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OXIDATIVE STRESS AND HEPATOTOXICITY IN RATS INDUCED BY POISONOUS PUFFERFISH (*Lagocephalus lagocephalus*) MEAT

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ABSTRACT: This study was undertaken to evaluate the effect of pufferfish (*Lagocephalus lagocephalus*) meat poisoning on hepatic functions of Wistar rats. For this purpose, groups of rats (Lcr, Lcu+b and Lcu-b) received diet supplemented with 10% of raw or cooked meat, respectively, with or without cooking water of *L. lagocephalus* while groups Mcr and Mcu+b received diet supplemented with 10% of raw or cooked meat of *Liza aurata*, which were used as a negative control. In Lcu+b group, ALT, AST and ALP rates (hepatic enzyme markers) decreased after two months of treatment, indicating liver damage. We also observed an increase of 54 and 65% of thiobarbituric acid reactive substances (TBARS) in their livers respectively 48 hours and two months after treatment compared to controls. The catalase (CAT) activity in group Lcu+b decreased ($p < 0.05$) after two periods of treatment, whereas metallothionein (MT) level significantly increased and decreased, respectively after 48 hours and two months. In fact, in the histological analysis of the livers from Lcu+b treated group, we observed an increase in vacuolisation, necrosis, hepatocytes ballooning and sinusoids dilation. These results indicate that *L. lagocephalus* meat cooked with water produces hepatotoxicity and oxidative damage.

KEY WORDS: hepatotoxicity, *Lagocephalus lagocephalus*, oxidative stress, tetrodotoxin.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Pufferfish belongs to the Tetraodontiformes order and is responsible for most poisoning cases along the Asian coasts (1). Their venom, tetrodotoxin (TTX), is a potent neurotoxin that acts on site 1 of voltage-dependent sodium channels in excitable membranes, blocking sodium influx and, consequently, action potential (2-5). In some regions, pufferfish species can be employed in human consumption. Food poisoning through consume of toxic puffers occurs especially in Japan and China where this fish meat is considered a delicacy (6, 7). In Japan, despite precautions, the annual death rate provoked by pufferfish poisoning is around 50 (8). On the other hand, *L. lagocephalus* is occasionally caught by fishermen but is always discarded, due to its venom.

Recent evidence has shown that some toxins induce oxidative stress and disruption of osmotic and ionic regulation in crustacean species (9, 10). A human distinctive form of ichthyosarcotoxism, acquired by eating flesh of fish containing toxic substances, was thought to be caused by blood poisoning and characterized by gastrointestinal, neurological and cardiovascular disorders (11, 12). Although it is now established that these symptoms, including death, are due to the increase in neurotransmitter release secondary to the binding of toxins to voltage-sensitive sodium channels, the mechanism of tissue damage remains unclear. Necrosis and myocytolysis have been observed in the intestine, thymus, spleen and lungs (13, 14).

Liver is a key organ actively involved in numerous metabolic and detoxifying functions. Consequently, it is continuously exposed to high levels of endogenous and exogenous oxidants that are the by-products of many biochemical pathways while the intracellular oxidant production is more active in liver than in other rat tissues (15, 16). The hepatic damage caused by microcystins (toxic blooms of cyanobacteria) can be measured by some clinical enzyme markers, such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). ALT is a transaminase that is employed as an important clinical indicator of hepatocellular injury (17).

Since a prolonged administration of toxins provokes the dysfunction of mitochondrial and monooxygenase systems, these alterations may be accompanied by an increase in reactive oxygen species (ROS) generation (13). An enhanced ROS production exceeding the antioxidant defense and repair capacity could lead to oxidative stress and cell damage (18). Lipid peroxidation can be either a cause or an effect of

reactions that produce toxicity (19). Late data have shown that some marine toxins provoke the production of ROS and lipid peroxides as well as DNA damage and antioxidant enzymes activity into hepatocytes by multispecific bile acid transporters (20). Moreover, this results in an increase in oxidative stress in the body, as evidenced by the enhanced rates of thiobarbituric acid reactive substances (TBARS), accompanied by a concomitant decrease in the rates of scavenging enzyme superoxide dismutases (SOD), catalase and glutathione peroxidase activity (GPx) in the liver, kidney and spleen (21). The physiological and oxidative effects of poisoning by pufferfish meat consumption – by humans or other animals – remain scarce.

The present study was conducted to evaluate the toxicity of pufferfish meat (*L. lagocephalus*) administered to rats and the incidence of oxidative stress and hepatotoxicity.

MATERIALS AND METHODS

Specimen Collection

Specimens of pufferfish *L. lagocephalus* (Linnaeus, 1766) were collected from different localities of the Tunisian coast between 2005 and 2007. Immediately after the collection, animals were eviscerated and samples immediately frozen at –20°C until use. For rat bioassay tests, raw or cooked meat were added to a standard commercial diet (SICO, Tunisia) and offered to experimental animals. Mullet *Liza aurata* meat was utilized as negative control.

Animals and Diets

Male Wistar rats (n = 72) weighing from 165 to 170 g were purchased from the Central Pharmacy of Tunisia. They were housed at 22 ± 3°C in a 12-hour-light-dark cycle, with a minimum relative humidity of 40%. After one week acclimatization to the laboratory conditions, animals were divided into six groups: C, control with free access to standard diet; Lcr, diet supplemented with 10% *L. lagocephalus* raw meat; Lcu+b and Lcu-b, diet supplemented with 10% cooked meat, with or without cooking water; Mcr and Mcu+b, diet supplemented with 10% raw or cooked *Liza aurata* meat, respectively, which were used as a negative control. All animal procedures were conducted in strict conformation with the guidelines of the local Ethics Committee for the care and use of laboratory animals.

At the end of the treatment (48 hours and two months), animals were killed and dissected.

Sample Collection

After animal anesthesia with chloral hydrate via intra-abdominal route, blood was collected by heart puncture and serum samples were obtained by centrifugation at 4,000 rpm for 15 minutes. Liver tissues were excised and washed with cold saline. Serum and organs were stored at –20°C for analysis.

Serum Analysis

Enzymatic activities including ALT, AST and ALP were determined using standard enzymatic kits (AST/TGO A03010, ALT/TGP A03020 and ALP A03000, Biotrol, France).

Lipid Peroxidation (LPO)

LPO was estimated by measuring the formation of TBARS according to the method of Buege and Aust (22). Briefly, one gram of liver tissue was directly homogenized in 2 mL of Tris buffer (50 mM Tris, 150 mM NaCl, pH 7.4) then mixed (for ten seconds) and centrifuged (9.000 rpm, 15 minutes, 4°C). An aliquot of supernatants was used for the assay of protein content according to the Lowry method (23). For the assay, 175 µL thawed supernatant was mixed with 175 µL of 20% trichloroacetic acid (TCA) containing 1% butyl-hydroxytoluene and then the mixture was centrifuged (1,000 rpm for ten minutes). Two hundred microliters of the resulting supernatant was mixed with 40 µL 0.6 M of HCl and 160 µL of 26 mM Tris (pH 7.4) buffer containing 0.72 mM thiobarbituric acid (TBA). The contents of the tubes were boiled for ten minutes at 80°C. After cooling, absorbance was measured at 530 nm using Jenway 6105 spectrophotometer (UK). TBARS concentration was estimated using the extinction coefficient of MDA-thiobarbituric acid complex (156 $mM^{-1}cm^{-1}$). Results were calculated as nmol MDA/mg protein.

Antioxidant Enzyme Activities (CAT and SOD)

Superoxide dismutase (SOD) activity

SOD activity was determined according to the method of Beyer and Fridovich (24). Enzyme activity was measured by mixing phosphate buffer (pH 7.8), containing 0.3 mM EDTA, L-methionine and nitroblue tetrazolium. Then, a 50-µL sample was added to the mixture, followed by the addition of riboflavin (22.6 µL). Next, the tubes were illuminated for 20 minutes. A control tube, in which the sample was replaced by a buffer, was also analyzed and the absorbance measured at 560 nm. One unit of SOD activity is defined as the amount of sample required to decrease by 50% SOD-inhibitable nitroblue tetrazolium.

Catalase (CAT) activity

CAT was calculated according to Aebi (25). A 20-µL hemolysate sample was added to a cuvette containing 780 µL (100 mM) phosphate buffer (pH 7.5). The reaction started by adding 100 µL of freshly prepared 500 mM hydrogen peroxide (H_2O_2). The decomposition of H_2O_2 was directly determined by the decrease in extinction at 240 nm and the difference in extinction (ΔE_{240}) per unit time was employed as a measure of the catalase activity. One unit of activity is defined as sample that can decompose 1.0 µmole of H_2O_2 per minute at 25°C and pH 7.5.

Hepatic Metallothionein (MT) Levels

MT was assayed as defined by Viarengo *et al*. (26). Liver tissue samples were homogenized in three volumes of 0.5 M sucrose, 20 mM Tris-HCl buffer, pH 8.6, by adding 0.006 mM leupeptine and 0.5 mM phenylmethylsulphonylfluoride (PMSF) as antiproteolytic agents. Next, the homogenate was centrifuged at 30,000 g for 20 minutes to obtain a supernatant containing metallothioneins that was treated with ethanol/chloroform. Subsequently, 1.05 mL of cold (–20°C) absolute ethanol and 80 µL of chloroform were added to aliquots of 1 mL of supernatant; the samples were then centrifuged at 6,000 g for ten minutes at 4°C. The collected supernatant was combined with 1 mg RNA and 40 µL 37% HCl and after with three volumes of cold ethanol (to a final concentration of 87%). The sample was maintained at –20°C for one hour and centrifuged in a swinging rotor at 6,000 g for ten minutes. The metallothionein-containing pellet was then washed with 87% ethanol and 1%

chloroform in homogenizing buffer. The pellet was re-suspended in 150 µL 0.25 M NaCl, subsequently in 150 µL 1 N HCl (containing EDTA 4 mM) and was then added to the sample. A volume of 4.2 mL of 2 M NaCl containing 0.43 mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) buffered with 0.2 M Na-phosphate, pH 8, was then added to the sample at room temperature. The sample was finally centrifuged at 3,000 g for five minutes; the supernatant absorbance was evaluated at 412 nm and metallothionein concentration was estimated utilizing reduced glutathione (GSH).

Histopathological Observations

For histological studies, liver samples were fixed in Bouin's solution, dehydrated through graded alcohols (50 to 100%) and embedded in paraffin. Thin sections (4 to 5 µm) were cut and stained with routine hematoxylin-eosin (HE) (27).

Statistical Analysis

Statistical analysis of data was performed using Student's t-test. All values were expressed as means ± SEM. Differences were considered significant if $p \leq 0.05$.

RESULTS

Clinical Observation

There was no mortality and no evident signs of neurotoxicity were recorded in rats after treatment. Only gastrointestinal disorders (diarrhea) were observed in Lcu+b group.

Serum Analysis

The main objective of this analysis was to evaluate possible hepatotoxic effects of fish meat (*L. lagocephalus*) by studying various hepatic enzyme markers in rat serum. Figures 1, 2 and 3 summarize the effects of *L. lagocephalus* meat, cooked or not, regarding biochemical variables. After two months, AST and ALT levels significantly decreased by respectively 40% and 35% in Lcu+b group. Additionally, a significant reduction in ALP activity (–30%) ($p < 0.05$) was observed in experiment Lcu+b group after treatment. The reduction in enzyme levels is indicative of hepatocellular lesion and tissue destruction. This outcome was not observed in Lcr and Lcu-b rats in all experimental conditions. No significant alterations were detectable in serum enzyme markers of Mcr and Mcu+b groups compared with the control group (T).

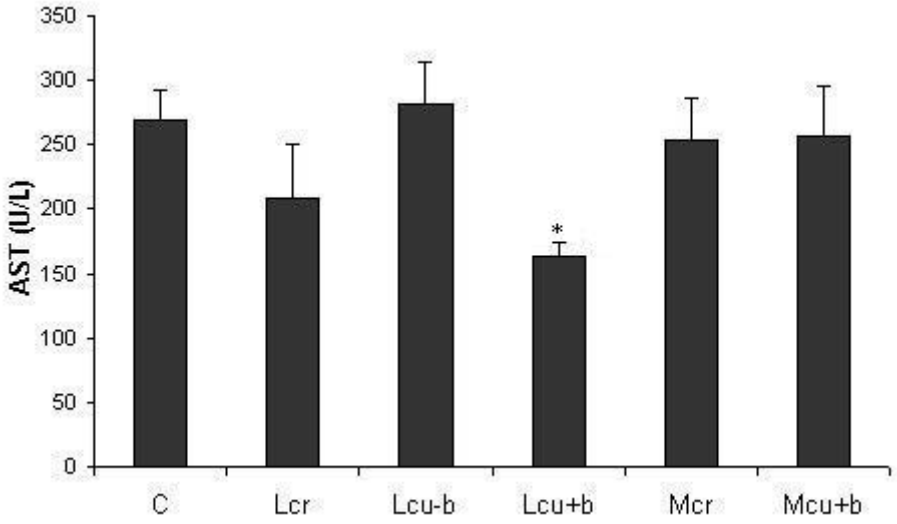

Figure 1. Activity of aspartate aminotransferase [AST (U/L)] in control and treated groups (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b) after two months of treatment. The values are expressed as mean ± SEM (n = 6). Significant levels observed are $^{*}p \leq 0.05$ compared with control group values.

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

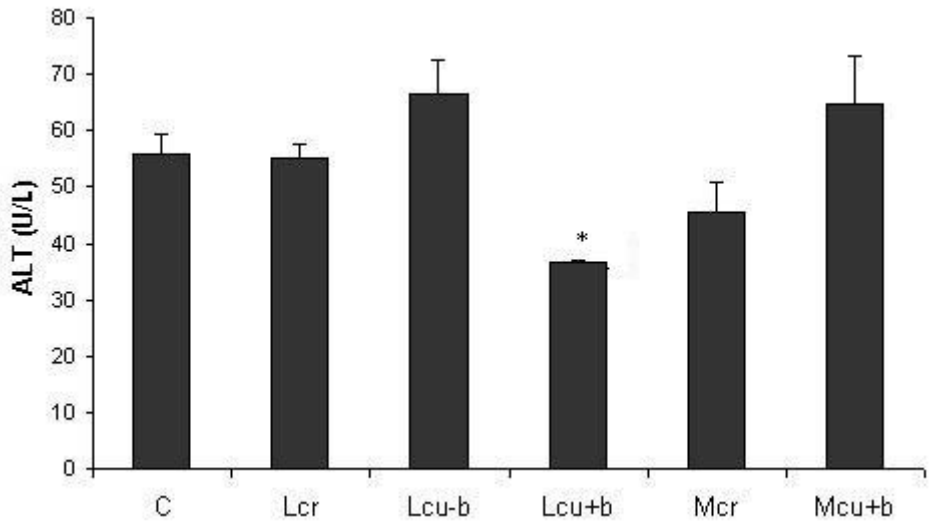

Figure 2. Activity of alanine aminotransferase [ALT (U/L)] in control and treated groups (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b) after two months of treatment. The values are expressed as mean ± SEM (n = 6). Significant levels observed are $^{*}p \leq 0.05$ compared with control group values.

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

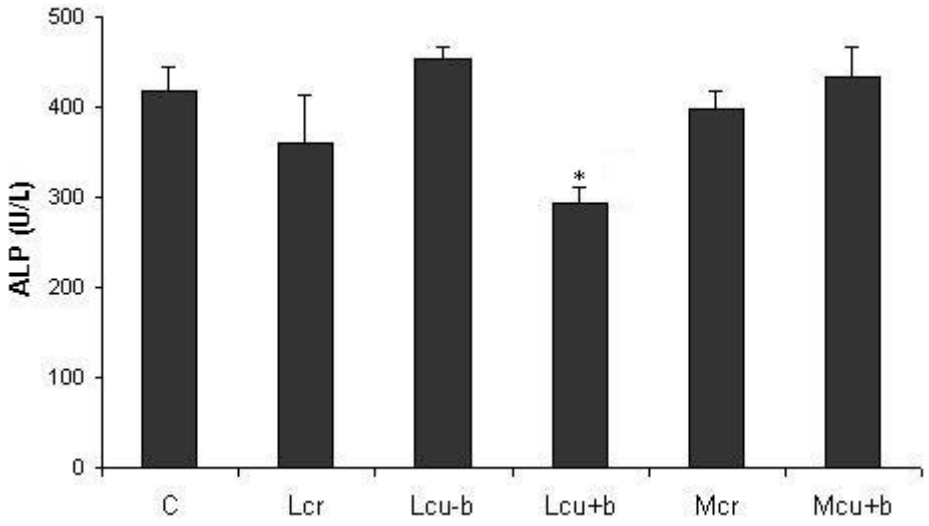

Figure 3. Activity of alkaline phosphatase [ALP (U/L)] in control and treated groups (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b) after two months of treatment. The values are expressed as mean ± SEM (n = 6). Significant levels observed are $^{*}p \leq 0.05$ compared with control group values.

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

Effect of *L. lagocephalus* Meat on Lipid Peroxidation Rates

In Lcu+b group, lipid peroxidation rates were more pronounced after a chronic exposure (two months) than after 48 hours of treatment (respectively, 54% and 65%). Thus, LPO effects appeared to be time-dependent. In the other groups (Lcr, Lcu-b, Mcr and Mcu+b), LPO presented no significant variation in the liver compared to the control group (Figure 4).

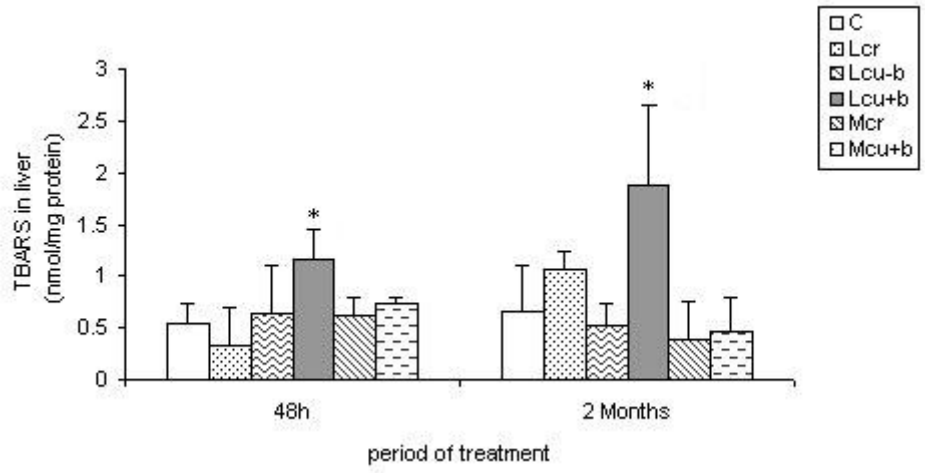

Figure 4. Lipid peroxidation values in the liver of control and treated rats (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b). The values are expressed as mean ± SEM (n = 6). Significant levels observed are $^{*}p \leq 0.05$ compared with control group values.

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

Effect of *L. lagocephalus* Meat on Antioxidant Enzyme Activities (SOD and CAT)

SOD activities remained unaltered in the liver throughout all experimental conditions (Figure 5). In Lcu+b group, CAT activity in the liver (Figure 6) decreased ($p < 0.05$) 48 hours and two months after the treatment. However it remained unaltered ($p > 0.05$) after short and long-term exposure experiments (48 hours and two months) in groups Mcr and Mcu+b (negative controls) and Lcr and Lcu-b (treated rats).

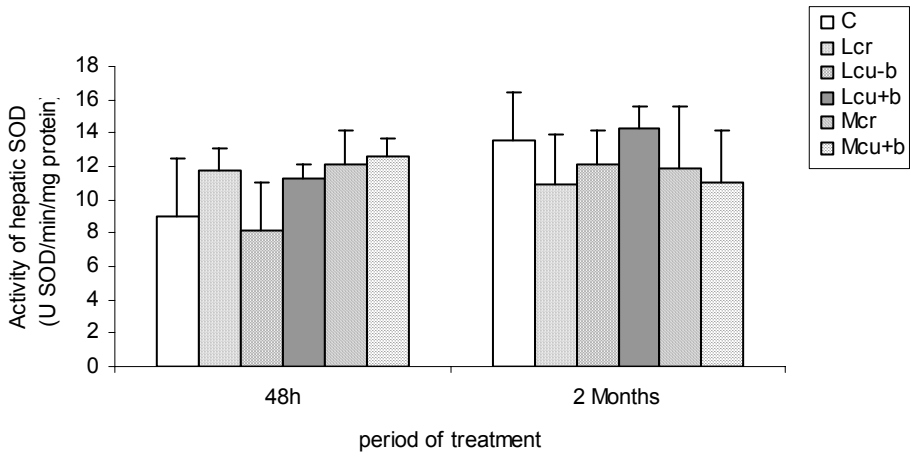

Figure 5. Superoxide dismutase activity in the liver of control and treated rats (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b). The values are expressed as mean ± SEM (n = 6).

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

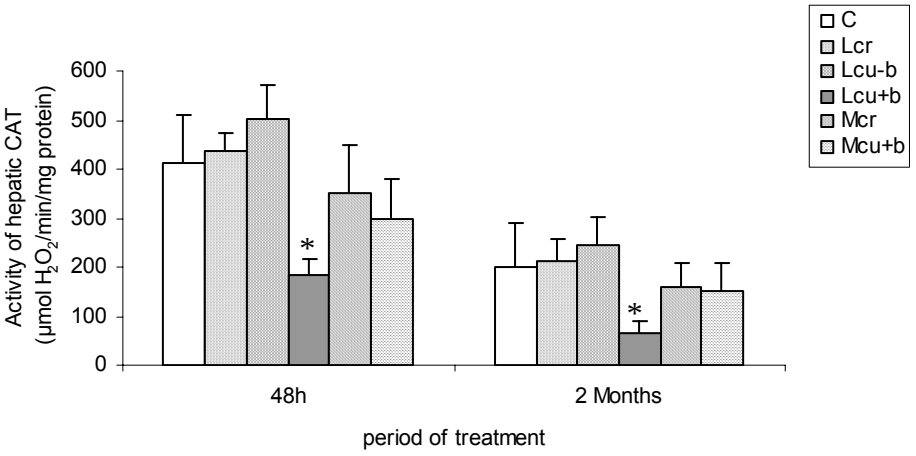

Figure 6. Catalase activity in the liver of control and treated rats (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b). The values are expressed as mean ± SEM (n = 6). Significant levels observed are $^{*}p \leq 0.05$ compared with control group values.

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

Hepatic Metallothionein (MT) Levels

The MT content of the treated groups is presented in Figure 7. During the short-term exposure experiment (48 hours), a higher ($p < 0.05$) MT level was observed in Lcu+b compared with controls. After two months, a significant reduction ($p < 0.01$) in the concentration of this metal-binding protein was observed in Lcu+b group. In other groups (Lcr, Lcu-b, Mcr and Mcu+b) no significant variations were registered.

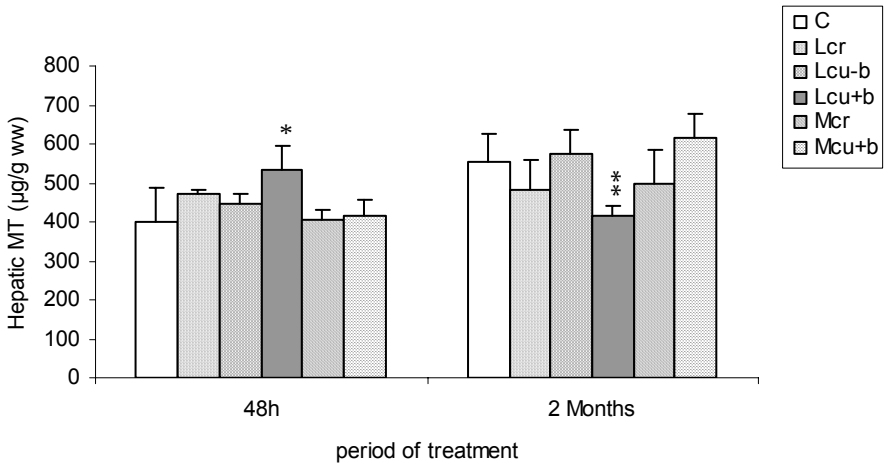

Figure 7. Metallothionein levels in the liver of control and treated rats (Lcr, Lcu-b, Lcu+b, Mcr and Mcu+b). The values are expressed as mean ± SEM (n = 6). Significant levels observed are $^{*}p \leq 0.05$ and $^{**}p \leq 0.01$ compared with control group values.

C: control group; Lcr: group that received diet supplemented with 10% *L. lagocephalus* raw meat; Lcu-b: group that received diet supplemented with 10% cooked meat without cooking water; Lcu+b: group that received diet supplemented with 10% cooked meat with cooking water; Mcr: group that received diet supplemented with 10% *Liza aurata* raw meat; Mcu+b: group that received diet supplemented with 10% *Liza aurata* cooked meat.

Hepatic Histopathology

Histological liver findings from treated groups are presented in Figure 8. Control animals revealed clear-cut hepatic lobules, separated by interlobular septa, crossed by portal veins. Hepatocytes were polyhedral, with round nuclei and sizes roughly uniform, except for a few binucleated cells. Similar observations were registered in Mcr and Mcu+b (negative control groups) as well as in Lcr and Lcu-b. Histopathological studies showed degeneration of hepatocytes and hepatic cords,

focal necrosis, congestion in central veins and sinusoids, as well as infiltration of lymphocytes in hepatic lobules in Lcu+b group.

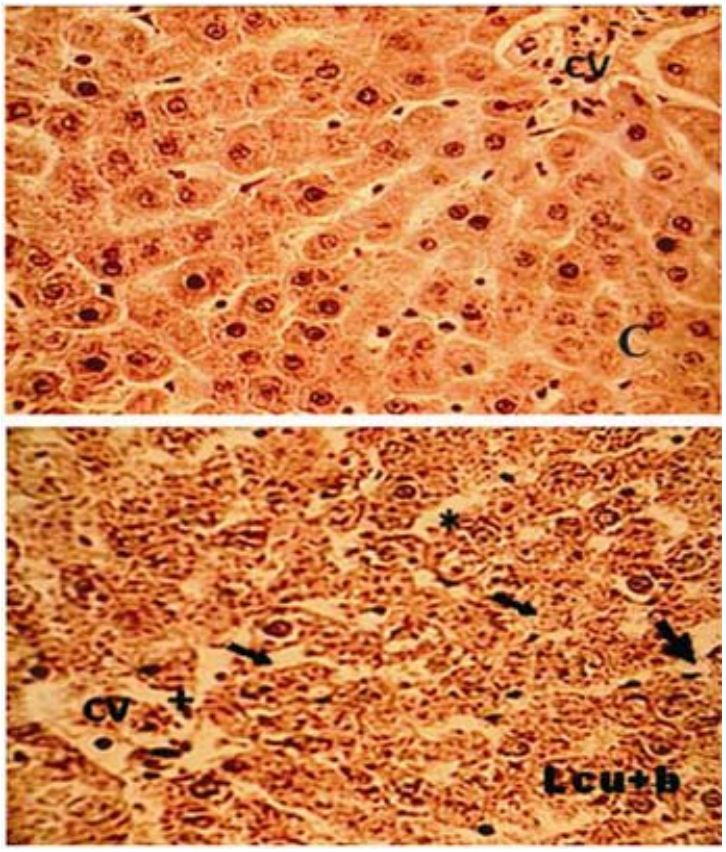

Figure 8. Liver sections of controls (C) and treated (Lcu+b) rats fed 10% *L. lagocephalus* cooked meat with cooking water.

Optical microscopy: HE (250x); cv: central vein; *: sinusoidal spaces; +: lymphocyte infiltration; ➡: vacuolization and regions of necrosis; ➡ : ballooned cells.

DISCUSSION

In our investigation, no mortality and no evident signs of neurotoxicity were recorded in experimental animals after treatment. Only gastrointestinal disorders (diarrhea) were observed in Lcu+b rats. These results agree with our previous work (28). The present study aimed to investigate the possible hepatic oxidative damage in rats fed pufferfish meat. The liver is a very active organ with a vital importance that is especially sensitive to toxins. Due to their metabolic function, high cytochrome P450 content and their major role in the biotransformation of xenobiotics, hepatocytes are

extremely exposed to oxidative stress *in vivo*, as indicated by the large number of liver pathologies that have been associated with oxidative stress (29, 30).

The current study examines the poison from pufferfish meat that induces changes of some biochemical parameters in rats, as confirmed in previous works (31). The determination of serum enzyme activity can be of great value for the detection of liver alterations. In this regard, we noted a significant reduction of serum transaminase activities (AST and ALT) and ALP in rats treated with a diet containing *L. lagocephalus* cooked meat and cooking water after two months of treatment. Our results show that prolonged treatment can cause liver damage and dysfunction (32). Similar results were reported in okadaic acid-treated mice that revealed a disturbance of AST and ALT levels in plasma (14, 33).

In previous studies, exposure to meat and liver extracts of *L. lagocephalus* induced a decrease of ALT and ALP activities in treated rats (28). After two months of treatment, the decrease of AST and ALT serum activities could be explained by the failure of enzyme defense system to overcome the influx of toxin accumulation in Lcu+b group, which promoted the lipid peroxidation, modulation of DNA, alteration in gene expression and cell death. It appears to comprise a cellular exhaustion of enzymatic activities (AST, ALT and ALP) after treatment.

The present study concludes that prolonged treatment of rats fed cooked meat with cooking water causes liver damage and toxicity. Our results showed that in Lcu+b rats, this diet provoked oxidative stress in the liver. Such evidence came from the increase of LPO levels in the liver of rats that received 48 hours of treatment (Lcu+b). Additionally, higher LPO rates in the liver were observed after two months in the same group. This modification could be due to several factors, since LPO products were consequences of higher oxygen free radicals (34). These results confirm previous findings that had shown an association between T-2 toxin and increased oxidative stress in experimental animals (35). A study by Letteron *et al*. (36) also showed a correlation between higher LPO and greater hepatic damage in rats and humans. Moreover, SOD activities remained unaltered in all experimental conditions in rat liver. Paradoxally, CAT activities decreased significantly 48 hours and two months after treatment in Lcu+b group. It seems that the toxic effect of *L. lagocephalus* is not due to the generation of free radicals based on singlet oxygen, which requires a dismutation, but acts directly by forming peroxides that require the

intervention of CAT activity. Also maybe TTX acts simply by inhibiting the expression of CAT activity, the origin of the toxic effect.

In fact, Tichivangana and Morrissey (37, 38) reported that ferrous ion level at 1 to 10 ppm acted as a strong pro-oxidant in cooked fish muscles. In this context, the hydrogen peroxide comes from the enzymatic dismutation of anion superoxide, resulting in a hydroxyl radical via the reduction of H_2O_2 with Fe^{2+} (39). On the other hand, Tichivangana and Morrissey (37, 38) have shown that the oxidation of muscle foods occurs in the following order: fish > poultry > pork > lamb. Furthermore, lipid oxidation is thermodynamically favorable; however, the direct reaction between oxygen and highly unsaturated lipids is kinetically hindered (39, 40). Hence, an activating factor is necessary to initiate free radical chain reactions followed by their self-propagation (39, 41). These effects are confirmed by the analysis of certain enzymes that justify the hepatic damage.

The hepatic level of MT behaves in two different ways according to acute or chronic exposure. These results are confirmed by an alteration of hepatic enzyme markers (AST, ALT and ALP) after prolonged treatment, indicating toxicity and liver damage. Although MT synthesis is known for being a response to various pathological and physiological agents, the present investigation also revealed a noticeable increase in MT content in Lcu+b rats 48 hours after treatment (42). This phenomenon could equilibrate the deleterious effects of TTX on hepatic MT synthesis. After a long time, a marked reduction in hepatic MT content was observed in rats fed *L. lagocephalus* cooked meat with cooking water.

Previous studies have proven that several sources of protein-bound iron exist in fish tissues, namely myoglobin, hemoglobin, ferritin and transferrin. The iron bound to these proteins may be released during cooking, thus activating oxygen and initiating lipid oxidation (43). Oxidative rancidity usually occurs more rapidly in cooked ground fish than in raw fish (44). Usually, this type of biochemical modification is connected with histological aspects. In our study, histological changes were observed in the liver of Lcu+b group, which were characterized by an increasing hepatocyte vacuolisation and sinusoidal space dilation in comparison to untreated controls. The number of binucleated cells also augmented in these animals. These observations indicated marked changes in the liver overall histoarchitecture in response to TTX, which could be due to its toxic effects.

Other studies had already mentioned that tetrodotoxin is heat-stable, water-soluble and a non-protein quinazoline derivative (45, 46). Our results also revealed that TTX was heat-stable and water soluble, indicating its generation during cooking process. Histopathological alterations in hepatocytes due to cyanotoxin exposure had already been described by some researchers, who studied cytoskeleton destruction caused by increased levels of protein phosphorylation after exposure to the toxin (47-49). Moreover, other authors observed dissociation and necrosis of hepatocytes after acute exposure to cyanotoxins. Ding *et al*. (50) indicated that cyanotoxin ROS generation may play an important role in the disruption of microfilaments structure in rat hepatocytes.

In conclusion, the present study showed that this Tunisian pufferfish meat (muscle and skin) presented toxicity, which clearly indicates the danger of using this fish as food.

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