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Histopathological Effects of NaCl Overconcentration in the Gills and Internal Organs of *Cyprinus carpio* L.

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Abstract: This study aimed to assess the toxic effect of the overconcentration of salt (NaCl) in farmed common carp fish, *Cyprinus carpio* L. in floating cages in Al-Saqlawiyah Rgion in Iraq. The salt was used at 13.8% which led to fish mortality reached to 70% through 96 hours of exposure. The fishes exhibited lethargy during 10 minutes with abnormal nervous signs and imbalance swimming with progressive decreases in opercular movement frequency. The gills appeared as cooked gill lesion with excessive mucus secretion and congestion in the hepatic, gastrointestinal and kidney tissues. Microscopical examination of gills showed hyper trophy in both pillar cells and mucus cells, variable in severity code of both epithelial hyperplasia and occlusion of lamellar space as well as epithelial cells lifting had been determined and vacuolar degeneration in hepatic cells with severe multifocal infiltration of inflammatory cells and necrosis in liver. Histopathological analysis of kidneys revealed interstitial nephritis while in the intestine, the microscopic examination exhibited hydropic degeneration of mucoid cells, hyperplasia of enterocytes and desquamation of the epithelial cells of villi. The conclusion of this study was that histopathological analysis can be used as biological indicators for evaluation of effects of NaCl overconcentration in fish organs.

Keywords: NaCl overconcentration, *Cyprinus carpio*, Histopathological alterations

Introduction

The best achievements of modern technologies in fish production is fish cultured in cages, more common in Iraq and Arab countries, depended on fishes as a protein for human and animal consumption (Arab Organization for Agricultural Development, 2013). These type of culture is characterized by rapid growth and high productivity of healthy and disease-free fishes. In addition, it is a good production method in areas which are difficult to use fish farming methods (Mwebaza-Ndawula et al., 2013).

Environmental effects represented 90% from cage problems, mainly physio-chemical properties of water and reducing water levels. These could be considered

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as stressor factors affecting fish growth, behavior and immune suppression which lead to disease (Morales et al., 2005; Masser, 2008).

Chemotherapeutic agents are mainly applied in fish culture to control and treatment of the diseases. One of them is the salt NaCl, which is defined as antiparasitic, antifungal and antibacterial agent (Roberts, 2001; Plante et al., 2002). The Food and Drug Administration (2002) considered the salt as little regulative potential, anti-stress compound maintains blood electrolyte balance and homeostasis, improves fish survival, reduces and prevents nitrite and copper toxicity (Tsuzuki et al., 2001; Kamunde et al., 2005).

These chemical substances are generally producing toxicity because lack of a weakness of their toxicity levels (King & Farrell, 2002). They may cause changes in behavioral and respiratory distress as a response to stress (Kane et al., 2005). Andrews et al. (2002) reported that application of salt at concentration above 1% was stressful to fishes.

Cytopathological examination is a main biological diagnostic method for estimation of invisible alterations (Gernhöfer et al., 2001). Several studies have been carried out to assess morphological changes in fish gills (Roberts, 2001). So, the aims of the present study were to evaluate behavior, clinical signs and microscopic alterations in gills, liver and kidneys of *C. carpio* reared in floating cages in Al-Saqlawiyah Region in Iraq exposed to overconcentration of NaCl.

Materials and Methods

Experimental Fishes

Twenty-five fishes (*C. carpio*) weighed 2 kg ±50 gm were reared in floating cages (4x3x3) in Al-Saqlawiyah Region in Iraq, the cages were raised to a height of two meters above the surface of the water, so the height of the water covering the fish's body was 30 cm. The net cage was surrounded by a plastic container for bathing the fish with antiparasitic salt solution at the concentration of 13.8% for one hour. Samples of treated fish were used to study the histopathological changes

Behavior Observation and Clinical Signs

Abnormal clinical signs and stress behavior (convulsion, hyperactivity, equilibrium status and mortality rate) were observed as suggested by Benli & Ayhan (2010).

Histopathological Study

For microscopic examination, samples were taken from lethargy fishes that exposed to salt overconcentration after 96 hours. Gills, kidneys, liver and intestine were fixed in 10% neutral buffer formalin for at least 72 hours. The tissue samples were prepared according to standard histopathological techniques suggested by Roberts (2001). Then, the tissues were stained with Hematoxylin and Eosin. All histopathological changes were evaluated and described under light microscope. The severity code for pathological changes in gills involved lamellar fusion and epithelial hyperplasia (Darwish et al., 2002) as indicated in Tables 1 and 2.

Table 1: Severity codes of lamellar fusion (LF) and epithelial hyperplasia (EH) in gill lamellae exposed to NaCl at 50 kg/cage for 96 hours.

Severity Code	LF	EH
0	Lamellae without notable fusion	Normal gill epithelial tissues
1	One-third lamellar fusion	Increase number (multifocal), up to 30% with slight inter lamellar proliferation
2	One-third to two thirds of lamellar fusion	Increase number (multifocal) from 31-60%
3	Greater than two thirds of lamellar fusion	Increase number (diffuse) above 61%

Another lesion in gills was cells present in the interfilamentous and inter lamellar spaces. This lesion is called epithelial lifting. The severity code is represented in Table 2.

Table 2: Severity code of epithelial lifting (EL) in an interfilamentous and inter lamellar spaces in gills exposed to NaCl at 50 kg/cage for 96 hours.

Severity code	Ratio of EL (%)
0	30
1	31
2	60
3	<60

Results

Effects of NaCl on Behavioral Response

Fishes were exposure to 50 kg/cage NaCl exhibited lethargy, motionless with stunned posture during the first 10 minutes. Then, they showed abnormal nervous signs as darts and abnormal swimming, excitation of some fishes attempted to jump with gulping swim and increase of air surface respiratory rate (ASR).

One of the most observations in this study was the increased opercular frequency (OF) at the first half hour of exposure to higher salt concentration, and then all fishes exhibited progressive decreases in OF which then was not possible to be determined during the other three hours. During the period of 96 hours of exposure, the mortality rate (MR) reached to 70%.

Macroscopic Examination

Fishes exhibit veil appearance as a result of excessive slimy mucus secretion which covered all the body surface and easily loss their scales, severe gill congestion with thick mucus layer on it with dead necrotic tissues which appeared as cooked gill lesions, while moderate to severe congestion in hepatic, gastrointestinal and kidney tissues.

Microscopic Examination

All examined carps, treated with high concentration of salt, exhibited the most common pathological alteration which was variable in severity code. The severity of lamellar fusion and epithelial cells lifting was determined. In comparison with the normal gill structure (Fig. 1), the severity of lamellar fusion and epithelial cells lifting ranked between codes 2 to 3 for lamellar fusion and 0 and 3 code for epithelial cells lifting with hyper trophy of pillar cells (Figs. 2 & 3).

Severity code of lamellar hyperplasia had been evaluated in this study, which was 3 grades as well as hyperplasia and hyper trophy of mucus with hydropic degeneration of chloride cells, increase infiltration of inflammatory cells and total occlusion of inter lamellar spaces (Fig. 4) as well as vacuolar degeneration in mucus cells (Fig. 5).

Also, there were variable degrees of hydropic degeneration from moderate to severe in hepatocytes (Fig. 6) accompanied with necrosis in hepatic tissue with severe multifocal infiltration of inflammatory cells (Fig. 7), while histopathological changes in intestine of fishes exposed to high salt concentration revealed necrosis in muscular layer, infiltration of inflammatory cells with hydropic degeneration of mucoid cells with hyperplasia of enterocytes and total occlusion of inter villi space. Also, there was sloughing of epithelia cells and lifting to intestinal lumen (Fig. 8).

One of the most important osmoregulatory organs in fishes are the kidneys, as they exhibit more severe histopathological alteration characterized by severe interstitial nephritis, coagulative necrosis and tubular kidney structure was lost with glomerular atrophy which accompanied with dilated Bowman's space (Fig. 9).

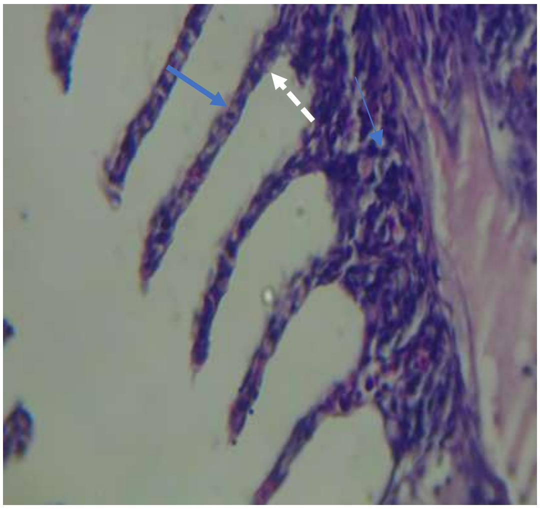

Figure 1: Normal gill structure of *C. carpio* showing lamellae (L) space between lamellae (thick row) and lamellar epithelial cells (narrow arrow) with pillar cells (white dotted arrow). Hematoxylin-Eosin (1.9 x 10X).

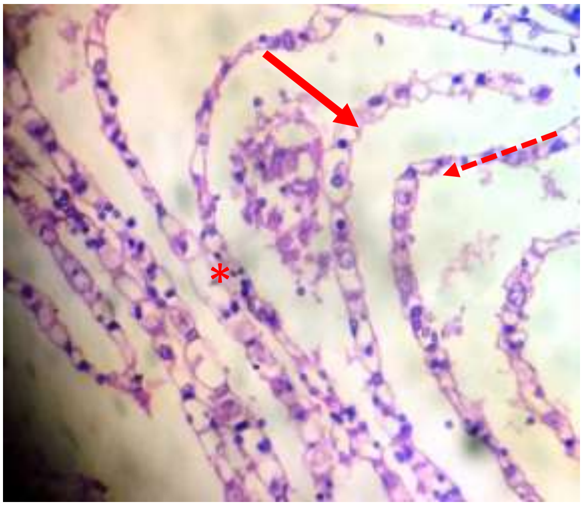

Figure 2: Gill structure of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing severe epithelial lifting (thick red arrow code) and lamellar fusion code 2 (*) with hypertrophy of pillar cells (red-dotted arrow). Hematoxylin-Eosin (1 x 40X).

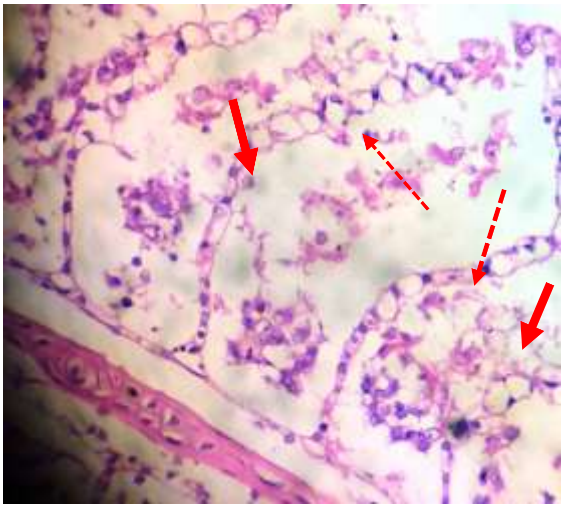

Figure 3: Gill structure of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing severe epithelial lifting (thick red arrow) code 3 and hyper trophy of pillar cells (red-dotted arrow). Hematoxylin-Eosin (1 x 40X).

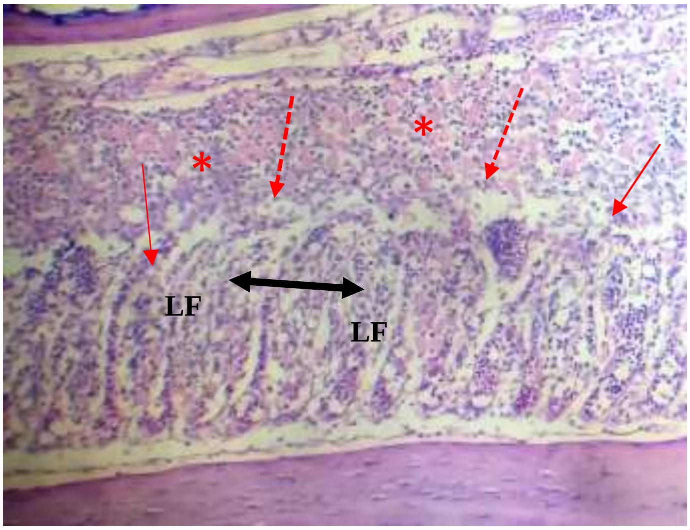

Figure 4: Gill structure of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing lamellar filament (LF), occlusion of inter lamellar space (thick arrow code 3), hyperplasia of mucus cells (red star) and hydropic degeneration of chloride cells (red dot arrow) with infiltration of inflammatory cells (red arrows). Hematoxylin-Eosin (1.9 x 10X).

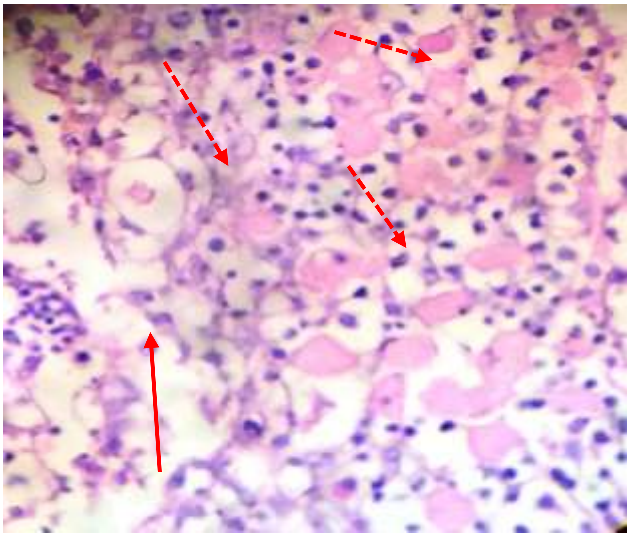

Figure 5: Gill structure of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing vacuolar degeneration of mucus (red dotted arrow) with infiltration of inflammatory cells (red arrow). Hematoxylin-Eosin (1 x 40X).

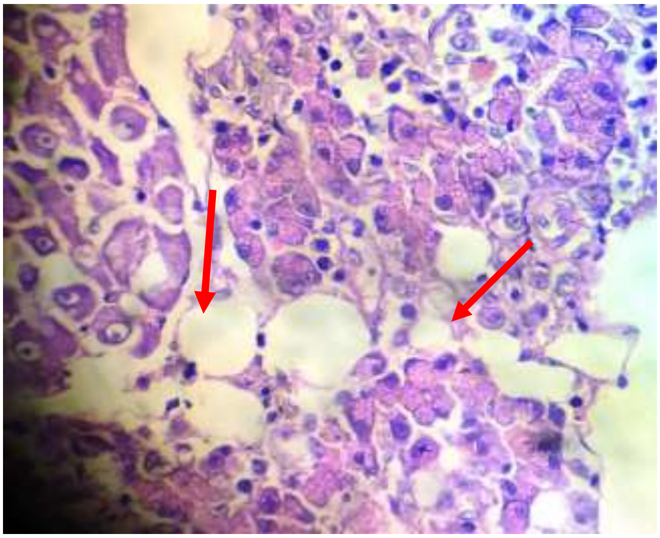

Figure 6: Liver of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing hydropic degeneration (red arrows). Hematoxylin-Eosin (1 x 40X).

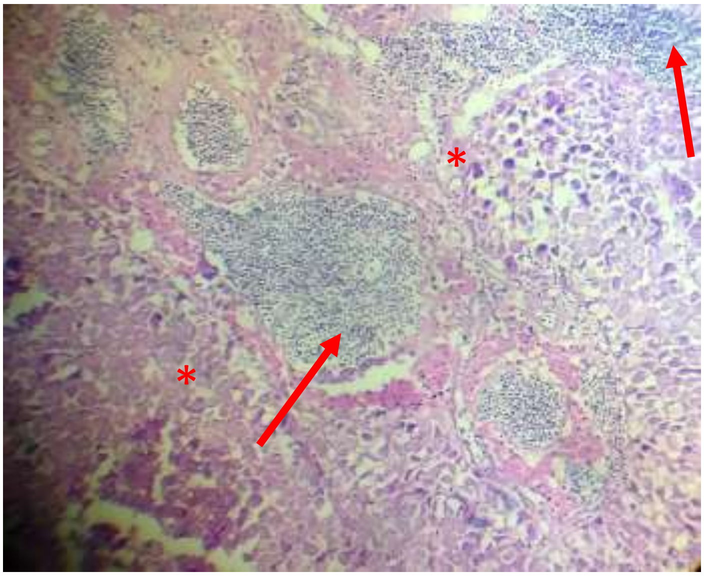

Figure 7: Liver of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing necrosis (red stars) with multifocal infiltration of inflammatory cells (thick red arrows). Hematoxylin-Eosin (2 x 10X).

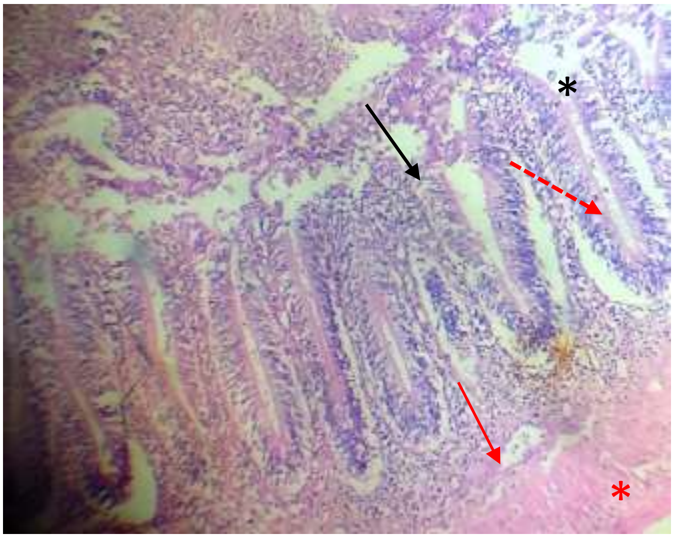

Figure 8: Intestine of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing necrosis of muscular layer (red star), infiltration of inflammatory cells (red arrows), hydropic degeneration (black star) of mucoid cells, hyperplasia of enterocytes (red dotted arrow) and desquamation of the epithelial of villi (black arrow). Hematoxylin-Eosin (1 x 10X).

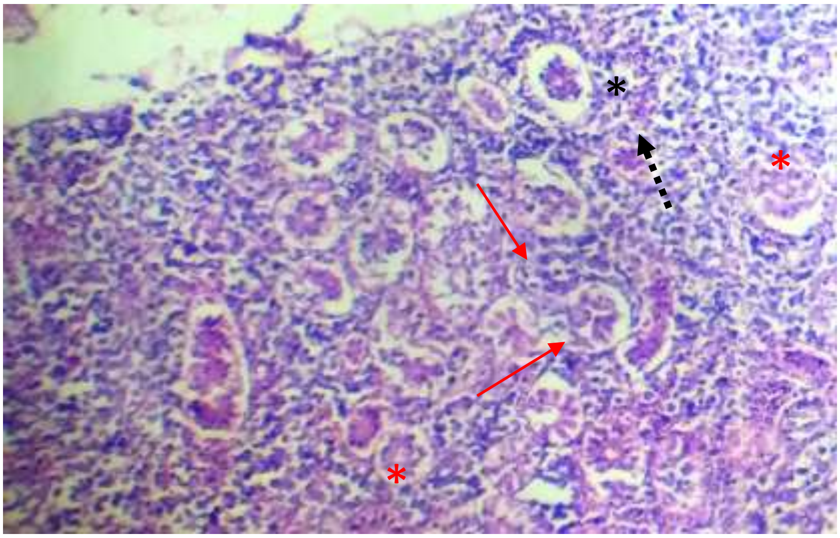

Figure 9: Kidney of *C. carpio* exposed to 13.8% of NaCl after 96 hours showing coagulative necrosis (red arrow), interstitial nephritis (red star) and atrophy of glomerular atrophy (black star) with dilated Bowman's space (black dotted arrow). Hematoxylin-Eosin (2.6 x 10X).

Discussion

Freshwater fishes are stenohaline which have the ability to tolerate salinity in aquatic environment at low concentration, but when they suddenly transferred to high salinity environment, they exhibited physiological changes and showed high mortality as a result of osmotic shock (Mubarik et al., 2019). From the results of this study, the mortality rate (MR) reached 70% after fish exposure to 13.8% of NaCl as during the first half hour to 96 hours of exposure. These results are in agreement with Hassan et al. (2013) who reported MR 100% in tilapia (*Oreochromis* sp.) exposed to salinity as 35 ppm. These may be due to osmotic and ionic regulatory losses. These mechanisms involve loss of water with concentrated plasma ions (Na^+, K^+, Cl^-) and with collapse and fish death (Koeypudsa & Jongjareanjai, 2011).

These variation in MR may depend upon fish age, species, and physiological status as well as on water column content from organic and inorganic materials. In addition to that ability of fishes to be acclimated to hyperosmotic environments experienced in two phases: Firstly, crisis phase in which there is an increase loss of water through gill-ion influx and elevated plasma ion and secondly due to regulatory or survival phase in which the ability of body fluid and function return to normal and plasma ion balance are restored (Evans et al., 2005).

Fish behavior plays important role as a bio-indicator of stress associated with exposure to toxic materials such as NaCl in which fishes have the ability to tolerate it at low concentration and loss these ability at high concentration (Al-Taee & Al-Hamdani, 2014). So, some of the present revealed investigation included abnormal behavior, nervous signs and hyperactivity, which are similar to findings demonstrated by Al-Taee (2007) in *C. carpio* when exposed to salt at a concentration of 1.5 ppm for 24 hours. High concentration of NaCl in aquaculture leads to hypernatremia which results from excessive uptake of Na^+ through apical membrane of gill epithelial tissue. Hypernatremia leads to hyper osmolality with loss of water from brain cells which caused shrinkage and lead to altering synaptic function (Castilla-Guerra et al., 2006).

Also, there was a respiratory distress represented by increase of opercular frequency (OF) as adaptive mechanism to salt concentration in aquatic environment (De Boeck et al., 2000). The reduction of OF is in agreement with the result obtained by Velasco-Santamaría & Cru-Casallas (2008). These may be due to reduction of salt uptake. Other adaptive mechanisms included excessive mucus secretion to reduce the salt toxicity, but they reduce oxygen intake, and hence fishes swim near water surface to increase oxygen intake (Soares et al., 2006).

Histological alteration in different organs give indicator about negative effects of xenobiotic impact (Yancheva et al., 2016). Gills are important organs to assess fish health and quantify aquatic environment. Gills have biological function in acid-base balance in fresh and marine water fishes (Strzyzewska et al., 2016).

In this study, when fishes were exposed to high NaCl concentration (13.8%), many pathological lesions had been detected in gills such as hypertrophy and hypersecretion of mucus cells and epithelial hyperplasia. These results are in agreement with Velasco-Santamaría & Cruz-Casallas (2008). It could be as a result of physiological functional compensation and considered as protective response to toxic irritants.

Severe damage in gill epithelial tissue leads to tissue hypoxia and increase capillary permeability and blood circulatory changes which lead to edema and severe epithelium lifting and lamellar damage (Roberts, 2001).

Liver is a vital organ for both metabolism and detoxification as well as it has a large blood supply which causes toxicant exposure (Mohamed, 2009). In the present study, the toxic pathological effects of NaCl were clear in the liver which were characterized by vacuolar degeneration and multifocal infiltration of inflammatory cells with necrotic area. Such lesions were represented in mrigal carp (*Cirrhinus mrigala*) as demonstrated by Velmurugan et al. (2007).

These alterations were resulted due to the denaturation of regulation of ATPase, which directly affecting or indirectly resulting from the processes for cellular energy transfer disruption, which led to impair ion regulation, inhibition of enzyme activity and changes in protein synthesis (Rajeshkumar & Munuswamy, 2011).

Intestine, kidneys and gills in fishes are main organs for elimination and preservation of the acid-base balance of body fluid (Ojeda et al., 2003). So, fish exposure to high concentration of sodium chloride causes histopathological changes. This result is in agreement with observation of Qin et al. (2017) who reported shrinkage of intestinal villi and pathological changes in *C. carpio* exposed to high dose (5%) of NaCl.

The kidneys in fishes, as in vertebrates, has important function due to electrolyte, osmotic balance and keeping stable internal environment. Additionally, they receive largest blood vessel supply so renal lesions which are considered as a good pointers of environmental contamination (Cengiz, 2006; Kurtović et al., 2008). So, fish exposure to high NaCl concentration leads to coagulative necrosis and interstitial nephritis with glomerular atrophy. All these changes can result from cell injury in the gills, which led to hypoxia. These cause disturbances in mitochondrial activity and decrease ATP and Na pump which led to increase Na^{+} and Ca^{+2} influx and efflux of K^{+}. As a result of consequence events, the hydropic degeneration, loss of villi, abnormality of cell membrane permeability led to tissue death (Zachary, 2017). It is concluded from this study that the use of NaCl in fish farms is within the permissible limits, it also concludes that the indiscriminate use of salt without relying on correct scientific methods may lead to physiological problems in fish that may lead to their death and the occurrence of histopathological changes, which are biological evidence for evaluate toxicity in the aquatic environment.

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