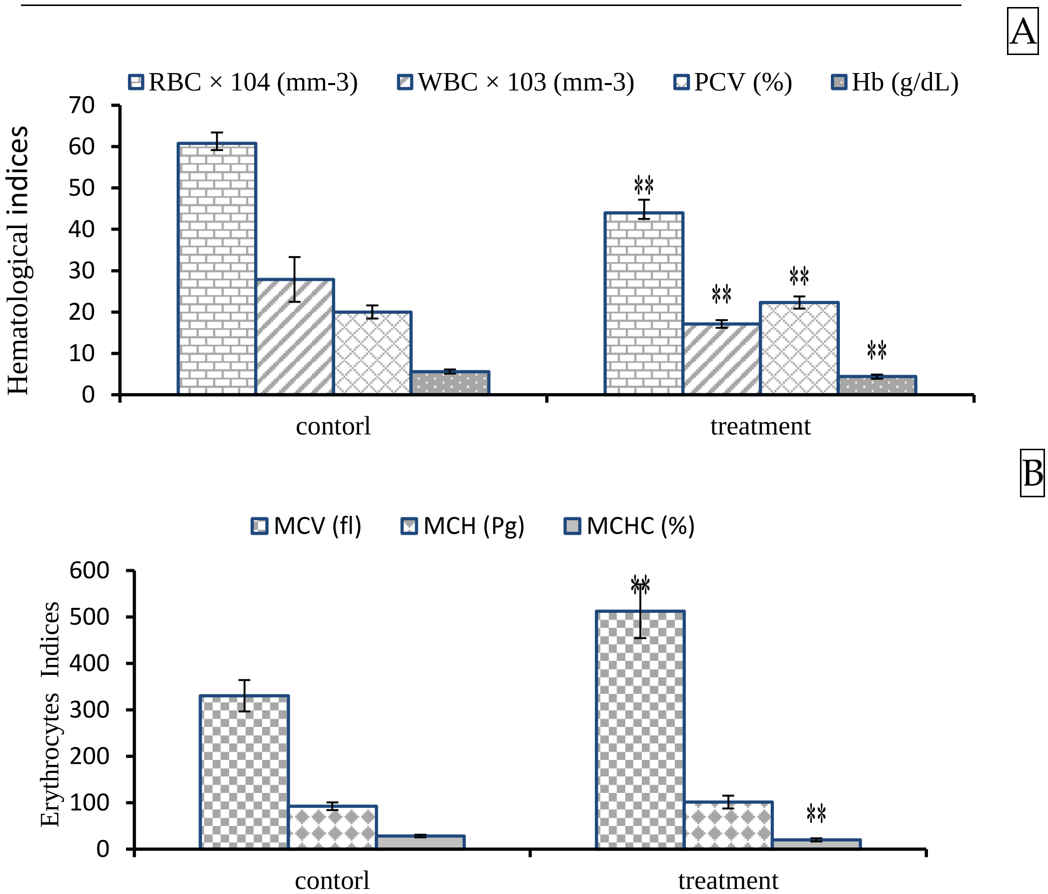

Fig. 1. Hematological indices of Persian sturgeon after acute exposing to crude oil (N = 10, concentration =16.5 ppm, **shows significant differences at α=0.01).

Differential leucocyte counting showed that lymphocytes and eosinophils decreased in group exposed to LC50 (96 h) of crude oil in comparison with control group (P<0.01). In contrast, mean frequency of neutrophils and monocytes were higher in treatment group significantly (P<0.01, Fig. 2).

Some serum biochemical indices showed a significant decline in fish exposed to LC50-(96 h) (P<0.01) including total protein, ALP, ALT, AST, and LDH enzyme activities, whereas serum glucose was significantly higher in control group (P<0.01, Table 2).

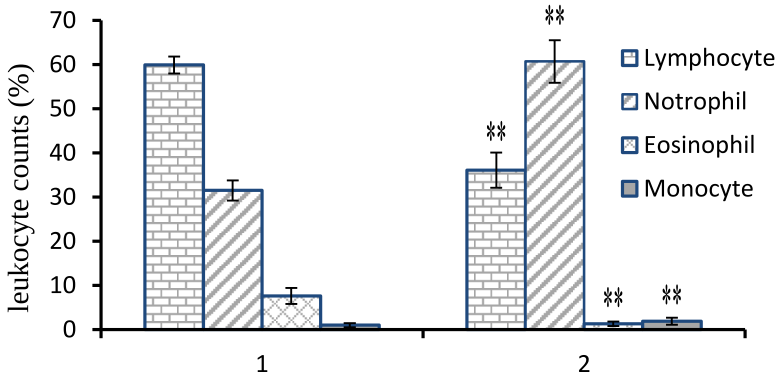

Fig.2. Leukocyte count of Persian sturgeon after acute exposing to crude oil (N = 10, concentration = 16.5 ppm, **shows significant differences at α=0.01.).

Table 2. Biochemical parameters of Persian sturgeon after acute exposing to crude oil (N=10, concentration=16.5 ppm).

Parameter	Control	Treatment	P value
Total protein (g.dL-1)	1.4 ± 0.2	0.9 ± 0.1	0.007 **
Serum glucose (g.dL-1)	41.6 ± 4.8	58.6 ± 6	0.000 **
ALP (IU.L-1)	775.4 ± 95.1	468.2 ± 53.9	0.000 **
ALT(IU.L-1)	3.7 ± 0.8	2.8 ± 0.6	0.003 **
AST (IU.L-1)	29.3 ± 2.3	22.2 ± 2.9	0.000 **
LDH (IU.L-1)	253.7.9 ± 23	159.1 ± 24	0.000 **

Data presented as mean ± standard deviation. **shows significant differences at α=0.01.

DISCUSION

Fish exposed to chemical agents can manifest a stress response that is mainly divided in primary (hormonal responses) and secondary (changes in plasma metabolites levels and hydromineral balance, as well as hamatological changes) response (Wendelaar Bonga 1977; Barton, 2002). In this work, a series of hematological and immunological parameters were examined in Persian sturgeon *A. persicus* after exposure to high concentration of crude oil. Hematological indices showed increase in PCV, MCV and MCH and decrease in RBC, WBC, Hb, and MCHC after acute exposure.

A progressive decrease in RBC, WBC counts and Hb level resulted in subsequent physiological stress. Decreases in the values of Hb and RBC could be attributed to hemolysis resulting in haemodilution, a mechanism for diluting the concentration of the pollutant in the circulatory system (Smith *et al.* 1979). Erythropaenia recorded in the exposed fish may also be caused by swelling of the erythrocytes (Annune & Ahuma 1998), damages to hematopoietic tissues in the kidneys and aggregation of cells at the gills thereby causing a decrease in the number of circulating cells of under stress fish (Fang, 1992). Hedayati & Jahanbakhshi (2013) reported similar results in juvenile *Huso huso* exposed to high concentration of crude oil. Also, a reduction of Hb and RBC in lead intoxicated common carp, *Cyprinus carpio,* was reported by Witeska *et al*. (2010).

Elevation of hematocrit after acute exposure indicates the importance of the route of diesel oil contamination. Results observed are in accordance with those of Chowdhury *et al*. (2004), who noted an increase in blood hematocrit and hemoglobin during environmental anoxia. Acute exposure to pollutants will increase blood oxygen-carrying capacity when impairment of gas exchange occurs (Savari *et al*. 2011). Exposure to various stressors elicits changes in the WBC (Wedemeyer & Yasutake 1977).

Leukopenia and/or leukocytosis are thus a normal reaction to stressors or irritants such as crude oil. Significant leukopenia was reported in *Heteropneutes fossilis* exposed to crude oil (Prasad *et al.* 1987) and *Clarias gariepinus* exposed to the 150 and 300 $mg.L^{-1}$ kerosene (Gabriel *et al*. 2007). Subpopulations of leukocytes changed in dealing with pollutant and other stressors (Ellis, 1977; Musa & Omoregie, 1999). In this study, neutrophils and monocytes increased while lymphocytes decreased after exposure to acute dose (LC_{50} concentration). Neutrophils and monocytes have phagocytic activity which might explain their increased percentage during exposure time. Therefore, the activity of first and second lines of defense against the cellular damage has been found after crude oil exposure.

The monocytes and neutrophils increased and lymphocyte decreased during different stressors in cultured fish *Oreochromis aureus* has been confirmed (Silveira Coffigny *et al.* 2004). Hedayati & Ghaffari (2013) reported similar leukocyte changes in silver carp *Hypophthalmichthys molitrix* exposed to copper sulfate. Lymphopaenia and/or neutrophilia have been observed as a result of sublethal diazinon and deltamethrin exposure in iridescent shark, *Pangasius hypophthalmus* (Hedayati & Tarkhani 2014), *Cyprinus carpio* (Svoboda *et al.* 2001), and *Oncorhynchus mykiss* (Banaee *et al.* 2013) exposed to diazinon, parathion and trichlorfon respectively. Likewise, neutrophilia was reported in *Huso huso* exposed to diazinon (Khoshbavar Rostami *et al.* 2006).

Normally, after exposure to a stressor agent, a significant increase in glycaemia occurs. Increase in glucose during stress supplies demanded energy to cope stress condition (Wendelaar Bonga, 1997).

In the present study, the tested animals showed a hyperglycemic response after 96h exposure to crude oil, indicating the provision of energy reserves for immediate utilization (Val *et al.* 2004).

Similarly, Alkindi *et al.* (1996) also observed significant elevated plasma glucose concentrations after 3h exposure to water soluble fraction of crude oil and an increase of over 50% after 48h in flounder (*Pleuronectes flesus*). However, glucose may not be the most important fuel in energy metabolism in other species (Pacheco & Santos 2001).

Protein metabolism can provide information on the general energy mobilization of an animal and show relationships with effects of contaminants in these organisms (Adams *et al*. 1990). In this study, crude oil caused significant decrease in serum levels of total protein at 96h post treatment.

It is known that the release of catecholamines and cortisol causes a variety of physiological and biochemical alterations, including hyperglycemia, glycogen depletion, and catabolism of plasma proteins, among others. These responses can be considered adaptive processes that help the organism with increased energy demand during exposure to stress factors (Martinez & Colus, 2002).

On the other hand, depletion of total protein content may be due to breakdown of protein into free amino acids under the effect of crude oil exposure (Shakoori *et al*. 1994). Our results are in accordance with Jahanbakhshi &

Hedayati (2013) and Simonato *et al*. (2008). AST, ALT, ALP and LDH are the enzymes that have been applied for evaluating hepatocellular damage (Gad 2007).

Results showed that crude oil inhibit all of the enzymes activities.

Gabriel *et al.* (2012) confirm that metabolic enzymes activities (AST, ALT, ALP and LDH) in gill, muscle, kidney, liver and plasma of *Clarias gariepinus* were inhibited by different concentrations of cypermethrin.

The lower values of AST, ALT and ALP enzyme activities when compared to the controls showed that inactive transamination and oxidative deamination have taken place. Similar results were reported by Adams *et al*. (1996).

CONCLUSION

In the present study, it is concluded that, crude oil has a profound influence on the hematological, biochemical, and enzymological profiles of fish. Our results confirmed, crude oil had a disruptive action on erythropoietic cells and inhibits all enzymatic activities. These parameters could be effectively used as potential biomarkers of crude oil toxicity to the freshwater fish in the field of environmental biomonitoring.

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بررسی سمیت حاد نفت خام بر پاسخ‌های اولیه فیزیولوژیک ماهی قره‌برون *Acipenser persicus*

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چکیده

پارامترهای خون‌شناسی نشانگرهای زیستی مناسبی برای ارزیابی خطرات بالقوه مواد شیمیایی هستند. هدف از این مطالعه، بررسی اثر سمیت حاد نفت خام بر عملکرد پایه فیزیولوژی ماهی قره‌برون *Acipenser persicus* بود. برای این منظور تعداد ۲۱۰ بچه ماهی قره‌برون (۱ ± ۹/۴ گرم) از مرکز پرورش ماهی رجائی مازندران، ایران تهیه شدند. بچه‌ماهیان در معرض نفت خام (۱۵، ۱۶، ۱۷، ۱۸ و ۱۹ میلی‌گرم در لیتر) قرار گرفتند. LC_{50}-96h تحت شرایط ایستا و با استفاده از نرم افزار SPSS تعیین شد. پارامترهای خونی و بیوشیمیایی بین گروه شاهد و تیمار LC_{50}-96h مورد مقایسه قرار گرفت. غلظت نیمه کشنده در آزمایش ۹۶ ساعته سمیت برابر با ۱۶/۵ میلی‌گرم در لیتر محاسبه شد. شاخص‌هایWBC ، RBC، Hb و MCHC بصورت معنی‌داری در تیمار LC_{50}-96h کاهش و مقادیر MCV، MCH و PCV آن نسبت به شاهد افزایش داشت ($P<0.01$). نتایج شمارش انواع گلبول سفید نشان داد فراوانی نسبی نوتروفیل و مونوسیت افزایش یافت، در حالی که درصد لنفوسیت و ائوزینوفیل در تیمار ۹۶ ساعته به طور معنی‌داری کاهش یافت ($P<0.01$). پارامترهای بیوشیمیایی نشان دهنده افزایش غلظت گلوکز سرم خون بود ($P<0.01$). دیگر پارامترهای خون‌شناسی شامل پروتئین کل، آنزیم‌های ALT، ALP و LDH در تیمار LC_{50}-96h به صورت معنی‌دار کاهش یافتند ($P<0.01$). نتایج ما شواهدی از اثر مخرب نفت خام بر روی بافت‌های خونی را نشان می‌دهد، به طوری که قابلیت بقای این سلول‌ها در اثر سمیت آن کاهش می‌یابد. نفت خام همچنین موجب بازدارندگی فعالیت آنزیم‌ها شده و افزایش قند خون (هایپرگلایسمیا) به علت القای شرایط استرس‌زا در فیل ماهیان مشاهده شد.

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