Bull Environ Contam Toxicol (2010) 84:657–661
DOI 10.1007/s00128-010-0022-0

Sub-Chronic Toxicity of *Senilia senilis* Hepatopancreas in Albino Wistar Mice

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Received: 10 November 2009 / Accepted: 21 April 2010 / Published online: 15 May 2010

Abstract This study investigates the sub-chronic effects of shellfish possibly contaminated by algal toxins in albino Wistar mice. Following in-feed treatment with hepatopancreas, there were significant ($p < 0.05$) differences in packed cell volume, haemoglobin concentration, red blood cell count, lymphocyte count and alanine aminotransferase between the different treatment groups and control. Post mortem revealed inflammation and congestion in the gastrointestinal tract of the middle and highest dose groups. The stomach of the mice fed on the highest dose of the hepatopancreas showed severe ulcerations, leaving behind deep cavities and necrotic tissues on the glandular epithelium of the mucosal wall.

Keywords Shellfish toxins · Sub-chronic toxicity · *Senilia senilis* · Nigeria

Diarrhetic shellfish poisoning (DSP) is a toxic episode caused by the consumption of shellfish contaminated by two main algal toxins, okadaic acid and dinophysistoxin I (DTX-1) (Vale and Sampayo 2002; Yasumoto et al. 1985; Hu et al. 1992). Other toxins associated with the syndrome include pectenotoxins (PTX), yessotoxins (YTX) and other unknown toxin(s) that are neurotoxic (Stabell et al. 1991; Ramstad et al. 2001). The first reported investigation into the toxicity of the shellfish found and consumed in Nigeria revealed that shellfish from Port Harcourt in Southeastern Nigeria was positive for the mouse bioassay in February, 2005 (Igboeli and Asuzu 2010) and studies have shown that DSP is seasonal in occurrence (Ninčević-Gladan et al. 2008). One of the main DSP toxins, okadaic acid, is a well known tumor promoter (Fujiki et al. 1991; Suganuma et al. 1989) and Cordier et al. (2000) associated consumption of shellfish supposedly negative for mouse bioassay with digestive cancers due to the regular intake of sub-lethal amounts of okadaic acid. The individual concentrations of some of the DSP toxins in a particular sample may be below the observable adverse effect level in man but when combined, could be positive in the mouse bioassay (Ninčević-Gladan et al. 2008). But consumption of low level of okadaic acid in shellfish over long periods has been identified as a possible cause of digestive cancers in humans (Cordier et al. 2000). The purpose of this study was to investigate the toxic effects of prolonged consumption of shellfish samples that are negative for the mouse bioassay.

Materials and Methods

The chemicals used for this work were of analytical grade and procured from Sigma–Aldrich, St. Louis, MO. Albino Wister mice aged 6–9 weeks were obtained from the Laboratory Animal House of the Faculty of Veterinary Medicine, University of Nigeria, Nsukka. They were maintained in metal cages with free access to water and food before the commencement of the study. Their feed was composed of the following: 14.5% crude protein, 4.8% crude fat, 7.2% crude fibre, 8% crude ash, 0.8% calcium,

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0.62% phosphorus, 0.33% available phosphorus, 0.6% lysine, 0.29% methionine, 0.52% methionine/cystine, 8,000 i.u. Vitamin A, 3,400 i.u. Vitamin D3, 25 mg Vitamin E, 4 mg Vitamin B2, 50 mg Vitamin C, 30 mg Manganese, 30 mg Zinc, 0.15% Sodium and 2300 kcal/Kg metabolisable energy.

Shellfish samples were collected from Port Harcourt in May 2005 for mouse bioassay and sub-chronic toxicity test. Shellfish samples have been identified as *Senilia senilis* (Igboeli and Asuzu 2010). The extraction method was according to Stabell et al. (1991) and Ramstad et al. (2001) while the mouse bioassay was carried out as described by Yasumoto et al. (1978). Details of both have been described elsewhere (Igboeli and Asuzu 2010).

For the sub-chronic toxicity tests, the shellfish sample was vapor-boiled, de-shelled and the hepatopancreas (HP) dissected out. Four groups (three treatment groups and a control) of seven albino Wistar mice (6–9 weeks of age) were placed on 35 g of treated/untreated feed per group per day, i.e. at 5 g feed/mouse/day. The HP was dried and incorporated in feed at three concentrations: 5 g HP/Kg feed, 25 g HP/Kg feed and 125 g HP/Kg feed, for the low (Group B), middle (Group C) and high dose (Group D) treatment groups, respectively. The treated mice were fed for 6 weeks. A separate group of mice, which served as control (Group A), was fed on untreated feed only. The mice were also closely observed for behavioral changes.

Post mortem was conducted at the end of the study to check for gross pathological lesions. Blood samples were also taken at the end of 6 weeks to determine the packed cell volume (PCV), hemoglobin concentration (Hb), white blood cell count (total and differential), red blood cell count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) levels. Serum enzyme analyses were adapted from Dorner et al. (1983) with slight modifications. Relative weights of the brain, liver, heart and kidneys were determined. Samples of the liver, kidney, brain, heart, stomach, duodenum, jejunum, ileum, colon, testes, adrenal, quadriceps femoris, femur, spleen, and lungs were collected and stored in 10% formalin for histopathological examination. Samples for histopathological examination were dehydrated by passing them through graded alcohol (70%, 90%, and 95% alcohol). Upon removal from alcohol, they were placed in xylene after which they were embedded in paraffin wax. Tissue sections (5 μm) were prepared and stained with haematoxylin and eosin (H&E) following standard procedures. The sections were examined under light microscope and interpretations were made in comparison with the controls.

Data collected (Tables 1, 2 and 3) were statistically analyzed using one way analysis of variance (ANOVA) or Student's *t*-test as the case may be. The level of significance was determined at $p < 0.05$.

Results and Discussion

In the mouse bioassay, no death was recorded 24 h post administration of any of the extracts (chloroform, diethyl ether and aqueous phase). However, the writhing indicating pain, congested gastrointestinal tract and fluid accumulation in the abdomen are signs of toxicity. The absence of death during the mouse bioassay with this batch unlike the February batch (Igboeli and Asuzu 2010) is suggestive of seasonal occurrence of the toxin (Amzil et al. 1999) and may be due to the seasonal differences in phytoplankton community and/or substances produced by them (Stabell et al. 1991; Stobo et al. 2008).

The HP did not cause any significant effect on weekly feed consumption and mean body weight gain of mice. However, 5 mg HP/kg feed caused a significant ($p < 0.05$) increase in the mean weight of the liver compared to the highest dose and the control (Table 1). Since the mean weight of the liver of the middle dose was not significantly different from any of the other groups, it appears that the HP exerted its effect at a low dose and its effect on the liver was not dose-related. No significant effect was seen in the relative weights of the brain, heart and kidneys (not shown) between the groups.

There was no significant ($p > 0.05$) difference in the AST activity among the groups (Table 2). The ALT activity of mice in Group B was significantly ($p < 0.05$) higher than the ALT activity in Group A, but was not significantly ($p > 0.05$) different from the ALT activities in Groups C and D. This suggests that the effect of HP on ALT was not dose-related as was seen in the effect on the

Table 1 Mean relative organ weights (g) of mice treated with different concentrations of hepatopancreas in feed

Organ	Group A (Control) Mean ± SE	Group B (5 g HP/Kg feed) Mean ± SE	Group C (25 g HP/Kg feed) Mean ± SE	Group D (125 g HP/Kg feed) Mean ± SE
Liver	$0.043^{a,b} \pm 0.001$	$0.048^{a} \pm 0.004$	$0.040^{b} \pm 0.001$	$0.042^{a,b} \pm 0.001$
Brain	0.022 ± 0.001	0.019 ± 0.001	0.019 ± 0.001	0.022 ± 0.001
Heart	0.044 ± 0.003	0.044 ± 0.004	0.045 ± 0.002	0.041 ± 0.002

Different superscript (a, b) denotes significant ($p < 0.05$) difference between two means

Table 2 Values of serum enzymes of mice treated with different doses of hepatopancreas

Blood enzymes	Group A (Control) Mean ± SE	Group B (5 g HP/Kg feed) Mean ± SE	Group C (25 g HP/Kg feed) Mean ± SE	Group D (125 g HP/Kg feed) Mean ± SE
AST (U/L)	27.43 ± 9.01	31.5 ± 4.21	38.0 ± 3.12	38.14 ± 6.82
ALT (U/L)	11.14^{b} ± 2.54	17.17^{a} ± 1.72	$15.29^{a,b}$ ± 1.61	$14.29^{a,b}$ ± 1.43
SAP (U/L)	19.57 ± 4.28	16.67 ± 2.5	20.14 ± 2.4	24.00 ± 3.46

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *ALP* alkaline phosphotase

Different superscript (a, b) denotes significant ($p < 0.05$) difference between two means

mean weight of the liver. The ALT activities of groups A, C and D were not significantly ($p > 0.05$) different. There was no significant difference between the ALP activities in Groups A, B, C and D.

The neutrophil counts were significantly ($p < 0.05$) higher in Groups A, C and D compared with Group B (Table 3), while an opposite pattern was observed for the lymphocyte counts (Table 3) which were significantly ($p < 0.05$) lower in Groups A, C and D compared with Group B. Significant ($p < 0.05$) increases were observed in the PCV, hemoglobin concentration and red blood cell count (Table 3), probably due to the diarrhea and consequent dehydration caused by the shellfish toxin in the middle and high dose groups. It is also possible that due to blood loss from stomach ulcers induced by the toxin, increased hematological values occurred through compensatory erythropoeisis. The toxin seemed to induce dose-related inflammation in the gastrointestinal tract since only the middle and high dose groups showed congestion, while no such lesion was found in the low dose and control groups. The dark colored, pasty and poorly formed feces in group D mice (high dose) 4 weeks into the sub-chronic toxicity test was suggestive of diarrhea and occult bleeding in the stomach, which was confirmed by the histopathological lesions observed. Since the pathological lesions observed occurred mostly in the high dose group, followed by the middle dose group, while none occurred in the low dose and control groups, it is concluded that the HP was responsible for the lesions observed in a dose-related manner. The mucosa of the stomach and intestines of mice in Groups C and D (middle and high dose groups, respectively) mice was inflamed and hyperemic. The gross appearance of the gastrointestinal mucosa of mice in Groups A and B was normal. The stomach of the mice fed on the highest dose of the HP showed serious ulcerations, leaving behind deep cavities and necrotic tissues on the glandular epithelium of the mucosal wall (Fig. 1). These pathologies are absent in the glandular epithelium of the control (Fig. 2), low and middle dose groups strongly suggesting that HP was responsible for the ulcerations at a high dose.

This study has revealed that locations that were previously positive for the mouse bioassay may become negative at other periods of the year thus suggesting a seasonal occurrence of the putative algal toxins (Ninčević-Gladan et al. 2008). Igboeli and Asuzu (2010) reported that shellfish samples from Port Harcourt were positive for the mouse bioassay in February 2005 but in the present study done in May 2005, they were negative. The absence of death in the mouse bioassay with this batch unlike in the February batch is suggestive of seasonal occurrence of the toxin and the differences observed may have resulted from seasonal differences in phytoplankton community and/or substances produced by them (Stabell et al. 1991; Stobo et al. 2008). Though the shellfish samples were negative for the mouse bioassay, when fed to mice, the numerous toxic effects observed suggests that the mouse bioassay could not detect the low level of shellfish toxins which may cause

Table 3 The effect of feeding hepatopancreas at different concentrations on the hematology of mice

Blood parameters	Group A (Control) Mean ± SE	Group B (5 g HP/Kg feed) Mean ± SE	Group C (25 g HP/Kg feed) Mean ± SE	Group D (125 g HP/Kg feed) Mean ± SE
PCV (%)	$26.29^{a,b}$ ± 2.8	24.17^{a} ± 1.08	33.29^{b} ± 3.57	$37.00^{b,c}$ ± 3.24
Hb (g/dL)	$8.63^{a,b}$ ± 0.9	8.05^{a} ± 0.36	$11.07^{b,c}$ ± 1.19	12.29^{c} ± 1.07
RBC ($\times 10^{6}/\mu L$)	$4.38^{a,b}$ ± 0.46	4.02^{a} ± 0.18	$5.547^{b,c}$ ± 0.6	6.14^{c} ± 0.53
TWBC ($\times 10^{3}/\mu L$)	11014.29^{a} ± 846.4	10000.00^{a} ± 656.25	$13157.14^{a,b}$ ± 648.76	11414.29^{b} ± 420.56
Neutrophil (%)	19.86^{b} ± 1.67	10.50^{a} ± 1.06	18.00^{b} ± 2.98	17.57^{b} ± 2.42
Lymphocyte (%)	80.0^{b} ± 1.64	89.50^{a} ± 1.06	82.00^{b} ± 2.98	82.43^{b} ± 2.42

PCV packed cell volume, *Hb* hemoglobin concentration, *RBC* red blood cell count, *TWBC* total white blood cell count

Different superscript (a, b, c) denotes significant ($p < 0.05$) difference between two means

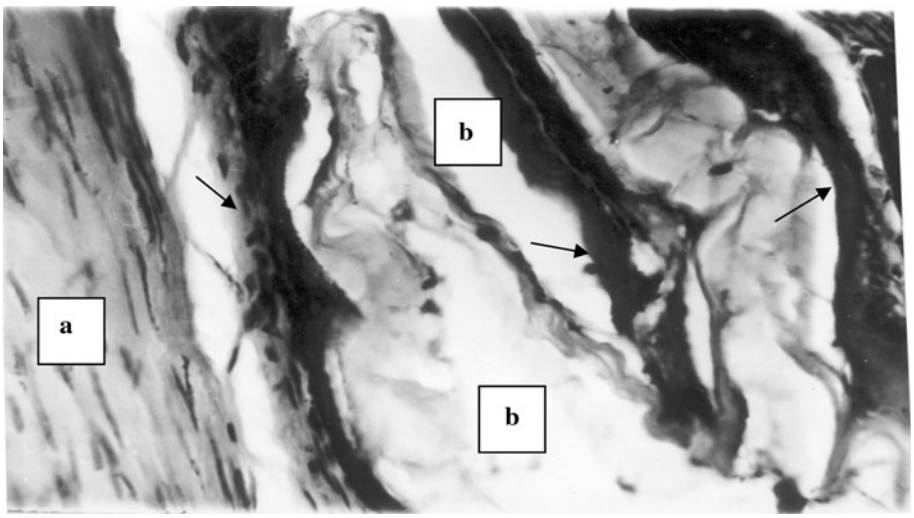

Fig. 1 Cross section of gastric glandular epithelium of Group D-highest dose group (**a**-smooth muscle; **b**-cavities in glandular epithelium of the gastric mucosa; the *arrows* are pointing at necrotic tissues)

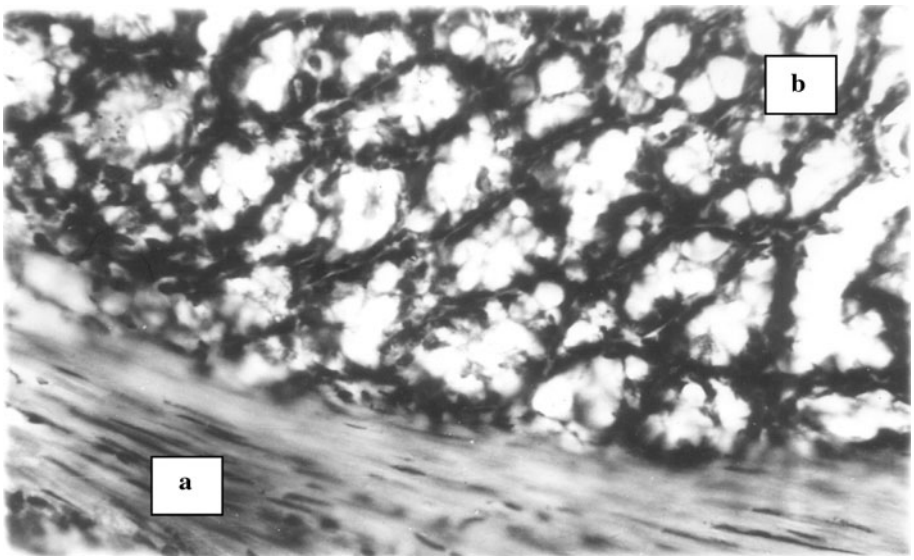

Fig. 2 Cross section of the gastric glandular epithelium of Group A-untreated group (**a**-smooth muscle; **b**-normal glandular epithelium of the gastric mucosa)

pathological conditions in unsuspecting public. This is in line with the observation of Cordier et al. (2000) that consumption of shellfish supposedly negative for mouse bioassay could cause digestive cancers due to intake of residual low level amounts of okadaic acid in shellfish over a long period. Thus, analytical and antibody methods should be used alongside the mouse bioassay in screening shellfish for toxins to ensure the safety of shellfish sold to the public (Stobo et al. 2008). The toxins responsible for the toxic effects of HP observed in this work were not investigated. But due to the tumor promoting effect of okadaic acid, we speculate that it may have been present in the shellfish samples used in this study but at a level that could not be detected by the mouse bioassay. Further studies should be carried out to identify the shellfish toxins that caused the pathologies observed in this study and their seasonality.

Acknowledgments The authors wish to thank Peter Okoye of the Histopathology Unit, University of Nigeria Teaching Hospital, Enugu; Austin Ngene, Medicine Laboratory, Department of Veterinary Medicine and Prof. J.A.O. Okoye, Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, University of Nigeria, Nsukka for their technical assistance. We are grateful to Drs. John Burka and Collins Kamunde, Department of Biomedical Sciences, Atlantic Veterinary College, University of Prince Edward Island, Canada for their corrections of the manuscript. Funding was from the Department of Veterinary Physiology and Pharmacology, University of Nigeria, Nsukka and the Samuel N.C. Igboeli family. We are grateful to both sources of funds.

References

Amzil Z, Quilliam MA, Hu T, Wright JLC (1999) Winter accumulation of paralytic shellfish toxins in digestive glands of mussels from Arcachon and Toulon (France) without detectable toxic plankton species revealed by interference in the mouse bioassay for lipophilic toxins. Nat Toxins 7:271–277

Cordier S, Monfort C, Miossec L, Richardson S, Belin C (2000) Ecological analysis of digestive cancer mortality related to contamination by diarrhetic shellfish poisoning toxins along the coasts of France. Environ Res Section A 84:145–150

Dorner JL, Hoffman WE, Fillipov MM (1983) Effect of in vitro hemolysis on values for certain porcine serum constituents. Vet Clin Pathol 12:15–19

Fujiki H, Suganuma M, Yoshizawa S, Nishiwaki S, Winyar B, Sugimura T (1991) Mechanisms of action of okadaic acid class tumor promoters on mouse skin. Environ Hlth Perspect 93:211–214

Hu T, Marr J, De Freitas ASW, Quilliam MA, Wright JLC, Pleasance S (1992) New diol esters (of okadaic acid) isolated from cultures of the dinoflagellates *P. lima* and *P. concavum*. J Nat Prod 55:1631–1637

Igboeli OO, Asuzu IU (2010) 'Diarrhetic' Type Shellfish Poisoning in Nigeria. Bull Environ Contam Toxicol 84:15–18

Ninčević-Gladan Ž, Skejić S, Bužančić M, Marasović I, Arapov J, Ujević I, Bojanić N, Grbec B, Kušpilić G, Vidjak O (2008) Seasonal variability in *Dinophysis* spp. abundances and diarrhetic shellfish poisoning outbreaks along the eastern Adriatic coast. Botanica Marina 51:449–463

Ramstad H, Hovgaard P, Yasumoto T, Larsen S, Aune T (2001) Monthly variations in diarrhetic toxins and yessotoxin in shellfish from coast to the inner parts of the Sognefjord, Norway. Toxicon 39:1035–1043

Stabell OB, Steffenak I, Pedersen K, Underdal B (1991) Diversity of shellfish toxins of "diarrhetic" type revealed by biological and chemical assays. J Toxicol Environ Hlth 33:273–282

Stobo LA, Lacaze J-PCL, Scott AC, Petrie J, Turrell EA (2008) Surveillance of algal toxins in shellfish from Scottish waters. Toxicon 51:635–648

Suganuma M, Suttajit M, Suguri H, Ojika M, Yamada K, Fujiki H (1989) Specific binding of okadaic acid, a new tumor promoter in the mouse skin. FEBS Letters 250:615–618

Vale P, Sampayo MAM (2002) First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Aveiro lagoon, Portugal and detection of okadaic acid esters in phytoplankton. Toxicon 40:989–996

Yasumoto T, Oshima Y, Yanaguchi M (1978) Occurrence of a new type of shellfish poisoning in the Tohoku district. Bull Jpn Soc Fish 44:1249–1255

Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J (1985) Diarrhetic shellfish toxins. Tetrahedron 41:1019–1025