CHANGES OF CHOLINESTERASE ACTIVITY, HISTOLOGY AND PROTEOME OF *Puntius javanicus* LIVER UPON EXPOSURE TO COPPER

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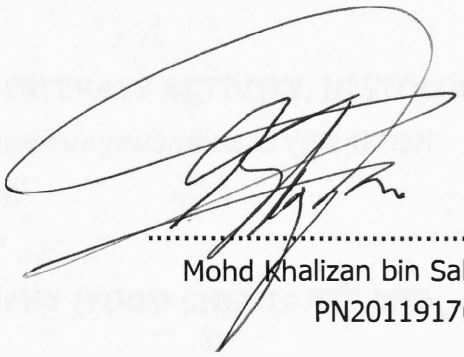

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ABSTRACT

The present study was carried out to investigate the effect of copper sulfate ($CuSO_4$) exposure on cholinesterase (ChE) activity, histology, and proteome of *Puntius javanicus* liver. Acute toxicity test to obtain lethal concentration (LC) values (LC_{50} and LC_{10}) of $CuSO_4$ was initially done by exposing eight groups of fish for 96 hours with 2.0, 4.0, 6.0, 8.0, 10, 13, 15 and 20 mg/L of $CuSO_4$, respectively. Based on Finney method (Probit analysis) of calculation, LC_{50} and LC_{10} of $CuSO_4$ were determined as at 10.30 mg/L and 6.11 mg/L, respectively. Sublethal $CuSO_4$ concentrations of 0, 0.1, 0.3, 0.5, 1.0 and 5.0 mg/L (Lower than LC_{10} value i.e. with 100% fish survival) were used to treat six groups of *P. javanicus* fish including a control for 96 hours. The liver of each treated and control fish was subjected to cholinesterase activity test, histology, and proteomic studies. For ChE activity study, an optimal assay condition of purified ChE was determined as at the pH 7.5 and at the temperature in the range of 25 to 35°C in 0.1M Sodium Phosphate buffer. As compared to other synthetic substrate, butyrylthiocholine iodide (BTCi) was selected as ChE specific substrate with the highest maximal activity (V_{max}), lowest biomolecular constant (K_m) and the highest catalytic efficiency ratio at the value of 53.49 (50.12 to 56.87)µmol/min/mg, 0.23 (0.16 to 0.31) mM, and 232.57, respectively. Storage condition showed that ChE need to be preserved in refrigerated condition. Metal ion *in-vitro* test showed that Cu, chromium and mercury had the capability to lowering the activity of ChE more than 50%. The combination of pairwise metal ion enhanced the inhibitory effect of more than 60%. Half inhibitory effect (IC_{50}) of Cu ion towards the ChE *In vitro* was found as at 0.0948 (0.06797 to 0.1628) mg/L. *In vivo* effect showed that at 0.1 mg/L of $CuSO_4$, the activity of ChE was increased significantly ($p<0.05$) by 6% compared to the control. However, the percentage activity of ChE was decreased to 95.41, 87.60, 84.60 and 73.00 % at the Cu concentration treatment of 0.3, 0.5, 1.0 and 5.0 mg/L, respectively. The toxicity effect of Cu on *P. javanicus* liver was visualized using light microscope and transmission electron microscope (TEM). Histology on the affected cells showed abnormalities of nucleus polygonal shape along with parenchymal vacuolation, dilation and congestion of sinusoid. At the higher $CuSO_4$ exposure (0.5,

1.0 and 5.0 mg/L), hepatostructure was significantly affected as indicated by the increasing number of dilation and congestion of sinusoids, vacuolation, macrophage activities and peliosis. The damage level and HSI value were increased and in contrast the number of hepatic nuclei per mm^2 were decreased as again associated with the increasing Cu treatment concentrations. Through an observation of selected hepatocyte ultrastructure (liver of treated goups with 0.5, 1.0 and 5.0 mg/L $CuSO_4$) using TEM, other abnormalities i.e. the development of pyknotic nucleus along with damaged organelles such as mitochondria, Golgi apparatus and endoplasmic reticulum disorientation were determined. Irreversible cell injury was also determined, in which hepatic nuclei had seen to undergo for karyorrhexis with the formation of apoptotic body that consisted of free scattered damaged organelles. Proteomic study based on second dimension electrophoresis (2D-PAGE) was performed whereby the patterns of resolved protein spots on the gels were visualized using calibrated densitometer G-800 after stained with a modified silver staining method. The estimated total number of protein spots of 1791 were matched and compared among the control and treated gels. Subsequently, 10 unique protein spots on the coomasie blue G-250 stained gels were selected (based on fold change more than 2.0) and subjected to identificaton by using MALDI-TOF-TOF mass spectrometry combined with data mining in SwissProt, UniProt and NCBInr. The identity and putative function of five upregulated (Gastrotropin, VAT-1L, hemogobin-β, two subunit of hemoglobin-α), four downregulated (Trypsin, ZC4H2, Islet-2A and hemoglobin-β A/B) and one up and downregulated (Parvalbumin) protein spots were determined in this study as shown in their individual bracket, respectively. In conclusion, Cu is evident to significantly affects the ChE activity, histology and proteome of *P.javanicus* liver. This study has generated several novel fundamental knowledge of the adverse effects of Cu on a fish model, which is potentially being used in future as an alternative biomarker or biosensor for the presence of contaminant especially Cu in the environment.

ABSTRAK

PERUBAHAN-PERUBAHAN AKTIVITI KOLINESTERES, HISTOLOGI DAN PROTEOM HATI Puntius javanicus TERHADAP PENDEDAHAN KEPADA KUPRUM

Kajian ini telah dijalankan untuk menyelidiki kesan-kesan kesan kuprum sulfat ($CuSO_4$) ke atas aktiviti kolinesteres (ChE), histologi dan proteom pada hati Puntius javanicus. Pengujian ketoksikan akut untuk mendapat nilai kepekatan letal (LC_{50} dan LC_{10}) mula-mula telah dijalankan dengan mendedahkan lapan kumpulan ikan untuk 96 jam dengan $CuSO_4$ iaitu masing-masing 2.0, 4.0, 6.0, 8.0 10, 15 dan 20 mg/L. Berdasarkan pada kaedah Finney (analisis Probit) $CuSO_4$ keatas kehidupan ikan, kesan LC_{50} dan LC_{10} masing-masing telah ditentukan pada 10.30 mg/L dan 6.11 mg/L. Kepekatan sublethal $CuSO_4$ pada 0, 0.1, 0.3, 0.5, 1.0 dan 5.0 mg/L (lebih bawah daripada nilai LC_{10}) telah digunakan untuk merawat enam kumpulan ikan P. javanicus termasuk satu kawalan untuk 96 jam. Hati daripada setiap ikan rawatan dan kawalan telah diteruskan untuk mengkaji aktiviti kolinesteres, histologi dan proteome. Untuk pengkajian aktiviti ChE, satu keadaan assai optimum ke atas ChE tulen telah ditentukan pada pH 7.5 dan pada suhu dalam julat 25 hingga 35°C di dalam 0.1M sodium phosphate buffer. Perbandingan pada subtrat sintetik yang berlainan, butirilthiokolin iodida (BTCi) telah dipilih sebagai substrat spesifik ChE aktiviti maksimum tertinggi (V_{max}), pemalar biomolekul (K_m) terendah dan kecekapan pemangkin tertinggi masing-masing pada 53.49 (50.12 ke 56.87)µmol/min/mg, 0.23 (0.16 ke 0.31) mM, dan 232.57. Keadaan penyimpanan menunjukkan ChE perlu untuk disimpan dalam keadaan yang sejuk. Ujian In vitro ion logam menunjukkan Cu, kromium dan merkuri mempunyai kemampuan untuk menurunkan aktiviti ChE lebih daripada 50%. Gabungan pasangan samatara logam ion telah meningkatkan kesan perencetan lebih daripada 60%. Nilai IC_{50} ion Cu ke atas In vitro ChE telah dijumpai pada 0.0948 (0.06797 to 0.1628) mg/L. Kesan in vivo menunjukkan pada 0.1 mg/L $CuSO_4$, aktiviti ChE meningkat secara ketara ($p < 0.05$) sebanyak 6% berbanding pada kawalan. Walau bagaimanapun, pada kepekatan $CuSO_4$ yang tinggi, peratusan aktiviti telah menurun ke 95.41, 87.60,

84.60 dan 73.00 % masing-masing untuk rawatan pada 0.3, 0.5, 1.0 dan 5.0 mg/L. Kesan keracunan Cu ke atas hati P. javanicus telah diperhatikan menggunakan mikroskop cahaya dan mikroskop elektron transmisi (TEM). Histologi ke atas sel yang terjejas menunjukkan bentuk poligon nukleus yang tidak normal selari dengan vacuolasi parenkimal, pengembangan dan kesesakan sinusoid. Pada pendedahan $CuSO_4$ yang lebih tinggi (0.5, 1.0 dan 5.0 mg/L), hepatostruktur telah terjejas secara ketara seperti ditunjukkan dengan meningkatnya beberapa pengembangan dan kesesakan sinusoids, vacuolasi, aktiviti macrophage dan peliosis. Tahap kerosakan dan nilai HSI telah meningkat dan berbeza dengan jumlah hepatik nukleus setiap mm^2 telah menurun lagi berkaitan dengan peningkatan rawatan kepekatan Cu. Melalui pemerhatian ke atas ultrastruktur (hati yang telah dirawat 0.5, 1.0 dan 5.0 mg/L $CuSO_4$) menggunakan TEM, terdapat kecacatan dengan kata lain pengembangan nukleus piknotic seiring dengan kerosakan organel seperti mitokondria, alat golgi dan disorientasi endoplasmik retikulum. Kecederaan sel tidak dapat dipulihkan juga telah ditentukan dimana nukleus hepatik telah mengalami karyorrhexis dengan pembentukan badan apoptotik terdiri daripada organel rosak yang bebas bertaburan. Kajian proteomik berdasarkan elektroforesis dua dimensi (2D-PAGE) telah dijalankan dimana corak tompok protein ke atas gel telah digambarkan dengan menggunakan densitometer ditentukur G-800 selepas diwarnakan dengan kaedah pewarnaan perak yang telah diubahsuai. Anggaran sejumlah 1791 tompok protein daripada telah dipadankan dan dibandingkan antara gel kawalan dan rawatan. 10 tompok yang unik pada gel yang diwarnakan dengan coomasie G-250 telah dipilih (berdasarkan perubahan lebih daripada 2.0 kali ganda) dan tertakluk untuk dikenalpasti dengan menggunakan spektrometri jisim MALDI-TOF-TOF digabungkan dengan penggalian data dalam pengkalan data SwissProt, UniProt dan NCBInr. Identiti dan fungsi putatif ke atas lima penjanaan naik (Gastrotropin, VAT-1L, hemogobin-β, dua subunit hemoglobin-α), empat penjanaan turun (trypsin, ZC4H2, Islet-2A dan hemoglobin-β A/B) dan satu penjanaan naik dan turun (Parvalbumin) telah ditentukan di dalam kajian ini seperti yang telah ditunjukkan di dalam kurungan individu masing-masing. Sebagai kesimpulan, Cu terbukti memberi kesan ketara terhadap aktiviti ChE, histologi dan proteome hati P. javanicus. Kajian ini telah menjana beberapa pengetahuan asas yang baharu ke atas kesan buruk Cu pada model ikan, yang berpotensi digunakan pada masa akan

datang sebagai biopenanda dan biosensor alternatif untuk mengesan kehadiran bahan cemar terutamanya Cu di dalam persekitaran.

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LIST OF ABBREVIATION

%	-	Percentage
°C	-	Degree celcius
µg/g	-	Microgram per gram
µg/L	-	microgram perlitre
μm^2	-	Micrometer square
1D	-	1 dimension
2D	-	2 dimension
Abs	-	Absorbance
Ag	-	Silver
Al	-	Aluminium
ANOVA	-	Analysis of variance
APS	-	Ammonium persulfate
As	-	Arsenic
ATC	-	Acetylcholine iodide
BSA	-	Bovine serum albumin
BTC	-	Butyrylthiocholine iodide
CBB	-	Commasie brilliant blue
Cd	-	Cadmium
ChE	-	Cholinesterase
Cr	-	Chromium
Cu	-	Copper
$CuCl_2$	-	Copper (II) chloride
$CuSO_4$	-	Copper (II) sulfate
d_c	-	Cosine distance
d_e	-	Euclidean distance
DNA	-	Deoxyribonucleic acid
DOE	-	Department of Environment
DTNB	-	5, 5-dithio-bis-2-nitrobenzoate
et al.,	-	and all
FAO	-	Food and agricultural organization

Fe	-	Ferum
g	-	Gram
GF	-	Gel filtration
GST	-	Gluthathione S-Transferase
HCl	-	Hydrochloric acid
Hept.nucl/mm^2	-	hepatocyte nucleus per mm^2
Hg	-	Mercury
HPLC	-	High performance liquid chromatography
HSI	-	Hepatosmotic index
IC_{50}	-	Initial concentration that cause 50% inhibition
ICP-OES	-	Inductively coupled plasma optical emission spectrometry
IEF	-	Isoelectrofocusing
KCl	-	Potassium chloride
kDa	-	Kilo Dalton
K_m	-	Biomolecular constant
L	-	Liter
LC_{10}	-	Lethal concentration at 10%
LC_{50}	-	Lethal concentration at 50%
LOD	-	Limit of Detection
LOQ	-	Limit of Quantitation
M	-	Molarity
mM	-	Milimolar
MALDITOFF	-	Matrix-assisted laser desorption/ionization-time of flight analysis
mg/L	-	Miligram perliter
min	-	Minute
MS	-	Mass spectrometry
MW	-	Molecular weight
NaN_3	-	Sodium nitrite