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The study of the changes in the thermal properties of *Labeo rohita* bones due to arsenic exposure

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Abstract The paper presents the changes in the thermal properties of control, arsenic exposed and DMSA treated *Labeo rohita* bones by using thermo analytical techniques. The result shows that the mass loss due to the thermal decomposition occurs in three distinct steps due to loss of water, organic and inorganic materials. The arsenic exposed bones present a different thermal behaviour compared to the control bones. The residue masses are increased due to arsenic exposure, while the DMSA treatment reduces the residue mass level. These thermal characteristics can be used as a qualitative method to check the metal accumulation in samples.

Keywords Arsenic · Bone · DMSA · *Labeo rohita* · TG-DSC-DTA · Thermal analysis

Introduction

Arsenic compounds are ubiquitous and widespread in the environment as a result of natural or anthropogenic occurrence. The United States Environmental Protection Agency has placed arsenic as one of the top pollutants of environmental concern [1]. Arsenic is present in water systems around the world as both a natural element and as a contaminant. This occurs as the result of complex geological processes and human disturbances created by industrialization. In aquatic environments, several species of microorganisms are able to either reduce or oxidize arsenic [2], allowing inter-conversion of forms and making arsenic biologically available to organisms including fish [3–5]. Fish is the major source of protein for human consumption. It is also a source of contamination, because of the amounts of heavy elements they can contain, some of which are highly toxic [6]. Further, there may be concern for metal uptake of human populations through ingestion of this fish. In polluted areas, exposure of fish to xenobiotics leads to interactions between these chemicals and biological systems, which give rise to biochemical disturbances [7]. Most of the metals in organisms accumulate in bones and can be liberated under some pathological conditions [8]. The earlier studies [9–11] have shown that bone tissue is an endogenic metal source reflecting the total metal content in an organism. In addition, those studies indicate that the bone content of heavy metals is mostly from environmental pollution.

Chelating agents have been used clinically as antidotes for acute and chronic metal intoxications. These compounds bind to and enhance the excretion of toxic elements. The water soluble meso-2,3-dimercaptosuccinic acid (DMSA) is employed in the treatment of acute and chronic poisoning by arsenic, lead, and mercury [12].

Fish bones consists of 10% water, 20% organic material (mainly collagen fibrils), and 70% inorganic material (predominantly carbonated hydroxyapatites) by weight. The organic phase or extra-cellular matrix is composed (90–95%) by collagen type I and non-collagenous proteins, glycoproteins, sialoproteins and phosphoproteins [13, 14]. Hydroxyapatites (HAP), one of the inorganic components of bones, have been used in medicine and dentistry because of its excellent biocompatibility with human tissue and fish bone is a cheaper source of HAP [15]. Thermal Analysis (TA) is a well-established set of techniques for obtaining qualitative and quantitative information about the effects of

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various heat treatments on materials of all kinds. Heating is performed under strictly controlled conditions and can reveal changes in structure and other important properties of the material being studied. Thermal analysis of bone can provide valuable information regarding the organic and mineral contents of bone [16]. Thermo analytical techniques measure the changes in the physical or chemical properties of the sample as a function of temperature. This technique involves monitoring the mass loss of the sample in a chosen atmosphere (usually nitrogen or air) as a function of temperature. Due to the sensitivity of thermal analysis, thermogravimetric analysis (TG) and differential scanning calorimetry (DSC) have been selected for the present work. TG is inherently quantitative, and therefore an extremely powerful thermal technique, but gives no direct chemical information. The ability to analyze the volatile products during a mass loss is of great value [17]. TG has been used extensively for the characterization of bones in a number of studies [14, 16–22]. The ability of TG and DTA to generate fundamental quantitative data has led to its widespread use in every field of science and technology. DSC has been established widespreadly in the research of biological systems [23]. Differential scanning calorimetric examination allows demonstrating the thermal consequences of local as well as global conformational changes in tissue elements. This technique has already proved to be applicable in the research of medical problems. DSC measures the differential heat flow between a sample and reference material.

DSC monitors the thermal response of a sample to temperature changes, or isothermally. The same temperature program is applied to a sample and a reference pan, and the difference in heat flow to each pan is measured. The transitions appear as endothermic peaks if heat is absorbed or as exothermic peaks if heat is given out. Foregoing examinations have demonstrated that DSC is a useful and well-applicable method for the investigation of the organs of the musculoskeletal systems [23, 24]. This method is particularly appropriate to the study of collagen-based tissues and materials like bone, because of an unusually large endotherm related to denaturation of type I collagen [25]. To see whether there are differences in thermal behaviour between the control, arsenic exposed and DMSA treated fish bones, TG and DSC measurements were performed in the present study. The aim of this paper is to study the changes in the thermal properties of control (healthy), arsenic exposed and DMSA treated *Labeo rohita* bones using thermo analytical techniques in order to monitor the transformation process suffered by the bones. In the current investigation, fish bones are selected because, it is rich in calcium, which is an essential mineral for normal body function (e.g. bone growth, blood clotting and neurotransmission).

Materials and methods

Test species

The freshwater fingerlings, *L. rohita* of length 6 ± 1 cm and weight 8 ± 1 g were procured from the Government fish farm at Lalpet (Tamil Nadu, India), 15 km away from the University campus. The collected fish were transported to the laboratory in an oxygen pack. The fish were first treated with 1% potassium permanganate solution for 15 min to avoid any infection and were then maintained in glass aquaria in the laboratory for 2 weeks prior to experimentation [26, 27].

Test chemicals

The AnalaR grade arsenic trioxide (As_2O_3) and *meso*-2,3-dimercaptosuccinic acid (DMSA) were purchased from Sigma Aldrich Company, Bangalore, India, and used without further purification.

Lethality studies

The 96 h LC_{50} value for arsenic was determined by using Litchfield and Wilcoxon [28] method and was found to be 124.5 ppm. Arsenic stock solution was prepared by dissolving 1.3202 g of As_2O_3 in 1 L of acidified water. A sub-lethal level of arsenic (41.5 ppm, one-third of the LC_{50} value) was used in the present study. The acclimated fish were stocked in 50 L glass trough of dimension 50 cm × 40 cm × 40 cm equipped with continuous air supply. Water characteristics such as pH, temperature, total hardness, total alkalinity, calcium and magnesium were measured according to standard methods [29] and maintained at optimum level. The mean composition of water in the aquaria was pH: 7.3 ± 0.2; temperature: 23 ± 2 °C; hardness as $CaCO_3$: 202 ± 5 mg L^{-1}; alkalinity: 127 ± 7 mg L^{-1}, calcium: 54 ± 4 mg L^{-1} and magnesium: 21 ± 3 mg L^{-1}.

Experimental study

The acclimated test fish were divided into three groups as group I, II and III, each containing 10 fish. The group I was used as control and reared in dechlorinated tap water (test water). The test fish belonging to group II were exposed to higher sub-lethal concentration (41.5 ppm) of arsenic for 14 days (sub-acute exposure). The test fish belonging to group III were exposed to 41.5 ppm of arsenic for 14 days followed by DMSA in water (5 mg kg^{-1} body weight of the fish) for 7 days [27]. All fish were fed with commercial fish pellets (Himalaya Company, India) once a day during the experimental period, which had no detectable arsenic

content. To maintain a constant concentration of the toxin, the water was renewed every 24 h and fresh toxin was added to obtain the sub-lethal level of 41.5 ppm. The fish were dissected after the completion of exposure. Bones were removed and used for the thermal analyses.

Thermal analysis

In the present study, the thermal analysis was carried out using a simultaneous thermogravimetry (TG) unit. The experiments were conducted in an air atmosphere using a simultaneous TG-DSC-DTA unit from TA Instruments, Model SDT Q600, available at the Central Electro-Chemical Research Institute (CECRI), Karaikudi, Tamil Nadu, India. The fish bone specimens were cleaned with a scalpel to remove any residues from the exterior of the specimens, washed with physiologic solution and treated in acetone to remove lipid residues. Then the specimens were dried in an air oven for 3 h to remove adsorbed water. Then the bones were powdered in an agate mortar and pestle in order to obtain bone powder. Platinum crucibles were used to hold the bone powdered samples and then heated from ambient temperature to 1,200 °C at a heating rate of 10 °C min^{-1} in an air atmosphere. Mass loss in samples was measured. Calorimetric measurements were performed using a differential scanning calorimeter using the same instrument. The peak temperature and enthalpy were determined numerically from thermograms for each exothermic process. The peak temperature was calculated as the temperature at minimum value of calorimetric signal within the exotherm. The enthalpy was determined from the area between exotherm and a baseline which was constructed by extrapolating to the scan beyond the exotherm.

Statistical analysis

Statistical analysis was performed using SPSS 11.5 software. The differences between the corresponding values between the control and expose/treated groups were established by the student's *t* test. A probability level (*p* value) of less than 0.05 is regarded as statistically significant.

Results and discussion

The TG data usually shows mass losses at particular temperatures that may be correlated with the organic and inorganic phases in the sample. The first step usually occurs between 50 and 260 °C and is associated with water loss. A second step from 260 up to 600 °C represents the combustion of the organic components to produce carbon dioxide, water and organic fragments. A third step is observed in the range 600–1,200 °C and is associated with the release of carbon dioxide from the carbonated hydroxyapatite, if the decomposition is carried out in an air atmosphere [30, 31].

Thermo gravimetric analysis

The TG and DSC curves for the control, arsenic exposed and DMSA treated *Labeo rohita* bones are presented in Figs. 1 and 2. As seen from the figures, these curves show the mass loss due to the thermal decomposition in an air atmosphere which occurs in three distinct regions. Region 1 is observed from ambient temperature to 215 °C with a peak at 94 °C. The mass loss in this region is characteristic

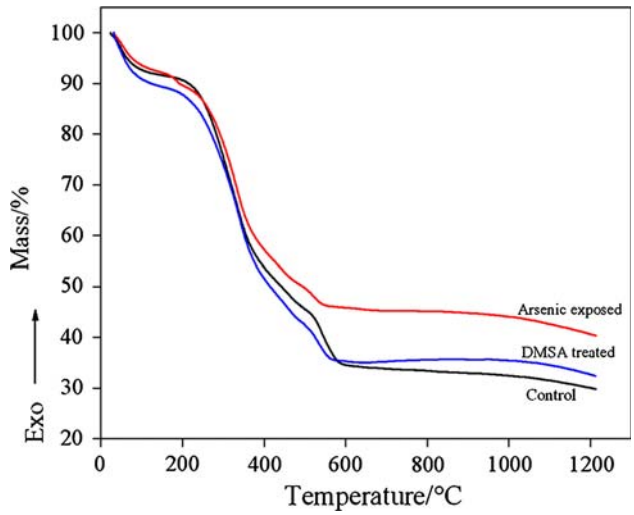

Fig. 1 The average TG curves for the decomposition of the control, arsenic exposed and DMSA treated bones of *Labeo rohita*

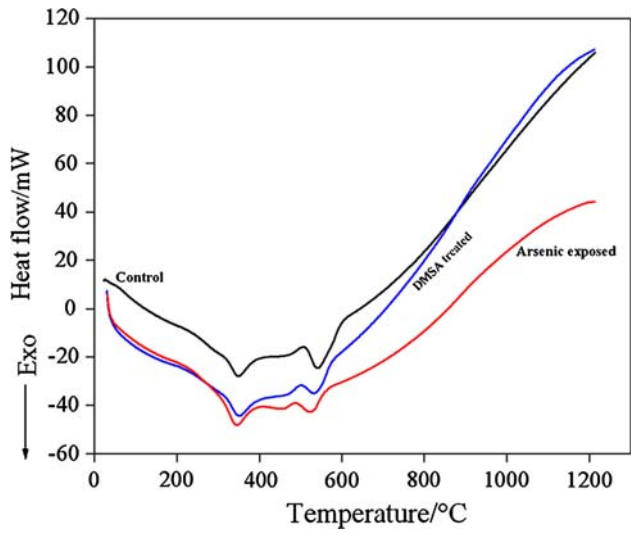

Fig. 2 The average DSC curves for the control, arsenic exposed and DMSA treated bones of *Labeo rohita*

of released hydroscopic water. The mass loss up to 215 °C is 10.05 ± 0.42%. Region 2 is characterized by a three step decomposition process of organic material. Between 215 and 371 °C, there is a first decomposition and can be attributed to the phosphate decomposition with mass loss of 32.72 ± 1.36%. Between 371 and 518 °C, there is a second decomposition probably due to lipid decomposition with 12.84 ± 0.64% of mass loss takes place. Between 518 and 585 °C there is a third decomposition with 9.40 ± 0.59% of mass loss, probably due to the mineral salts' decomposition [32]. In an oxidizing atmosphere the organic phase is expected to be removed up to 600 °C. The mass loss between 215 and 585 °C is 54.96 ± 2.11% of the original mass. Region 3 is observed between 585 and 1,200 °C. The mass loss in this latter temperature range was measured to be 5.21 ± 0.28% based on the original mass, giving a total mass loss of 70.22%. The total mass loss was 59.70% for arsenic exposed bones and 67.74% for DMSA treated bones. The mass loss of last region agrees with the reported values of 5% [30] and can be attributed to decomposition of the inorganic phases, notably the removal of carbon dioxide from the carbonated apatite as has been observed elsewhere [16].

Table 1 presents the mass losses in different regions for the control, arsenic exposed and DMSA treated bones. As seen from the table, the mass lose is increased due to arsenic exposure when compared to the control. This might be due to the degeneration of organic matrix due to arsenic exposure. Similar behaviors were observed by Utech et al. [14] in pathologic human bone samples.

Differential scanning calorimetry

The calorimetric study is based on the assumption that the macromolecules of biological systems are in complex interactions with their environment. Any change in the external chemical-physical variables (e.g., temperature) results in characteristic changes of the system, which can be detected by calorimetry [23]. To see whether there are differences in thermal behavior between the control, arsenic exposed and DMSA treated fish bones, DSC measurements were performed.

As seen from Fig. 2, the DSC curves show three well defined exothermic peaks due to three effects of mass loss. Region 1 is associated with the evolution of water. The observed mass loss may be accounted for by the onset of decomposition processes which evolve water as well as organic species. Loss of water content is presented in the TG curves (Fig. 1) for all the three samples, starting from ambient temperature to 215, 192 and 214 °C, respectively, for the control, arsenic exposed and DMSA treated fish bones. The underlying reason could be that the thermal heat capacity of any biologic system is basically dependent on the amount of water tied.

Region 2 is characterized by a decomposition process of the organic material identified with the sharp exothermic peaks in the DSC curves at 349, 469 and 544 °C for the control bone samples. Similar results were observed by Utech et al. [14] in healthy and pathologic human bones.

The thermal decomposition of the samples is observed throughout region 3 as the TG curves are not observed to reach zero even at 1,200 °C. In this region only 5.21 ± 0.28% of mass loss is observed in the control bones. This mass loss may be due to the evolution of organic products as suggested by Onishi et al. [31]. In the arsenic exposed and DMSA treated bone samples, an increase of the slope is observed in the TG curves in the region 800–1,200 °C with the peak centered around 900 °C indicating a broad exothermic band. Although there is likely to be some decomposition of the mineral phase in this region, the mass increase appears to be predominantly associated with the organic products or may be due to the oxidation of free arsenic cation as suggested by Rincón et al. [33].

From the TG curves, the maximum loss is observed in the second region for all the samples. In general, maximum mass loss is observed in the control bones and minimum in the arsenic exposed bones. This may be due to arsenic intoxication; The DMSA treated bones show a mass loss nearly equal to that of the control bones. This suggests that the arsenic accumulated in the bones is effectively removed by the chelator, DMSA.

From the Fig. 2, it is observed that the peak at 544 °C gives an enthalpy of 1569 ± 35.2 J g^{-1} for the control. For

Table 1 Total mass losses in different regions for the control, arsenic exposed and DMSA treated *Labeo rohita* bones

Samples	Percentage of mass loss			Residue mass percentage at 1,200 °C
	Region 1	Region 2	Region 3	
Control	10.05 ± 0.42	54.96 ± 2.11	5.21 ± 0.28	29.78 ± 1.24
Arsenic exposed	9.97 ± 0.36	43.40 ± 1.94	6.33 ± 0.81	40.30 ± 1.51
DMSA treated	13.07 ± 0.55	51.35 ± 1.76	3.22 ± 0.12	32.36 ± 0.97

Water and organic contents are calculated as a percentage of the starting mass; each value is the mean ± SD of 10 individual observations. The degree of significance was denoted as: $p < 0.05$

Table 2 The calorimetric enthalpy changes observed in the control, arsenic exposed and DMSA treated *Labeo rohita* bones

Samples	Peak 1 (°C)	ΔH (J g^{-1})	Peak 2 (°C)	ΔH (J g^{-1})	Peak 3 (°C)	ΔH (J g^{-1})
Control	349	759.4 ± 21.5	469	164.5 ± 4.4	544	1569 ± 35.2
Arsenic exposed	344	1278 ± 30.6	456	144.0 ± 3.9	528	604.5 ± 19.1
DMSA treated	350	764.5 ± 25.7	467	177.0 ± 4.0	538	993.6 ± 21.8

Each value is the mean ± SD of 10 individual observations. The degree of significance was denoted as: $p < 0.05$

arsenic exposed bones, the DSC peak is shifted to lower values and gives an enthalpy of 604.5 ± 19.1 J g^{-1}, which is almost 2.6 times lower than that in control bones (Table 2). For DMSA treated bones, the peak is observed at 538 °C and gives the calorimetric enthalpy of 993.6 ± 21.8 J g^{-1}. Note that ΔH values corresponding to control and DMSA treated bones appear similar, suggesting they were trended the same way. These results suggest that DMSA appears to aid the removal of arsenic and a partial recovery of bone structure apparently occurs. It further suggests that DMSA not only forms stable and soluble complexes with arsenic, but also ensures restorative reaction of the organisms.

Further, as seen from the Fig. 2, in the control bone samples, there is smaller difference between the thermal heat capacities of the starting and ending conditions than the arsenic exposed one. The underlying reason could be that the thermal heat capacity of any biological system is basically dependent on the amount of water tied [23]. The other evident is the fact that the exothermic reaction shows a narrow peak in the case of control bone. The reaction takes place as a single compact unit. This may be due to the reduced quantity of collagen, its abnormal structure or the deviations of the surrounding basic material [23]. It is supported by the calorimetric enthalpy which is almost twice (1.7) higher than in the control bone (Table 2). In DMSA treated bone, the observed peak is very nearer to that of control bones and the calorimetric enthalpy is almost equal to that of the control bones (Table 2). These results confirm the recovery of bone structure in the DMSA treated bone samples.

The thermal decomposition of bone mineral appears to depend on the organic content of the bone. In the present study, the residue masses for the control, arsenic exposed and DMSA treated bones are 29.78 ± 1.24%, 40.30 ± 1.51% and 32.36 ± 0.97%, respectively. The increased residue content in the arsenic intoxicated bones might be due to the accumulation of arsenic. Further, in the DMSA treated bones the residue masses decreased nearer to the control values. This may be due to the removal of arsenic from the arsenic exposed bones by DMSA. This is further evidenced by the ICP-OES studies [27]. The amount of arsenic in the arsenic exposed bones is 13.55 ppm and in DMSA treated bones, arsenic content is decreased to 2.33 ppm.

The DSC results clearly proved that definitive differences are present between the thermal characteristic of the normal (control) and arsenic exposed *Labeo rohita* bones. The significant change in the calorimetric enthalpy in the arsenic exposed bones may be due to the change in the amount of collagen, and in the amount of secondary bindings between the collagen fibers [34].

Conclusion

The arsenic exposed bones present a different thermal behaviour compared to the control bone. The residue masses increase due to arsenic exposure, while the DMSA treatment reduces the residue mass level. In addition, the calorimetric enthalpy values are changed drastically due to arsenic exposure, whereas the DMSA treatment increases these values significantly. These thermal characteristics can be advantageously used as a qualitative method to check the metal accumulation/intoxication in biological samples.

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