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Toxic Impact of Exposure to Calcium Hypochlorite and Granular Activated Carbon on African Catfish (*Clarias gariepinus*): A Study of the Alterations in Hemato-Biochemical Profile and Oxidative Indices

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ABSTRACT

This study aimed to evaluate the hemato-biochemical parameters, oxidative stress indices and histopathological alterations in different organs after exposure of fish to calcium hypochlorite ($Ca(OCl)_2$) as well as granular activated carbon (GAC) (unrinsed) as dechlorinator. A total of 96 *Clarias gariepinus* was divided equally into four groups in triplicates: Group 1 was kept as a control, while groups 2, 3, and 4 were exposed (daily) to $Ca(OCl)_2$ at a concentration of 0.045mg/L water, GAC at a concentration of 50mg/L water and $Ca(OCl)_2$ plus GAC at a same concentration of previous groups, respectively. The exposures were conducted for 96h after that blood and tissue samples were collected for performing experimental tests. The results revealed that significant increase in erythrogram and leukogram parameters, besides increase activities of serum alanine aminotransferase and aspartate aminotransferase and concentrations of bilirubin fractions, total proteins, globulins, sodium, chloride, calcium, phosphorus and ammonia in all experimental groups. Fourth group showed insignificant increase in red blood cells and monocytes counts. Hyperalbuminemia was observed in $Ca(OCl)_2$ group alone. Serum alkaline phosphatase activity and creatinine concentration were significantly decreased in all experimental groups. Malondialdehyde and hydrogen peroxide levels showed significant increase, besides superoxide dismutases activity was decreased significantly in all experimental groups in the liver, kidneys and gills tissues. In conclusion, chlorine caused hematological disturbances, hepato-renal impairment with oxidative stress. Despite the importance of GAC as dechlorinator, it caused several adverse results under the condition of this experiment, and this sheds light into the importance of safely use of GAC on aquatic organisms.

Key words: Hematological, Biochemical, Oxidative indices, Calcium hypochlorite, Activated carbon.

INTRODUCTION

Clarias gariepinus is a principal clarid catfish in Africa and has been used largely as a laboratory fish model by many scientists to perform different scientific studies (Ibrahem 2012). Fish have a very close relationship with their surrounding environment, so poor water quality and waterborne toxicities most of the time kill-fish more than infectious agents (Roberts and Palmeiro 2008). So, it can be said that the ideal aquatic environment depends mainly on the water with high physicochemical quality (El-Sherbiny et al. 2019).

Elemental chlorine (Cl_2) and other chlorine compounds were used for many years in drinking water disinfection and wastewater treatment, where it plays an important role in prevention of waterborne infectious diseases spread, when used appropriately (Ghernaout 2017). Also, chlorination is one of the common and widely used practices in aquaculture for disinfecting fish hatcheries besides disinfection of fish production ponds by sterilization of tanks and standing water in drained ponds (Pons and Boyd 1998; Parker 2002). It is used in a liquid state as sodium hypochlorite (NaOCl; bleach) or granular/powdered form as calcium hypochlorite ($Ca(OCl)_2$) for water sterilization (IPCS 2000). In case of aquacultural purposes, calcium hypochlorite is preferred to use in comparing with sodium hypochlorite. This is due to more available chlorine (about 65%) in calcium hypochlorite, while the sodium hypochlorite has a low percentage of the active ingredient and less stable compound (Khan et al. 2008; Noga 2010; Hamdullah et al. 2010). In water, chlorine can be present as

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hypochlorous acid and hypochlorite ions and is defined as "free available chlorine" or it may react with natural organic matter and form chlorinated disinfectant by-products (DBPs), which fall under the combined chlorine category (IPCS 2000; Schmittinger 2000; Wu et al. 2021).

Despite the importance of chlorine as a disinfectant, the discharging of drinking water, heavily chlorinated wastewater and industrial chlorinated effluents into the aquatic system or addition of high concentrations of chlorine to fishponds for reducing bacterial population may leave high chlorine concentrations in aquatic environment (Cooke and Schreer 2001; Fisher et al. 2003). Chlorine can induce toxic effects to aquatic creatures such as gill damage which lead to respiratory difficulty, behavioral disturbances and some hematological abnormalities (Comfort et al. 2019). According to the most research reports, free chlorine is the most toxic form and therefore different dechlorination methods were established (Ganesh et al. 2006).

In the past years, more interest has been given to the potential use of activated carbon for dechlorination (Ganesh et al. 2006). Activated carbon (AC) is the common term for describing the family of carbonaceous adsorbents, commonly made from coal, wood, coconut shell and lignite and is available basically in three forms (granular, powder and pellet), which has been treated in a special way that makes their surfaces highly adsorbent with a massive surface area (Menéndez-Díaz and Martín-Gullónb 2006; Aly et al. 2016). It is being used in aquaculture to remove impurities out of water and removing the halogens such as chlorine, ozone and bromine, besides removing metabolic by-products in recirculating systems (Aly et al. 2016). According to previous studies, granular activated carbon (GAC) was used for chlorite removal by adsorption and chemical reduction, where the chemical reduction process on the surface of GAC becomes the main removal mechanism when the adsorptive sites are occupied (Collivignarelli et al. 2006).

Although several studies have reviewed chlorine toxicity of fresh and marine water fish which represented in studying different generalized physiological and behavioural responses for fish and most of the data in literature were in the 1970s, 1980s, and 1990s, but the pathological changes of chlorine toxicity are discussed rather briefly in previous literature (Cooke and Schreer 2001; Mahjoor and Loh 2008; Batley and Simpson 2020).

Furthermore, there are a lot of studies focusing on the positive effects of AC on pollutant removal in addition to both of its physical properties and the filtering effectiveness (Millward et al. 2005). On the other hand, the available information on possible deleterious effects of AC on the different biological parameters of aquatic species besides ecotoxicological effects are so limited and confined to aquatic invertebrates and other benthic organisms in sediments (Jonker et al. 2009). Limited studies have been performed on fish which suggested that GAC might be capable of causing head and lateral line erosion and the carbon dust (known as fines) was thought to be the causative agent for that. Exposure of fish to the carbon dust problem may happen for several reasons such as using low quality carbon (soft and dusty) or accidental occurrence of carbon crumbling and ejection into aquariums or use dry carbon, which unrinsed or soaked in distilled water before applying to the aquarium (Hemdal 2010; Hemdal and Odum 2011).

This study aimed to evaluate the hemato-biochemical parameters besides oxidative stress indices and histopathological alterations in the different organs to determine the possible pathological changes after exposure of fish to calcium hypochlorite as well as granular activated carbon (unrinsed) as a dechlorinator.

MATERIALS AND METHODS

Tested Agents

Calcium hypochlorite ($Ca(OCl)_2$) (technical grade) was used in granular form with (65%) available chlorine, which is a product of (Sree Rayalaseema HI-Strength Hypo Limited, Andhra Pradesh, India). Granular activated carbon (GAC) was bought under the commercial name (NORIT GAC 830 PLUS), which is a product of Cabot Corporation USA and was purchased from the Egyptian distributor (Redachem Egypt Ltd.). It has a very high purity level with an iodine number 975 and a total surface area $1075 m^2/g$.

Fish

A total of 96 apparently healthy Nile catfish (*Clarias gariepinus*) with a body weight (200±10g) were used in this study. The fish were obtained and transported from the ponds of the Central Laboratory of Aquaculture Research, Abbassa, Abou-Hammad, Sharkia, Egypt to the fish unit in the Faculty of Veterinary Medicine, Zagazig University, Egypt. Fish were acclimatized for two weeks in stock ponds then randomly divided and maintained in 120-litre rectangular glass aquaria (100 x 40 x 30cm) of dechlorinated tap water. Continuous aeration was provided for each aquarium. Fish were fed during the experiment on a basal diet, which formulated to provide the proper nutrient needs of *C. gariepinus* in accordance with NRC (2011) recommendations. During the course of the experiment, the mean values of water quality indicators were temperature 28±2°C, pH 7±0.5 and dissolved oxygen 5-6mg/L water. The photoperiod was 14h light: 10h darkness.

Ethical Approval

The study was approved by Zagazig University Institutional Animal Care and Use Committee "ZU-IACUC", Egypt (Approval No: ZU-IACUC/2/F/141/2019).

Experimental Design

Fish were divided equally into four groups in a random manner (three replicates for each group, 8 fish/replicate). Group 1 was kept as a control without any exposure, group 2 was exposed to $Ca(OCl)_2$ daily at a concentration of 0.045mg/L water (Kolawole and Olukunle 2014) for 96h period according to Halder et al. (2014). The exposure concentration of $Ca(OCl)_2$ was selected in order to be representative of environmental concentrations encountered in the polluted water, where concentrations ranging from 0.04-0.2mg/L were considered to be toxic to most of fish species according to Batley et al. (2021). Group 3 was exposed to GAC daily

which was used directly as received without soaking or rinsing in distilled water and was applied into aquaria in a plastic mesh at a concentration of 50mg/L water for 96h period according to Hatt et al. (2013) and group 4 was exposed to $Ca(OCl)_2$ plus GAC daily at a same concentration and duration of previous groups. Water in aquaria was replenished at 80% each 24h to maintain constant exposure to tested agents and to remove unconsumed food and fecal matter by siphoning (static-renewal exposure regime). Also, the clinical signs and mortality of fish were observed during the experimental period.

Sampling

Blood and tissue samples were collected at random from the fish in the different experimental groups after 96h from starting the experiment. The fish were anaesthetized before sampling by using of clove oil at a concentration of 50mg/L water through immersion in a separate container (Javahery et al. 2012). Blood samples were collected from a fish caudal vein (6/group) by using sterile disposable plastic syringe. The first part of collected blood, dispensed into sterilized tubes containing dipotassium salt of ethylene diamine tetra acetic acid (EDTA) for performing the different hematological tests. The second part of collected blood was dispensed into sterilized plain tubes, and then the serum was separated from clotted blood, which was centrifuged at 3000rpm for 15min for performing the different biochemical analysis. Ten fish from each group were euthanized by decapitation and immediately the abdominal cavity of fish was opened for organs collection for oxidative stress indices estimation and histopathological evaluation.

Hematological Studies

Red blood cells (RBCs) and total leukocytes counts, hemoglobin (Hb) concentration and hematocrit (Ht) value were determined using an automated blood cell analyzer (Sysmex XT-2000iV, Kobe, Japan) (Harvey 2012). Giemsa-stained blood films were prepared for estimation of differential leukocytic count (Campbell 2015).

Biochemical Studies

The separated serum was analysed to determine the activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) and concentrations of bilirubin (total and direct), total proteins, albumin, creatinine according to the methods described by Burtis and Ashwood (1999) and ammonia concentration according to the method stated by Neely and Phillipson (1988) and these parameters were determined using semi-auto chemistry analyzer (Chem-7 manufactured by Erba Diagnostics, Germany). Also, serum was used for the measurement of sodium (Na) level according to Henry et al. (1974) and chloride (Cl), calcium (Ca) and phosphorus (P) levels according to the methods described by Burtis and Ashwood (1999) and these parameters were measured using (ADVIA 1800 chemistry system manufactured by Siemens, Japan). Globulins concentration was obtained mathematically by take away the value of albumin from total proteins, and also indirect bilirubin was obtained by calculation via subtracting direct bilirubin from total bilirubin. All of these parameters were measured in kinetic or colorimetric way by using specific commercial kits (Spinreact, Spain).

Oxidative Stress Indices in Tissues Homogenates

Pieces (0.25g) of the liver, kidneys and gills were weighed separately and rinsed with ice-cold saline to take off any blood clots then were grounded with cold phosphate buffered saline (PBS) solution (pH 7.4). Finally, the prepared homogenates from the different organs were centrifuged and the collected supernatants were aliquoted for the oxidative stress indices estimation. Malondialdehyde (MDA) level in the various tissue homogenates was determined spectrophotometrically by using the method described by Ohkawa et al. (1979) by using a commercial kit (Biodiagnostics, Egypt). Tissue hydrogen peroxide (H_2O_2) level was measured according to Halliwell and Gutteridge (1989) by using a colorimetric kit (Oxis International, Portland, OR, USA). Tissue superoxide dismutases (SOD) activity was estimated calorimetrically according to the method of Nishikimi et al. (1972) by using the kit (Biodiagnostics, Egypt). All these parameters were measured by using of photometer 5010 (Robert Riele GmbH and co-kg, Germany).

Histopathological Study

The selected organs for histopathological examination (liver, kidneys and gills) were collected, then fixed in (10%) buffered neutral formalin solution, following by process of dehydration in a graded ethanol (70 to 100%), after that cleared in xylene and embedded in paraffin. Paraffin sections (5 µm thick) were prepared, and finally stained with hematoxylin and eosin dyes, and then examined microscopically according to Suvarna et al. (2013).

Statistical Analysis

Data were analyzed by using SPSS statistical analysis package (version 21.0). One-way analysis of variance (ANOVA) was significant at $P<0.05$ (Snedecor and Cochran, 1994) and Tukey's HSD post-hoc descriptive was used to test the significance differences between the mean values. All data in study are presented in the form of mean±SE.

RESULTS

Clinical Observations and Survival

Both of fish groups which were exposed to $Ca(OCl)_2$ and GAC alone exhibited the signs of loss of reflexes, sluggish movement and restlessness besides covering of fish body and gills with a layer of mucus. While, the fish group exposed to $Ca(OCl)_2$ plus GAC showed signs of restlessness, loss of movement coordination, air gulping and peeling of skin with excessive amount of mucus on body surface and gills. No mortality was observed in the control group, while fish exposed to $Ca(OCl)_2$ alone recorded 3 mortalities, the group exposed to GAC alone recorded 4 mortalities and the group exposed to $Ca(OCl)_2$ plus GAC recorded 5 mortalities.

Effects on Hematological Markers

The hematological markers are presented in Table 1. Comparing with the control group, RBCs count showed significant increase in both groups exposed to $Ca(OCl)_2$ and GAC alone, the highest value was observed in GAC-exposed group. While, the group exposed to $Ca(OCl)_2$ plus GAC showed insignificant increase in comparison with control group. Also, the significant increase in Hb concentration and Ht value was observed in all experimental groups compared to the control, while the more pronounced change in Hb concentration was observed in the group exposed to GAC alone followed by group exposed to $Ca(OCl)_2$ alone then $Ca(OCl)_2$ plus GAC group. With regard to Ht value, the highest value was detected in the group exposed to GAC alone.

Total leukocytes, heterophils and lymphocytes counts were significantly increased in all experimental groups compared to the control group, the highest values of these parameters were observed in the group exposed to GAC alone followed by group exposed to $Ca(OCl)_2$ alone, while group exposed to $Ca(OCl)_2$ plus GAC showed the lowest values. Compared with the control group, monocytes count showed significant increase in both of groups exposed to $Ca(OCl)_2$ and GAC alone and insignificant increase in the group exposed to $Ca(OCl)_2$ plus GAC.On the other hand, the eosinophils and basophils showed non-significant statistical change in all experimental groups.

Effects on Biochemical Markers

Table 2 illustrates a significant increase in the serum ALT and AST activities, bilirubin (total, direct, and indirect), total proteins, globulins, sodium, chloride, calcium, phosphorus, and ammonia concentrations in all experimental groups in comparison with the control group. The highest values of serum ALT and AST activities and bilirubin (total, direct, and indirect), sodium, chloride and ammonia concentrations were observed in the fish group exposed to both of $Ca(OCl)_2$ and GAC followed by the fish group exposed to GAC alone and finally the group exposed to $Ca(OCl)_2$ alone. On the other hand, the highest value of serum total proteins concentration was observed in the group exposed to $Ca(OCl)_2$ alone followed by the group exposed to $Ca(OCl)_2$ plus GAC, while the fish in the GAC group displayed the lowest value in this parameter, besides that serum globulins concentration appeared more pronounced in both groups exposed to $Ca(OCl)_2$ alone and $Ca(OCl)_2$ plus GAC. Serum calcium concentration was more obvious in group exposed to $Ca(OCl)_2$ plus GAC. While, serum phosphorus concentration was more pronounced in the group exposed to GAC alone. Serum albumin concentration showed a significant increase in the group exposed to $Ca(OCl)_2$ alone and non-significant change in other groups in comparison with the control group. Compared with the control group, serum ALP activity and creatinine concentration showed a significant decrease in all experimental groups. The lowest value of serum ALP activity was observed in the group exposed to $Ca(OCl)_2$ alone.

Effects on Oxidative Stress Indices

Regarding the results of MDA and H_2O_2 levels in the liver, kidneys and gills tissues, significant increase was found in all experimental groups relative to the control group in all tested tissues (Figs. 1 and 2). The highest values in all tested tissues were detected in the group exposed to $Ca(OCl)_2$ plus GAC. Conversely, SOD activity was decreased significantly in all experimental groups compared with the control group in the liver, kidneys and gills tissues (Fig. 3). The lowest values in all tested tissues were observed in the group exposed to $Ca(OCl)_2$ plus GAC.

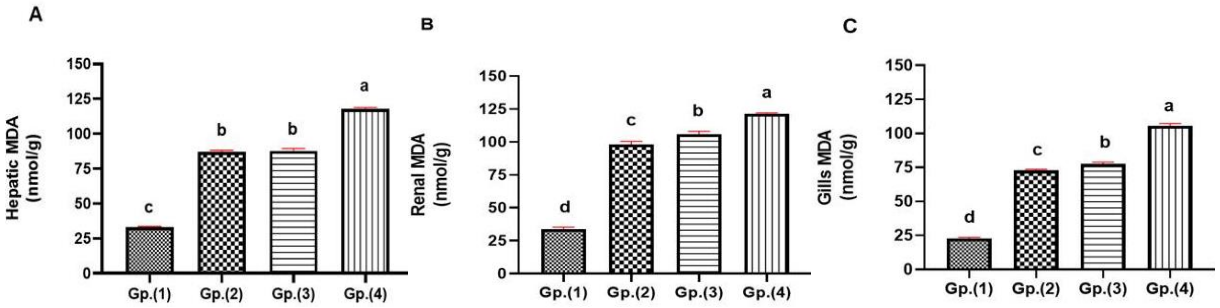

Fig. 1: MDA level in the liver, kidneys and gills in the different fish groups after 96h from starting the experiment. Data are expressed in the form of mean±SE. Bars with different letters are significantly different (P<0.05) and the highest value was represented by the letter (a); same letters indicate no differences. Gp. (1) Control group, Gp. (2) $Ca(OCl)_2$ group, Gp. (3) GAC group, Gp. (4) $Ca(OCl)_2$ plus GAC group.

Table 1: Some hematological markers in groups (1-4) after 96h from starting the experiment

Analytes	Experimental groups				P Value
	Gp. (1)	Gp. (2)	Gp. (3)	Gp. (4)	
RBCs ($\times10^6$/µl)	2.55±0.025c	3.17±0.049b	4.44±0.280a	3.00±0.032bc	<0.001
Hb (g%)	8.98±0.064d	11.92±0.036b	12.60±0.291a	10.90±0.118c	<0.001
Ht (%)	27.57±0.117c	33.24±0.506b	38.00±0.707a	32.00±0.547b	<0.001
TLC ($\times10^3$/µl)	25.78±0.265d	48.02±0.654b	58.13±0.671a	38.00±0.707c	<0.001
Heterophils ($\times10^3$/µl)	11.80±0.251d	19.02±0.317b	25.49±0.636a	16.49±0.492c	<0.001
Lymphocytes ($\times10^3$/µl)	11.47±0.201d	24.84±0.382b	27.86±0.540a	18.16±0.387c	<0.001
Monocytes ($\times10^3$/µl)	2.11±0.126c	3.46±0.222ab	4.20±0.262a	2.89±0.131bc	<0.001
Eosinophils ($\times10^3$/µl)	0.25±0.079	0.48±0.145	0.43±0.113	0.39±0.123	0.595
Basophils ($\times10^3$/µl)	0.15±0.062	0.22±0.108	0.15±0.114	0.07±0.078	0.750

Data were presented in the form of mean±SE. Means bearing different alphabets within the same row are significantly (P<0.05) different. No letters indicate (P>0.05). Gp. (1) Control group, Gp. (2) $Ca(OCl)_2$ group, Gp. (3) GAC group, Gp. (4) $Ca(OCl)_2$ plus GAC group. RBCs, red blood cells; Hb, hemoglobin; Ht, hematocrit; T.L.C., total leukocytic count.

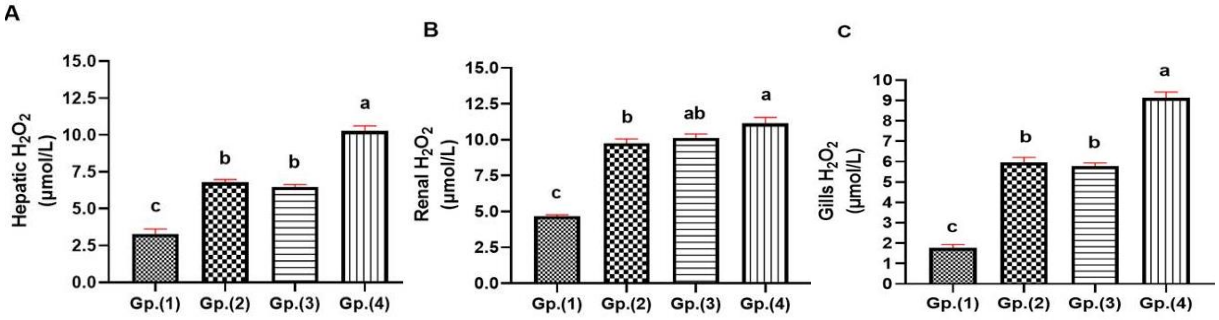

Fig. 2: H_2O_2 level in liver, kidneys and gills in the different fish groups after 96h from starting the experiment. Data are expressed in the form of mean±SE. Bars with different letters are significantly different (P<0.05) and the highest value was represented by the letter (a); same letters indicate no differences. Gp. (1) Control group, Gp. (2) $Ca(OCl)_2$ group, Gp.(3) GAC group, Gp. (4) $Ca(OCl)_2$ plus GAC group.

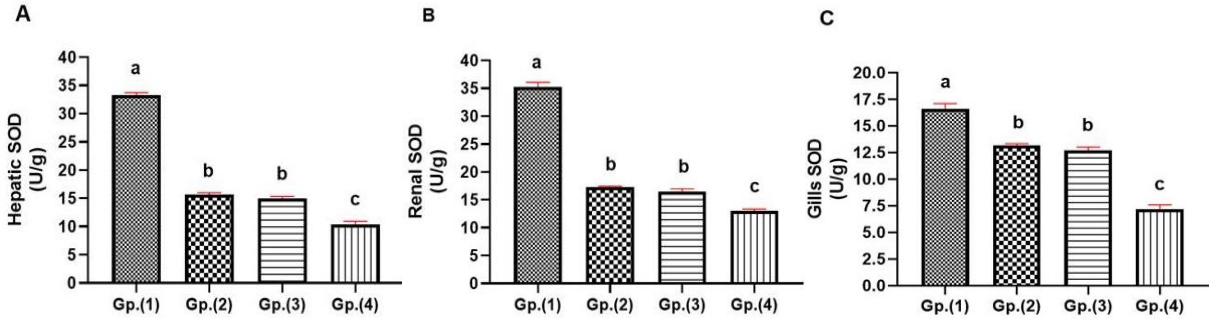

Fig. 3: SOD activity in liver, kidneys and gills in the different fish groups after 96h from starting the experiment. Data are expressed in the form of mean±SE. Bars with different letters are significantly different (p<0.05) and the highest value was represented by the letter (a); same letters indicate no differences. Gp. (1) Control group, Gp. (2) $Ca(OCl)_2$ group, Gp. (3) GAC group, Gp. (4) $Ca(OCl)_2$ plus GAC group.

Table 2: Some serum biochemical markers of groups (1-4) after 96h from starting the experiment

Analytes	Experimental groups				P Value
	Gp. (1)	Gp. (2)	Gp. (3)	Gp. (4)	
ALT (U/L)	17.53±0.701d	22.72±0.794c	27.77±0. 731b	73.76±1.185a	<0.001
AST (U/L)	117.46±2.911d	217.78±2.536c	274.36±2.751b	293.68±2.091a	<0.001
ALP (U/L)	20.61±0.903a	9.84±0.546d	17.41±0.677b	13.60±0.325c	<0.001
Total bilirubin (mg/dl)	0.42±0.022d	0.62±0.016c	0.71±0.013b	0.78±0.008a	<0.001
Direct bilirubin (mg/dl)	0.10±0.005c	0.13±0.006b	0.15±0.004ab	0.17±0.005a	<0.001
Indirect bilirubin (mg/dl)	0.32±0.019d	0.49±0.010c	0.56±0.011b	0.61±0.004a	<0.001
Total proteins (g⁄dl)	3.21±0.055d	5.12±0.066a	3.71±0.044c	4.31±0.078b	<0.001
Albumin (g⁄dl)	1.19±0.044b	1.85±0.027a	1.24±0.032b	1.29±0.035b	<0.001
Globulins (g⁄dl)	2.02±0.058c	3.27±0.073a	2.47±0.055b	3.02±0.075a	<0.001
Creatinine (mg/dl)	0.52±0.013a	0.43±0.010b	0.38±0.010c	0.36±0.012c	<0.001
Sodium (mmol/l)	123.06±0.864d	142.30±0.964c	155.40±1.164b	179.79±2.500a	<0.001
Chloride (mmol/l)	82.89±1.643d	93.22±1.521c	106.42±1.701b	118.12±1.167a	<0.001
Calcium (mg/dl)	8.79±0.174c	9.61±0.142ab	9.46±0.124b	10.18±0.077a	<0.001
Phosphorus (mg/dl)	9.82±0.182c	11.66±0.092b	12.87±0.190a	11.68±0.213b	< 0.001
Ammonia (mg/dl)	0.02±0.001d	0.04±0.001c	0.06±0.002b	0.07±0.001a	< 0.001

Data were presented in the form of mean±SE. Means bearing different alphabets within the same row are significantly (P<0.05) different. Gp. (1) Control group, Gp. (2) $Ca(OCl)_2$ group, Gp. (3) GAC group, Gp. (4) $Ca(OCl)_2$ plus GAC group. ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

Histopathological Findings

Liver: The examined liver of fish in the control group showed normal lobular arrangement, central venules, hepatocytes, sinusoids and reticulio-endothelial system are well formed and histologically normal (Fig. 4a). Furthermore, the photomicrographs of liver regard to the group exposed to Ca $(OCl)_2$ alone showed degenerative vasculitis with associated perivascular edema. Also, the hepato-portal area revealed remarkable edema and lymphocytic aggregations, peri-portal and interstitial aggregations of melano-macrophages and or lymphocytes are detected (Fig. 4b and 4c). On the other hand, the hepatopathological assessment of the fish group exposed to GAC alone revealed that moderate hepato-portal vascular congestion, perivascular edema beside hepatocytes apoptotic and degenerative changes in a moderate number of cells (Fig. 4d and 4e). The hepatic histological architecture of fish group, which exposed to both $Ca(OCl)_2$ and GAC showed severe dilatation and erythrocytic-leukocytic cytosis of the hepato-portal veins with perivascular lymphocytic aggregation. Mild to moderate numbers of hepatocytes were degenerated and or apoptotic (Fig. 4f and 4g).

Kidneys: Histology of renal tissue of fish in the control group showed normal renal glomerular, tubular and interstitial structures with minimal round cells infiltrations in the latter (Fig. 5a). On the other hand, the renal photomicrographs of fish group exposed to $Ca(OCl)_2$ alone showed severe congestion of the blood vessels, massive renal tubular atrophic, degenerative and necrotic changes, extensive interstitial lymphocytic infiltration,

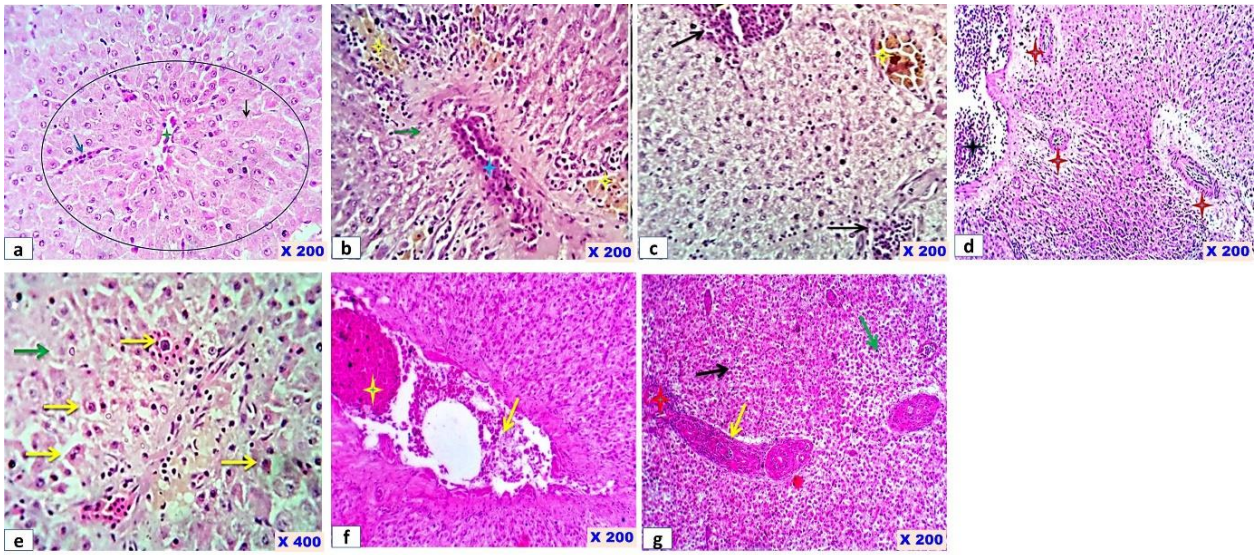

Fig. 4: Photomicrograph of H&E-stained sections. (a) Liver section of control group showing normal lobular arrangement (circle), central venules (star), the hepatocytes (black arrow), sinusoids (blue arrow) and reticulio-endothelial system are well formed and histologically normal. Liver sections of group exposed to $Ca(OCl)_2$ alone showing (b, c) degenerative vasculitis with associated perivascular edema (blue stars and green arrow), the hepato-portal area shows remarkable edema and lymphocytic aggregations (black arrows) and peri-portal and interstitial aggregations of melano-macrophages and or lymphocytes are seen (yellow stars). Liver sections of group exposed to GAC alone showing (d, e) moderate hepato-portal vascular congestion (black star), perivascular edema (red stars) beside hepatocytes apoptotic and degenerative changes in a moderate number of cells (yellow and green arrows). Liver sections of group exposed to $Ca(OCl)_2$ plus GAC showing (f, g) severe dilatation and erythrocytic-leukocytic cytosis of the hepato-portal veins (yellow arrows and stars) with perivascular lymphocytic aggregation (red star) and mild to moderate numbers of hepatocytes were degenerated and/or apoptotic (green and black arrows).

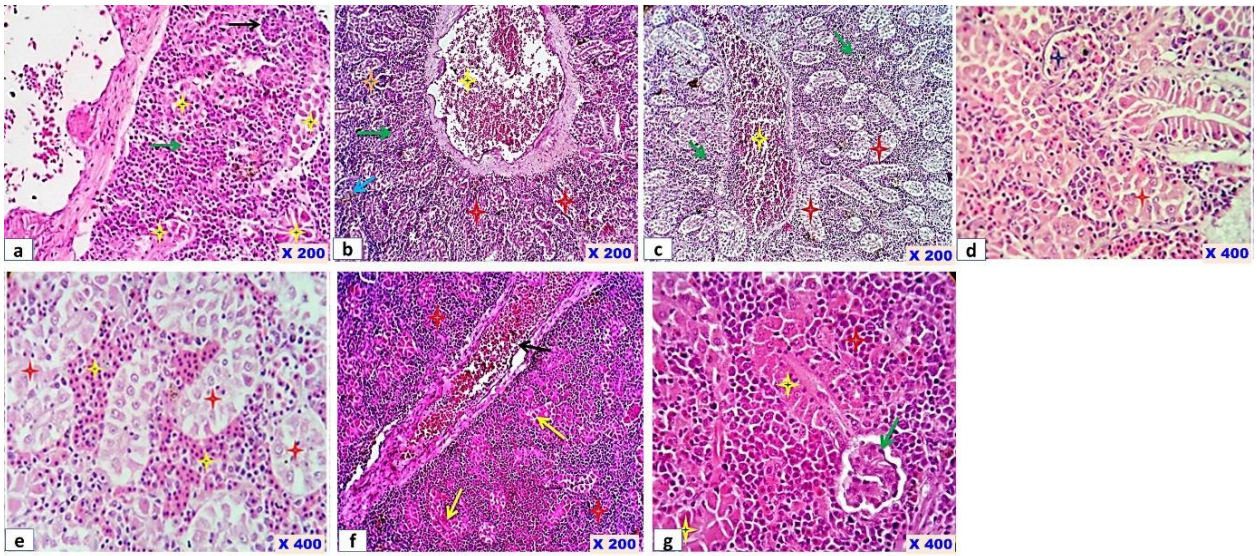

Fig. 5: Photomicrograph of H&E-stained sections. (a) Kidney section of control group showing normal renal glomerular (black arrow), tubular (yellow stars) and interstitial structures with minimal round cells infiltrations in the latter (green arrows). Kidney sections of group exposed to $Ca(OCl)_2$ alone showing (b, c) severe congestion of the blood vessels (yellow stars), massive renal tubular atrophic, degenerative and necrotic changes (red stars), extensive interstitial lymphocytic infiltration (green arrows), glomerular shrinking and or lobulation (orange star) and focal aggregations of melano-macrophages (blue arrow). Kidney sections of group exposed to GAC alone showing (d, e) massive renal-tubular degenerative and early necrotic and/or apoptotic changes (red stars), hyperemia of the blood vessels and extensive interstitial lympho-plasmacytic infiltration and aggregation (yellow stars), while the glomeruli showed unremarkable changes (dark blue star). Kidney sections of group exposed to $Ca(OCl)_2$ plus GAC showing (f, g) tubular degenerative and early necrotic changes beside a few cells suffering apoptotic changes (yellow arrows and stars), the blood vessels were moderately congested (black arrow), the interstitium showed moderate aggregations of lympho-plasmacytes (red stars) and some glomeruli are atrophic (green arrow).

glomerular shrinking and/or lobulation and focal aggregations of melano-macrophages (Fig. 5b and 5c). Looking to the examined kidney sections of fish group exposed to GAC alone, massive renal-tubular degenerative and early necrotic or apoptotic changes were found. Also, hyperemia of the blood vessels and extensive interstitial lympho-plasmacytic infiltration and aggregation were observed. Focal renal tubular neoplastic change was seen in a few examined slides. The glomeruli showed unremarkable changes (Fig. 5d and 5e). In fish group exposed to both of $Ca(OCl)_2$ and GAC, renal photomicrographs showed tubular degenerative and early necrotic changes beside a few cells suffering apoptotic changes. The blood vessels were moderately congested. The interstitium showed moderate aggregations of lympho-plasmacytes. Some glomeruli are atrophic (Fig. 5f and 5g).

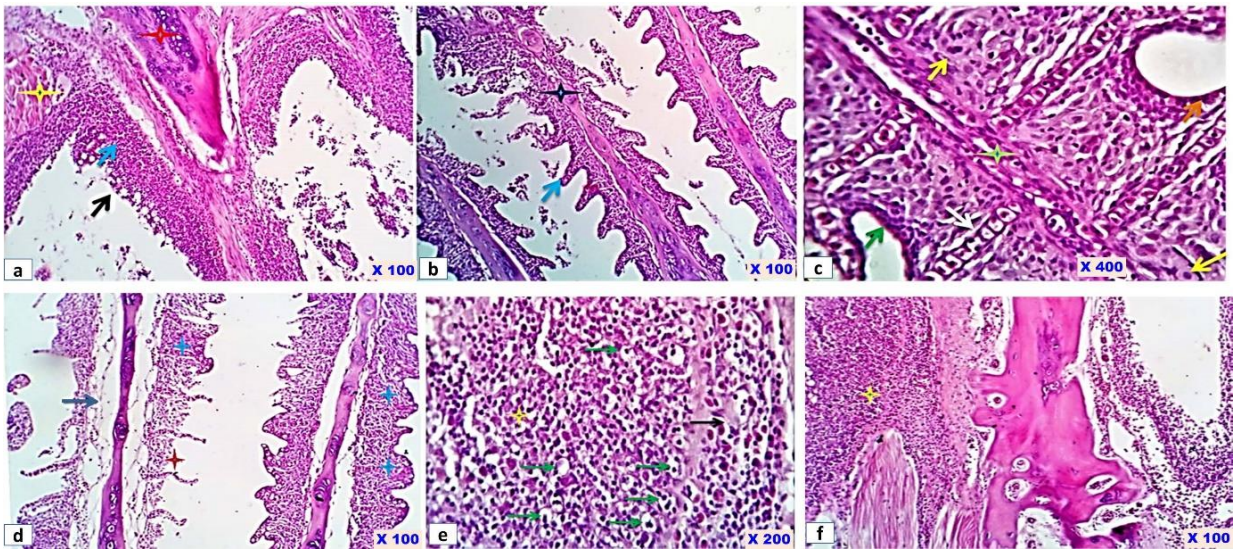

Fig. 6: Photomicrograph of H&E-stained sections. Gills sections of control group showing the histomorphological structures of (a) gill arch (Fibro-masculaar elements, yellow star), carteliigenous terminals (red star) and stromal cells (eosinophilic granular cells and round cells, black and blue arrows), (b) primary and secondary gill filament (blue arrow and star), (c) lamellar pavement cells (green arrow), epithelial cells (orang arrow), chloride cells (yellow arrow) and efferent venule (green star). Gills sections of group exposed to $Ca(OCl)_2$ alone showing (d-f) focal epithelial lifting, denudation and necrosis (red star) or epithelial hyperplasia with subepithelial aggregations of eosinophilic granular cells, lymphocytes, and many chloride cells, mostly with clear vacuolated cytoplasm (blue stars and green arrows),besides congestion of the efferent gill venules, which are stuffed by lymphocytes (black arrow) and sometimes accompanied perivascular edematous changes (dark blue arrow). The gill arch showed large collections of lymphocytes, eosinophilic granular cells and macrophages (yellow star).

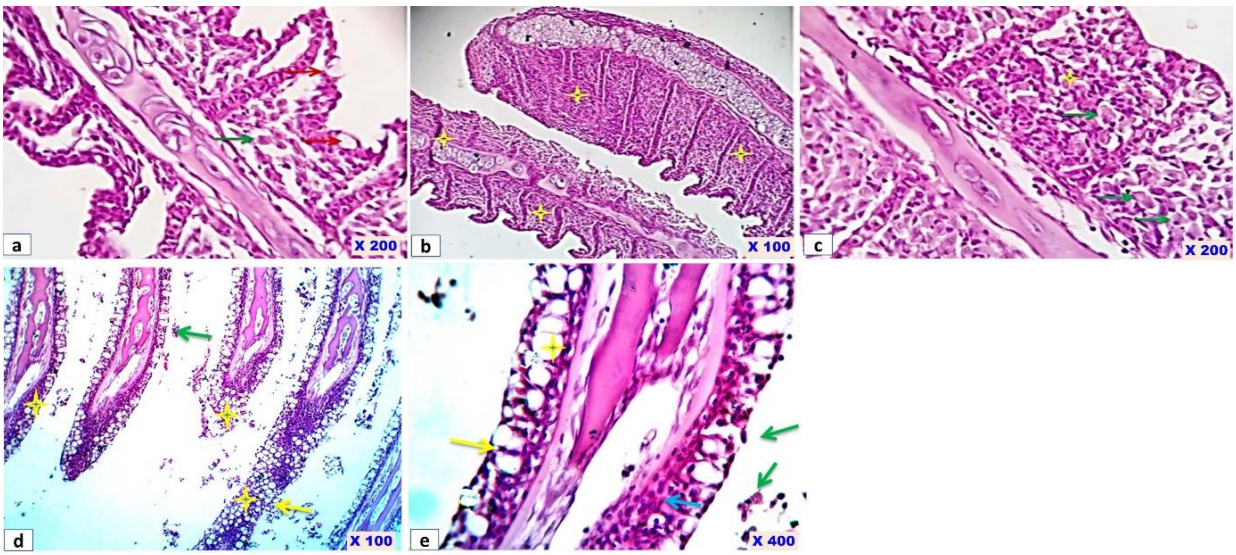

Fig. 7: Photomicrograph of H&E-stained sections. Gills sections of group exposed to GAC alone showing (a-b) gill filaments either focally denuded from their epithelial lining with superficial vacuolations and subepithelial aggregation of macrophages and chloride cells (red and green arrows) or they are of proliferative nature (yellow stars) with (c) secondary gill filament adhesion due to chloride cell hyperplasia (green arrows) and lympho-plasmacytic infiltration and epithelial hypertrophy and hyperplasia. Gills sections of group exposed to $Ca(OCl)_2$ plus GAC showing (d, e) extreme vacuolation of the filament epithelial lining (spongiosis) (yellow stars and arrows), epithelial lifting (green arrow), hyperemia of the efferent venules and mild lymphocytic infiltration (blue arrow).

Gills: Examined serial sections from the gills of control fish revealed normal histo-morphologic structures, including the gill rockers with its epithelial lining that comprise mucus cells, chloride cells and the subepithelial stromal cells (eosinophilic granular cells and some lymphocytes). The gill arch showed normal fibro-mascular elements, osteo-cartilagenous terminals and stromal structures comprising some mononuclear cells and eosinophilic granular cells. The primary and secondary gill filaments were apparently normal with normal pavement cells, lamellar epithelial cells, pillar cells, mucus secreting cells (goblet cells), chloride cells, capillary channels (afferent and efferent venules) and a few mononuclear cells (Fig. 6a-c). Examined gills sections of the group exposed to $Ca(OCl)_2$ alone denoted remarkable changes particularly in the secondary filaments represented by focal controversial epithelial lifting, denudation and necrosis or epithelial hyperplasia with subepithelial aggregations of eosinophilic granular cells, lymphocytes, and many chloride cells, mostly with clear vacuolated cytoplasm. Congestion of the efferent gill venules, which were stuffed by lymphocytes sometimes, accompanied perivascular edematous changes were recorded. The gill arch showed large collections of

lymphocytes, eosinophilic granular cells and macrophages (Fig. 6d-f). Highly sophisticated changes were noticed in the group exposed to GAC alone as the gill filaments were either focally denuded from their epithelial lining with superficial vacuolations and subepithelial aggregation of macrophages and chloride cells or they were of proliferative nature with secondary gill filament adhesion due to chloride cell hyperplasia and lympho-plasmacytic infiltration and epithelial hypertrophy and hyperplasia (Fig. 7a-c). Examined serial sections of gills in fish group exposed to $Ca(OCl)_2$ and GAC revealed extreme vacuolation of the filament epithelial lining (spongiosis), epithelial lifting, hyperemia of the efferent venules and mild lymphocytic infiltration. The gill arch showed massive infiltration and aggregation of lympho-plasmacytes and eosinophilic granular cells (Fig. 7 d-e).

DISCUSSION

Despite the big and important role of chlorine as a disinfectant, sensitivity of fish to chlorine as well as its toxicity has been recorded by several studies (Heath 1997; Yonkos et al. 2000; Kowalska et al. 2006). For this reason, a number of methods have been developed for removing or reducing free chlorine residuals and activated carbon was the most common way between different dechlorination processes (Salama et al. 2016). Quality of water can be identified by the fish living in it, whereas fish health condition gives a good indication about the health situation of the aquatic ecosystem. Early toxic effects of chemicals or pollutants are visible on the level of cells or tissues, even before the appearance of specified or significant changes in fish external appearance or behavior (Aswale et al. 2019).

Measurement of hematological parameters can be used as indicators for critical changes following exposure to the environmental stressful conditions (Kumar et al. 2011). In this study, the significant increase of RBCs count, Hb concentration and Ht value was observed in all experimental groups except the group exposed to $Ca(OCl)_2$ plus GAC. This may be related to the hypoxia due to gills dysfunction and/or dehydration status besides fish response to the stressful conditions (Zeitoun 1977; Smith 2019). Alterations in the hemoglobin concentration under any type of exposure to chemicals or poisonous stressors are indicator to the oxygen utilization and metabolism, as oxygen transport in the blood depends upon the hemoglobin content of RBCs in the blood of fish; therefore, Hb concentration and RBCs count are considered as reflectors of the pollution stress. Physiological responses of fish to different stressors include of primary, secondary and tertiary responses in accordance with the stage and degree of stress (Ahmed et al. 2020), so insignificant increase of the mentioned hematological parameters in the group exposed to $Ca(OCl)_2$ plus GAC may reveal that the combined exposure of fish to both of agents may induce negative impacts on the hematopoietic system and start to lower the different hematological parameters.

According to the results of leukogram, leukocytosis occurred as a consequence of the significant increase of heterophils, lymphocytes and monocytes counts in all experimental groups except the group exposed to $Ca(OCl)_2$ plus GAC, which showed insignificant increase in the monocytes count in comparison to the control group, these changes may have occurred as inflammatory response from heterophils and monocytes to antigenic stimulation. Also, lymphocytosis is a suggestive of immunogenic stimulation in body (Thrall et al. 2012). These results are similar to that previously reported by Sakthika and Felicitta (2017) and Comfort et al. (2019), where it was observed that leukocytosis after exposure of fish to chlorine as well as lymphocytosis, where chlorine enhanced release of lymphocytes from lymphoid tissues to deal with the toxic condition.

Fish liver is considered a good model for the interaction between the environmental factors and hepatic structure and functions. Where many environmental stressors have an effect on fish liver and induce metabolic disturbances and structural damage (Datta-Munshi and Dutta 1996). In this study, there was a marked elevation in the serum ALT and AST activities in all experimental groups. These results may relate to hepatocellular damage or cellular injury and leakage of transaminases into the circulation (Rudneva 2014). On the other side, serum ALP activity showed marked reduction in all experimental groups, and this could be attributed to the damage or disturbance in cell organelles such as, cell membrane transport system and endoplasmic reticulum where ALP is responsible for membrane transport (Al-Ghanim et al. 2020). Also, the exposure of fish to chemicals can inhibit the activity or synthesis of ALP enzyme (Goetz 1980), so the decrease in ALP activity may be considered as an index of damage liver parenchyma and hepatocellular necrosis (Onikienko 1963). According to the results of serum bilirubin, a significant increment in (total, direct and indirect) bilirubin level was observed in all tested groups and this may be due to the hepatic parenchyma injury which resulted from xenobiotic exposure and lowering the hepatic ability to conjugate and excrete the bilirubin leading to a build-up of indirect and direct bilirubin in the blood (Luu 2013).Various observed histopathological lesions in the liver in the different experimental groups in this study confirmed the results of the measured biochemical tests.

Proteins are a major component in the blood and needed to build and repair tissues and raise an immune response in the body. As represented in this study, serum total proteins concentration showed significant increase in all experimental groups and this change may relate to globulins concentration, which was comparatively high in the different groups and this may reflect the high immunological defense response due to chemical exposure challenges (Javed et al. 2017). Regarding the results of serum albumin in the group exposed to $Ca(OCl)_2$ alone and according to the result of previous study of Zeitoun (1977), the marked increase in this parameter may be due to the hemoconcentration.

In fish, the functions of excretion and osmoregulation are closely related and performed by both of gills and kidneys (Smith 2019). Generally, the fish produce a small amount of creatine, uric acid and creatinine (Thrall et al. 2012). Creatine kinase activity in the liver produce creatine phosphate, which is broken down in the muscles to convert ADP into ATP, creatinine is a product of creatine phosphate breakdown, which is finally excreted

by the kidneys (Stoskopf 1993). The significant decrease in the level of serum creatinine in all experimental groups in this study may be due to lowering the activity of creatine kinase in the dysfunctional liver, which leads to decrease creatine phosphate concentration and this in turn leads to decrease the creatinine concentration in the blood. Also, the reduction in the basal metabolism may consequently reduce ATP and creatinine production (Mirghaed et al. 2018).

Blood concentrations of electrolytes such as Na^+ and Cl^- are indicative of a fish's ability to osmoregulate (McDonald and Milligan 1992). Significant increase of serum sodium and chloride ions levels in all fish groups in comparison with control one may be attributed in general to kidney dysfunction and gills injury, which in turn affects the osmoregulatory ability (Gabriel et al. 2019). According to previous studies on animals, the exposure to hypochlorite can result in hypernatremia and hyperchloremia with metabolic acidosis (Plumlee 2003). According to some previous studies in animals, the activated carbon can induce the increase in sodium concentration in blood (Drobatz et al. 2019).

Regarding the results of serum calcium and phosphorus in this study, hypercalcemia and hyperphosphatemia, which observed in all experimental groups, may be due to the impairment of renal function and poor calcium excretion alongside with phosphorus (Randels-Thorp and Liss 2017). Also, according to a study of Groff and Zinkl (1999) on Cyprinids (freshwater fish), hypoxia leads to reduction of glomular filtration rate which leads to hypercalcemia and hyperphosphatemia. Also, increasing of serum phosphate concentration might be because of different tissues damage that causing the phosphorous molecules release and the consequent elevation of its level (DiBartola and Willard 2011).

Ammonia is a prime end product of amino acid metabolism in the fish and most of fish excrete it as a main nitrogenous waste product. According to the results of this study, a marked increase in serum ammonia concentration in all experimental groups may be due to the swelling and inflammation that happened combined with gills damage which leading to increase diffusion distance between blood and water and lack of passive diffusion of ammonia to water (Thrall et al. 2012), where ammonia excrete mainly by the gills in fish (Evans et al. 2014). Numerous observed histopathological lesions in the kidneys and gills in the different experimental groups in this study confirmed the results of the measured biomarkers (serum Na, Cl, Ca, P and ammonia).

Measurement of antioxidants and oxidative stress markers could be used as a diagnostic tool for fish exposure to pollutants or deleterious chemicals (Acton 2013). MDA is a major degradation product of lipid hydroxides (LPO) and is often used as an effective biomarker for evaluating LPO when aquatic species are exposed to pollutants xenobiotics (Huang et al. 2020). According to the results of this study, MDA levels in the liver, kidneys and gills were significantly elevated in all experimental fish groups. Such increase in the level of MDA in different tissues reflects the excessive ROS production and the severity of free radical attack on body tissues (Dong et al. 2018). In another context, significant increase of H_2O_2 level in different tested tissues (liver, kidneys and gills) in all experimental groups may indicate increasing level of oxidative stress in these tissues, which triggers different deleterious biochemical reactions that will participate in lowering cellular function (Farooqui and Farooqui 2012; Biller and Takahashi 2018). In the normal health condition, antioxidant defense system of any organism, can remove ROS and protect the variety numbers of biological molecules from ROS attack. However, when ROS production under the influence of xenobiotics exceeds the scavenging capability of antioxidant defense system in the body, the protection system imbalance will be occurred, and consequently weakening the activity of antioxidant enzymes (Huang et al. 2020). As shown, in the present study the activity of SOD was significantly decreased in the liver, kidneys and gills in all experimental groups may be attributed to the excessive superoxide ions production in the fish tissues which exceeds the capacity of SOD to remove or neutralize it (Zahran et al. 2018).

Regarding the results of the most measured parameters, the pronounced changes were observed in the group exposed to both of $Ca(OCl)_2$ and GAC followed by group exposed to GAC alone and finally the group exposed to $Ca(OCl)_2$ alone. So that, these results shed light on the possible adverse effects of activated carbon on aquatic creatures biological system and therefore that the combined negative impacts of both $Ca(OCl)_2$ and GAC produced marked changes in all biological parameters in this group in comparison with the other groups especially the group exposed to GAC alone revealed significant changes. Few previous studies recorded that some negative impacts of activated carbon on aquatic invertebrates and certain benthic organisms in sediment which may confirm the results of this study. Jonker et al. (2009) found that addition unwashed activated carbon to sediments can be toxic to some aquatic invertebrates (*Lumbriculus variegatus, Daphnia magna, and Corophium volutator*) based on different mechanisms of toxicity including of physical or chemical stress on organisms. Also, some negative effects for AC were tested in the different forms and ways and have been reported by some studies and represented in the inhibition of the growth, reduction of the weight, decreased body lipid content and the behavior disturbance (such as feeding behavior) in different aquatic invertebrates (Nybom et al. 2012; Janssen and Beckingham 2013; Abel et al. 2017). According to the results of the study of Kupryianchyk (2011), addition of AC to clean and unpolluted sediment, caused 100% mortality of *Gammarus pulex* (benthic specie), whereas AC did not cause complete mortality in *G. pulex* when used in polluted sediment. So can say that AC may be effective and useful in reducing toxic substances in certain environment, but also may be the cause of different adverse effects on the lived creatures in this environment.

Conclusion

Under the light of this study, it can be concluded that the presence of $Ca(OCl)_2$ in *Clarias gariepinus* aquatic environment caused negative impacts on the fish biological markers which appeared in the form of hematological disturbances, hepato-renal impairment and oxidative stress in the different organs as well as

histopathological alterations in the tested organs, which provide both supplementary and supportive information on potential target organs toxicity of chlorine. On the other hand, in spite of importance of GAC for chlorine removal and reduction of pollutants exposure for aquatic organisms in general, this study found several adverse results after using activated carbon on the different tested parameters in fish under the condition of this experiment. Finally, it can be said that these results are very important in assessing the seriousness and toxicity of chlorine and its compounds and also important for the references of future studies. Also, the proper precautions need to be taken for efficient and safe use of AC in the aquatic environment as well as the there is need for the additional investigations on possible secondary effects of improper use of it.

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