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Histopathological and cholinesterase changes in the gills of *Clarias gariepinus* as a result of cadmium exposure

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Abstract

Aim : The cholinesterase (ChE) based inhibition and histopathological studies from fish were investigated and represented in this study to develop as one of the great potential biomarkers for heavy metals monitoring.

Methodology : In this study, the histopathological study of gills were observed a under microscope. The capability of ChE extracted from the gills of *Clarias gariepinus* was assessed for declining Cd. ChE was purified through affinity chromatography and continued with the optimisation and inhibition study (IC_{50}) of cholinesterase.

Results : Histopathological study of gills was carried out and several changes such as aneurysm, necrosis and lamella fusion were noted. Purification fold obtained from purified enzyme was 1.15 with 30% a yield specific activity 20.726. The optimum temperature for purified AChE was 35°C along with acetylthiocholine iodide (ATC) as a preferable substrate that had the highest V_{max} value of 0.5452 U mg^{-1} and the lowest K_m value of 0.0311 mM. The optimum pH was observed to be 10 of Tris-HCl as a medium. Meanwhile, the IC_{50} of cadmium was 6.808 $mg\ l^{-1}$ with R^2 value of 0.9532.

Interpretation : The result of the study can be used as a tool for further developing a biomarker for the detection of heavy metals in aquatic ecosystems. In addition, the baseline data provided can also be used for designing a kit, which would give rapid and accurate result.

Key words: Acetylcholinesterase activity, Biomarkers, Cadmium, *Clarias gariepinus*, Histopathological study

Cholinesterase activity and histopathological
changes as a biomarker

Dissection of gills from *Clarias gariepinus*

Extraction of cholinesterase from
gills of *Clarias gariepinus*

Purification of cholinesterase from
gills of *Clarias gariepinus*
• Affinity chromatography

Optimisation of cholinesterase
• Substrates
• pH
• Temperature

Inhibition study (IC_{50})

Histopathological
study by Light inverted
microscope

Development of biomarker
for the detection of heavy
metals in aquatic ecosystems

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Introduction

In recent years, pollution of aquatic environment by heavy metals has become a global problem. Heavy metals enter the aquatic environment either by natural or anthropogenic sources. The natural sources include aerial fallout, industrial/urban effluents, as well as surface run off from the anthropogenic sources. The food chain is endangered as marine life is the main protein and top consumption source (Förstner *et al.*, 2013). These problems exacerbate since metals get transported with sediments, persistent in the environment and can bio-accumulate in the food chain. Pollutants significantly affect the aquatic life and all trophic levels from primary producers to predators, ultimately humans. It may disturb the organisation of many freshwater fish species and other aquatic communities and hampering the normal balance of the ecosystem (Padrilah *et al.*, 2017; Todd *et al.*, 2010; Agtas *et al.*, 2007).

Cadmium is regarded as one of the most toxic heavy metals as it causes sublethal and behavioural effects on the organisms at lower concentrations (Eisler, 1985). This has been proved in an experiment showing the ability of heavy metals to modify the chemical communication between individuals, which can result in info-disruption or false communication affecting ecological relationships within and between the species (Boyd, 2010). Moreover, cadmium is capable to accumulate in each organ, especially liver and kidney. The gills of fish are the primary target to toxicant before being transported to other organs, deposit in the muscles or are partially eliminated via excretion. Due to high accumulation of cadmium in muscles, the fish is considered unsafe for consumption. Continuous exposure to polluted water bodies such as river, ocean and sea which also contributes to the accumulation of heavy metals in fish (Sulaiman and Mei, 2013). A study conducted at Strait of Malacca demonstrated the presence of Cd in fish sample (Alina *et al.*, 2012).

Fish are known to be an excellent biomarker due to its sensitivity towards toxicants (Hayat *et al.*, 2016; Sabullah *et al.*, 2015b; Tham *et al.*, 2009). The gills of freshwater fish are the largest area of the body, direct in contact with the water (Kumar *et al.*, 2015). Pollutants study revealed that gills are the first targets, which are involved in the breathing mechanism of fish (Hayat *et al.*, 2015). Pollutants enter fish through gills and release their primary toxic effects on the brachial epithelium (Playle *et al.*, 1992), therefore gills are selected parts to study the response of environmental stress and to indicate physical and chemical stresses (Au, 2004). *Clarias gariepinus* (catfish) is used as a biomarker for observing the effects of cadmium on the enzyme cholinesterase extracted from the gills. It is widely used in aquaculture and has become the subject of numerous studies in biochemistry (Hee and Kottelat, 2008).

Cholinesterase (ChE) inhibition has been used extensively as a biomarker for detecting heavy metal pollution (Ahmad *et al.*, 2016c; Hayat *et al.*, 2017). In this study, the potential of ChE from the gills of *C. gariepinus* as an alternative

biosensor for heavy metals was evaluated. Previous studies have reported that there can be more than one ChE present in the tissues of fish and these different forms may vary in their sensitivity to anticholinesterase agents (Monteiro *et al.*, 2005; Padrilah *et al.*, 2017). In the present study, attempts were made to elucidate the effect of cadmium on the histopathology and cholinesterase enzyme activity in gills of *Clarias gariepinus*. Moreover, the objective of this study was to determine the physical and behavioural changes of *C. gariepinus* inhibited by cadmium using in vivo method and to identify the minimal concentration and half maximal inhibitory concentration (IC₅₀) of cadmium that would produce significant changes in *C. gariepinus*.

Materials and Methods

Acclimatization and treatment: Irrespective of sex, healthy *C. gariepinus* weighing 100 g body weight and 20 cm long were procured from the Department of Fisheries, Semenyih, Selangor, Malaysia, were brought alive and acclimatized to laboratory conditions for five days in large glass aquariums supplemented with 40 l of filtered dechlorinated tap water with full aeration. *C. gariepinus* were placed in glass aquariums containing filtered water and fed with commercial fish pellets (floating type) twice a day where the total pellet mass was 2% of the fish weight that contained 28-32% of protein level prior to the study. Fish were randomly distributed in eight groups of six fishes in each aquarium, with three replicates. Fish were treated with different concentrations of Cd (1, 5, 10, 20, 30, 40 and 50 mg l⁻¹), for 96 hrs to generate LC₅₀ data. These concentrations were selected to provide a baseline data on the concentration that trigger negative effects on the species. The water was filtered through the sponge layer filter to trap the remaining food waste and excrement, while hygiene and cleanliness of the water was maintained by renewing the filters daily. After the end of treatment, physical behaviour and morphological changes in the fish were observed.

Histopathological studies : Gills of treated and control fish were fixed in 10% neutral buffered formalin for 48 hrs to inhibit decaying or autolysis of tissues so that the samples could be preserved for future study. Samples were then placed in the cassette and fixed again in 10% formalin. Samples were then subjected to tissue processing in which the cassette containing samples were immersed in alcohol with a series of ascending concentrations at a given time (80% alcohol for 2 hrs, 95% alcohol for 2 hrs and 100% alcohol for 3 hrs), followed by chloroform and paraffin for 3 and 5 hrs and 30 min, respectively. The samples were then embedded into the wax and placed on a cold plate for 15 min until they became a block. After being embedded in wax, the samples were sectioned at 5 µm thickness using a microtome and then stained using haematoxylin and eosin staining.

Extraction and purification: Gill samples were homogenised by adding 0.1 M sodium phosphate buffer (pH 7), containing 1 mM of phenylmethylsulfonyl fluoride (PMSF) (gill: buffer ratio is 1:4) using an Ultra-Turrax T25 homogeniser. The homogenate was centrifuged at 15,000 x g at 4°C for 20 min. The supernatant

collected after centrifugation was kept at -25°C in a clean Falcon tube for further purification. The purification process was carried out by loading 15 ml supernatant into the affinity column containing procainamide sepharyl 6B. 20 mM of sodium phosphate buffer with pH 7.0 (washing buffer) was added onto the column with a flow rate calibrated at 1.0 ml min^{-1} for washing. This stage is crucial as it eliminates the unbound protein from the column to the matrix. The absorbed enzyme was then eluted using 20 mM sodium phosphate buffer containing 1 M NaCl. Fractions of 1 ml were collected starting from washing stage until the end of elution stage. Each fraction was tested for enzyme activity and protein content following the method of Ellman *et al.* (1961) and Bradford (1976), respectively.

Estimation of acetylcholinesterase (AChE) activity and protein: AChE activity was estimated following the method of Ellman *et al.* (1961) with slight modification. A 96-well microplate was selected for examining the enzyme activity at 405 nm wavelength. Each microplate comprised 200 μl sodium phosphate buffer (0.1 M, pH 7.0), 20 μl of DTNB (0.1 mM) and 10 μl ChE, which were mixed and incubated for 15 min. Later, 20 μl of the substrates comprising 5.0 mM of acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) was added to the mixture and incubated for 10 min for the reaction to take place.

Optimal substrate specificity, pH and temperature: Purified ChE activity was tested with three synthetic substrate of 0.1 M ATC, BTC and PTC of different concentrations (0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 mM) to determine the substrate specificity for ChE in sodium phosphate buffer (0.1 M, pH 7.0). Michaelis-Menten constant (K_m) and maximal velocities (V_{max}) of ChE activity were determined using GraphPad Prism 5 (Miller and Miller, 2000). The optimum pH was determined by incubating ChE with an overlapping buffer system that contained 0.1 M acetate buffer (pH 3.0, 4.0, 5.0, 5.5), sodium phosphate buffer (pH 6.0, 7.0, 8.0) and Tris-HCl buffer (pH 7.0, 8.0, 9.0, 10.0). To determine the optimal temperature for the enzyme, ChE sample was incubated for 15 min at 15, 20, 25, 30, 35, 40 to 50°C .

Half maximal inhibitory concentration (IC₅₀): The effect of cadmium on *C. gariepinus* ChE were studied for a period of 96 hrs. The reaction mixture contained 150 μl of Tris-HCl buffer (0.1 M, pH 9), 50 μl metal ion with a final concentration of 1 mg l^{-1} , 20 μl DTNB (0.1 mM) and 10 μl ChE. All mixtures were incubated for 15 min 20 μl of ATC (2.5 mM) was loaded into the mixtures followed by incubation with 10 min period. The absorbance was read at the end of incubation period at 405 nm. The concentration of cadmium ($1\text{--}50\text{ mg l}^{-1}$) inhibition profile was analysed and IC₅₀ cadmium was obtained by GraphPad Prism software.

Statistical analysis: GraphPad Prism Software (Version 5) was used to analyse the data. In the characterisation study, values are represented as mean $\pm$ SD. Student's t-test and one-way analysis of variance followed by post-hoc analysis by Tukey's test was used in evaluating statistical significance ($p < 0.05$) between groups.

Results and Discussion

During the study, the behaviour of fish was observed to be normal during the acclimatization period and at lower concentration of cadmium (1, 5 and 10 ppm), the swimming rate and rate of excretion of fish were similar to control, considering no significant effect ($p < 0.005$) (Table 1). The change in terms of decrement in excretion was observed from 20 to 50 ppm of cadmium exposure. Visible change observed in most body parts of fish were damaged scales and lower swimming performance, which may be due to loss of energy or lethargy.

The fish were constantly found to remain at the surface, which may be due to difficult breathing as their gills were damaged. The gills may undergo loss of cellular architecture and cell degeneration (Chavan *et al.*, 2014). Gills are the primary site of osmoregulation and respiration in aquatic vertebrates involved in the abundant secretion of mucus in a way to adapt to their own habitat (Athikesavan *et al.*, 2006). The presence of heavy metals in the aquatic environment may increase mucus secretion which is one of the defence mechanism towards pathogen or commensals. Gills are the main target organ to be affected easily when an organism is exposed to dissolved heavy metals, affecting the fish in terms of behaviour, morphology and histology.

The fish exposed to 1, 5, 10, 20, 30, 40 and 50 ppm of cadmium showed several histopathological alterations (Fig. 1) such as aneurysm, lifting of respiratory epithelium, necrosis and fusion of secondary lamella. In a study conducted by Butchiran *et al.* (2012) similar changes as epithelial hyperplasia with lamellar fusion, epithelial hypertrophy, telangiectasia, oedema, general necrosis, increased mucous production and degeneration of primary and secondary gill lamellae were observed in the gills of *Labeo rohita* exposed to phenol. Aneurysm results due to the accumulation of blood in the secondary lamellae to protect the gill epithelia from infection and mechanical abrasions (Mabika *et al.*, 2013) which might be used as a sensitive and reliable biomarker for acute toxicant exposure to metals, including cadmium and copper.

Epithelial lifting is one of the common acute inflammatory responses from the gills to pollutants by increasing the distance of pollutant and blood diffusion, impairing oxygen uptake. However, under these conditions, the fish increases its respiration rate by compensating for low (Mabika *et al.*, 2013). Zeitoun and Mehana (2014) reported that the fusion of secondary lamella occurred in *Tilapia nilotica* exposed to environments that are heavily polluted with heavy metals as the fish were trying to limit the surface area of gills exposed to pollutant. That can be considered as a protective method. Necrosis occur when cells are highly damaged and can not perform common biochemical process, forcing it to die and rupture.

Acetylthiocholine indicates the highest amount of enzyme activity (29.75 U) in crude extract that shows the most preferable substrate in gills. Acetylthiocholine as the most

Table 1: Behavioural changes observed in *C. gariepinus* for 96 hrs during acclimatization and treatment

Observation	Acclimatization		Heavy metal treatment (ppm)						
	For 6 Days	0	1	5	10	20	30	40	50
1) Fish behavior									
Swimming performance	****	****	****	***	***	***	**	**	**
i. Swimming rate	UD,B	UD,B	SASB,B	SASB,B	SASB,B	S	S	S	S
ii. Swimming pattern									
(b) Fin movement	BW	BW	BW	BW	BW	BW	BW	BW	BW
(c) Excretion	***	***	***	***	***	**	**	**	**
(d) Mucus like secretion from gill	*	*	**	**	**	**	***	***	***
(e) Scale condition	****	****	***	***	***	***	**	**	**
2. Food Intake	****	****	***	***	***	**	**	**	**

Indicators: *: very little, **: little, ***: moderate, ****: high; UD = up & down, SASB = some above, some below, B = at the base of water, S = at the surface of water, BW = backward

Fig. 1: (A) Normal gill tissue of *C. gariepinus* (4x magnification). a) Normal primary lamella, b) Normal secondary lamella. B) Gill tissue exposed to 5 mg l⁻¹ of cadmium (4x magnification). a) Aneurysm effect on the primary lamella, b) Necrosis effect on the secondary lamella, c) Lifting of the respiratory epithelium. (C) Gill tissue exposed to 10 mg l⁻¹ of cadmium (4x magnification). a) Aneurysm, b) Necrosis, c) Lifting of respiratory epithelium, d) Fusion of secondary lamella. (D) Gill tissue exposed to 30 mg l⁻¹ of cadmium (4x magnification). a) Aneurysm, b) Necrosis, c) Fusion of lamella. E) Gill tissue exposed to 50 mg l⁻¹ of cadmium (4x magnification). Completely degraded and damaged gills by the heavy metal.

Fig. 2 : Inhibition of cholinesterase activity against varying cadmium concentrations. Error bar represent mean of three replicates $\pm$ S.D.

abundant form of cholinesterase in gills has been reported earlier (Jebali *et al.*, 2013; Mahboob *et al.*, 2014). In the present study, the enzyme activity decreased by almost half at 1 ppm and became stable at 5 ppm and 10 ppm and drastically reduced at 20 ppm (Fig. 2) because the gills were damaged due to cadmium toxicity. Purification fold obtained from purified enzyme by affinity chromatography of Procainamide-Sepharose 6B was 1.15 with 30% yield (Table 2), shows the ability of affinity chromatography to purify the protein. Mehrani (2004) and Li *et al.* (2008) demonstrated that Procainamide-Sepharose affinity chromatography is more suitable and effective way to purify cholinesterase and could eliminate a remarkable amount of proteins including albumin. The study conducted by Tham *et al.* (2009) on purification of *Clarias batrachus* cholinesterase

showed that the partial purification of enzyme was successful with approximately 5-fold purification and a good yield of nearly 50%. Fig. 3 shows Michaelis–Menten kinetics curve formed using GraphPad Prism 5. The curve represents the result of hydrolysis by ChE for the substrates. Table 3 demonstrates different values for the enzyme activities when reacted with three types of substrates. Substrate acetylthiocholine recorded the highest V_{max} value of 0.5452 and the lowest K_m value of 0.0311, suggesting the highest efficiency of 17.53 as compared to others. This result corr. Nigam *et al.* (2014). Hayat *et al.* (2016) recorded that ChE from *L. calcarifer* gills was capable of breaking down acetylthiocholine at a faster rate compared to other two synthetic substrates, *i.e.*, butyrylthiocholine iodide and propionylthiocholine iodide. Three types of buffer used for optimisation

Table 2 : Purification of ChE using ATC as substrate

Sample	Volume (ml)	Enzyme activity (U)	Total activity (U)	Protein content (mg ml ⁻¹)	Total protein (mg)	Specific activity (U mg ⁻¹)	Purification fold	Yield (%)
Crude	50	29.754	1487.745	2.551	127.563	16.561	1	100
Affinity	15	26.335	395.028	0.787	20.726	20.726	1.151	30

Table 3: Comparison of maximum velocity (V_{max}) and biomolecular constant (K_m) for ATC, BTC and PTC of *C. gariepinus* ChE

Substrate	V_{max} Specific activity (U mg ⁻¹ protein)	K_m , mM	Catalytic efficiency (V_{max}/K_m)
Acetylthiocholine iodide	0.5452	0.0311	17.5305
Butyrylthiocholine iodide	0.2111	0.4169	0.5063
Propionylthiocholine iodide	0.5052	0.0450	11.2267

Fig. 3 : Substrate specificity profile of three synthetic substrates, namely, acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) at different concentration ranging from 0 to 2.5 mM incubated with partially purified ChE from gill extract of *C. gariepinus*. All values are mean of three replicates $\pm$ S.E.

process were acetate, phosphate and Tris-HCl buffers at different pH range. Fig. 4A shows comparison between three types of buffer showing the most optimum parameter for highest AChE activity at pH 10 using 0.1 M Tris-HCl. Similar results were recorded by Sabullah *et al.* (2014), who demonstrated that pH 8 of Tris-HCl was most optimum for ChE activities in different types of fish used for the study. The assay for the most optimum buffer and pH is important for the later stage of the study as unfavourable condition can negatively impact the enzyme. Masson *et al.* (2002) reported that pH change can cause protonation of imidazole group of histidine that exist at catalytic triad, thus affecting the formation of enzyme-substrate complex.

The optimum temperature for AChE activity in gills was 35°C using pH 10 Tris-HCl buffer (Fig. 4B). When temperature was low, the enzyme activity was found to be lowest. However, with increase in temperature upto 35°C, the enzyme activity increased producing a bell shaped curve. The stability of protein was lost due to denaturation process, which explained decrease in enzyme activity above 35°C. Hayat *et al.* (2015), reported the maximum ChE activity in gills of *L. calcarifer* at temperature ranging of between 20 to 30°C. This result is also in accordance with the previous study of Sabullah *et al.* (2014), who reported that the optimum temperature for ChE extracted from *Puntius javanicus* ranged between 25°C to 35°C. In an

Fig. 4 : Optimisation of (A) pH and (B) temperature for ChE from *C. gariepinus*. Error bars represent mean of three replicates $\pm$ S.E.

another study Ahmad *et al.* (2016a) observed maximum activity of ChE in *Anabas testudineus* muscles in the range of 25°C to 40°C, while the maximum activity of blood ChE was observed at 30°C. It can be concluded that this particular enzyme works best at temperature ranging from 20°C to 40°C that prevails in Malaysia, a tropical country with warm temperature, high humidity and copious rainfall and average temperature of around 27°C (Hii *et al.*, 2016). The results this study indicate that cadmium induces toxicity on *C. gariepinus*, and AChE present in the gills of *C. gariepinus* may serve as a significant biomarker of cadmium induced neurotoxicity.

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