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Original Article

Evaluating Heavy Metal Contamination Effects on the Caspian Pond Turtle Health (*Mauremys caspica caspica*) Through Analyzing Oxidative Stress Factors

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Abstract

Background and aims: Antioxidant defense plays a vital part in organism protection against oxidative stress which is produced by reactive oxygen species (ROS). Oxidative stress represents a serious threat to the animals facing with heavy metals. This study was designed to analyze the habitat suitability for Caspian pond turtles, namely, *Mauremys caspica* in Mazandaran province by measuring lead (Hg) and mercury (Pb) tissues concentrations and heavy metals' effects on the health status of Caspian pond turtles through quantifying the oxidative stress factors.

Methods: Hg and Pb were measured in kidney and liver tissues of 20 sampled Caspian pond male turtles (treatment group) using atomic absorption spectrometry (AAS) and a Caspian pond male turtle was included in the control group. Moreover, glutathione (GSH) level, catalase (CAT), and superoxide dismutase (SOD) activities were investigated in kidney and liver tissues.

Results: The mean (SD) concentration of Pb and Hg were 35.83 (4.20), and 0.604 (0.03) mg/kg for the sampled livers and also 31.01 (3.42) mg/kg and 0.316 (0.04) mg/kg for the sampled kidneys, respectively. Levels of trace elements, CAT, and SOD activities were found to be higher in the liver. Totally, GSH levels, as well as, CAT, and SOD activities were found to be higher and lower, respectively, in the control turtle as compared with the contaminated Caspian pond turtles. Trace-element levels had a positive correlation with CAT and SOD activities while having a negative association with GSH levels in contaminated Caspian pond sampled turtles.

Conclusion: According to the results, it was inferred that high Hg and Pb concentrations in the turtles were due to the heavy metal contamination of their habitat in Mazandaran province. Based on the positive correlation between the heavy metal concentration of the tissue and dysfunction of oxidative stress defense markers, it can be concluded when the Caspian pond turtles are faced with heavy metal contamination risk, these markers can act as a bioindicator of their health status. No doubt, more studies are required to prove this hypothesis.

Keywords: Caspian pond turtle, Mercury, Lead, Oxidative stress, Trace elements

Introduction

Technological growth of human society has resulted in trace-element discharge increase in the environment and consequently metal pollution. Industrial and agricultural activities and hospital wastes are some of the important sources of heavy metal pollution caused by humans in the environment. Ubiquity, long persistence, and their bioaccumulative characteristics make the animals constantly exposed to such pollutions.[1]

Trace elements are divided in two groups, namely, the essential elements, such as copper, zinc, and selenium which have an important role in normal biological activities of the cells; and the non-essential elements (i.e., the toxic elements) like the mercury (Pb) and lead (Hg) which are destructive for most animals especially at very high levels.[1]

The Pb can cause hypertension, atherosclerosis, nephropathy, neuropathy, and neoplasia in the animals.[2] It changes the hematologic system by preventing the function of several enzymes that play a vital role in the biosynthesis of the heme. Like most of the trace elements, after absorption, Pb concentrate in tissues especially those with a high metabolic activity including the liver and kidney. Moreover, Pb can impair the immune system[3].

Furthermore, in its organic and inorganic forms, Hg is considered as one of the most toxic metals for the organisms. In general, methylmercury as one of the Hg organic forms, is more toxic to the animals than the other ones.[4] It suppresses steroid hormones' levels, leading to reduced reproduction in cold-blooded animals such as fish.[5,6]

Heavy metals diversely affect antioxidant defense by the production of reactive oxygen species (ROS) and impairment in the balance between antioxidant defense and ROS levels.[7] High levels of ROS can damage the structure of cells through oxidative damage and may lead to tissue dysfunction.[8,9] Meanwhile, catalase (CAT), superoxide dismutase (SOD), and glutathione reductase (GR) are important members of the antioxidant defense the concentrations and also activities of which change with the production of additional ROS.[10] Recently, factors associated with the antioxidant defense such as SOD, CAT, and GR have been introduced as biochemical signs of oxidative stress activities in animals facing with trace-element pollutio.[11,12]

The Caspian pond turtle, *Mauremys caspica*, which is a medium-sized turtle, is common in the Middle East and uses habitats near the source of freshwater such as the lakes, rivers, and brackish water. Caspian pond turtles exist in the North, West, and Southwest of Iran with high numbers. Many factors such as landscape alteration and heavy metal pollution threat their survival.[13]

Although a precise investigation of antioxidant enzymes related to heavy-metal contamination of the animals can provide specific information about the toxic effects of these elements on the health of each species, few studies have examined the impact of trace elements on oxidative stress components in turtles naturally exposed to metals. As turtles are long-living animals and their home range in not so wide, they can be an indicator of heavy metal pollution of their habitat. In this study, the concentrations of Pb and Hg in Caspian pond turtles and also some stress factors were assessed in order to analyze water quality sources in Mazandaran province. Moreover, the possible effects of heavy metals on their health status were evaluated through analyzing the oxidative stress factors.

Materials and Methods

Study Area and Sampling

This study was performed in Mazandaran province, located in the northern part of Iran which is one of the most severely metal polluted areas in Iran. To decrease the effects of sexuality, weight, season, and mating on oxidative stress factor, only 20 male turtles with 20-22 cm size (curved carapace length) from regions with same climate condition and geographic situation were sampled during a short period of time after mating (2014-2017).[14]

All the sampled turtles were found died because of incidental fisheries captured along the rivers that flow into the Caspian Sea. Samples were only obtained from carcasses for which the time of death could be assessed as <24 hours. Liver and kidney tissues were collected in the laboratory after necropsy.

Trace-Element Estimation

To determine the trace-element concentration in kidney and liver tissue samples, wet-tissue weight was measured. After digestion with concentrated nitric and perchloric acid (1:1), the mixture was slowly boiled for 1 hour (FAO, 1993). The digested samples were analyzed twice to estimate Hg and Pb concentrations with an atomic absorption spectrophotometer (GBC Scientific Equipment, Melbourne, Australia). Negative and positive controls were included as well.

Biochemical Analysis

Biomarkers of oxidative stress were measured immediately after sample collection to avoid alteration of the results due to the storage. Kidney and liver tissue samples were homogenized and solved in ice-cold phosphate-buffered saline (pH of 7.4) and sonified with an ultrasonifier by 6 cycles. The homogenate was centrifuged (15 000 x G, 10 minutes, 4°C), and the supernatant was immediately used for enzyme evaluation.[15]

The CAT activity was also determined in supernatants by determining the absorbance variations at 240 nm in a reaction medium containing 10 mM H_2O_2, and 50 mM sodium phosphate buffer (pH of 7.0). One unit of the CAT activity is defined as the amount of enzyme needed to reduce 1 μM H_2O_2 per minute and the specific activity is reported as units/mg protein[16]. To analyze the sensitivity and specificity of the protocol, negative and positive samples were included as well.

In addition, the level of SOD activity was determined according to the kit protocol (Sigma-Aldrich, GmbH, Munich, Germany). The xanthine and xanthine oxidase generate superoxide radicals which bind to 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) and produce a red formazan dye. The SOD activity is measured by inhibition degree of this reaction. One unit of SOD activity is defined as the amount of enzyme needed to inhibit the reaction by 50%.[17]

The GSH and total protein were also measured by the spectrophotometer using bovine serum albumin as the standard protein.[18] Sensitivity and specificity of the process were assessed through negative and positive control.

Statistical analyses were conducted employing the SPSS (statistical package for the social sciences), version 18 and Excel (2010) software. Kolmogorov–Smirnov test was also used to analyze the normality of the results. As all the results were normally distributed, differences in the levels of trace elements and the biomarker values among tissues were measured by *t* test. Moreover, Pearson correlation coefficient was applied to analyze the biomarkers association with Pb and Hg concentration.

Results

Concentrations of Pb and Hg in Sample Tissues

Pb concentrations as compared with Hg concentrations were higher in sampled tissues. Besides, higher absorptions of Hg and Pb were detected in the liver rather than kidney tissues (Table 1).

Oxidative Stress Factors in Tissues

The GSH mean concentration and mean of SOD and CAT activities of kidney and liver tissues are shown in Table 2.

Correlation Between Oxidative Stress Factors and Heavy Metals

Kidney and liver trace-element concentrations were significantly and positively correlated with CAT and SOD activities but negatively associated with glutathione (GSH) level (Table 3).

Discussion

As Caspian pond turtles are placed at the upper trophic levels of ecosystems, trace-element contamination data on this species can provide evidence regarding the extent of pollution throughout the food chain and can be a good surrogate for human exposure as well.[19]

As previously mentioned, the mean concentration of Pb and Hg were 35.83 ± 4.20 mg/kg and 0.604 ± 0.03 mg/kg (in the liver), as well as, 31.01 ± 3.42 mg/kg and 0.316 ± 0.04 mg/kg (in the kidney). Investigating the literature, only one similar study was found on this species in Iran. Yadollahvand et al measured the concentration of some trace elements in the tissues of 15 Caspian pond turtles in Golestan province, which is located in the proximity of Mazandaran province. They reported a mean concentration of 32.4 µG/g d.w. for Pb in the liver tissues. As their results were represented in dry weight, it seems that heavy metal contamination was higher in sampled Caspian pond turtles of this study which means that they were living in more contaminated areas as compared to the species living in the Gharasoo River.[20]

Table 1. Hg and Pb Concentrations (ppm) in Kidney and Liver Tissues of 20 sampled Caspian Pond (Mazandaran Province) and Control turtles

Tissues		Metal	
		Hg	PB
Sampled kidney	Mean (SD)	0.316 (0.04)	31.01 (3.42)
	Min-Max	0.260- 0.370	25- 38.7
Sampled liver	Mean (SD)	0.604 (0.03)	35.83 (4.20)
	Min-Max	0.557- 0.651	27.9- 43.7
Control kidney	Mean (SD)	0	0
Control liver	Mean (SD)	0	0

Table 2. Mean Concentrations (SD) of GSH µM/g Level and SOD and CAT Activities (U/g protein) in Sample and Control Turtle Tissues

Tissues		Antioxidant		
		CAT	SOD	GSH
Sampled kidney	Mean (SD)	305.3 (7.34)	7.54 (0.92)	104.8 (8.20)
	Min-Max	270-328	6-9	95-120
Sampled liver	Mean (SD)	255.2 (7.72)	3.56 (0.79)	84.55 (8.08)
	Min-Max	245-270	2.5-5.2	70-102
Control liver		217	3.5	82
Control kidney		185	2.1	59

Besides, there exist some similar studies on other turtle species in the world the results are not exactly comparable with the result of this study. However, such investigations show the important use of turtles as biological indicators of their ecosystems. Seem et al, for example, reported lower concentrations of Hg in liver tissues of the loggerhead (2.41 µG/g) and green (0.55 µG/g) turtles, respectively. Concentrations of Pb were below the limits in both species.[21]

Storelli et al studied Hg and Pb contamination in loggerhead turtles (i.e., *Caretta caretta*) of the eastern Mediterranean Sea. They observed mean concentration of 0.43 µG/g and 0.16 µG/g (wet weight) for Hg in liver and kidney tissues, respectively. In addition, Pb mean concentrations were found to be low and often below the limit of detection.[22]

As compared with the results of the existing literature, Pb and Hg concentrations in the analyzed tissues were apparently higher than those reported in other freshwater turtle specie.[23-26] Trace-element levels in the tissues and organs of the turtles can be related to many environmental and physiological factors. Thus, differences observed in the current study as compared to those of other similar studies can be the consequence of diet preferences among turtle species as well as different levels of exposure to environmental pollution.[27,28]

The findings of the present study regarding higher Pb and Hg concentrations in liver tissues are in line with the results of Storelli et al in loggerhead turtles of the eastern Mediterranean Sea.[22] According to some studies, the liver is the main detoxifying and metabolizing organ for many xenobiotics including trace elements. This characteristic can partly explain why the liver has a higher heavy metal concentration rather than kidney.[28,29]

There is no information on maximum permissible Hg and Pb levels in turtle tissues. Since the health status of the sampled Caspian pond turtles could not be assessed, Hg and Pb contamination effects on sampled animals are not clear. However, as compared with the findings of other similar studies on other freshwater species regarding Pb level, it was found that Pb levels of the liver were several times higher than those found to be correlated with a reduction in white blood cell counts in red-eared sliders in Kentucky, USA.[26] Therefore, it is likely that a similar reduction could have been occurred in Caspian pond turtles, increasing their

Table 3. Pearson's Correlation Coefficient (r) Between Trace-Element Concentration and Antioxidant Factors in Liver and Kidney of Sampled Caspian Pond Turtles

	Metal	Biomarker		
		CAT	SOD	GSH
Liver	Hg	0.893**	0.948**	- 0.980**
Kidney	Hg	0.966**	0.975**	- 0.913**
Liver	Pb	0.512*	0.574**	- 0.651**
Kidney	Pb	0.831**	0.841**	- 0.813**

SOD, superoxide dismutase; CAT, catalase; GSH, glutathione.
*Correlation is significant at $P < 0.05$; **Correlation is significant at $P < 0.01$.

susceptibility to the infectious diseases.

Similarly, Hg concentrations are comparable to those observed in snapping turtles from New Jersey (0.6 & 0.39 mg/kg in liver and kidney, respectively) and Maryland (0.9 & 0.44 mg/kg in liver and kidney, respectively), USA. In these turtles, no clear correlation was found between Hg and population reduction. It is said that, high levels of Pb and Hg in Caspian pond turtles can be dangerous, especially in that they can cause severe damage to species' health.[23] Detection of high concentrations of Pb and Hg in internal organs of Caspian pond turtles revealed discharge and presence of these trace elements in their living ecosystem in Mazandaran province, which is characterized by the presence of fruit trees and high agricultural activities[25]. Furthermore, based on these results, it can be concluded that sources of Pb and of Hg contamination in the studied regions were continuous and persisten.[30]

Although there are some published papers respecting heavy metal concentrations in the turtles, there is no basic data on GSH level, as well as, SOD and CAT activities in Caspian pond turtles. The general trends in the current study were an increase in SOD and CAT activities and a decrease in GSH level associated with increasing Pb and Hg concentrations compared with one Caspian pond turtle as the control sample. Oxidative status was also highly related to Hg and Pb levels in kidney and liver tissues.

Correlations between antioxidant enzyme activities and concentrations of Hg and Pb were indicative of physiological sensitivity of Caspian pond turtles to chemicals and protective activity of antioxidant defense. The GSH is a non-enzymatic antioxidant that has been identified in almost all kinds of organisms and its regulation can be affected by heavy metal contamination. Besides, it binds to metals with sulfhydryl groups, thereby prevents them from creating ROS.[31,32]

If metal concentrations overcome the defensive capability of GSH, then cells also use certain enzymatic antioxidants such as SOD and CAT. The CAT in conjunction with SOD serves as an effective scavenger of ROS that prevents cellular damage. These scientific data can justify higher SOD and CAT activities and reduced levels of GHS in contaminated tissues compared with the control turtle.

Investigations regarding the correlation between the heavy metal concentration and oxidative stress have yielded conflicting results. Some researchers have reported increased oxidative stress factors with high heavy metal concentration while others found no apparent association in this respect.[33-35] As similar research on turtles is very scarce. Accordingly, the results of the present study could only be compared with those of other cold-blooded animals.

Cappello et al, for instance, showed that an increase in Hg level caused depletion of GSH in gills tissues of the wild golden grey mullet, namely, *Liza aurata*.[36] However, Monteiro et al reported that Hg induced a significant increase in GSH levels, as well as, SOD and CAT activities in the livers of neotropical fish.[37]

Similarly, Hermenean et al analyzed histopathological alterations and oxidative stress in liver and kidney of chubs (*Leuciscus cephalus*) following exposure to heavy metals. They confirmed a positive correlation between liver and kidney Pb concentrations with SOD and CAT activities and a negative association between Pb concentration and GSH levels in the tissues[38]. Moreover, Hermenean et al found similar results regarding the existence of a positive relationship between Pb concentration in liver and kidney tissues with SOD and CAT activities but a negative correlation between Pb concentration and GSH levels.[36] In the current study it was found that SOD and CAT activities in liver tissues were higher than kidney tissues, showing that liver as compared with kidney had a higher capacity and adaptability to counteract ROS.

Furthermore, in another study by Valdivia et al on basic oxidative stress metabolites in eastern Pacific green turtles (*Chelonia mydas agassizii*), the liver was found to have the highest CAT and GST activities.[39]

It is generally accepted that, the enzymatic antioxidant concentration of the liver is higher than the kidney in the vertebrates.[40] As most metabolic and anabolic actions of the body occur in the liver, larger amounts of peroxides are generated in it. Hence, the liver requires more enzymatic antioxidant to counteract the produced peroxides.[29]

There are many pollutant and pressure factors affecting oxidative stress mechanisms. A number of these factors including exposure to a mixture of heavy metals, age, season, and sex of the organism can modulate a response to oxidative stress[41]. For example, an increase in total SOD levels in muscle and liver tissues (59% & 118% elevations, respectively) were observed in garter snakes (*Thamnophis sirtalis*) following facing with anoxia for 10 hours. This is why sampling was concentrated on a short-time period and males of approximately the same size in this study so that to avoid possible biases due to the modulation of oxidative stress by these factors.[42]

Conclusion

Since it was impossible to analyze all the factors affecting oxidative stress, it cannot be concluded with certainty that Hg or Pb were the only factors that changed the antioxidant defense factors in this study. While some evidence was found in this regard, more dose-dependent field and in vitro studies are needed to identify the exposure levels of Hg and Pb that may cause increased oxidative stress in Caspian pond turtles, as well as determining how to separate the variation caused by pollution exposure from that caused by other ambient factors. Comparing the results of the current study with those of other similar studies revealed that the effects on oxidative status observed in one species cannot be generalized to other species. The results of this study provide basic information about the possible relationship between trace-element contamination and oxidative stress. Nevertheless, more research is required to confirm that antioxidant endpoints can be reliably used as biomarkers to assess the heavy metals risk in Caspian pond turtles.

Ethical Approval

Not applicable.

Conflict of Interest Disclosures

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