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Histopathological and Biochemical Biomarker Response of Mussel, *Unio Pictorum*, to Carbamate Pesticide Carbaryl: A Laboratory Study

Shelan Mustafa Khudhur and Yahya Ahmed Shekha*

Environmental Sciences Department,
College of Science, Salahaddin University-Erbil, Kurdistan Region, Iraq.
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ABSTRACT

An experiment was conducted to assess the effect of different concentrations of the insecticide carbaryl on histological and biochemical parameters including (SOD, GSH, rGSH, CAT and MDA) on gills tissue of freshwater mussel *Unio pictorum* for 96 hours. Significant increase in SOD and rGSH activities was observed in a concentration- dependent manner. However, statistically significant decrease in GSH levels was observed only at highest concentration. MDA levels reached higher rate at high concentration of carbaryl treated group. Mussels show behavioral responses during exposure by exhibiting increase in duration for shell closure and increase in mucus secretion. The histopathology of gills indicated that higher doses of carbaryl resulted in massive destruction in normal architecture of gill tissue. Molluscs accumulate contaminants in their body tissues and thus are used as bio-indicator for evaluating water quality and habitat degradation.

Key words: Water pollution, Pesticide, Carbaryl, Mussels, Histological, Biochemical assessment.

INTRODUCTION

Water pollution causes serious problems in the aquatic environment through the use of indiscriminate pesticides including fertilizers, herbicides and insecticides (Mundhe *et al*., 2016). Consequently, pollution affects the species composition and biodiversity of the aquatic community (Xu *et al*., 2014). Bivalve molluscs have a major role in the evaluation of contaminant levels and are widely employed in toxicity assessment worldwide. This is due to several reasons including, easy collection, appropriate size, their distribution and relatively sedentary habits (Kumar *et al*., 2012). Histological alterations and biochemical changes of gill tissues produced by the chemical stress causes disturbed metabolism, enzyme inhibition, retardation of growth, fecundity reduction and longevity of the organism, which will affect balance in the ecosystem (Solé *et al*., 2018). Carbaryl is one of the most common pesticides used in agricultural fields around freshwater reservoirs for pest control in northern Iraq. Therefore, the objective of this study was to investigate the effects of carbaryl on histopathological changes, antioxidant status and oxidative stress biomarkers in freshwater mussel gills. There was also an attempt to gain access to the utility of these parameters as biomarkers of carbaryl exposure.

MATERIALS AND METHODS

Physicochemical parameters: Physico-chemical parameters of surface water were measured according to standard methods of APHA (Rice, 2017).

Sampling: Adult freshwater mussels, *Unio pictorum* were collected from Greater Zab River which is located in the mountain area about 60 Kilometers northeast of Erbil city, Iraq. They acclimated for 10 days in a glass tank each of which containing 20 animals. The water in the aquarium was renewed daily and ventilated mechanically. The natural photoperiod of 13:11(Light: Dark) hours were kept.

Selection of Pesticides: The commonly used insecticides carbaryl (WP %85) (1-naphthyl methyl carbamate) was chosen for this study.

Sub-lethal studies: The effects of different increasing concentrations of Carbaryl was conducted by exposing the mussels (6 groups containing 10 mussels in each group) to sub-lethal concentrations of Carbaryl (0, 0.5, 1, 1.5, 2 and 2.5 mg/L) of the test chemical under laboratory conditions for 1, 24, 48, 72, and 96 hours. At the end of the experiments, the animals were dissected and gill tissues collected for the histological and biochemical analysis (Stalin *et al*., 2017).

HISTOLOGICAL ANALYSIS

The histological examination was carried out at the laboratory of the Department of Biology, Salahaddin University/ College of Science. The gill tissue samples were placed in Bouien's fluid for 24 hours and followed by dehydration using a gradient of ethanol in increasing concentrations (50, 70, 95 and 100%). Then the samples, were immersed in xylene and, later embedded in paraffin wax. Five micrometer thick tissue sections were cut using rotary microtome (Bright, MIC) and stained by haematoxylin

*Corresponding author's e-mail: shelan.khudhur@su.edu.krd , yahya.shekha@su.edu.krd

and eosin (H&E) (Suvarna *et al*., 2018). The paraffin tissue sections were examined under a light microscope mounted with a camera and Image Analysis Software utility.

BIOCHEMICAL ASSAYS

Tissue homogenization: Pieces gill tissues of each bivalve were washed in ice-cold normal saline solution. The homogenization was then carried out by handheld glass homogenizer in a 20 mM phosphate buffer. Homogenates were centrifuged at 4,000 rpm at 4°C for 10 minutes. The supernatants were collected and stored at -80°C until for later assay. Each parameter was measured in triplicate.

The concentrations of each malondialdehyde (MDA) as a marker of lipid peroxidation, reduced glutathione (GSH) concentrations, glutathion peroxidase (GSH-PX), catalase (CAT) and super oxide Dismutase (SOD) activities were measured by using Elabscience kits (Lohiya *et al*., 2018, Alnahdi *et al*., 2018).

Statistical analysis: All statistical analyses were carried out using IBM SPSS statistica 25 software. Mean values of the experiments were tested for differences using one-way analysis of variance (ANOVA), with Duncan's post hoc test at the 0.5% probability level ($p<0.05$). All the results were expressed as Mean ± Standard Error (S.E).

RESULTS AND DISCUSSION

Physicochemical properties or the quality of the river water investigated did not change throughout the period of the study (Table 1) and this was within the recommended range for freshwater mussel culture as mentioned by Pandey *et al.* (2016).

Histological alteration: Histological examinations are quick, easy to perform, reliable, sensitive and relatively cheap techniques used to interpret tissue changes as indicator against stress caused by pollutant (Yavaşoğlu *et al.,* 2016). Histology of gill tissues of mussel in control group showed uniform arrangements of the lamellae (L) with uniform inter-lamellar space (ILS) and healthy epithelial cells. Cilia covered the surface of each lamellae or filament and connective tissue was integrated into the base of the gill lamellae (Plate1; Fig 1). Gill tissue consists of two plates on each side of the body; each of these plates consists of a number of filaments lined by columnar epithelial cells with ovoid nuclei and the core of the plates consists of loose connective tissue (El-Shenawy *et al.,* 2009).

Table 1: Physico- chemical characteristics of water used in the experiment.

Characteristics of water	Mean ± S.E.
Dissolved oxygen (mg/L)	8.750 ± 0.02887
pH	8.030 ± 0.006
Water Temperature (°C)	16.97± 0.033
Total Hardness (mg/L)	174.0 ± 4.619
Total Alkalinity (mg/L)	9.400 ± 0.05773
total ammonia (mg/L)	6.057 ± 0.05544

In the present study histopathological examination showed morphological alterations in gill tissues of *Unio pictorum* revealing different changes consistent with increment in the concentration levels of the pesticide. During the investigation, excessive mucus secretion and increase in shell closure duration were noticed throughout treated groups. These findings may explain a defensive reaction from the mussels under investigation as similar results were studied by other investigators. Thus, the results of our study are in agreement with Kumar, *et al*. (2012) who studied the effects of dimethoate toxicity and behavioural responses in freshwater mussel *Lamellidens marginalis*. The molluscs increased mucus secretion as common reaction to stress (Pandit and Mundhe, 2013).

Following 96 hours of carbaryl treatment mussels by exposure of the experimental groups to 0.5 mg/L carbaryl, the epithelial lining exhibited alteration due to swelling of the gill filaments. Hypoplasia of epithelial cells and interlocking clumps of cilia which covers gill filaments were also damaged. At certain points in connective tissue, the core has been changed such as expansion in capillaries, normal structure breakdown and vacuolization (Plate 1; Fig 2). Whereas organism exposed to 1 mg/L carbaryl indicated total loss of gill architectures and damaged inter-lamellar junctions. In spite of severe disruption of gill rods, small tufts of cilia were noticed on the top and lateral sides and their shapes were disrupted because of necrosis. There has been a severe loss of epithelial cells by cell death. Also, tissue ruptures in connective tissue and atrophy of the structure of haemolymph channels have been observed in many cases (Plate1; Fig 3). Organism exposed to 1.5 mg/L carbaryl displayed change in the length of gill lamellae with lengthening gill rods toward inside and clubbing of their shapes was along with epithelial cell hyperplasia. Swelling and hyperplasia of the epithelium in haemolymph channels have been observed in the inner parts. In the inner sides of the gill lamellae, granulated cells also appeared. In connective tissue, regular cell structure disintegration and tissue rupture were observed (Plate1; Fig 4). Histolopathological changes including granulated cell appearance, elongation of gill rods toward inside, vacuolization in connective tissue, appearance of inflammatory cells, nuclear hypertrophy, swelling and shortening changes were all observed in gill tissues of bivalves after exposure to 2mg/L carbaryl pesticide (Plate1; Fig 5). The epithelial cells showed total structure alteration in organisms treated with 2.5 mg/L of carbaryl. The epithelial cell shapes were lost, reduced in length with swallowed lumen and pycnotic changes in nuclei. Moderate necrotic changes in interlamellar epithelial cells were also observed. The connective tissue showed vacuolization and disintegration (Plate1; Fig 6).

The observations made in the present study indicate that mussels exposed to carbaryl exhibited several alterations

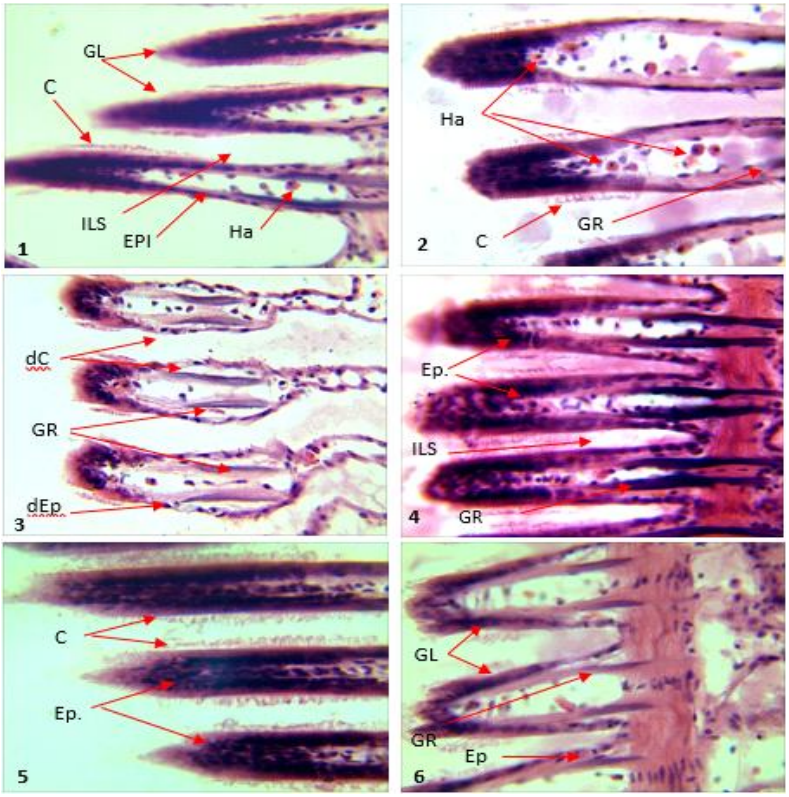

Fig 1: Micrograph of transverse sections (5-7 µm thickness, stained with H and E) through gill tissues of *U. pictorum*. Control (1) and carbaryl exposed (2, 3, 4, 5 and 6) specimens. (GL): gill lamellae, (ct): connective tissue; (c): cilia, (ep): epithelium; (ha): haemocyte; (tr): tissue rupture; (dc): damaged cilia; (Ep): epithelium rupture; (d-ep): damaged epithelium;(gl-j): junction of adjacent gill lamellae.

in the tissue architecture of the gills. Similarly, these alterations were reported previously by (Katalay *et al.*, 2016; Yavaşoğlu *et al.*, 2016). They showed changes in gill epithelium such as pycnotic nuclei of epithelial cells and connective tissue necrosis, decrease in intrallamilar space, elongation of gill filaments and, damage of the supporting chitinous rods.

The histopathological observations in the current study confirm that carbaryl has affected gill cells and the damage in the gill tissue architecture is more severe with higher concentrations.

Biomarkers of Oxidative Stress: The effect of carbaryl on oxidative stress related toxicity was studied including measurement of SOD, GSH, rGSH, CAT activities (nmol min^{-1} mg^{-1} protein) and MDA content (nmol mg^{-1} protein) in gills tissue of freshwater mussels *Unio pictorum* (Figs 1-5), respectively.

Results showed that with increased carbaryl concentrations, the activity of SOD was significantly ($P \leq 0.05$) changed in the exposed duration of 1–96 hours in the treated mussels. There was a significant elevation of SOD activity after exposure to the different carbaryl concentrations compared to control as shown in Fig 2 A. Different organisms can promote their antioxidant enzymes like SOD in order to protect their tissue against free-radicals harmful effects such as O_2 (Ighodaro and Akinloye, 2018). Increase in SOD activity can stimulate the dismutation reaction of the superoxide anion and stimulation of antioxidant defense system. Thus, SOD is known to play a key role in the antioxidant protection of invertebrates (Pandey *et al.*, 2018, Basopo and Naik, 2016).

Glutathione related metabolism was the most frequently affected among antioxidant-based biomarkers. The highest decrease in total activity of glutathione in gills has been observed in mussels treated with 2.5 mg/L carbaryl as presented in Figs 2 B compared to control. Significant increase in rGSH activity was observed in high - dose carbaryl treated groups (2.5 mg/L), however the increase was not significant in other treated groups (Fig 2 C). in agreement to our study, similar results were observed for other mussel species when the activity of glutathione was decreased significantly in digestive glands and gills after cypermethrin exposure (Pandey *et al.*, 2016). However, significant decrease in GSH levels was observed only at high concentration of permethrin (Khazri *et al.*, 2017). Also Köprücü *et al.*, (2010) reported that the reduced GSH is the main non protein thiol and one of the key reductants found in cells. GSH have antioxidant properties, and its protective

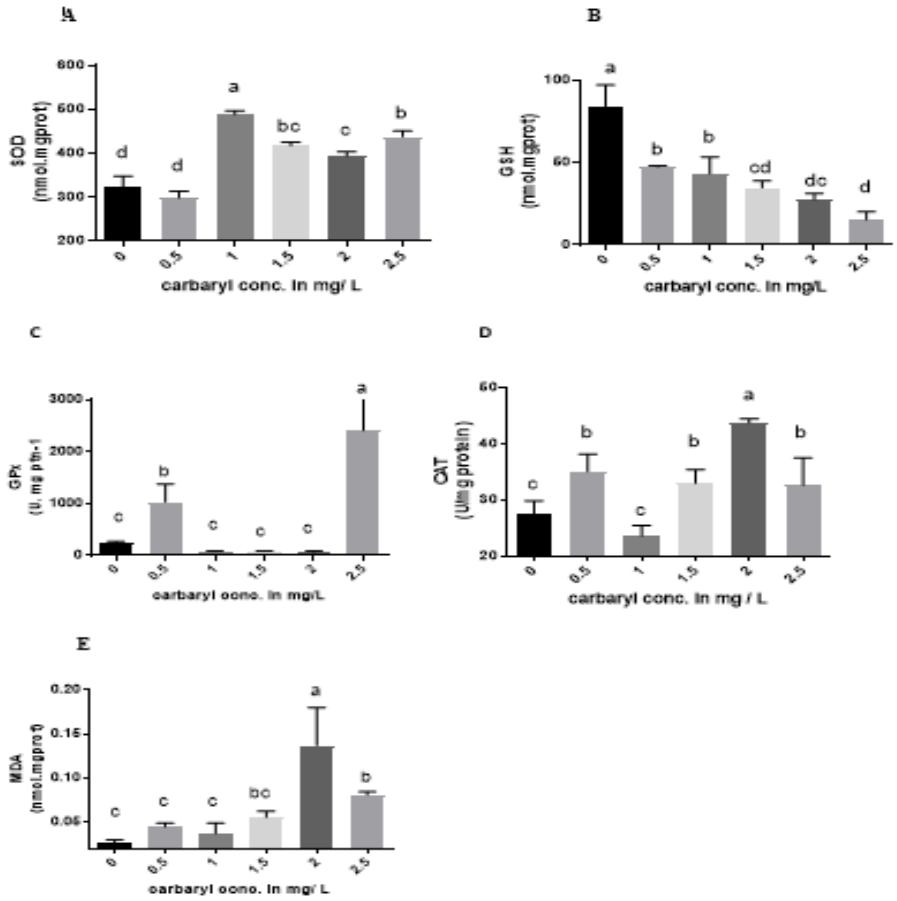

Fig 2: Activity of (A) Superoxide dismutase (SOD), (B) Total glutathione (GSH), (C) Glutathione peroxidase (GPx), (D) Catalase (CAT) and (E) Malondialdehyde (MDA) in the gills of U. pictorum exposed to different doses of carbaryl. Values are presented as mean ± ESM. Different letters indicate significant differences between the control and the exposure groups of the treatment ($p<0.05$).

role against oxidative stress-induced toxicity in aquatic animals is well studied. The present study showed that the GSH content in some treated groups was rather stable during the exposure period. It may be an evidence that GSH is being produced by GSSG reduction and not because its need due to involvement in other metabolic processes including, ascorbic acid metabolism, maintenance of intercellular communication and prevention of protein thiol (– SH) groups from oxidizing and cross-linking (Matos *et al*., 2007).

Furthermore, variation in the activity of catalase (CAT) was observed due to exposure and the effects of carbaryl in mussels *Unio pictorum* gills (Fig 2 D). There is a clear variation between the treated and control groups. Significant increases in CAT activity obtained with increases in carbaryl dose exposure. The highest CAT activity (2 mg/ L) recorded in 2 mg//L, which was statistically significant from other treatments. The trend of CAT activity is in accordance with previous studies in which some pesticides caused increase in CAT activity. This elevation of CAT activity in gills helps in detoxification of the pesticide (Khazri *et al*., 2017). CAT is known to be of particular importance when the clearance of H_2O_2 in high concentrations is required. The early activation of CAT and GPX and the high intensity of their response indicate that these enzymes are the front line of defense against carbaryl induced oxidative stress in *C. apertus* and that peroxide over production represents one of the main mechanisms of carbaryl induced pro-oxidative condition (Matos *et al*., 2007). In present study, decreased CAT activities in some treated groups could be due to flux of superoxide radicals generated during oxidative stress, which have been reported to inhibit CAT activity as mentioned by Pandey *et al*., (2016).

Regarding MDA, an increase in MDA levels in gill tissues of mussels *U. pictorum* was observed especially at the last two concentrations of the pesticide with highest statistically significant ($P\leq0.05$) value recorded in 2 mg/L (Fig 2 E). These findings with regard to the MDA levels are compatible with other study results (Khazri *et al*., 2017), who discovered that MDA increased in gill cells treated with high concentration cypermethrin (CYP) in freshwater mussels (*Unio elongatulus eucirrus*). The explanation of increasing in MDA may suggest that CYP may penetrate in

to the cellular lipid membrane that may disturb the phospholipid orientation and may cause changes in membrane fluidity. When a tissue or cell cannot prevent oxidative damage, lipid peroxidation increases, measured as an increase in level of MDA (Al-Fanharawi *et al*., 2018).

CONCLUSION

From the present study, we can conclude that exposure to sub- lethal carbaryl concentration leads to histological alteration in structure of gill tissue and biochemical changes (observed as oxidative stress) in gills of freshwater mussel *U. pictorum*. We can indicate from our results the potentially negative impacts of carbaryl insecticide on freshwater invertebrate in general and *U. pictorum* in particular. River pollution by carbaryl insecticide from agricultural activities, create unsafe condition for the aquatic life in the rivers.

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