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Biochemical responses in mice induced by saxitoxins extracted from the cockles *Acanthocardia tuberculatum*

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ABSTRACT

Harmful algae blooms have increased in frequency and geographic range in recent decades, and they produce toxins strains such as saxitoxins (STXs). they block voltage-gated sodium channels and can lead to several poisonings and the death of organisms that pose a significant risk to public and environmental health. The study of STXs toxicity has been carried out but little is known about the response of antioxidant enzymes activities to STXs in mice. The purpose of this study was to evaluate biochemical responses and oxidative stress induced by STXs extracted from *Acanthocardia tuberculatum.* To this end, daily, mice were treated orally for 7 days with sublethal concentrations (10 µg/100 g mouse). The animal's liver was assessed using biomarkers such as activities of catalase (CAT), thiobarbituric acid reactive substances (TBARS), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and succinate dehydrogenase (SDH). In the blood, plasmatic markers were analysed as glutamic oxalic transaminase (GOT), glutamic pyruvic transaminase (GPT), creatinine phosphokinase (CPK), lactate dehydrogenase (LDH), urea and creatinine. Globally, test toxicity test showed a significant decrease in the weight at 10 µg /100 g mouse, and the results showed an increase of GPT, GOT, CPK, LDH, CAT and TBARS activities and the inhibitory effect of GAPDH activities but creatinine, urea and SDH activities showed no significative difference from the control. We concluded that STXs induce oxidative stress breaking in mice the balance of the defence system and causing oxidations reactions. Moreover, STXs affect energy metabolism in mice, however, renal function in mice is not affected by exposure to STXs.

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Introduction

Cyanobacterial blooms create severe practical problems for public health (1) and increase evidently worldwide due to the production of toxin strains such as saxitoxins (STXs). STXs are one class of well-known neurotoxins associated with harmful algal blooms and are receiving great interest due to their high acute toxicity and wide geographic distribution (2).

STXs are considered one of the most toxic neurotoxins (3) and a group of more than 24 STX analogues closely related to tetrehydropurines, that block sodium channels of neurons that halts the transmission of electrical impulses and paralyses the neuromuscular system, leading to some cases death due to the respiratory fault (4).

STXs are also known as paralytic shellfish toxins (PSTs) and are produced naturally by certain species of marine dinoflagellates and freshwater cyanobacteria (5) that are present in tropical, subtropical and temperate climatic regions. These toxins are accumulated and sometimes metabolized into toxin derivatives in many species of filter-feeding bivalves, such as mussels, clams, cockles and scallops, making them potentially toxic to humans (6).

Bivalves ingesting the microalgae are important vectors for transferring STXs along the food chain; the capacity to accumulate STXs differs among bivalve species (7, 8). Many bivalves can accumulate high concentrations of STXs without showing any observable adverse effects and are relatively resistant to the harmful effects (9, 10).

Acanthocardia tuberculatum is among the slowed detoxifying cockles (11) that showed persistent contamination with high levels of toxicity, decarbamoyl saxitoxin (dcSTX) and saxitoxin (STX) are the toxins that account for most of this toxicity. Relative partitioning of STXs among tissues is variable and the foot is the most toxic organ followed by others organs (12; 13). Prolonged retention (several months to years) of STXs as dcSTX and STX is a characteristic of *A. tuberculatum* that can be explained not only by the specific retention of dcSTX (12) and differential accumulation of STXs in non-visceral organs (13) but also by the presence of soluble toxin-binding protein (PSPBP) in *A. tuberculatum* mainly in the foot (14).

Acanthocardia tuberculatrum, a cockle living on the western Mediterranean coast of Morocco, was chosen for extraction of STXs for the following reasons; this cockle is an appropriate organism to study the biochemical effects

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of the STXs extracted due to its remarkable retention of high persistent levels of STXs for several years in its tissues even when the potential toxin-producing microalgae are not present (15), also, because it is mainly exploited in the canning industry in Morocco and Spain and even leading to overexploitation of their natural banks (16).

The research has mainly focused on the accumulation of biotoxins. Some studies have also focused on analysing metal concentrations, shell structure and gene organization of the mitochondrial genome. Distribution data have been provided in several publications but few studies have been conducted on the response of antioxidant enzymes activities to STXs in mice. Antioxidants are substances that delay and/or prevent the oxidation of cellular substratum at low concentrations. Several enzymes are normal by-products of metabolism that could contribute to the knowledge of the states of the animal and could serve as biomarkers of oxidative stress by the effect of foreign compounds such as STXs. This study was undertaken to examine the effect of STXs after oral administration of toxic cockle extract on the metabolic markers, stress biomarkers, and clinical parameters of Swiss albino mice. Hence, the activities were measured after experimental exposure to sublethal concentrations of STXs.

Materials and Methods

Samples

Specimens of the cockle (*A. tuberculatum*) were collected from Kaa Srass on the Mediterranean coast of Morocco. The cockle tissues were kept at -20°C until use. NAD+ (free acid) was purchased from Boehringer (Mannheim, Germany) and all other chemicals were of analytical grade.

Extraction of paralytic shellfish toxins and mouse bioassay

Toxicity analysis was carried out by mouse bioassay according to the AOAC method (1990) (17): 100g homogenized tissues collected from toxic cockles (Kaa Srass) were mixed with 100ml 0.1M hydrochloric acid and boiled for 5min, pH adjusted to 2–3. The volume of the mixture was brought to 200 ml with double-distilled water, stirred and centrifuged at 3000rpm for 10min. The PSP mouse bioassay involves acidic aqueous extraction of selected organs. One milliliter of the supernatant was injected intraperitoneally into each of the three albino mice (20± 2g). The mice are observed for classical PSP symptoms, such as jumping in the early stages, ataxia, ophthalmia, paralysis, gasping and death by respiratory arrest. The time from initial injection to mouse death is recorded and the values of toxicity are expressed in terms of STX.

Animals and administration of STXs

Swiss albino mice were adapted to laboratory conditions at a temperature of 22°C with food and water *ad libitum*. The light cycle during the entire experiment was set to 14 h light and 10 h dark.

Forty-five animals were randomized into five groups of nine mice each, and the STXs extract was administered daily by oral injection for 7 days. Mice were given STXs at 10 µg /100 g of mice's weight while corresponding groups were given sterile double distilled water serving as a control in each treatment.

Blood analysis

The determination of the CPK, GOT, GPT, LDH and urea were carried out by " Laboratoire des analyses médicales du Centre National de la Sécurité Sociale (CNSS)", Casablanca.

Crude extract preparation

All procedures were carried out at 4°C. Samples of the liver were quickly weighed and then homogenized 1/3 (w/v) in 50 mM potassium phosphate buffer pH 7.4 containing 1mM EDTA, 1mM DTT. The homogenates were then filtered and stored at -20°C until use.

Biochemical assays

All assays were conducted at 25°C using Jenway 6405 UV/Visible spectrophotometer.

Catalase

CAT activity was determined by the decrease in absorbance at 240 nm, using 7.5 mM H_2O_2 in 50 mM potassium phosphate buffer at pH 7 and adjusting the absorbance of this solution to 0.05± 0.01. Total CAT enzyme activity was expressed in terms of units (µmoles substrate converted to protein min^{-1}) / mg total protein (18).

Thiobarbituric acid reactive substances

The assessment of the extent of hepatic lipid peroxidation relied on the determinations of malondialdehyde (MDA) equivalent content in the crude liver homogenates. Duplicate determinations from each liver were made and the average of the three measurements was used in the subsequent statistical analysis of the data. Lipid peroxidation was estimated by the formation of thiobarbituric acid reactive substances (TBARS) and quantified in terms of malondialdehyde (MDA) equivalents according to the method described by Samokyszyn and Marnett (1990) (19).

One milliliter of samples is added to 1 ml solution (0.375% thiobarbituric acid and 15% trichloracetic acid in 0.25 M hydrochloric acid). The tubes are heated at 100°C for 15 min. Then, they are cooled in the ice to stop the reaction. A centrifugation was then carried out with 1000g for 10 min. The reading of supernatant is made to 535 nm, TBARS were calculated from a standard curve (8-50 nmol) of malondialdehyde (MDA), freshly prepared by acidification of TEP (1,1,3,3- tetraethoxypropane). Results are expressed as TBARS (nmol/mg protein) using $\varepsilon = 1.56 \times 10^5\ M^{-1}\ cm^{-1}$.

Glyceraldehyde 3-phosphate dehydrogenase

GAPDH activity in oxidative phosphorylation was determined by monitoring NADH generation at 340 nm (20). The reaction mixture of 1 ml contained 50 mM Tricine-NaOH buffer pH 8.5, 10 nM sodium arsenate, 1 mM NAD+ and 2 mM D-G3P.

Succinate dehydrogenase

The enzyme was assayed according to King (1967) (21) with assay conditions: 100 mM potassium phosphate buffer (pH 7.4), 0.3 mM EDTA, 0.053 mM DCIP and 100 µg of protein. The mixture was preincubated for 10 min at 37°C before to added 50 µl of KCN-Succinate (containing 3.25 mg/ml of KCN in 0.5 M succinate). The measure of activity was done at 625 nm.

Table 1. Growth parameters from control and STXs treated mouse with 1µg/100g mouse.

Growth parameter	Control	STXs
Initial body weight (g)	19.4±1.24	19.3±1.16
Final body weight (g)	19.6± 1.01	19.2±1.06
Specific growth rate (% day)	0.41±0.12	-0.10±0.63*
Food intake (% body weight/ day)	1.52±0.30	1.06±0.38*

*Variations statistically different from control ($p < 0.05$).

Table 2. Effect in vivo of STXs on the response of oxidative stress and metabolic biomarkers.

	Control	STXs
Catalase (µmol/min/mg of protein)	3.51±0.76	6.44±1.73* (×1.83)[a]
Thiobarbituric acid reactive substances (nmol/mg of protein)	0.42±0.16	0.85±0.13* (× 2.02)[a]
Glyceraldehyde-3-phosphate dehydrogenase (µmol/min/mg of protein)	0.50±0.15	0.19±0.095* (-62%)[b]
Succinate dehydrogenase (Absorbance/min/mg of protein)	1.48±0.76	0.76±0.29

Values (mm) are expressed as mean± standard deviations (n=9). [a] indicate how many times the values have increased compared with the control values. [b] Indicate the percentage of decrease of the values compared with the control values. * $p < 0.05$ (student t-test).

Protein quantification

Protein content in the liver was measured according to the Bradford procedure, using bovine serum albumin (BSA) as standard (22).

Statistical analysis

All experiments were replicated four times and tests were duplicated, the experimental data represent the means ± standard deviations. Means were compared using the Student t-test, using SPSS statistical software Version 12.0. Differences were considered significant at the level $p<0.05$ and very significant at the level $p<0.01$.

Results

Toxicity test

The general state and the mice mortality were followed during the 7 days of treatment. No sign of stress or difficulties breathing in the treated orally mice STXs was observed.

No changes were observed with the amounts 0,1 and 1 mg/100 g mice during 7 days of treatment. On the other hand, a significant decrease in the weight was observed (25%) and (33%) at 10 and 100 mg/100 mice, respectively. Neither mortality nor visible disease signals were observed in the mice during the 7 days of treatment (Figure 1). A statistically significant decrease in food intake and specific growth rate (SGR) was observed in STXs treated mice with 1mg/100 g mouse when compared with control mice (Table 1). However, no differences were seen in the final gross body mass between control and treated oral mice (Table 1).

Effect of STXs on stress and metabolic biomarkers

The impact of STXs on liver function was evaluated by the assays of metabolic enzymes and stress biomarkers (Table 2). For stress biomarkers, the treatment of the mice showed that STXs induced a significant increase in the level of TBARS (×2.02) and CAT activity (×1.83) compared with the control (Table 2).

For the metabolic markers, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was very sensible to STXs, it strongly decreased (-62%). No change was observed in mitochondrial succinate dehydrogenase activity (Table 2).

Effect of STXs extract on plasmatic parameters

The blood analysis indicated an important increase in the level of CPK (× 2.79), GOT (x 1.51) and GPT (× 1.56). LDH is also increased (× 1.76). No significant changes were observed in creatinine and urea activities (Table 3).

Discussion

There are differences among the bivalves in the way they deal with and respond to the toxic cells and their toxins, as determined by neurological, physiological and behavioural responses. In general, bivalves are not affected

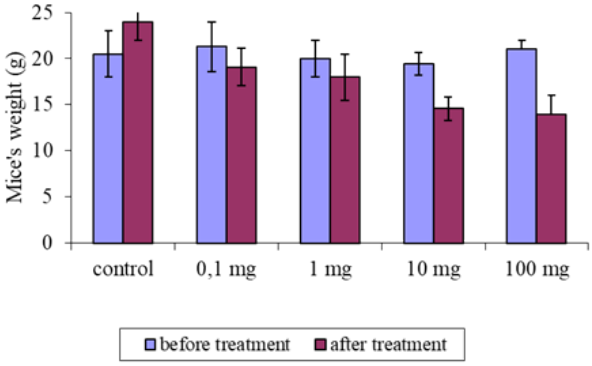

Figure 1. Weight variation of the Swiss albinos mice before and after oral injection (7 days) with 0.1 µg/100g mouse, 1 µg/100g mouse, 10 µg/100g mouse, 100 µg/100g mouse of STSx extract. Values are means of standard deviations. *p< 0,05 (student t-test), indicates the percentage of decrease or increase of the values compared with the control.

Table 3. Plasmatic parameters from control and STXs treated mouse.

	Control	STXs
GOT (U/l)	223±26.44	337±35.37* (× 1.51)[a]
GPT (U/l)	45.6±14	71.5± 13.86* (×1.56)[a]
CPK (U/l)	800±171.11	2232±277* (×2.79)[a]
LDH	800.25±248.19	1413.5±257* (×1.76)[a]
Urea	0.32±0.10	0.41±0.049
Creatinine	8.66± 1.52	9.5±1.41

Values (mm) are expressed as mean± standard deviations. [a] Numbers in brackets indicate how many times the values have increased compared with the control values. * $p<0.05$ (student t-test).

by exposure to toxic dinoflagellates that produce STXs. However, Estrada et al in 2010 reported that after injection with STXs extracted from the *Gymnodinium catenatum*m, *Nodipecten subnodosus* (giant lions-paw scallop) is paralyzed, indicating that STXs provokes effects similar to what is observed in vertebrates, including paralysis and metabolic stress. Paralysis stress was accompanied by negative scallop responses, based on visible effects, generation of lipid peroxidation, and changes in antioxidant enzymes in haemocytes and tissues (23).

The antioxidant defences of marine molluscs have been the subject of several studies, focusing on fish and bivalve species, but little else is known about the biochemical effect of STXs in mammals. Previous data has shown that STXs could induce phase II xenobiotic metabolizing enzymes (XMEs) like glutathione S-transferase (GST) in Atlantic salmon (*Salmo salar*), and suggested a potential role for XMEs in STXs metabolism (24). Another study reported that in mammals, the phase II XMEs could also be induced by STXs as in fish. Thus, phase II XMEs may serve as detoxifiers in STXs intoxication in mammals as has been found in aquatic animals (25).

In our study, responses to the oxidant system in mice can be found after oral administration to STXs extracted from *A. tuberculatum*. No difference was observed in gross body mass but food intake and specific growth rate were decreased in STXs treated mice at 1mg/100 g mouse. The decrease in SGR may be related to the concomitant decrease in food intake. However, the body weight of the mice was altered by STXs at 10 and 100 mg/100 g mice. The same results have been reported in both male and female zebrafish, in effect, a significant impairment in body weight and length was observed in response to saxitoxin (26).

For stress biomarkers, a significant increase in lipid peroxidation (TBARS) was observed, Similar results were obtained in green algal (*Chlamydomonas reinhardtii*) that suggest that high concentrations of STX can affect the algal defense system causing reactions of oxidations (27). Moreover, STXs exposure has been reported to induce oxidative stress, cellular damage, and immunotoxicity indiscriminately in both oysters and scallops (28). A recent study showed that, in areas with a high incidence of blooms, shellfish showed a high activity of antioxidants, however, during the stages involving the distribution and bioconversion of toxins, there is decreased activity of antioxidant enzymes resulting in oxidative damage (29).

This increase in TBARS shows the beginning of oxidative stress in the liver and could indicate damage to tissue cells. Effectively, the stress response is a catabolic reaction and oxidative stress occurs as a consequence of the imbalance between the formation of oxygen free radicals and the inactivation of these species by the antioxidant defense and inactivation of these species by the antioxidant defense system. Indeed, living tissues are endowed with innate antioxidant defense mechanisms, such as the presence of the activity of CAT, GST, peroxide dismutase, ethoxyresorufin-O-deethylase (EROD)… Reduced activity of these enzymes is associated with the accumulation of free radicals (30). One consequence of the overproduction of free radicals is lipid peroxidation and damage to membranes (31). Lipid peroxidation (LPO) is an important feature in cellular injury and has often been used as a biomarker of environmental stress, increasing membrane rigidity, osmotic fragility, reducing erythrocyte survival and perturbations in lipid fluidity and reflecting damage to cell membranes from free radicals (32).

Free radical-induced oxidative stress has been implicated in the pathogenesis of a wide variety of clinical disorders, resulting usually from a deficiency of natural antioxidant defenses. Potential antioxidant therapy should therefore include either natural free radical scavenging antioxidant enzymes or agents which are capable of augmenting the activity of these enzymes which include CAT. Indeed, CAT activity is induced by the production of hydrogen peroxide in the cells and catalyses the reaction, which reduces this compound to water and oxygen (33).

The increase of CAT in this study suggested that the liver was under oxidative stress as a result of exposure to the toxin. It seems that the rise of lipid peroxidation was ameliorated by the rise in CAT activity because CAT detoxifies H_2O_2 and lipid hydroperoxides. Effective induction of CAT in tissues and cells would help to clear the peroxides accumulated after exposure to STXs. High levels of oxidative damage can result not just from oxidative stress, but also from the limitation of the cellular repair system, and this dysfunction may cause deregulation of the cell defense system, leading to cell death (34). Previous studies have shown that mussels (*Mytilus chilensis)* exposed in vivo to saxitoxin reported a significate higher gene transcription in superoxide dismutase and CAT levels in (35). Our results were consistent with the toxicological studies that reported a significant increase in the levels of malondialdehyde, together with decreased enzymatic activities of catalase and superoxide dismutase, was observed in fish of both sexes exposed to 1 $\mu g \cdot L^{-1}$ saxitoxin, indicating the occurrence of lipid peroxidation and oxidative stress (26).

For the metabolic biomarkers, GAPDH was strongly suppressed. This decreased GAPDH activity, suggests that the recovery process increased the production of energy in the liver, and the metabolism energy in the heart and liver are perturbed which can be caused for a long time serious problems, such as cirrhoses of the liver and myocardial infracts. No significative change of SDH activity was recorded during 7 days of exposure to STXs and this kind of enzyme appeared less interesting biomarker than the above parameters for short-term exposure to the toxin.

For the serum marker enzymes, CPK is an important enzyme in the energetic metabolism found mainly in the heart, brain, and skeletal muscle and is an indication of muscle damage. This increased CPK level means there has been injury or stress to muscle and cell necrosis in the heart. The transaminase enzymes (GOT, GPT) are important in the production of various amino acids, with a high metabolic activity within cells. The increase of transaminases in our study reflects cellular injury and is an indicator of liver damage. This increase is often associated with disease infarction (heart attack) or liver (necrosis). The liver biochemical alterations have been also reported in fish exposed to *Cylindrospermopsis raciborskii* culture containing 97% STX (36).

LDH is usually used as a general cytotoxicity, necrosis indicator (37) and a marker of myocardial infarction. LDH activities in the mice receiving STXs were significantly increased than the control. The same results have been reported in the previous study that report that the lung tissues of mice were exposed to higher doses of STXs (38). The increase in LDH activity in our study shows that

tissues are damaged by injury or disease, consequently, they release more LDH into the bloodstream (39). There appeared to be no renal toxicity in the mice because we found no significant change in the renal function of urea and creatinine.

In summary, though the STXs are known to be sodium channel blockers, our results indicated that they could also affect the antioxidant enzymatic systems and plasmatic markers in mice injected with STXs. Indeed, STXs in mice act as an exogenous source of oxidative stress, yielding reactive oxygen species that are responsible probably for LPO. The results showed also cellular injury, liver damage and necrosis heart in mice caused by exposure to STXs. To our knowledge, this is the first report showing the induction of enzymatic systems in mice by STXs extracted from *Acanthocardia tuberculatum*.

Abbreviations

CPK, creatinine phosphokinase; GOT, glutamate oxalate transaminase; GPT, glutamate pyruvate transaminase; PSTs, paralytic shellfish toxins; STXs, saxitoxins; TBARS, thiobarbituric acid reactive substances; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SDH, succinate dehydrogenase; STX, saxitoxin ; dcSTX, decarbamoyl saxitoxin; PSPBP, paralytic shellfish poisons binding protein; LDH, lactate dehydrogenase; LPO, lipid peroxidation; XMEs, xenobiotic metabolizing enzymes; SGR, Specific growth rate; BSA, bovine serum albumin; CAT, catalase.

Author contribution

N. T: Conceived, designed and performed the experiments, analyzed the data, contributed analysis tools, and wrote the paper. H. A: revised the final version of the paper. M. N. B: revised the final version of the paper. M. B: Contribute to the design and analyzed the data, correcting the paper. M. M. E: Conceived, designed and supervised the experiments and the analysis of the data, critical of writing the paper and coordinating the work
All authors approved the final version of the paper.

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Conflict of interest

The authors declare that they have no conflict of interest

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