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Characterisation of cholinesterase and histopathological features of brain of *Clarias gariepinus* following exposure to cadmium

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Abstract

Aim : Cholinesterase (ChE) inhibition and histopathological features of brain tissues of *Clarias gariepinus* were investigated with a purpose to develop biomarkers for monitoring heavy metals.

Methodology : The effects of cadmium toxicity on *C. gariepinus* were assessed by ChE inhibition and characterisation of ChE by affinity chromatography and histological variations in brain tissues were studied by H&E staining under light microscope.

Results : The purified enzyme gave 10.58 fold purification with the recovery of 12% with specific activity of 159.729 U mg⁻¹. The Michaelis-Menten constant (K_m) and V_{max} value obtained was 1.985 mM and 0.4479 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. The enzyme showed the ability to hydrolyse acetylthiocholine iodide (ATC) at a faster rate compared to other two synthetic substrates, propionylthiocholine iodide (PTC) and butyrylthiocholine iodide (BTC). ChE showed highest activity at 35°C in Tris-HCl pH 7.5. The metal inhibition study also found the maximal inhibitory concentration (IC_{50}) value for cadmium to be 0.6517 mg l⁻¹. Histological study revealed decrease in the number of cells, degeneration of Purkinje cells, increased aggregation area and cells necrosis.

Interpretation : Inhibition of cholinesterase activity and degeneration of Purkinje cells observed in the present study can be used as a tool for further developing a biomarker for detecting heavy metals in the aquatic ecosystems.

Key words: Biomarker, Cadmium, Cholinesterase, *Clarias gariepinus*, Histopathology

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Introduction

Clarias gariepinus (catfish) is considered as one of the most significant fish species. Globally, African catfish is commercially grown due to its popularity as a common fresh water fish that is widely consumed. It has been recorded as a commercially important fish being resistant to several diseases (Mdegela *et al.*, 2010). They are particularly vulnerable and heavily exposed to aquatic pollution (Saleh and Marie, 2014). These organisms are often at the top of the aquatic food chain and may concentrate a large amount of metals from the surrounding waters (Rajkowska *et al.*, 2011). As known, the concentration of heavy metals in the aquatic environment have increased dramatically due to industrial, mining, agricultural and domestic activities (Abbas *et al.*, 2002). The metals gets bioaccumulated at different levels in various organs of aquatic organisms (Zaki *et al.*, 2016). Cadmium is a common environmental metal contaminant, and dispersion of cadmium in the environment has increased over the past decades due to its extensive use in nickel-cadmium battery production and metal plating (Sharma *et al.*, 2014). In general, cadmium is biologically non-essential, non-biodegradable, persistent type of heavy metal known to have high toxic potentials (Jaishankar *et al.*, 2014).

Some of the physiological effects of chronic exposure to cadmium at sublethal levels are manifested in the form of disturbances in respiration, reduction in growth, changes in hematology and enzyme activity that reveal the stress response in fish (Lacroix and Hontela, 2004). A study conducted by Al-sawafi *et al.* (2017) on zebra fish (*Danio rerio*) exposed to cadmium showed a significantly exponential increase over time and peaked at day 25, plus obvious tissue chaos was noticed in the treated fishes compared to the control group. Rani *et al.* (2015) also reported cholinesterase enzyme activity as an early warning biomarker for the presence of cadmium pollution of Indian major carps.

Fish are also ideal sentinels for behavioural assays of toxic chemical exposure due to their constant and direct contact with the aquatic environment due to the chemical exposure occurs over the entire body surface and ecological relevance in many natural systems (Halappa and David, 2009). Most behavioural study in fish under toxic media are swimming performance which exhibit irregular, erratic and darting swimming movements, hyper excitability, and loss of equilibrium and sinking to the bottom (Sharbidre and Patode, 2012). The behavioural changes may be due to the inhibition of acetylcholinesterase (AChE) activity. This is because inactivation of AChE activity results in excess accumulation of acetylcholine (ACh) in cholinergic synapses leading to hyper stimulation and cessation of neuronal transmission (paralysis).

Catfish is a prime cultured and an important staple freshwater fish, generally found in rivers, ponds and reservoirs. Contamination of aquatic biota at sublethal levels by cadmium is common in Malaysia, mostly in Langat River (Shazili *et al.*, 2006).

In view of this, the present study was undertaken to evaluate the behavioural responses, brain histology features and cholinesterase activity of *C. gariepinus* exposed to various concentrations of cadmium.

Materials and Methods

Fish sampling and treatment of CdCl₂: Healthy *Clarias gariepinus* weighing 100 ± 20 g and 20 ± 2 cm in length were bought from a commercial supplier in Semenyih, Selangor. They were brought to laboratory in well-aerated container to avoid physical injuries and stress to the fish. *C. gariepinus* were acclimatised under laboratory conditions for 5 to 10 days in a glass aquarium containing 40 l of dechlorinated water. During this time, fish were fed with commercial fish pellet to sustain fish health. After acclimatisation, *C. gariepinus* was exposed to different concentrations of CdCl₂ (1-50 mg l⁻¹) for 96 hrs, based on the study conducted by Okareh and Akande (2015) with LC₅₀ value of 16.3 mg l⁻¹.

Physiological and behavioural study: Behavioural responses, clinical signs and mortality rates were recorded. Behavioural pattern such as swimming, fin movement and food intake by fish were monitored. After 96 hrs of treatment, the number of dead fish in each group was recorded, and the remaining fish were removed from the aquarium, placed in an ice bath, and then sacrificed by decapitation.

Histopathological study: The brain tissue of fish were dissected and fixed in 10% neutral buffer formalin. The samples were then subjected to dehydration before being embedded in wax. A 5 µM thick sections of brain were cut and stained in hematoxylin-eosin. The stained sections were observed under microscope.

Preparation of crude homogenate: *C. gariepinus* was freeze-killed by immersing the fish in ice for about 30 min. The brain was removed and weighed for homogenising process. The brain collected was homogenised in 0.1 M sodium phosphate buffer (pH 7) containing 2 mM phenylmethylsulfonyl fluorides with the sample and buffer in 1:4 (w/v) ratio. Homogenisation was carried out using Ultra-Turrax T25 Homogenizer. Homogenate was then centrifuged at 10,000 xg in a Sorvall Ultra Pro-TH-641 centrifuge for 20 min at 4°C (Sabullah *et al.*, 2015a). The supernatant was collected and stored at -25°C in clean polycarbonate tubes.

Determination of cholinesterase (ChE) activity: The ChE activity of *C. gariepinus* was estimated following the method of Ellman *et al.* (1961) with slight modification. The enzyme assay was carried out by adding 10 µl crude sample into 96-well microplate containing 200 µl of sodium phosphate buffer (0.1 M, pH 7.0) and 20 µl of 5, 5'-dithiobiss (2-nitrobenzoic acid) (0.1 M). The sample was then incubated for 15 min before 20 µl substrates (5.0 mM acetylthiocholine iodide, butyrylthiocholine iodide and propionylthiocholine iodide) was added into the mixture and incubated for another 10 min to allow for the reaction to occur. ChE activity was expressed as the amount of substrate (mM) broken down by ChE per min (U) with the

extinction coefficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$ and the specific activity was expressed as $\mu \text{ mole min}^{-1} \text{ mg}^{-1}$ of protein or U mg^{-1} of protein (Ellman *et al.*, 1961). Protein content was determined by the method of Bradford, taking bovine serum albumin as standard (Bradford *et al.*, 1976).

Purification of ChE through affinity chromatography and SDS-PAGE : The purification process was carried out by affinity chromatography, which is routinely used to purify ChE from several sources. Partial purification of the enzyme was carried out. Procainamide-Sepharose 6B resin was packed into a column with dimension of 16 mm diameter and 50 mm height using 20 mM sodium phosphate buffer at pH 7.0 prior to loading the brain extract of *C. gariepinus*. The resin was prepared based on the method of Hodgson and Chubb (1983) with slight modification. The crude brain extract (240 μl) was loaded into the column. Washing was done by passing 10 ml of washing buffer (20 mM sodium phosphate buffer, pH 7.0) into the column at a flow rate of 1 ml min^{-1} . The elution process was then carried out using 20 mM sodium phosphate buffer (pH 7.0) containing 1.0 M sodium chloride. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure described by Laemmli (1970) for monitoring purification. The polyacrylamide gel was set up using combination of stacking gel (4%) and resolving gel (12%).

Effect of temperature, pH and substrate on enzyme activity : Purified ChE activity was tested by incubating enzyme with three synthetic substrates viz., acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) at 0.1 M (pH 7.0) and different concentrations of substrate (0.1, 0.5, 1, 2 and 2.5 mM). Maximum velocity (V_{max}) and Michaelis-Menten constant (K_m) values were determined using Graphpad Prism 5.0. The effect of pH was elucidated by equilibrating purified AChE for 15 min with different buffers and different pH conditions namely 0.1 M each of acetate buffer (pH 3.0, 4.0, 5.0, and 5.5), sodium phosphate buffer (pH 5.0, 6.0, 7.0, 7.5 and 8.0) and Tris-HCl buffer (pH 7.0, 7.5 and 8.0). The effect of temperature was determined by incubating the assay mixture at 15, 20, 25, 30, 35, 40 and 50°C for 15 min.

Half maximal inhibitory concentration (IC_{50}) : The half maximal inhibitory concentration (IC_{50}) served as the sensitivity coefficient of fish to inhibitors. IC_{50} study of partially purified ChE activity from *C. gariepinus* was conducted using different concentrations of Cd ranging from 1.0 to 10.0 mg l^{-1} . The heavy metal inhibition profile of ChE activity was analysed and the IC_{50} value of heavy metal was obtained using GraphPad Prism to access *in vitro* effect of cadmium.

Results and Discussion

Fish behavior represents the fish physiological response towards the environmental factor (Sabullah *et al.*, 2015b). Alteration in behavior is considered as a sensitive biomarker to evaluate the toxic exposure and effect of status of fish body (Gerhardt, 2007). The parameter for behaviour alteration

measurement such as swimming performance, avoidance behavior and feed intake was studied during a 96 hrs treatment with cadmium. The swimming behaviour exhibited a decrease in swimming as cadmium concentrations were increased, and the started fish to swim to the water surface together with increased frequency of opercula and mouth movement indicating difficulty in breathing. This proved that cadmium toxicity caused loss of resistance in swimming performance of the fish.

Eissa *et al.* (2006) reported that Cd^{2+} caused changes in the spatial distribution of *Cyprinus carpio*, which reflected as alterations in the preferred swimming depth when exposed to sublethal concentration of cadmium. Meanwhile, Padrilah *et al.* (2017) reported vertical and downward swimming patterns and sudden movements of *C. gariepinus* exposed to low concentration of copper; the fish began to sink to the bottom and became motionless at high concentration (10.0, 15.0 and 20.0 mg l^{-1}). Meanwhile, in the control group there were no behavioural changes, and no mortality was recorded throughout the experiment. The abnormal swimming pattern of *C. gariepinus* under stressful conditions may be due to the obstructed functions of neurotransmitters, which may have disrupted the nervous system (Adakole, 2012).

Swimming performance was found to affect the appetite in fish groups exposed to increasing concentrations of cadmium which may be due to the inhibition of dopamine and serotonin involved in locomotion, aggression and feeding behaviour, in the presence of heavy metals. Study on the role of some feed additives in fish fed on diets contaminated with cadmium also showed that daily feed intake decreased significantly ($p < 0.001$) with increasing dietary cadmium level Ayyat *et al.* (2017).

The general histological screening indicated a low to high cadmium damage on brain tissue of *C. gariepinus* after 96 hrs. Histopathological alterations observed in the brain tissue is shown in Fig. 1. The control group did not show any histological changes in any of the tissues examined (Fig. 1A) and constituted of normal neuronal cell, pyramidal cell and nissl substances. In contrast, exposure to 5 mg l^{-1} Cd (Fig. 1B) showed neuronal cell degeneration, dystrophic changes, swelling of pyramidal cells, loss of nissl substances and vacuolisation. Dark-stained degenerating neurons and vacuolar changes with empty spaces, which appeared as moth-eaten area, increased in number at 10 mg l^{-1} Cd exposure (Fig. 1C). Cerebral edema started appearing due to alteration in brain cell. Exposure to 30 mg l^{-1} Cd (Fig. 1D), increased in the formation of neuronal cell degeneration, vacuolisation and cerebral edema. The cell was totally damaged, and the formation of generalised cell edema and cell vacuolization increased (Fig. 1E) at 50 mg l^{-1} Cd exposure.

Cd is a persistent neurotoxic contaminant that can cause behavioural changes, impairing the subsequent survival or reproduction of the exposed organisms (Beauvais *et al.*, 2001). In the present study, Cd treated fish group revealed neuronal cell degeneration and swelling of pyramidal cells (Fig. 1B), which

Fig. 1: Characteristic histopathological changes in the brain of *Clarias gariepinus* revealed by H&E staining (400X) after 96 hrs treatment: A) Control. Pictomicrograph of brain tissue of *C. gariepinus* showing pyramidal cell (PC), neuronal cell (NC) and Nissls substances (NS); (B) Treated with 5 mg l⁻¹ cadmium showing loss of Nissl, substance (LNS), dystrophic changes (DC), vacuolisation (V), neuronal cells degeneration (NCD) and swelling of pyramidal cells (SPC); (C) Treated with 10 mg l⁻¹ of cadmium showing neuronal cells degeneration (NCD), vacuolisation (V) and swelling of pyramidal cells (SPC); (D) Treated with 30 mg l⁻¹ cadmium showing neuronal cells degeneration, vacuolisation and cerebral edema (CE) and (E) Treated with 50 mg l⁻¹ cadmium showing increased vacuolisation and cerebral edema.

Fig. 2 : Percent inhibition of cholinesterase in the brain extract of *Clarias gariepinus* exposed for 96 hrs. Error bars represent mean $\pm$ SE (n=3).

corroborates with the previous study of Patnaik *et al.* (2011) on *Cyprinus carpio communis* exposed to sublethal concentration of Cd (4.3 and 1.6 ppm). The incidence of severe necrosis by neuronal cells degeneration indicates loss of nissl granules in cerebrum and mild vacuolar changes (Loganathan *et al.*, 2006) in *Labeo rohita* exposed to 10 ppm of Zn and *Catla catla* exposed to copper and iron (Bose *et al.*, 2003). Vacuolisation in brain tissue may be due to glycolysis leading to microsomal and mitochondrial dysfunction (Ramudu and Dash, 2015), which can be observed clearly in Fig. 1C. Similar results were reported by Babatunde *et al.* (2015) on *Oreochromis niloticus* brain

exposed to 2 mg l⁻¹ paraquat for 10 weeks. Severe cerebral edema exhibited in the present study (Fig. 1D) indicates Cd toxicity that has also been implicated on increasing the permeability of blood brain barrier leading to cerebral edema (Wang and Du, 2013; Gonçalves *et al.*, 2010).

Total ChE activity was determined to study the activity of enzymes from the brain extract of *C. gariepinus* using three different types of substrate : Acetylthiocholine iodide, Butyrylthiocholine iodide and Propionylthiocholine iodide. Present study showed ATC with highest significant enzyme

Fig. 3 : Elution profile of partially purified acetylcholinesterase from the brain extract of *Clarias gariepinus* using Procainamide-Sepharyl 6B affinity chromatography method.

Fig. 4 : Molecular weight of purified cholinesterase from *Clarias gariepinus* by interpolation of retention factor (rf) of protein markers. Purified ChE indicated by green dotted.

activity ($p < 0.05$) with a total ChE activity of 31.0938 U compared to other two substrates; BTC (9.97549 U) and PTC (19.74265 U). High ChE activity in ATC was probably due to its biochemical role in the brain. AChE is abundant in the brain, and its main function is to regulate cholinergic neurotransmission by catalysing the hydrolysis of acetylcholine at neuromuscular junction and central synapses (Rodrigues *et al.*, 2011). Supported by Ferrari *et al.* (2007), AChE accounts for more than 97% of the total brain cholinesterases in most teleosts. Nunes (2011) also claimed that the major cholinesterasic form in the brain tissues of *Dicentrarchus labrax* is AChE.

The results obtained from the substrate profile is also in agreement with the study conducted by Ding *et al.* (2011), where they extracted the brain ChE from *Oreochromis aurea* that showed specific affinity towards ATC as its substrate. Thus, the highest ChE activity for ATC was used for subsequent purification. The *in vivo* study of ChE inhibition by Cd is shown in Fig. 2 where higher concentrations of Cd inhibited ChE activity from *C. gariepinus* brain exposed to Cd (1-50 mg l⁻¹), likely due to the bioaccumulation of Cd in fish affecting enzyme directly by binding to catalytic functional group in enzyme, and also the ability of Cd to bind with lipid-rich structures of mitochondria (Rani *et al.*, 2015).

Fig. 3 shows the purification data of *C. gariepinus* ChE from the procainamide-based affinity chromatography. A significant amount of protein and a low ChE activity were detected at the washing stage probably due to column overloading. ChE was isolated at the eluting stage of purification with high ionic strength 20 mM of sodium phosphate buffer pH 7.5, containing 1.0 M NaCl (fractions 46-58). Fractions with the highest ChE activity were pooled together for the next step of enzyme characterisation. The affinity

purification on Procainamide-Sepharose 6B matrix resulted in ChE purification fold of 10.58 times relative to the specific activity of crude extract and with 12% recovery. Affinity chromatography was found to be the most preferred method as it uses specific binding properties. It has a specific ligand, procainamide for ChE to bind. The enzyme activity was observed to decrease throughout the purification process. A decrease in enzyme activity is probably due to the denaturation of ChE and unavoidable loss of ChE during purification (Dako *et al.*, 2012).

Purification of ChE using Procainamide-Sepharose 6B as matrix in affinity chromatography aided to exclude the undesired proteins and retention of only desired protein, *i.e.*, ChE. Based on the SDS-PAGE gel profile, the molecular weights of the unknown protein bands, after partial purification through affinity chromatography, appeared within the range of 100 to 37 kDa. Three bands were observed and the molecular weight of each unknown protein band was calculated using the calibrated protein marker standard curve (Fig. 4). The diamond bullets indicate the position of protein bands from the partially purified AChE fraction. Fish AChE usually has a molecular weight of about 60 kDa (Tripathi and Srivastava, 2008). For example, ChE from *Colossoma macropomum* has a size of 62 kDa (Silva *et al.*, 2015), however, Assis *et al.* (2015) reported a molecular weight of 220 kDa for AChE extracted from the fish, *Arapaima gigas*. The results of SDS-PAGE in this study showed a probable molecular weight of AChE that brackets the 60 kDa theoretical molecular weight of AChE from fish; however, further purification is needed to correctly assess the molecular weight of AChE from *C. gariepinus*. Although a single band was not obtained from the experiment, the presence of ChE was verified by enzyme assay. Protein analysis through SDS-PAGE helps to elucidate the

Fig. 5: Effects of (A) substrate specificity profile of three synthetic substrates, (B) pH and (C) temperature on the enzyme activity of partially purified AChE from the brain extract of *C. gariepinus*. Error bars indicate mean of three replicates $\pm$ SE (n=3).

Fig. 6 : Percent inhibition on the enzyme activity of partially purified acetylcholinesterase from the brain extract of *Clarias gariepinus*.

effectiveness of purification on AChE from the brain extract of *C. gariepinus*.

The rate of enzymatic hydrolysis of ATC, BTC and PTC increased from 0 to 2.5 mM, thus proving the potential of enzyme to hydrolyse substrates at different frequencies (Fig. 5A). All the substrates showed significant difference in term of catalytic reaction ($p < 0.05$). Purified ChE also showed the potential to hydrolyse ATC with highest rate as compared to BTC and PTC with maximal velocity (V_{max}) reaching to $1.985 \mu\text{mol min}^{-1} \text{mg}^{-1}$ and substrate affinity based on the apparent biomolecular constant, K_m value of 0.4479. The V_{max}/K_m ratio showed catalytic efficiency towards purified sample. In the present study, ATC showed highest V_{max}/K_m ratio of 4.4318 among the substrates. AChE is abundant in brain tissue contain abundance of AChE (Tsim and Soreq, 2012). Thus, AChE hydrolyses ATC more efficiently than other substrates. Therefore, ATC with a concentration of 2.5 mM was selected as the preferred substrate for further analysis. Fig. 5B shows the influence of pH on the enzyme activity by using different buffer.

The figure indicates that the enzyme activity was relatively low at pH 3 and increased to pH 5.5 in sodium acetate buffer. Enzyme activity also showed similar results under sodium phosphate buffer at pH 5.5 and increased at pH 7, with a sharp increase in the enzyme activity beyond pH 7 to 7.5 in Tris-HCl buffer and decreased at pH 8. The result showed that AChE activity in *C. gariepinus* has a broad pH range and pH 7.5 was selected as optimum pH as mean point was highest among others. At this point, the neutralisation of imidazole group in the AChE structure led to the stabilisation of the enzyme-substrate complexes. It is also an ideal state for AChE to hydrolyse its substrate. Optimum condition near to neutral pH from other sources of AChE has been reported (Ding, 2011). The result was similar to a study conducted by Hayat et al. (2016), where

the maximum ChE activity in freshwater fish, *Lates calchifer* was observed between pH 7.0 and 8.0 with equal activity using either potassium phosphate or Tris-HCl buffers. Tris-HCl buffer was selected as the best buffering system for the characterisation of AChE. The result can also be compared with that of AChE assessment from Toman (*Channa micropeltes*), where its partially purified AChE displayed the highest activity on ATC using 0.1 M Tris-HCl buffer at pH 7 (Sharif et al., 2014). Thus, Tris-HCl with pH 7.5 was used as optimum buffer for further analysis of AChE activity.

Fig. 5C shows that the activity of purified AChE increased until it reached an optimal temperature of 35°C due to increase in the kinetic energy between AChE and its substrate that further increased AChE activity, hence driving the high rate of collision between molecules (Ahmad et al., 2016). The result of high enzyme activity leads to the efficient formation of enzyme-substrate complexes and the increase in the amount of products. Moreover, similar reports were elucidated by Sabullah et al. (2014) and Assis et al. (2015) where optimum temperature for AChE was 35°C in *Rachycentron canadum* and *Puntius javanicus*. Beyond 50°C AChE activity was completely lost.

Screening of heavy metals showed that brain AChE of *C. gariepinus* was sensitive towards Cd as it inhibited the enzyme activity, greater than 50%. The IC_{50} value of partially purified AChE activity was determined to be 0.6517 mg l^{-1} (Fig. 6). Sarkarati et al. (1999) reported that the inhibition of ChE by metal ions is due to the attraction of negative charge of amino acid side chains that contain carboxyl groups, such as glutamate and aspartate present at the catalytic triad of ChE, leading to structural change of the active site. Recently, Hayat et al. (2017) also reported that metal ions inhibition is caused by the affinity binding towards the amino acid side chain. Thus, it is suggested that the partially purified AChE from the brain extract

of *C. gariepinus* has a potential to inhibit the activity of metal cons, and can act as a biomarker for monitoring other contaminants, such as pesticides.

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