



Biochemical and physiological responses in Wistar rat after administration of puffer fish (*Lagocephalus lagocephalus*) flesh

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Abstract

The aim of this study was to evaluate the toxicity of the puffer fish *L. lagocephalus* after administration to Wistar rats. For this purpose, male wistar rats were fed during two periods of treatment, 48 h and 2 months, with a standard rat diet supplemented with the *L. lagocephalus* flesh. Various diet compositions were tested: 100% standard rat diet (T); 10% of raw flesh of *L. lagocephalus* (Lcr); 10% of cooked flesh of *L. lagocephalus* with or without water of cooking (Lcu+b and Lcu-b respectively). The values were validated by concerning flesh of raw and cooked mule *Liza aurata* (Mcr and Mcu+b respectively). The evaluation of hematologic parameters showed that Lcr and Lcu+b foods induced in the rats haemolytic anaemia (for the two treatment periods) as well as macrocytic anaemia accompanied with swelling and interruption of erythrocytes maturation. For Lcu+b rats, serum analysis showed disturbance of transaminase activity (aspartate aminotransferase AST and alanine aminotransferase ALT) which could indicate an uncompensated hepatic cellular lesions. Indeed, a significant reduction of serum alkaline phosphatase (ALP) rate was noted after 2 months of treatment. These results were confirmed by a hyperproduction of thiobarbituric acid reactive substances (TBARS) in the liver tissue suggesting hepatic oxidative damages. However, the serum cholinesterase activity (AChE) showed no significant variation between rat groups, suggesting that brain was not altered. These findings were not similar to those of Lcu-b and Lcr. Hence, the cytotoxic factor of *L. lagocephalus* flesh seems to be water-soluble and heat-stable. In raw flesh, the extraction is less to cause only hematologic effects, whereas, in cooked flesh with the use of water of cooking, the extraction is more important and causes more effects. These results suggested that in diet containing the cooked flesh with water of cooking, the toxic effect is more pronounced than in raw flesh.

Key words: Biochemical parameters, hematotoxicity, hepatotoxicity, *L. lagocephalus*, puffer fish.

Introduction

Ingestion of flesh, viscera or skin of puffer fish belonging to the family Tetraodontidae causes considerable intoxication. Generally, skin, viscera and ovaries are the most toxic tissues. Muscle of some species may also be toxic. Toxicity results from the action of the sodium channel adduct tetrodotoxin (TTX) ¹. TTX is a potent neurotoxin acting on Site 1 of the voltage-dependent sodium channels of excitable membranes, blocking sodium influx and consequently, action potential ². At the time of intoxication, the distribution of toxin by blood around the body is rapid. TTX can induce serious histopathological and metabolic damage. In mouse, TTX induces diarrhoea, dyspnoea, hyper salivation and may cause hyper-secretion and stomach ulcers. Neurological effects and transitory cardiovascular events were also observed ³⁻⁶. In addition, the haemolytic properties of marine toxins were demonstrated in red fish and humans ⁷. However, toxic effects are reported after chronic intoxication following consumption of flesh from the poisoning puffer fish (*L. lagocephalus*) ⁸. Recent evidence suggests that certain marine toxins, including TTX, also induce oxidative stress and disruption of osmotic and ion regulation in

crustacean species. These toxins can disturb enzymatic function and hematologic parameters ^{9,10}. Toxicity was dependent on the quantity of introduced toxins or free radicals and the detoxifying capacity of the liver. This relationship was clearly demonstrated during intoxication by repetitive consumption of *L. lagocephalus* flesh, which revealed that toxins were metabolized by the liver, inducing hepatic necrosis ¹¹. However, it was not clear if hepatic or nervous lipoperoxidation occurred after consumption of *L. lagocephalus* flesh.

Previous studies on fish toxicity are limited to the effects of injecting extracted TTX on animals, whereas reports on the physiological and cytotoxic effects of consumption of the toxic fish by animals or humans remained scarce. Hence, the present study was conducted to evaluate the effects of acute or chronic consumption of *L. lagocephalus* flesh on haematology factors, enzymatic and metabolic biomarkers and on lipid peroxidation products in adult rats.

Material and Methods

Specimen collection: Specimens of puffer fish (*L. lagocephalus*, Linnaeus, 1766) were caught at different localities in the Tunisian coast between 2003 and 2005. Directly after collection, the fresh samples were eviscerated and immediately frozen at -20°C until used. For the rat bioassay tests, the raw and cooked flesh were added to the standard commercial diet (SICO, Sfax, Tunisia) and administered to the rats. The flesh of mule *Liza aurata* was utilized as a negative control.

Animals and diets: Male Wistar rats (n= 72) weighing 165-170 g were purchased from Central Pharmacy of Tunisia. They were housed at 22±3°C with light-dark periods of 12 h and minimum relative humidity of 40%. Water was provided *ad libitum*. After acclimatization to the laboratory conditions for 1 week, animals were divided in six groups of six animals each: Group 1: control with free access to standard diet (T); Group 2: diet supplemented with 10% of raw flesh of *L. lagocephalus* (Lcr); Group 3: diet supplemented with 10% of cooked flesh free of the cooking water (Lcu-b); Group 4: diet supplemented with 10% of cooked flesh with water of cooking (Lcu+b); Groups 5 (Mcr) and 6 (Mcu+b): diet supplemented respectively with 10% of raw and cooked flesh of *Liza aurata* used as a negative control. At the end of treatments (48 h and 2 months) animals were sacrificed and dissected.

Sample collection and haematologic parameters: After animal anaesthesia with chloral hydrate by intra-abdominal way, blood samples were collected with heparin by heart puncture to determine haematological parameters (RBC, WBC, Ht, Hb, MCV) using routine laboratory methods. Serum samples were obtained by centrifugation at 4000 rpm for 15 min and stored at -20°C until analysis. Liver and brain tissues were excised, washed with cold saline and stored at -30°C until analysis.

Enzymatic and metabolic analysis: Enzymatic activities including aspartate aminotransferase (AST/TGO A03010), alanine aminotransferase (ALT/TGPA03020), alkaline phosphatase (ALP A03000), creatinine (creatinine A02335) and cholinesterase (AChE 0053) were determined in serum using commercial reagents kits purchased fromELITROL and Biotrol (France).

Lipid peroxidation (LPO): LPO was estimated by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust¹². Briefly, one gram of tissue (liver or brain) was directly homogenized in 2 ml of Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4), then mixed for 10 s and centrifuged (9000 rpm, 15 min, 4°C). An aliquot of the supernatant was used to assay for protein content according to the method of Lowry *et al.*¹³. For the assay, 175 µl thawed supernatant was mixed with 175 µl of 20% trichloroacetic acid (TCA) containing 1% butyl-hydroxytoluene and centrifuged (1000 rpm for 10 min). 200 µl of the resulting supernatant was mixed with 40 µl 0.6 M HCl and 160 µl 26 mM Tris (pH 7.4) buffer containing 0.72 mM thiobarbituric acid (TBA). The contents of the tubes were boiled for 10 min at 80°C. After cooling, absorbance was measured at 530 nm using a spectrophotometer (JENWAY 6105). TBARS concentration was estimated using the extinction coefficient of malondialdehyde (MDA)-thiobarbituric acid complex (156 mM⁻¹cm⁻¹). Results were calculated as nmol MDA/mg protein as indicated by Buege and Aust¹².

Statistical analysis: Data were analysed using the Student's *t*-test. All values are expressed as means ± SD. Differences were considered significant if p≤0.05.

Results

Effects of *L. lagocephalus* flesh on haematological parameters: Results on the effects of *L. lagocephalus* flesh on haematological parameters are summarized in Table 1. Rats fed on the standard diet supplemented with 10% *L. lagocephalus* flesh, either raw (Lcr) or cooked (Lcu+b) had significantly lower number of red blood cells (RBC) compared with the control group during the study period (48 h and 2 months). The hematocrit (Ht) content also decreased significantly over the 48 h treatment period. The mean corpuscular volume (MCV) in animals with the toxic diet was significantly increased after 2 months compared to the control. Haemoglobin concentration (Hb) was unchanged among the groups. In the Lcr and Lcu+b groups, a significant decrease in white blood cell count (WBC) was observed after 2 months. Haematological parameters remained unchanged for Lcu-b, Mcr and Mcu+b rat groups.

Table 1. Effects of *L. lagocephalus* flesh on haematologic parameters in rats.

Parameter and treatment	RBC	HT	MCV	WBC
48 h after treatment				
T	6.412 ± 0.235	0.345 ± 0.008	55.11 ± 2.59	3.01 ± 0.123
Lcr	3.773 ± 0.136 **	0.297 ± 0.009 **	66.57 ± 0.714	2.7 ± 0.063
Lcu-b	7.083 ± 0.186	0.324 ± 0.014	47.65 ± 15.07	3.145 ± 0.202
Lcu+b	4.25 ± 0.34 **	0.22 ± 0.002 **	55.69 ± 4.66	3.541 ± 0.507
Mcr	5.716 ± 0.229	0.314 ± 0.012	54.69 ± 2.807	2.7 ± 0.151
Mcu+b	6.841 ± 0.228	0.308 ± 0.007	43.73 ± 2.687	2.75 ± 0.275
2 months after treatment				
T	5.879 ± 0.205	0.379 ± 0.01	66.00 ± 2.561	2.975 ± 0.108
Lcr	4.350 ± 0.075 **	0.348 ± 0.015	77.66 ± 3.377 *	2.286 ± 0.085 **
Lcu-b	6.008 ± 0.184	0.336 ± 0.011	55.85 ± 17.662	3.270 ± 0.274
Lcu+b	4.225 ± 0.222 **	0.333 ± 0.025	85.03 ± 5.916 **	1.875 ± 0.125 **
Mcr	5.272 ± 0.136	0.351 ± 0.006	68.892 ± 2.538	2.472 ± 0.232
Mcu+b	5.488 ± 0.08	0.34 ± 0.019	62.74 ± 5.94	2.406 ± 0.101

Values are expressed as mean ± S.D. (n= 6). The significance levels observed are * p<0.05 and ** p<0.01 in comparison to control group values; RBC as 10⁶/mm³ of blood; HT as %; MCV as µm³/RBC of blood and WBC as 10³/mm³ of blood.

Table 2. Effects of *L. lagocephalus* flesh on enzymatic and metabolic biomarkers in rats.

Parameter and treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	Creatinine ($\mu\text{mol/L}$)	ACHe (U/L)
48 h after treatment					
T	302.77 \pm 28.4	49.11 \pm 3.26	661.62 \pm 61.07	40.22 \pm 2.37	148.58 \pm 31.28
Lcr	351.8 \pm 99.09	58.2 \pm 11.92	507.8 \pm 26.38	45 \pm 2.7	148.58 \pm 56.39
Lcu-b	321.66 \pm 38.9	63.66 \pm 6.14	475.6 \pm 46.67	44 \pm 1.41	140.76 \pm 46.92
Lcu+b	550.2 \pm 124.65*	63.75 \pm 3.97*	532.2 \pm 60.22	33.2 \pm 3.69	125.12 \pm 20.68
Mcr	382.4 \pm 86.37	53 \pm 2.66	640.6 \pm 101.16	34 \pm 3.87	175.95 \pm 11.73
Mcu+b	284.33 \pm 16.04	48.75 \pm 7.66	851.25 \pm 45.76	42 \pm 3.41	152.4 \pm 11.73
2 months after treatment					
T	269 \pm 23.45	55.83 \pm 3.59	417.77 \pm 26.42	43.88 \pm 3.49	172.04 \pm 15.64
Lcr	208.25 \pm 42.3	55 \pm 2.76	360.4 \pm 53.6	42.6 \pm 2.71	93.84 \pm 23.46
Lcu-b	280.8 \pm 32.91	66.5 \pm 5.89	453.25 \pm 12.55	36.6 \pm 2.13	199.41 \pm 11.73
Lcu+b	163.33 \pm 11.18*	36.66 \pm 0.47*	293.33 \pm 17.04*	46.33 \pm 3.29	211.14 \pm 46.92
Mcr	253 \pm 32.47	45.66 \pm 5.35	397.33 \pm 20.74	51.66 \pm 2.18	175.95 \pm 35.19
Mcu+b	256.5 \pm 38.98	64.66 \pm 8.56	432.66 \pm 34.79	43 \pm 3.39	129.03 \pm 11.73

Values are expressed as mean \pm S.D. (n= 6). The significance levels observed are * p<0.05 and ** p<0.01 in comparison to control group values.

Effects of *L. lagocephalus* flesh on enzymatic and metabolic biomarkers:

Table 2 shows the effects *L. lagocephalus* flesh on AST, ALT, ALP, creatinine and AChE activities. Lcu+b rat group showed a significant increase in transaminase activities (AST and ALT) after 48 h of treatment. AST and ALT levels increased by 45 and 23%, respectively, in Lcu+b compared to control. However, these activities were significantly reduced by 40 and 35%, respectively, after two months of treatment, whereas no significant variations in ALP were observed after 48 h of treatment. After treatment for 2 months, ALP activity was 30% lower in the Lcu+b group than in the control group (P>0.05). There was no significant change in AChE activities among the groups. In the Lcr, Lcu-b, Mcr and Mcu+b groups, no significant variation was observed in the activities of AST, ALT and ALP compared with the control group.

Effects of *L. lagocephalus* flesh on the rate of thiobarbituric acid reactive substances:

In the Lcu+b group, TBARS levels in the liver at 48 h and 2 months were significantly higher (54 and 65%, respectively). In brain tissues, this parameter remained unchanged compared to the control group. However, TBARS levels were unchanged among the groups (Lcr, Lcu-b, Mcr and Mcu+b) in liver and brain compared to control group (Table 3).

Table 3. Effects of *L. lagocephalus* flesh on lipid peroxidation products as thiobarbituric acid reactive substances in rats.

Parameter and treatment	48 h	2 months
LPO (nmol TBARS/mg protein in liver tissue)		
T	0.54 \pm 0.09	0.66 \pm 0.12
Lcr	0.22 \pm 0.03	1.06 \pm 0.17
Lcu-b	0.64 \pm 0.36	0.53 \pm 0.1
Lcu+b	1.17 \pm 0.28 *	1.87 \pm 0.85 *
Mcr	0.62 \pm 0.18	0.39 \pm 0.06
Mcu+b	0.83 \pm 0.06	0.46 \pm 0.03
LPO (nmol TBARS/mg protein in brain tissue)		
T	0.18 \pm 0.04	0.18 \pm 0.02
Lcr	0.18 \pm 0.05	0.14 \pm 0.02
Lcu-b	0.11 \pm 0.02	0.29 \pm 0.05
Lcu+b	0.16 \pm 0.03	0.21 \pm 0.04
Mcr	0.18 \pm 0.02	0.25 \pm 0.07
Mcu+b	0.25 \pm 0.06	0.22 \pm 0.02

Values are expressed as mean \pm S.D. (n= 6). The significance levels observed are * p<0.05 and ** p<0.01 in comparison to control group values.

Discussion

Standard rat food supplemented with *L. lagocephalus* raw (Lcr) and cooked flesh with the water of cooking (Lcu+b) induced anaemia. Similar pathologies have been reported in other toxicity experiments using sea products^{14, 15}. The maitotoxin (MTX), isolated from viscera of *Ctenochaetus straiatus* fish, was reported to cause parallel effects *in vivo* assays using small freshwater fish *Tanichthys albonubes*¹⁴. Sea cucumbers also displayed several toxic properties including hemolysis and ichthyotoxicity, suggesting the presence of saponins¹⁶. In the present work, after acute exposure of rats to diet supplemented with raw and cooked flesh with water of cooking, haemolytic anaemia was observed. This anaemia was characterized by a decrease in RBC number and Ht with an increase of MCV and unchanged Hb values. These observations, also reported in other studies, were explained by the presence of TTX in sea products. The anaemia persisted after two months of treatment. Enhancements of MCV, stable hematocrit rate (Ht) and haemoglobin concentration (Hb) were observed indicating a macrocytosis anaemia with swelling of erythrocytes. These results suggested that the toxic compounds in *L. lagocephalus* present haemolytic and interruption of erythropoiesis after two months of treatment. The phenomenon of erythrocyte swelling has been described previously by Sauviat *et al.*⁷ with ciguatoxins which are similar to TTX present in *L. lagocephalus* flesh^{3,4}. Haematologic parameters were not changed in the Lcu-b, Mcr and Mcu+b groups. It seems that flesh of the mule fish does not have active substances which cause anaemia. Similar results were obtained with flesh of *L. lagocephalus* free of the water of cooking (Lcu-b).

The haemolytic activity of saponins is due to the interaction between the toxins and the free 5(6)-sterols of the plasma membrane¹⁷. This interaction causes an alteration in membrane selective permeability to the K⁺ ion and results in cytolysis. In our study, cytotoxicity by flesh of *L. lagocephalus* was observed in groups of rats (Lcr and Lcu+b) receiving diet supplemented with raw and cooked flesh containing the water of cooking. Therefore, as suggested by Hwang *et al.*¹⁸, the *L. lagocephalus* flesh toxin seems to be both water-soluble and heat-stable. Results obtained in the present work suggested that toxic compound altered RBC number more than WBC, particularly in the Lcr and Lcu+b treatment groups. This observation indicates that the haemolytic activity of the tetrodotoxin against RBC occurs at lower

concentrations of toxins compared with other cell model¹⁹. This effect can be explained by the higher content of sterols in RBC compared with other cell models. In fact, the presence of sterols favours the action of these toxins on RBC.

Certain metabolic and enzymatic biomarkers can be used in the diagnosis of some cases of accidental and experimental poisoning. For example, variations in enzymatic activity parameters in certain lysed tissues were also reported by Correa *et al.*²⁰. The evaluation of these parameters remains important in the accurate identification of the toxicity and in suitable treatment²¹.

Lcu+b rat group showed a significant enhancement in transaminase activities (AST and ALT) after 48 h of treatment. These activities depend on the number of altered cells and damaged tissues²⁰. Similar findings (increases in creatine kinase rate and transaminase activities) were observed after human intoxication caused by *Tityus serrulatus* envenomation²². After 2 months of treatment, significant reductions in AST, ALT and ALP activities in the Lcu+b group was probably associated with some intoxication effects. However, the creatinine rate in serum remained constant, suggesting that the kidneys were not affected. Moreover, AChE activity was not altered in any rat treatment groups. It is recognised that damage caused by repeated consumption of small quantities of toxins may be of greater importance than that caused by acute intoxication²³. Hence, chronic and subchronic exposure to toxins has been demonstrated in animal studies to lead to the appearance of tumours²⁴. Results indicated that serum cholinesterase activity (AChE), as a neurotoxic biomarker, was unchanged. It appears therefore, that this intoxication did not affect the brain. Ito *et al.*²⁵ reported that azaspiracid (AZP) induced necrosis and fatty change in liver, intestine and lymphoid tissues, such as the thymus and spleen. Similar ultrastructural modifications were observed in liver following treatment with okadaic acid (OA), including atrophic signs of hepatocytes²⁶.

The production of free radicals following exposure to some xenobiotics and toxic foods can produce various cytotoxic effects such as enzyme system inactivation, damage to proteins and DNA and, in particular, cell membrane destruction as a result of lipid peroxidation. Generally, lipid peroxidative damage can be evaluated by the measurement of TBARS rate. In this study, an increased hepatic TBARS rate occurred in the Lcu+b group after 48 h of treatment with a further increase after two months. These results confirmed that the food containing the flesh cooked with water of cooking was more toxic than the other diet treatments. A correlation between enhanced lipid peroxidation and serious hepatic damage in rat and human was suggested in previous work²⁷. However, the cerebral TBARS rate presented no significant variations in any treatment group in the current work, as confirmed by the stable AChE activity; this lack of effect probably results from the presence of a haemato-encephalic barrier protecting the mammal brain against toxic compounds.

We hypothesize that in muscle of *L. lagocephalus*, and probably in all species, there are a natural antioxidants in their red-colored flesh, which were sensible during heat treatment²⁸. Cooking treatment produced important changes in the muscle components (water, muscle fibres, connective and adipose tissues). Hence, structural changes induced by heating influences texture and other parameters associated to flesh quality^{28, 29}, the quantity of coagulated proteins in the interstices, and the gel formed by

collagen and lipids. Moreover, it seems that the raw flesh of *L. lagocephalus* contained antioxidant compounds which could be destroyed by heat treatment (100°C) causing toxicity enhancement.

These observations on the toxicity of the Tunisian puffer fish (*L. lagocephalus*) revealed the presence of toxicity in flesh and enhanced toxicity of cooked flesh and clearly indicate the danger of using this fish as food.

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