October 2007 Volume 1 Issue 3

Trade Science Inc.

BIOCHEMISTRY
An Indian Journal

Regular Paper

BCAIJ, 1(3), 2007 [139-143]

Biochemical And Histopathological Effects Of Tetrodotoxin Isolated From Puffer Fish *Tetraodon Patoca* Available In Bangladesh

Sohel Hasan[1], F.Nikkon[1], F.Pervin[1],M.M Rahman[1], T.Hossain[1], S.K.Sarker[3], Ashik Mosaddik[2]*, N.Absar[1]

[1]Department of Biochemistry and Molecular Biology, University of Rajshahi, Rajshahi-6205, (BANGLADESH)

[2]Department of Pharmacy, University of Rajshahi, Rajshahi-6205, (BANGLADESH)

[3]Laboratory of Bioscience Biotechnology, TIT,(JAPAN)

Tell : 880-721-750294 ; Fax: 880-721-750064

E-mail: shasanbiochem05@yahoo.com

Received: 12th March, 2007 ; Accepted: 17th March, 2007

ABSTRACT

Puffer fish toxin, tetrodotoxin(TTX) was isolated and purified from liver of puffer fish(*Tetraodon patoca*) by thin layer chromatography and elucidated by IR, ^{1}H-NMR, ^{13}C-NMR and mass spectroscopic data. The tetrodotoxin,(2.25μg) was administrated daily intraperitoneally for 14 days. There was a significant difference observed between weight loss in rats receiving TTX and control rats(103.0±0.5 vs 123.0±0.5 respectively). A significant changes detected in haematology RBC, 3.40±0.25 vs 5.05±0.05(cells mL^{-1})×10^{6}; WBC, 5.05±0.005 vs 6.525±0.051(cells mL^{-1})×10^{6}; Hb, 10.20±0.89 vs 14.50±0.20(%); platelet, 367.0±0.05 vs 335.0±1.25(cells mL^{-1})×10^{6} and ESR, 16.5±1.20 vs 11.25±1.29(mm/h), in blood parameters (SGPT, 10.0±0.50 vs 8.75±0.82 int. units L^{-1}; SGOT, 11.70±0.05 vs 10.0±0.70 int. unitsL^{-1}; SALP, 43.0±0.075 vs 37.4±0.027 int. unitsL^{-1}; bilirubin, 0.38±0.025 vs 0.31±0.048 mmolL^{-1}; creatinine, 1.92±0.075 vs 1.57±0.018mgdL^{-1} and urea, 21.25±0.50 vs 17.75±0.84mmolL^{-1} for experimental and control rats, respectively. In histopathological study, it showed that all the tissues such as liver, lung, heart and kidney of rats were affected after treatment with the toxins but the changes were more pronounced in liver as compared to the other tissues.

KEYWORDS

Puffer fish;
Tetrodotoxin;
Tetraodon patoca;
Hhistopathology.

INTRODUCTION

Poisoning due to ingestion of toxic puffer fish have frequently occurred in Japan and also in Taiwan, Hongkong, China, Thailand, Singapore Malaysia, Kiribali, Figi Australia, Papua NewGuinea, Bangladesh and U.S.A. with much fewer victims[1,2]. In Bangladesh on 16 November 1998 a food poisoning incident due to ingestion of puffer fish of related species, *Takifugu oblongus* occurred, affecting 8 people died among 15 victims[3]. There is very little information available on the freshwater puffers responsible for the poisoning, their toxicity scores and toxin composition. In order to clarify this situation, freshwater puffer specimens of *Tetraodon*

Regular Paper

sp. were collected from Bangladesh and their toxicity was assayed. Since human intoxications caused by consumption of a little toxin seemed to be an intriguing matter, which motivated us to conduct a biochemical and histopathological investigation on the freshwater puffers poisoning.

Two species of freshwater puffer fish, *Tetraodon cutcutia* and *Chelonodon patoca*, inhabiting Bangladesh, possessed considerable amounts of paralytic toxins, which were composed of saxitoxin(STX), decarbamoylsaxitoxin(dcSTX), gonyautoxins 2 and 3 (GTX2,3), decarbamoylgonyautoxins 2 and 3 (dcGTX2,3), and three unidentified components (designated STX-uk, GTX-uk1 and GTX-uk2)[4].

Recently, some researchers reported that freshwater puffers in Thailand, which once caused a food poisoning incident[5], were highly toxic, and that the toxic principle was tetrodotoxin(TTX), as in marine puffers[6,7], although Kungsuwan *et al.* detected paralytic shellfish poison(PSP) from a Thai freshwater puffer *T.leiurus* as the main toxin[8]. Zaman et al., also reported the occurrence of a methyl derivative of saxitoxin in Bangladeshi freshwater puffers, *Tetraodon cutcutia*[9]. Therefore, the present study on puffer fish available in Bangladeshi river was analyzed to obtain more information about the nature of toxin or other toxin substances.

This paper described biochemical and histopathological toxic effect of tetrodotoxin(TTX) isolated from puffer fish *Tetraodon patoca*, available in Bangladeshi river in rats.

MATERIALS AND METHODS

Fish collection

The fish were collected after catching from Rupsha river sourrounding Shundarban area that is located in Southwest part of Bangladesh in month of November 2003. The fish were identified by Fisheries Laboratory, Zoology Department, Rajshahi University, Bangladesh.

Chemicals

All organic solvents used in extraction and isolation was analytical grade supplied by Marck, Germany. The PTLC Silica gel-60 plates were collected from Fluka, Switzerland. IR-spectrum was measured by KBr using Shimadzu FTIR 8400 spectrophotometer. ^{1}H NMR and ^{13}C-NMR spectra were recorded using Bruker 400MH$_z$ spectrophotometer and CD_3COOD/D_2O as solvent. Normal saline, haematoxylin and eosin(H & E) were all analytical grade.

Animals

Long Evan's rats(110-113g) were collected from the Animal Resources Branch of The International Center for Diarrhoeal Research, Dhaka, Bangladesh. The experiment was performed at the Department of Pathology, Rajshahi Medical College. The rats were kept in numbered iron cages for two weeks before treatment. They were fed a balanced diet[10] and tap water, under standard conditions of a 12-h dark-light cycle, 60±10% humidity and a temperature of 21.5±1.0^{0}C. These protocols were approved by the Institutional Animal Care and Use Committee of UNICAMP which follows the recommendations of the Canadian Council on Animal Care.

Isolation and purification

The liver(100gm) was taken in a container containing cold distilled water(300ml) and mixed uniformly with slow stiring for 2h at room temperature. After centrifugation at 8000rpm for 8min at 10^{0}C, the supernatant (aq.) extract was washed with different organic solvents such as n-hexane, chloroform and ethyl acetate successively to remove organic solvents soluble materials and the aqueous fraction was freezed dried. Then it was developed with PTLC on Silica gel-60(Fluka) using 1-butanol: acetic acid: water(8:1:1) as developing solvent. Then the corresponding band was scrap off under UV at 254nm and eluted with water followed by freeze dried to afford the desired compound, TTX.

Administration

Compound, TTX(0.25mg) was dissolved in 3.3ml water to get a stock solution and administered 300µL intraperitoneally at a dose of 2.25µg/day for 14 consecutive days to experimental group and control group received only water. Four rats were injected in each group.

EXPERIMENTAL

A measured amount of fresh food was supplied daily at 10.00a.m and the general well-being and behavior

Regular Paper

of the animals were observed daily, throughout the study. For the haematological study, blood was drawn from the tail vein of both the groups before administration of the compound and after the experimental period, to estimate the total and differential blood count, platelet count, and percent haemoglobin. For the biochemical study, blood was collected from each rat sacrificed at day 14 from the jugular veins of each of the animals. Serum glutamic-oxaloacetic transaminase, serum glutamate pyruvate transaminase, serum alkaline phosphatase, urea, uric acid and creatinine were determined using standard procedures and reagents supplied by Boehringer Mannheim GmbH.Diagnostica. Histopathological studies of the liver, kidney, heart and lung were performed using haematoxylin, eosin stain and D.P.X mounting fluid. The samples were observed under a microscope at the department of pathology, Rajshahi Medical College, Rajshahi, Bangladesh.

Statistical analysis

Results are presented as the mean±s.d. Student's s t-test was used for comparison between the experimental and control groups. $P<0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Characterization of TTX

The IR spectra of presently purified TTX showed characteristic absorption at 3405 cm^{-1}(OH), 1623cm^{-1} (guanidium), 1559cm^{-1}(COO^-) and at 1120cm^{-1} due to C-O stretching bond which were similar with the reported data for TTX[14]. It also gave a identical peck at 279nm similar to TTX[12]. In the ^{13}C-NMR data 11 signals were observed including the carbonyl group at δ_C 156.6 and the ^{1}H-NMR data showed characteristic peaks at δ_H 5.42(d, J= 9.4Hz) and δ_H 2.65(d, J=9.6Hz) for H-4 and H-4a of a guanidium ring system, that is the typical spin-spin coupling constant between these two protons. All other ^{13}C-NMR and ^{1}H-NMR data were very close agreement with the published data of TTX(Figure 1), previously isolated from puffer fishes which gave a molecular ion peak$(M+H)^+$ at m/z 320 and at m/z 302 due to$(M+H–H_2O)^+$ ion that corresponding to the molecular formula of TTX($C_{11}H_{17}N_3O_8$) [13,14]. To best of our knowledge, this is the first occurrence of tetrodotoxin from freshwater puffer fish available in Bangladesh.

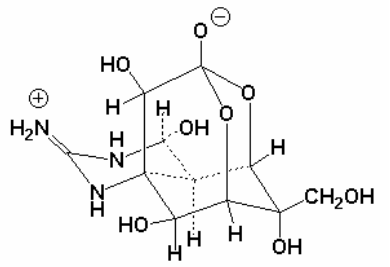

Figure 1: Structure of TTX

TABLE 1 : Changes in body weight of control and toxin treated rats

Group	Body weight		
	Before experiment	After experimental period	Change (%)
Control	110.17±1.45	114±1.87	+3.47±0.28
Toxin treated	112.12±1.34	94.25±2.29	-15.93±0.70

Values are mean±s.d n=4. There was significant difference between body weight of control and experimental rats

Gross general observation

The control group rats did not show any abnormalities and their food intake was also observed to be

TABLE 2 : Haematological profiles of control and TTX(2.25µg/day) treated rats of the experimental periods for 14 days

Parameters	Control			TTX treated rats	
	1st day M±SD	7th dayM±SD	14th day M±SD	7th day M±SD	14th day M±SD
RBC(cellsm$L^{-1}\times10^6$)	5.05±0.05	5.10± 0.25	5.0± 0.05	3.60? 0.05	3.40±0.25
WBC(cellsm$L^{-1}\times10^6$)	6.525±0.51	6.41± 0.50	6.45± 0.2	5.80? 0.10	5.07±0.005
Neutrophil (cellsm$L^{-1}\times10^{6)}$	43.5±2.29	43.0± 2.25	42.5± 2.29	40.2? 0.05	35.5±0.50
Lymphocyte(cellsm$L^{-1}\times10^{6)}$	53.20 ±2.121	52±1.01	53.0 ±2.1	41.20? 0.05	33.05 ±0.20
Monocyte(cells $mL^{-1}\times10^6$)	4.75±0.43	4.0±0.45	4.25± 0.40	3.85? 0.25	3.50± 0.025
Eosinophil (cells $mL^{-1}\times10^6$)	4.25±0.020	4.5±0.025	4.85±0.005	4.20± 0.005	4.5±0.025
Platelet(cells $mL^{-1}\times10^6$)	335.0±1.25	333.0±1.58	336.0±1.25	352.0±0.52	367.0±05
Hemoglobin(%)	14.50±0.20	14.80±0.24	15.70±0.50	12.75±0.344	10.20±0.898
ESR(mm/1st hour)	11.25±1.29	12.5±1.05	12.86±1.15	13.5±1.25	16.5±1.20

Values are means±s.d., n=4. There were significant differences between control and experimental values for all experiments

Regular Paper

normal. On the other hand, the experimental group rats(TTX treated) showed some noticeable signs, such as tremor, convulsions, reflex abnormalities, muscular paralysis, muscular numbness of the hind and four legs as well as salivation. Further the food intake per-day was also found to be much less than that of control rats.

Body weight

TABLE 1 shows the individual and average body weight of all rats before and after administration of the toxin. The body weight of TTX treated rats was decreased by 15.93% while that of the control group was increased by about 3.47% after the experimental period.

Haematological profiles

As given in TABLE 2, the haematological profiles such as total RBC, total WBC and differential count of WBC and haemoglobin were decreased in TTX treated rats as compared to those of control group. Remarkably, the platelet count and ESR were increased in treated rats after the experimental period.

Biochemical parameters of blood

TABLE 3 : Biochemical parameters of control and TTX treated rats after the experimental periods of 14days

Parameters	Control $n=4M_1\pm SD_1$	TTX treated rats	% of change
SGPT (int.unitsL^{-1})	8.75±0.82	10±0.50	+14.28±0.39
SGOT (int.unitsL^{-1})	10±0.70	11.70±0.50	+17±0.28
SALP (int. unitsL^{-1})	37.4± 0.027	43.57±0.075	+16.48±0.17
Serum bilirubin (m mol/L)	0.317±0.048	0.38±0.025	+19.87±0.047
Creatinine (mgdL^{-1})	1.57±0.018	1.92±0.075	+22.29±0.31
Urea (mgdL^{-1})	17.75±0.84	21.25±0.05	+21.25±0.94

Values are means±s.d., n=4. There were significant differences between control and experimental values for all experiments

TABLE 3 shows the biochemical parameters of blood. All the parameters such as SGPT(serum glutamic-pyruvate transaminase), SGOT(serum glutamic-oxalo-acetic transaminase), SALP(serum alkaline phosphatase), bilirubin, creatinine and urea levels of serum were increased significantly in experimental group in comparison to that of control group indicating that the tetrodotoxin(TTX) had toxic effects on liver and kidney functions.

Histopathological observations

A marked detectable histopathological difference among the tissues of control(water only) and TTX treated rats(2.25µg/rat/day for 14 consecutive days) were observed after the experimental period(Figure 2). It was found that the tissues such as liver, kidney, lung and heart of the toxin treated rats were affected and the changes were summarized in TABLE 4. Although all

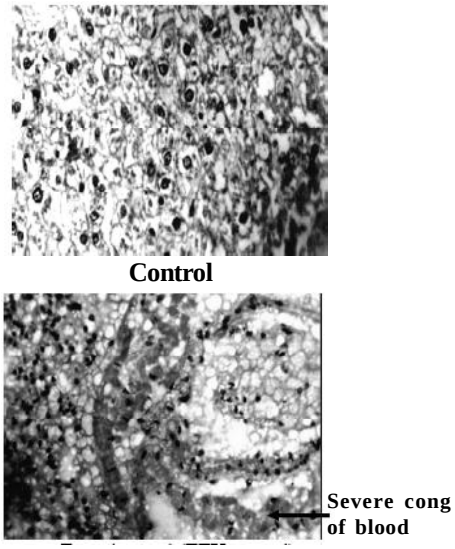

Figure 2 : Microphotograph showing histopathological changes in the liver tissues of control and experimental (TTX treated) rat after intraperitoneally injection of 2.25µg/rat/day of the toxin

TABLE 4 : Changes observed in different tissues after treatment with TTX for 14 days in rats

Sample	Tissue	Types of effectiveness TTX(Treated)
Concentration of TTX (2.25µg/rat/day)	Liver	Severe congestion of blood vessels. No inflammation and necrosis.
	Heart	Mild congestion of blood vessels and inflammation .
	Kidney	Inflammation, stoma edema and vascular congestion.
	Lung	Mild congestion of blood vessels and inflammation .
Control(300 µL of distilled water injected intraperitoneally)	All the tissues	There was no inflammation, necrosis, stoma edema and congestion of blood vessels of the liver, lung, heart and kidney.

the tissues of the experimental rats(toxin treated) were severely affected but the changes were more noticeable in liver as compared to that of others.

CONCLUSION

It can be concluded from the present data that the tetrodotoxin, TTX has high toxic effects in rats at dose and duration used in this study. It was also reported that symptoms of poisoning patients resembled partly those caused by tetrodotoxin(TTX) or paralytic shellfish poison(PSP)[15]. Puffer fish possesses paralyzing/ palytoxin(Tetrodotoxin, TTX and analogues) that are secreted upon stimulation[13]. It was also described that an endogenous origin of tetrodotoxin in puffer fish. Puffer fish accumulates TTX at an extremely high concentration in their tissues among with saxitoxin(STX)[11,23,16-19]. Furthermore, some species of puffer fish have been reported to accumulate saxitoxin(STX) as the principal toxin[17,20]. It was also reported that TTX is one of the most potent molecules that selectively blocks the voltage sensitive sodium channels of excitable tissues[21].

The histopathological study indicates that all the tissues as well as metabolic systems(based on changes in the biochemical parameters and histopathological changes) were affected after administration with the toxin, TTX. The changes were observed to be more pronounced in liver as compared to the other tissues examined. Similar liver toxicity was previously found from Bangladeshi puffer fish[4]. But the toxicity showed in this study is not only PSP/ TTX, but might be other toxin(s). Therefore, the origin, mechanism of toxicity, or metabolic pathway of this component remains to be studied.

ACKNOWLEDGEMENTS

The authors are thankful to the Head, Department of Pathology, Rajshahi Medical College, for providing laboratory facilities and BCSIR Lab. Dhaka, Bangladesh for supplying spectroscopic data of TTX.

REFERENCE

[1] C.S.Reddy, A.W.Hayes; Principles and Methods of Toxicology, in 'Food-born toxicants', Ed by Hayes AW.Raven, New York, **2**, 81**(1989)**.

[2] W.R.Lange; Am.Fam.Physician, **42**, 1029 **(1990)**.

[3] Y.Mahmud, M.B.Tanu, T.Noguchi; J.Food Hyg.Soc.Japan, **40**, 473-480 **(1999)**.

[4] L.Zaman, O.Arakawa, A.Shimosu, Y.Onoue; Toxicon, **35**, 423-431 **(1997)**.

[5] S.Laobhripatr, K.Limpakarnjanarat, O.Sangwongloy, S.Sudhasaneya, B.Uchatvorakul, S.Leelasitorn, K.Saitanu; Toxicon, **28**, 1372-1375 **(1990)**.

[6] M.Kodama, Y.Ogata; Nippon Suis.Gakk, **50**, 1949-1951 **(1984)**.

[7] K.Saitanu, S.Laobhripatr, K.Limpakarnjanarat, O.Sangwongloy, S.Sudhasaneya, B.Anuchatvorakul, S.Leelasitorn; Toxicon, **29,** 895-897 **(1991)**.

[8] A.Kungsuwan, O.Arakawa, M.Promdet, Y.Onoue; Toxicon, **35 ,**1341-630 **(1997)**.

[9] L.Zaman, O.Arakawa, A.Himoru, Y.Hida, Y.Onoue; Toxicon, **36**, 627-630 **(1998)**.

[10] P.B.Hawk, L.Oser, W.H.Summerson; 'Practical Physiological Chemistry', 13th edn, McGraw Hill Book Company, USA, 136-139 **(1954)**.

[11] A.M.Kodama, Y.Hokama, T.Yasumoto, M.Fukui, S.J.Manea, N.Sutherland; Toxicon, **27**, 1051-1053 **(1989)**.

[12] Yamashita; Toxicol.Toxin Reviews, **20(1)**, 51-66 **(2001)**.

[13] M.Nakamura, T.Yasumoto; Toxicon, **23,** 271-276 **(1985)**.

[14] T.Noguchi, Y.J.Mahmud; Toxin Reviews, **20**, 35-50 **(2001)**.

[15] Y.Mahmud, O.Arakawa, T.Noguchi; J.Nat.Toxins, **9(4)**, (in press) **(2000)**.

[16] K.Matsumura; Toxicon **33**, 1239-1241 **(1995)**.

[17] E.J.Schantz; Ann.NY Acad.Sci., **479,**15-23 **(1986)**.

[18] Y.Onoue, T.Noguchi, K.Hashimoto; 'Tetrodotoxin Determination methods', Ed by Ragelis EP, American Chemical Society, Washington D.C., 345-355 **(1984)**.

[19] O.Arakawa, T.Noguchi, Onoue; Fish.Sci., **61**, 659-662 **(1995)**.

[20] Y.Oshima, K.Sugino, 'Mycotoxins and Phycotoxins', Amsterdam, Elsevier, 319-326 **(1989)**.

[21] H.S.Mosher; Ann.N.Y.Acad.Sci., **479**, 32-43 **(1986)**.

[22] N.Lagos, H.Onodera, P.A.Zagatto, D.Andrinolo, S.M.Azevedo, Y.Oshima; Toxicon, **37**, 1359-1373 **(1999)**.

[23] S.Sato, T.Ogata, V.Borja, C.Gonzales, Y.Fukuyo, M.Kodama; Toxicon, **38,** 1101-1009 **(2000)**.