METHALOTHIONEIN AND LIPID PERIOXIDATION: COMBINATIONAL BIOMARKER PROTOCOL IN BIOMONITORING OF SOME HEAVY METALS EFFECTS IN MUS MUSCULUS (MICE)

Supported by tetfund

***Osuala[1], Fidelia I. and Igwo-Ezikpe, Miriam. N. [2]**

[1]Department of Zoology, Faculty of Science, University of Lagos, P.M.B. 101017, Lagos, Nigeria
[2]Department of Biochemistry, Faculty of Basic Medical Sciences, College of Medicine, University of Lagos, P.M.B. 12003, Lagos, Nigeria
*Corresponding author:fhilosu@yahoo.com

Received: January 11, 2023 **Accepted:** March 12, 2023

Abstract:

The study evaluated the sublethal effects of selected heavy metals; Cd, Fe, Mn, Pb and Zn utilising metallothionein (MT) induction and Lipid perioxidation (LPO) as biomarker indice using Albino mice model, *Mus musculus*. The assessment of MT and LPO was evaluated using standard methods. Mice were orally feed with sublethal ($1/10^{th}$ of 96 hrLC_{50}) concentrations of heavy metals. Iron induced the highest concentration of MT in exposed mice followed by Cd > Mn > Pb > Zn based on the 28^{th} day exposure periods, relative to the control. The lipid peroxidation assay revealed that the levels of melanoaldehyde (MDA) in the liver, kidney, heart and brain of exposed mice was significantly ($P < 0.05$) higher than levels detected in control group. On the 28^{th} day of exposure, it was established that Zn induced the highest MDA in the liver and heart whereas Cd induced the highest levels in the kidney and brain. The implication of this study is that biological responses such as metallothionein induction, and lipid peroxidation can act as battery of biomarkers for early detection and diagnosis of heavy metals complex pollution in mammals.

Keywords: Methallothionein, Lipid perioxidation, Mus musculus, Heavy metals, melanoaldehyde, Kidney

Introduction

Heavy metals are naturally occurring metallic elements with high atomic weight and density greater than 5 g/cm^3 that are toxic at relatively low concentrations (Falusi and Olanipekun, 2007; Raymond and Felix, 2011). Heavy metals have high persistence in the environment long after the source of emission has been removed. According to DeVagi and Arfiziah (2009) heavy metals could therefore be grouped as important sources of pollution. Numerous studies have reported toxic effect induced in humans and animals exposed to certain heavy metals especially arsenic (Bargagli, 2000) chromium (Dayan and Paine, 2001), mercury (Lee *et al*., 2001) cadmium (Watanabe *et al*., 2003) and lead (Flora *et al*., 2007). Heavy metals generate reactive oxygen species (ROS) that exert oxidative stress (Vinodhini and Narayanan, 2008) that inhibits biological detoxification or repair (Agarwal *et al*., 2003). In addition, heavy metals bind strongly to thioneins, amino acids, enzymes, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), oxidize proteins and polyunsaturated lipids causing oxidative deterioration of biological macromolecules (Flora *et al*., 2008; José *et al*., 2010). Metallothioneins are cysteine rich, low molecular weight metal binding proteins of about 7 kDa and apparent molecular weight of 13 kDa whose synthesis is induced by heavy metals. Metallothioneins are believed to be important in detoxification and homeostasis of heavy metals such as copper and zinc (Christopher and Peter, 2002). Their induction confers metal tolerance to organisms (Klaasseen *et al*., 1999) due to their ability to bind some heavy metals such as cadmium and copper (Coucelo *et al*., 2000). Studies have reported that MT play priority role in free radical scavenging in vitro (Milena, 2002, Illuminati *et al*., 2010). Thus the induction of metallothioneins in animals due to exposure to xenobiotics has resulted in the proposition of metallothioneins as a potential biomarker for heavy metal exposure (De Smet *et al*., 2001). These heavy metals induce the synthesis of MT could also result in lipid peroxidation (LPO), enzyme inactivation, DNA damage and even cause cell death (Robert and Ernest, 2008). Numerous studies have focused on metal-induced toxicity and carcinogenicity, emphasizing their role in the generation of reactive oxygen species in biological systems and their significance. Therefore, toxicological assessment of heavy metals in living tissues can be monitored by measurement of some biochemical parameters induced by exposure such as biomarkers. This has gained immense support and research in developed countries (Viarengo *et al*., 1999; Handy *et al*., 2003; Moore *et al*., 2004; Ringwood *et al*., 2004) but their usage in developing countries especially in Nigeria is has not sufficiently gained adequate attention. Additionally a comprehensive monitoring programme combining various biomarkers for early detection of the adverse effects of exposure to heavy metal pollution is currently inadequate in Nigeria and many parts of the developing world.

The significance of this study was to assess heavy metal impact using metallotheinein and Lipid perioxidation as biological markers to monitor physiological disruption.

Thus the aim of the study was to access some selected heavy metals by utilizing the induction of MT and LPO in mice at sub lethal concentrations over a period 28 days.

Materials and Methods

Test Chemicals

The heavy metals investigated in this work were obtained as metallic salts from Fisons Laboratory Reagents, Analar grades. Solutions of Zn^{2+}, Cd^{2+}, Pb^{2+}, Mn^{2+} and Fe^{2+} were prepared using zinc chloride ($Zncl_2.3H_2O$), cadmium sulphate ($CdSO_4.8H_2O$), lead sulphate ($PbSO_4$), magnesium sulphate ($MnSO_4$) and Iron sulphate ($FeSO_4$) respectively.

Test Animals

Male Albino mice, *M. musculus* of similar size (19 - 24 g body weight and 10 - 15 weeks old) used in this study were purchased from the Nigerian Institute of Medical Research (NIMR) Yaba, Lagos. Nigeria.

Acclimatization and Maintenance

Mice were fed with 35 g standard rodent laboratory chow purchased from Ladokun feeds Ltd. Ibadan per day and had free access to drinking fluid (distilled water). The mice were kept in ventilated plastic cages (20 x 12 x 9 cm) with wood shavings to acclimatise for 14 days to experimental conditions (30^{o}C ± 2^{o}C and Relative Humidity - 70% ± 2%) before commencement of bioassay. Mice was used due to its sensitivity.

The animals were handled with care in accordance to the Institute for Laboratory Animal Research (ILAR) guidelines.

Ecotoxicology: Sublethal Studies

Chronic Toxicity

Mice were divided into five experimental groups (n = 12 per group) and a control group (n = 3) for the sublethal experiment. They were administered with salts of the heavy metals $CdSO_4$, $FeSO_4$, $MnSO_4$ $Pb(NO_3)_2$ and $ZnSO_4$ at concentration of 0.05 mM, 0.45 mM, 0.57 mM, 0.24 mM, and 24 mM respectively while the control group was administered distilled water only.

Collection of Serum Samples

Blood was collected from mice and drained into plain bottles. The blood was allowed to clot and then centrifuged at 3000 rpm for 10 minutes after which the clear supernatant (serum) was separated from the pellet and kept frozen until required.

Collection Liver, Heart, Kidney, Brain Samples and Preparation of Tissue Homogenates

The animals were fasted for 24 hours after the last administration for the various exposure days and subsequently dissected and the tissues (liver, heart kidney and brain) harvested. Tissue homogenates were prepared by suspending the 1 mg of each tissue in ice-cold 4 ml of 50 mM phosphate buffer, pH 7.2 to give a final volume of five times dilution and then homogenized using Teflon homogenizer. The homogenates were kept frozen at -20^{0} C overnight before analysis.

Measurement of Methallothionein

Methallothionein (MT) levels were assessed in the serum by evaluating the sulphyhydryl (-SH) residue according to Ellman (1959) method as modified by Viarenge *et al*. (1993). The amount of MT was defined assuming a cystein content of 23. The MT concentrations of 3 replicates for each measurement were calculated using reduced gluthathione (GSH) as a standard and expressed as (nmol MT) g^{-1}. Chemicals were obtained from Ikzus Environment, Italy.

Reagent and Working Solutions Preparation

Sulfhydryl reference (reduced glutathione) standard was prepared by adding 0.725 µl of Sol C_2 (resuspension buffer Component 2) to a microtube. The reaction buffer (Sol D) was obtained by mixing 200 ml stabilized Ellman's buffer and 3.4 ml concentrated Ellman's reagent that was equilibrated at 23 ^{o}C. Absolute ethanol was also equilibrated at -20 ^{o}C. The homogenising buffer was obtained by adding 1 part of buffer. The resuspension buffer was prepared by mixing equal amounts of Sol C_2 (resuspension buffer, Component 2). The blank solution was obtained by mixing 50 µl Sol C (resuspension buffer Component 1) and 1.950 µl Sol D (reaction buffer).

Procedure for the Extraction and Evalution of ***Metallothionein Content***

Serum (0.5 ml) was homogenized at 0 - 4^{o}C using the already prepared homogenating buffer and centrifuged at 3000 rpm for 20 minutes. The protein content was then determined from the supernatant by Bradford method (Bradford, 1976). The supernatant was collected in a 2 ml tube. Cold absolute ethanol of 1.5 ml was added to the tube and incubated at -20 ^{o}C for 30 - 60 minutes. The mixture was centrifuged at 12,000 - 16,000 g at 4^{o}C for 5 minutes. To this was added 1.95 ml of the previously prepared reaction buffer, which was equilibrated at room temperature. The resulting mixture was mixed, incubated for 2 minutes at room temperature and then centrifuged at 12-16,000 rmp. Absorbance was also read for samples at 412 nm. To calculate methalothionein concentration, the $ABS^{MT}{}_{412}$ value of sample was interpolated over the standard curve to obtain the concentration (nmol) of sulfhydryl groups that is cysteine residues (nmol Cys^{MT}), due to metallothionein present in the sample. To obtain the concentration of methalothionein (nmol MT) per gram of tissue, the following formula was applied.

$$\textbf{(nmol MT)g}^{-1} = \frac{\textbf{(nmol Cys}^{MT}\textbf{)}}{\textbf{0.1 g.n}^{ocys}} \qquad (1)$$

Where: 0.1g is the amount of tissue equivalent to 0.3 ml of supernatant subjected to precipitation while n^{ocys} is the number of cysteine residue present in the investigated metallothionein. To express metallothionein concentration per mg of the total protein present in the supernatant, the following equation was applied:

$$\textbf{(nmol MT)g}^{-1} = \frac{\textbf{(nmol Cys}^{MT}\textbf{)}}{\textbf{mg.n}^{ocys}} \qquad (2)$$

Where: mg is the amount of protein present in the 0.3 ml supernatant subjected to ethanol precipitation.

Measurement of Lipid Perioxidation (MDA)

Lipid peroxidation (LPO) was estimated based on the formation of TBARS (thiobarbituric acid reactive substances) as an index of lipid peroxidation according to the method of Niehaus and Samuelson as described by Usoh *et al*. (2005). To 2 ml of Trichloric acid – Thiobabutoric acid – Hydrochloric acid (TCA-TBA-HCL) reagents in a test tube, 1 ml of crude enzyme extract was added, mixed thoroughly and heated for 15 minutes in boiling water. The flocculent precipitate after cooling was removed by centrifugation at 10,000 × g, for 10 minutes and absorbance read at 535 nm against reagent blank. LPO index, malonaldehyde (MDA) was calculated with extinction coefficient of 1.5 x 10^5 $M^{-1}cm^{-1}$.

Determination of Total Protein

This was determined using Biuret method (Gonall *et al*., 1949). Blank Biuret reagent of 5.0 ml was prepared by dissolving 1 g of $CuSO_4$ $5H_20$ crystal in 500 ml of distilled water and added to sample. These were mixed properly and allowed to stand for 20 minutes at temperature 25 – 27 ^{o}C. Absorbance was read for one test and standard against a blank at 540 nm. The concentration of protein was calculated using the formulae:

Optical density for standard × concentration of standard

Statistics Analysis

Data was analyzed with oneway analysis of variance (ANOVA). Differences at $P < 0.05$ were considered significant. This was used to compare several treatment means in appropriately designed experiments. Further analysis was carried out only when there was a significant difference at the 5 % ($P < 0.05$) level of significance (taken as minimum requirement) based on Duncan multiple range test at 0.05 levels of significance using SPSS 10.0 computer software package (SPSS Inc., Chicago, U.S.A).

Results and Discussion

Mice (*M. musculus*) exposed to Cd at 0.05 mM per body weight induced 7.60 x 10^{-4} nmol g^{-1} of MT on 7th day that decreased to 6.56 x 10^{-5} nmol g^{-1} on the 14th day but increased to 1.91 x 10^{-3} nmol g^{-1} on the 28th day (Fig. 1). These were significantly ($P < 0.05$) different from 2.64 x 10^{-4} nmol g^{-1} obtained for control mice group.

Results showed that Fe induced significant increase in MT level from 1.45 x 10^{-3} nmol g^{-1} on the 7th day with a subsequent increase of 2.61 x 10^{-4} nmol g^{-1} on the 21st day to 313.00 x 10^{-3} nmol g^{-1} on the 28th day (Fig. 1). These were significantly ($P < 0.05$) different from 2.64 x 10^{-4} nmol g^{-1} obtained for control.

Manganese induced MT value was 1.92 x 10^{-3} nmol g^{-1} on the 7th day which decreased to 2.21 x 10^{-4} nmol g^{-1} on the 14th day but increased to1.62 x 10^{-3} nmol g^{-1} on the 21st day and finally decreased to 1.39 x 10^{-4} nmol g^{-1} on the 28th day (Figure 1). These were significantly ($P < 0.05$) different from 2.64 x 10^{-4} nmolg^{-1} obtained for control mice.

Metallothionein induced by Pb decreased from 4.92 x10^{-4} nmol g^{-1} on the 7th day to 3.72 x 10^{-4} nmol g^{-1} on 14th day but increased to 9.32 x 10^{-4} nmol g^{-1} on the 28th day (Fig. 1).

The highest level of MT induced by Fe was in concordance with the findings of Petering *et al*. (1984), Ann and Hill (1987), Ruriko *et al*. (2002). The increased MT synthesis has been associated with increased capacity for binding with these metals and protection against metal toxicity. The level of MT synthesis that increased was similar to the findings by Laura *et al*. (2011). The authors investigated MT in the blood samples from autistic children and observed that the plasma concentration of thirteen (13) heavy metals (Al, As, Ca, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Si and Zn) induced a higher expression of MT than in healthy individuals which represented the control group and concluded that heavy metals induce MT in varied amount and in diverse tissue. Likewise, Ann and Hill (1987) observed that female broiler chicks receiving an intra peritoneal injection of Fe (10 mg kg^{-1} BW) were found to have a greatly increased level of hepatic metallothionein.

The most pronounced effect of MT synthesis as induced by all the selected test heavy metals, which was observed to have increased on the 7th day but was later suppressed on the 14th day of exposure and subsequently increased from the 21st - 28th day could probably be attributed to the fact that mice on first contact with the heavy metals responded by inducing a defensive mechanism, triggering the production of metallothionein (MT) to mop up the stress. This is in agreement with the findings of Luqing and Hongxia (2006) These were significantly ($P < 0.05$) different from 2.64 x 10^{-4} nmol g^{-1} obtained for control.

Zinc induced an increase in MT levels from 1.07 x 10^{-3} nmol g^{-1} on the 7th day with a sharp decrease to 2.76 x 10^{-4} nmol g^{-1} on the 14th day which slightly increased significantly ($P < 0.05$) to 4.85 x 10^{-4} nmol g^{-1} on the 28th day in relation to control at 2.64 x 10^{-4} nmol g^{-1} (Figure 1). The trend in Zn induced MT was in contrast to that observed in cadmium and manganese treated groups. Based on the 28-day exposure period the levels of MT induced by each metal in descending order were as follows: Fe >Cd > Mn > Pb > Zn

Throughout the duration of exposure for all the selected heavy metals there were significant ($P < 0.05$) differences amongst all the treated groups (ANOVA, $P < 0.05$). Post hoc test with Duncan indicated that for all treated groups there were significant ($P < 0.05$) differences in MT concentration on all sampling days.

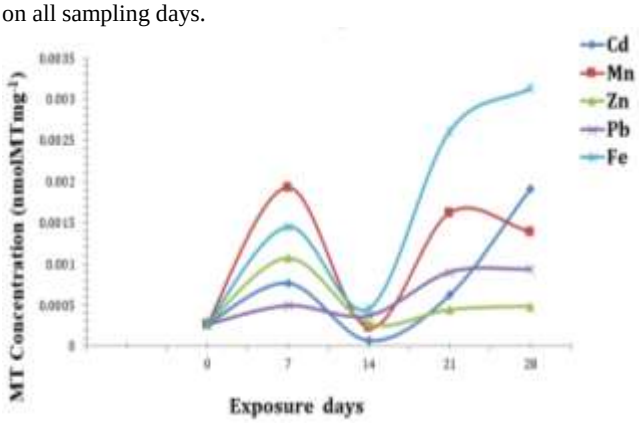

Fig. 1: Metallothionein concentration in the serum of mice exposed to heavy metals

who investigated the effect of two Cd concentrations (the final Cd concentration of 0.025 and 0.05 mg L^{-1}, prepared with $CdCl_2 \cdot 5H_2O$) on metallothioneins (MT), in the gills and hepatopancreas of the Portunid crab Charybdis japonica in a 15 days' exposure period and observed that MT was significantly induced after 3 days. Similarly, Ryan and Hightower (1994) postulated that all organisms from microbes to humans respond to chemicals and physical stresses by increasing the synthesis of small group of stress protein such as metallothionein.

Metallothionein constitute a class of low molecular weight cysteine rich metal binding proteins which are biosynthetically regulated at the level of gene transcription in response to heavy metals, hormones, cytokines, physiological and environmental stress (Liu and Thiele, 1996). The suppression on the 14th day could be attributed to the mice system acclimatizing to the heavy metal exposure but with increasing exposure to the heavy metals on the 28th day, the MT induced level was overwhelmed prompting the system to generate the production of enzymes and protein which enhanced the ability to produce more MT. A number of studies have shown that MT scavenge a wide range of ROS including superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide at high concentrations in animals (Xiaoyan *et al*., 2004; Kate *et al*., 2006). Evidence indicated that MT is important in regulating cell signaling in mice

through interactions with these oxygen radicals (Sato and Kondoh, 2002). Mohamed *et al*. (2010) in a study of the effects of heavy metals inducing metallothionein observed a similar finding with that of this research. The molecular assay showed a decreased trend of MT-1 in liver of rat treated to Cd (200 ppm) in drinking water for 30 days. The authors observed that, although there was a dynamic equilibrium in binding of metal among cytosolic ligands, once metal accumulation exceeds metallothionein production, the detoxification system can become saturated and excess metal may bind to sensitive enzymes and exert toxic effects. This has been referred to as spillover (Brown *et al*., 1977). This might probably be a second reason for the suppressed level of MT on the 14th day of exposure.

The ability of relatively low exposure (sublethal / chronic toxicity) to non-essential trace metal contaminants (for example, cadmium) to induce metallothionein has generated interest in the use of metallothionein as a biomarker for metal pollution (Hyne and Maher, 2002). Iron is an indirect MT inducer that is mediated primarily by stress response. The induction of MT by iron as was observed in this study was in agreement with the results of other workers such as Fleet *et al*. (1990). These authors showed that the administration of iron caused a 10-fold increase in hepatic MT and a reduction in serum Zn plasma. Thus it could be implied that increased induction by iron could cause a depression in plasma zinc. This will further initiate MT synthesis and cause a sequestration of Zn from the Plasma. However, various studies have attempted to establish various mechanisms of MT induction but it has been traditionally believed that MT induction is a secondary reaction caused by xenobiotics and an established inducer, specifically glucocorticoids that is contrary to the views of Tomoki and Norio (2008).

Zinc induction of metallothionein (MT) which was the least compared to the other heavy metals may be due to the fact that, MT contains zinc-binding proteins in mammalian cells which would rather maintain intercellular metal homeostatis in the system. It has also been hypothesized that Zn plays a role in the function of zinc-dependent signal transduction proteins and transcription factors. However, the implication of the present findings regarding Zn - MT induction suggested that on mice exposure to sublethal concentrations of the heavy metal, may have channeled energy towards the production of MT to fight the stressed environmental condition induced by heavy metal. This would have a negative feedback on the system as it affects other physiological activities in the body system such as growth and reproduction. It is now clear that at least in mammals, insects and crustaceans that MT is induced under many other conditions such as in metal exposure. Cellular toxicities may ensue after the metal-binding capacity of MT has been exceeded. Therefore, based on the findings of this is study, it could be safely proposed that MT measurements may provide considerably more information about potential health hazards of metals in exposed animals than tissue metal residues alone.

Lipid Peroxidation (LPO)

The level of MDA detected based on the 28th day, in relation to control showed that Cd elicited a decrease from 2.32 x 10^{-9} n Mg^{-1} to 5.62 x 10^{-10} n Mg^{-1} in the liver; while in the heart decreased from 1.8 x 10^{-8} n Mg^{-1} to 4.22 x 10^{-10} n Mg^{-1}; in the kidney it decreased from 1.94 x 10^{-9} n Mg^{-1} to 9.82 x 10^{-10} n Mg^{-1} whereas in the brain the decrease was from 1.11 x 10^{-9} n Mg^{-1} to 1.36 x 10^{-9} n Mg^{-1}. There were no significant differences ($P > 0.05$) in the MDA induction levels in the liver, heart and kidney except in the brain ((Fig. 2). Thus with respect to organs the levels of MDA descended as follows: Brain > Kidney > Heart > Liver.

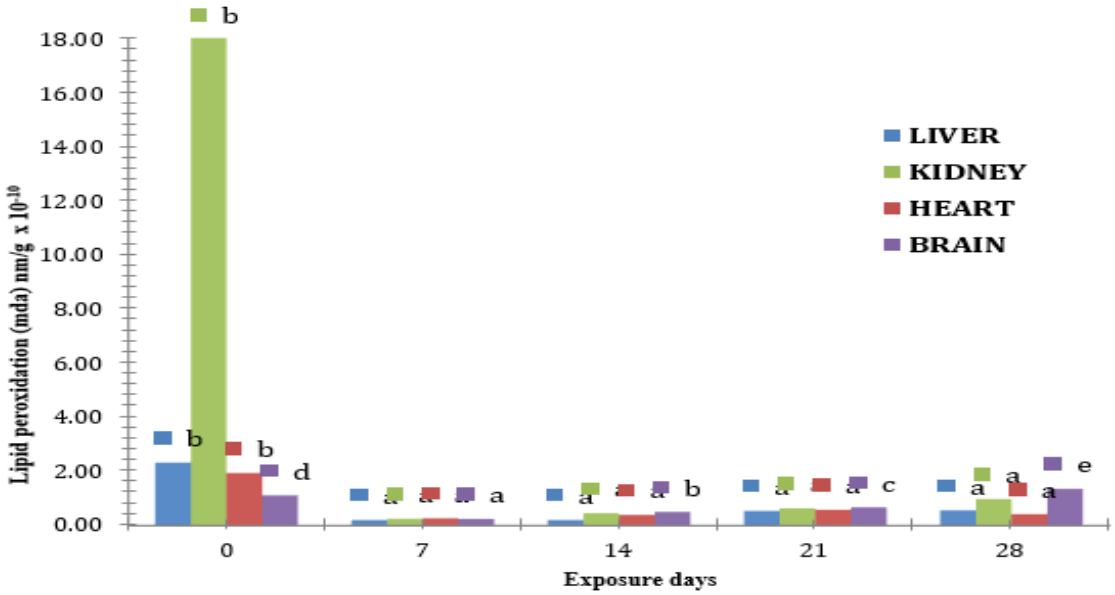

Fig. 2: Malonaldehyde (MDA) level in liver, kidney, heart and brain of *Mus muculus* exposed to cadmium (Cd). Values with dissimilar letters (a, b, c, d and e) are significantly (P < 0.05) different from each other with respect to the organs. Values are expressed as means (n = 3).

The level of MDA in the liver, heart, kidney and brain of *M. musculus* exposed to Fe decreased significantly (P < 0.05) when compared to control group (Fig. 3). The level of MDA induction based on the 28th day exposure with reference to control, showed a decrease from 2.32 x 10^{-9} n Mg^{-1} in control to 4.47 x 10^{-10} n Mg^{-1} in the liver; while in the heart decreased from 1.8 x 10^{-8} n Mg^{-1} to 2.94 x 10^{-10} n Mg^{-1}; in the kidney it decreased from 1.94 x 10^{-9} n Mg^{-1} to 2.85 x 10^{-10} n Mg^{-1} whereas in the brain decreased from 1.11 x 10^{-9} nMg^{-1} to 6.99 x 10^{-10} nMg^{-1}. However, the 7th day MDA induction in the heart showed that there were slight increases which were significantly (P < 0.05) different from that induced on the 14th, 21st and 28th day although, not higher that in control. Thus with respect to the organs, the levels of MDA induced in descending order were as follows: Brain > Liver > Heart > Kidney.

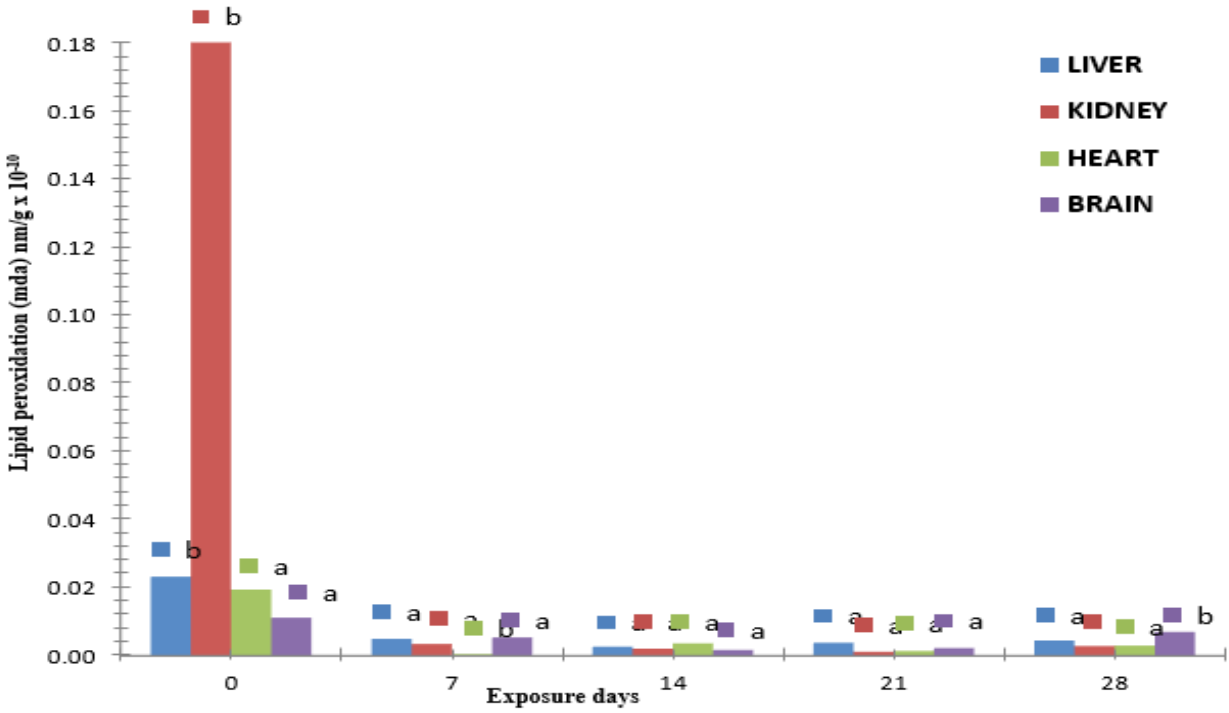

Fig. 3: Malonaldehyde (MDA) level in liver, kidney, heart and brain of *M. musculus* exposed to iron (Fe). Values with dissimilar letters (a, b and c) are significantly (P < 0.05) different from each other with respect to the organs. Values are expressed as means (n = 3).

The result of the lipid peroxidation assay showed that the level of MDA in the liver, heart, kidney and brain of *M. musculus* exposed to Mn decreased significantly (P < 0.05) when compared to control (Fig. 4). The level of MDA induction based on the 28th day exposure with reference to control, showed that the level decreased from 2.32 x 10^{-9} n Mg^{-1} l to 4.7 x 10^{-10} n Mg^{-1} on 28th day in the liver; while in the heart decreased from 1.8 x 10^{-8} n Mg^{-1} to 4.47 x 10^{-10} n Mg^{-1}; in the kidney it decreased from 1.94 x 10^{-9} n Mg^{-1} to 4.96 x 10^{-10} n Mg^{-1} whereas in the brain, decreased from 1.11 x 10^{-9} n Mg^{-1} to 7.10 x 10^{-10} n Mg^{-1}. Thus with respect to Mn exposure the levels of MDA in the organs decreased follows: Brain > Kidney > Liver > Heart.

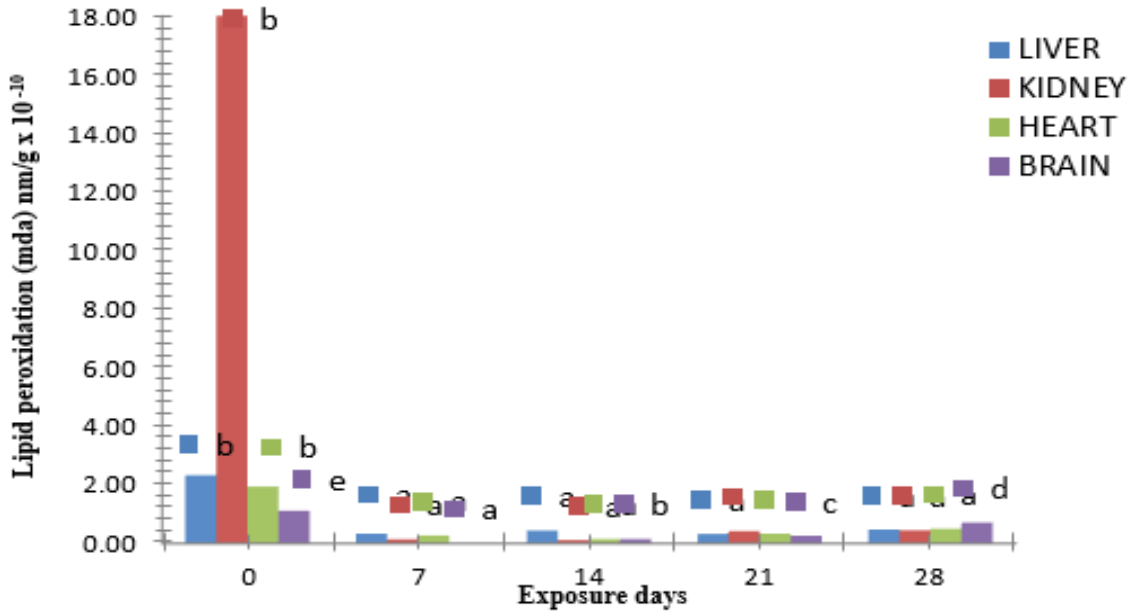

Fig. 4: Malonaldehyde (MDA) level in liver, kidney, heart and brain of *M. musculus* exposed to manganese (Mn). Values with dissimilar letters (a, b, c, d and e) are significantly (P < 0.05) different from each other with respect to the organs. Values are expressed as means (n = 3).

Exposure of mice to Pb induced a decrease in the MDA induction in the liver, heart, kidney and brain of mice that decreased significantly (P < 0.05) when compared to control. Comparism of the level of MDA induction based on the 28th day in relation to control showed that it decreased from 2.32 x 10^{-9} n Mg^{-1} to 5.60 x 10^{-10} n Mg^{-1} in the liver; while in the heart decreased from 1.8 x 10^{-8} n Mg^{-1} to 4.36 x 10^{-10} n Mg^{-1}; in the kidney it decreased from 1.94 x 10^{-9} n Mg^{-1} to 5.38 x 10^{-10} n Mg^{-1} whereas in the brain the decrease was from 1.11 x 10^{-9} n Mg^{-1} to 2.77 x 10^{-10} n Mg^{-1} (Fig. 5). There were no significant differences (P < 0.05) in the induction level MDA in the liver, heart and kidney except in the brain when compared to each exposure days. Thus with respect to Pb the levels of MDA induced in descending order were as follows: Heart > Liver > Kidney > Brain.

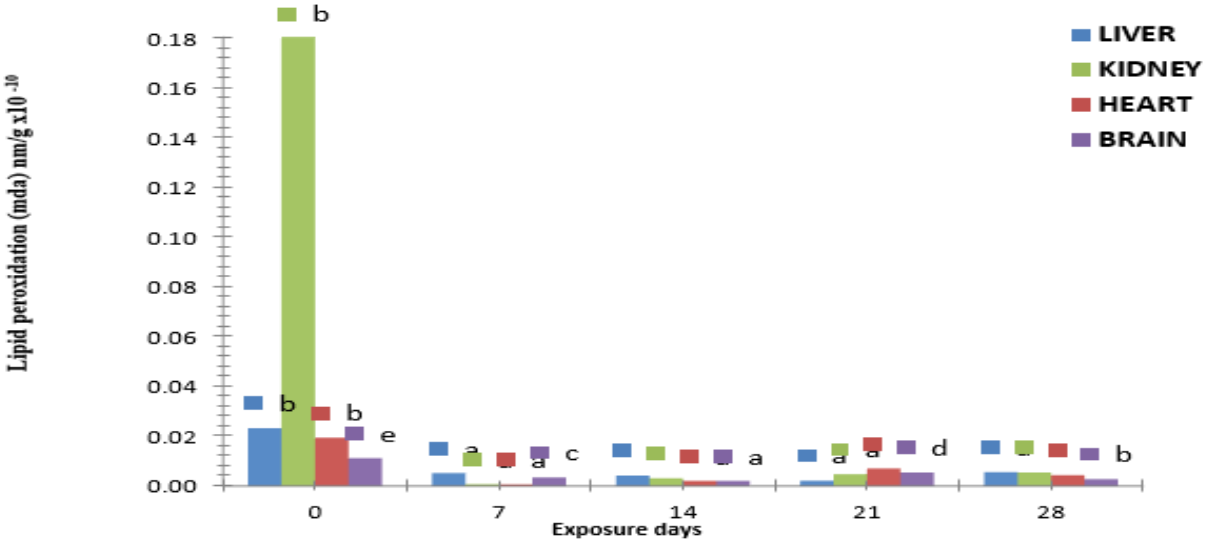

Fig. 5: Malonaldehyde (MDA) level in liver, kidney, heart and brain of *M. musculus* exposed to Pb. Values with dissimilar letters (a, b, c, d and e) are significantly (P < 0.05) different from each other with respect to the organs. Values are expressed as means (n = 3).

Zinc induced MDA showed that the level in the liver, heart, kidney and brain of mice, decreased significantly ($P < 0.05$) when compared to that in the control group. Comparism of the of MDA induction level based on the 28th day, in relation to control showed that it decreased from 2.32 x 10^{-9} n Mg^{-1} in control to 4.45 x 10^{-10} n Mg^{-1} on 28th day in the liver; while in the heart decreased from 1.8 x 10^{-8} n Mg^{-1} to 4.77 x 10^{-10} n Mg^{-1}; in the kidney it decreased from 1.94 x 10^{-9} n Mg^{-1} to 5.26 x 10^{-10} n Mg^{-1} whereas in the brain the decrease was from 1.11 x 10^{-9} n Mg^{-1} to 8.09 x 10^{-10} n Mg^{-1}. There were no significant differences ($P < 0.05$) in the MDA induction levels in the liver, heart and kidney except in the brain when compared to the 7th day ((Fig. 6). Thus with respect to Zn the levels of MDA induced in organs in descending order were as follows: Heart > Brain > Liver > Kidney

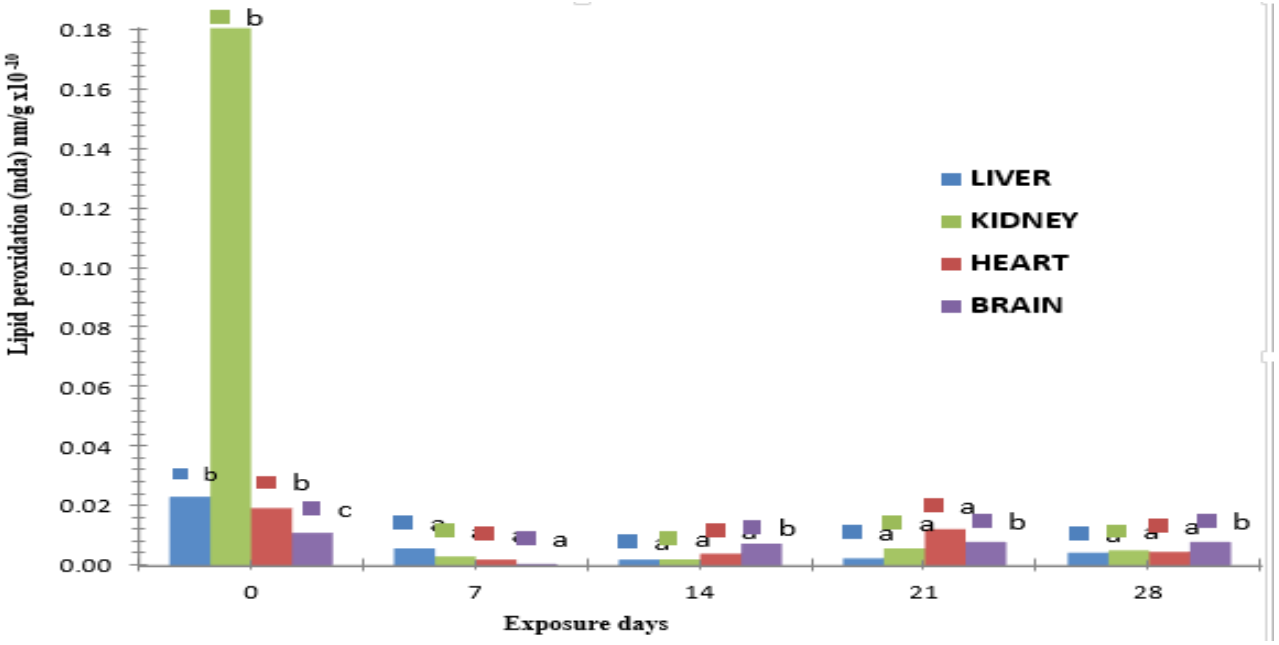

Fig. 6: Malonaldehyde (MDA) level in liver, kidney, heart and brain of *M. musculus* exposed to zinc (Zn). Values with dissimilar letters (a, b and c) are significantly ($P < 0.05$) different from each other with respect to the organs. Values are expressed as means (n = 3)

The MDA level as induced in the organs of treated mice by the selected heavy metals showed that the heavy metals induction were in descending order as follows:
Liver (Zn > Cd > Pb > Fe > Mn)
Kidney (Cd > Zn > Pb > Mn > Fe)
Heart (Zn > Pb > Cd > Fe > Mn)
Brain (Cd > Zn > Fe > Mn > Pb)
A noticeable increase in MDA levels in the brain following cadmium treatment as observed in the study, demonstrated the fact that this heavy metal was able to impair the function and ultrastructure of the central nervous system by producing oxidative stress. This is in agreement with Sarkar *et al*. (1997), El-Demerdash *et al*. (2004) and Yu-Mei *et al*. (2009). This could also be due to attack of free radicals generated by the highly toxic Cd on the fatty acid component of the membrane lipid content of the brain cells. Another probable explanation is that heavy metal ions are toxic to the central nervous system because blood-brain-barrier (BBB) was overwhelmed by the ROS and therefore the protein complexes were unable to mop these radicals hence an increase in the MDA levels in the brain. The exposure of the mice to Fe, Cd, Mn, Zn and Pb found to cause slight insignificant increases in level of lipid peroxides, in the brain, heart and liver, with increased treatment time is indicative of a commencement of oxidative damage in these tissues. This result is in agreement with Khan (2009), Malhotra *et al*. (2010) and Leena *et al.* (2011) who reported increase in LPO in tissues of mice exposed to *Terminalia chebula*, *curcumin*, resveratrol and lead nitrate. This slight increase in LPO might be due to inhibitory effect on mitochondrial electron transport system leading to stimulation in the production of intracellular ROS (Hales 2002; Heena *et al*., 2007; Cristina and Ana, 2007). A comparism of the susceptibility of the exposed tissues revealed that Cd induced the highest level of MDA in the kidney and brain is in agreement with the findings of Xiao *et al.* (2002) and Tandon *et al*. (2003) who reported a higher level of MDA in blood, liver and brain of Cd intoxicated rats. These findings also indicated that LPO induction is an early and sensitive consequence of Cd exposure.
Elevation of ROS levels leads to cellular damage when the rate of its generation surpasses the rate of decomposition by antioxidant defence systems. The measurements of lipid peroxides levels in plant and animal tissues exposed to different pollutants have been recognized as reliable early warning signal of exposure to an environmental stress and integration to environmental monitoring programmes (Fatima and Ahmed, 2005). The slight and weak increases observed in this study relative to the control showed that with prolonged exposure time, the level of LPO might probably increase above that of the control. This further justifies the need of LPO as a biomarker of heavy metal pollution in the environment and as an early warning signal in diagnosing health effects.

Conclusion

The general induction of MT and LPO by the selected heavy metals to mop up free radical scavenger is due to the interaction of sublethal concentration of these selected heavy metals directly with the metal ions, which is dependent on the subcellular origin. To quantify the interaction with xenobiotics and its potential on living organisms such as humans, one of the methods of monitoring is the use of biomarkers. They provide measures of exposure, toxic effect and individual susceptibility to environmental chemical compounds The variation in the induction of MT and LPO in the organs of mice exposed to heavy metal compounds can therefore, serve as a good battery of biomarkers for early detection of pollution associated with heavy metals.

Recommendation

The induction of metallothionein and Lipid perioxidation by heavy metals as shown in this study is an indication that combination of these biomarkers could be an excellent environmental diagnostic tool that should be incorporated into heavy metal monitoring programmes.

Acknowledgement

The authors are grateful to Dr O. A. Abiodun for editing the write up and for providing technical support.

References

Agarwal A., Saleb RA & Bedaiwy MA 2003. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril., 79: 829 – 843.

Ann P & Hill CH 1987. Response of Hepati Metallothionein to Iron Administration: Lack of Correlation with Serum Corticosterone. Biol. Trace Elem. Res., 14 (3): 225-263.

Bargagli R 2000. Trace Metals in Antarctica Relate to Climate Change and Increasing Human Impact. Rev Environ Contam and Toxicol., 61: 129 – 173.

Bradford M 1976. A Rapid And Sensitive Method for the Quantitation Of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal Biochem., 72: 248–254.

Brown DA, Bawden CA, Chatel KW & Parsons TR 1977. The Wildlife Community of Iona Island Jetty, Vancouver, B. C. and Heavy-Metal Pollution Effects. Environ Conserv., 4(3): 213-216.

Christopher C & Peter G 2002. Phytochelatins and Metallothioneins: Roles in Heavy Metal

Detoxification and Homeostasis. Annu. Rev. Plant Biol., 53: 159-182.

Coucelo J, Joachim N. & Coucelo J 2000. Calculation of Volumes and Systolic Indices of Heart Ventricle from Halobatrachus didactylus: Echocardiographic Noninvasive Method. J Exp Zool., 286: 585-595.

Cristina T & Ana C 2007. Melatonin as Antioxidant under Pathological Processes. Recent Pat on Endocr Metab Immune Drug Discov., 1(1): 63-82.

Dayan AD & Paine AJ 2001. Mechanisms of Chromium Toxicity, Carcinogenicity and Allergenicity, Review of the Literature from 1985-2000. Hum Exp Toxicol., 20(9): 439-451.

De Smet H, De Wachter B, Lobinski R & Blust R 2001. Dynamics of (Cd, Zn) –Metallothionein in Gill, Liver and Kidney of Common *Carp Cyprinus* during Cadmium Exposure. Aquat Toxicol., 52: 269- 281.

Devagi K & Arfiziah A 2009. Accumulation and Depuration of Lead and Chromium Using *Nerita lineate*. *World Appl Sci J*., 6(9): 1205-1208.

El-Demerash FM, Yousef MI, Kedwany FS & Baghdadi HH 2004. Cadmium-Induced Changes in Lipid Peroxidation, Blood Haematology, Biochemical Parameters and Semen Quality of Male Rats in: Protective Role of Vitamin E and Beta Carotene. Food Chem Toxicol., 42(10): 1563-1571.

Ellman GL 1959. Tissue Sulfhydryl Groups. Arch Biochem Biophys., 82(1): 70-77.

Falusi BA & Olanipekun EO 2007. Bioconcentration Factors of Heavy Metals in Tropical Crab (*Carcinus* Sp) from River Aponwe, Ado-Ekiti, Nigeria. J Appl Sci Environ Manag., 11(4): 51-54.

Fatima RA & Ahmad M 2005. Certain Antioxidant Enzymes of Allium Cepa as Biomarkers for the Detection of Toxic Heavy Metals in Wastewater. Sci Total Environ., 346(1-3): 256–273.

Finney DJ 1971. Probit Analysis 3rd Edition London: Cambridge University Press. 80 pp.

Fleet YC, Andrews GK & McCormick CC 1990. Iron-Induced Metallothionein in Chick Liver, a Rapid, Route-Dependent Effect of Zinc Status. J Nutr., 120 (10): 1214-1222.

Flora SJS, Saxena G & Mehta A 2007. Reversal of Lead-Induced Neuronal Apoptosis by Chelation Treatment in Rats: Role of ROS and Intracellular Ca^{2+}. J pharmacol Exp Ther, 322(1): 108-116.

Flora SJS, Mittal M & Mehta A 2008. Heavy Metal Induced Oxidative Stress and its Possible Reversal by Chelation Therapy. Indian J Med Res., 128(4): 501-523.

Gornall AC Bardwawill CJ & David MM 1949. Determination of Serum Protein by Means of the Biuret Reaction. J Biol Chem., 177: 751-756.

Hales DB 2002. Testicular Macrophage Modulation of Leydig Cell Steroidogenesis. J Reprod Immunol., 57(1 -2): 3-18.

Handy RD, Galloway, TS & Depledge MH 2003. A Proposal for the Use of Biomarkers for the Assessment of Chronic Pollution and in Regulatory Toxicology. Ecotoxicol., 12(1-4): 331-343.

Heena T, Suhel P, Hasibur R, Basu DB & Sheikh, R 2007. Catechin as an Antioxidant in Liver Mitochondrial Toxicity: Inhibition of Tamoxifen-Induced Protein Oxidation and Lipid Peroxidation. J Biochem Mol Toxicol., 21(3): 110–117.

Hyne RV & Maher WA 2002. Macroinvertebrate Biomarkers: Links to Toxicosis and Changes in Populations or Communities.*CRCFE*, 4: 1-26.

IIIuminati S, Truzzi C, Annibaldi A, Migliarini B, Carnevali O & Scarponi 2010. Cadmium Bioaccumulation and Metallothionein Induction in the Liver of the Antarctic Teleost Trematomus bernacchii during an On-Site Short-Term Exposure to the Metal via Seawater. *Toxicol Environ Chem.*, 92(3): 617-640.

José MM, Juan AS, Francisco JA & Javier M 2010. Roles of Dioxins and Heavy Metals in Cancer and Neurological Diseases Using ROS-Mediated Mechanisms. Free Radic Biol Med., 49(9): 1328-1341.

Kate L, Hsing-Ju W,Stephen PK, Michael AA, Michael PJ & Alastair GM 2006. Defences against Oxidative Stress in Neisseria gonorrhoeae: A System Tailored for Challenging Environment. Microbiol Mol Biol Rev., 70(2): 344-361.

Khan KH 2009. Terminalia Chebula Reduces the Oxidative Stress Induced by *Salmonella typhimurium* in Mice and may reduce the risk of getting Typhoid. Adv Biol Res., 3(1-2): 01-08.

Klaassen CD, Liu J & Choudhuri A 1999. Metallothionein: An Intracellular Protein to Protect Against Cadmium Toxicity. *Annu Rev Pharmacol Toxicol*., 39: 267-294.

Laura V, Lanza C, Rivaro P, Abelmoschi M, Genti S, Veneselli E, Minniti G, Grasselli E, Canesi L. & Voci A 2011. Metals, Metallothioneins and Oxidative Stress in Blood of Autistic Children. *Res Autism Spectr Discord*., 5(1): 286-293.

Lee YW, Ha MS & Kim YK 2001. Role of Reactive Oxygen Species and Glutathione in Inorganic Mercury-Induced Injury in Human Glioma Cells. Neurochem Res., 26: 1187-1193.

Liu XD & Thiele DJ 1996. Oxidative Stress Induced Heat Shock Factor Phosphorylation and HSF-Dependent Activation of Yeast Metallothionein Gene Transcription. Genes Dev., 10(5): 592-603.

Luqing P & Hongxia Z 2006. Metallothionein, Antioxidant Enzymes and DNA Strand Breaks as Biomarkers of Cd Exposure in a Marine Crab, *Charybdis*

japonica. Comp Biochem Physiol Part C: Toxicol Pharmacol. 144(1): 67-75.

Malhotra A, Nair P & Dhawan DK 2010. Modulatory Effects of Curcumin and Resveratrol on Lungs Carcinogenesis in Mice. Phytother Res., 24(9): 1271-1277.

Milena P 2002. Metallthionein Expression and Roles in Central Nervous System. Biomed Rev., 13: 1-15.

Petering DH, Loftsgaarden J, Schneider J & Flower B 1984. Metabolism of cadmium, zinc and copper in the Rat Kidney: The Role of Metallothionein and other Binding Sites. Environ. Health Perspect., 54: 73-81.

Ringwood AH, Hoquet J, Keppler CJ & Gielazyn M 2004. Linkages between Cellular Biomarker Responses and Reproductive Success in Oysters (*Crassosirea virginica*). Mar. Environm Res., 58: 151-155.

Ruriko N, Naoko K & Koichi M 2002. Concentrations of Cd, Zn, Fe, and MT in Liver and Kidney of Nonhuman Primates. Biol Trace Elem Res., 87(1-3): 95-111.

Ryan JA & Hightower LE 1994. Evaluation of Heavy-Metal Ion Toxicity in Fish Cells Using a 291 Combined Stress Protein and Cytotoxicity Assay. Environ Toxicol Chem., 13: 1231-1240.

Sarkar S, Yada VP & Bhatangar D 1997. Cadmium-Induced Lipid Peroxidation and the Antioxidant System in Rat Erythrocytes: The Role of Antioxidants. J Trace Elem Med and Biol., 11(1): 8-13.

Sato M & Kondoh M 2002. Recent Studies on Metallothionein: Protection against Toxicity of Heavy Metals and Oxygen Free Radicals. Tohoku J. Exp. Med., 196: 9-22.

Tandon SK, Singh S, Prasad S, Khandekar K, Dwivedi VK, Chatterjee M & Mathur N 2003. Reversal of Cadmium Induced Oxidative Stress by Chelating Agent, Antioxidant, or their Combination in Rat. Toxicol. Lett., 145: 211-217.

Tomoki K & Norio I 2008. Function of Metallothionein in Gene Expression and Signal Transduction: Newly Found Protection Role Metallothionein. J Health Sci., 54(3): 251-260.

Usoh IF, Akpan EJ, Etim EO & Farombi EO. 2005. Antioxidant Actions of Dried Flower Extracts of *Hibiscus sabdariffa*. Pak J Nutr., 4: 135-141.

Valavanidis A, Vlahogianni T, Dassenakis M & Scoullos M 2006. Molecular Biomarkers of Oxidative Stress in Aquatic Organisms in Relation to Toxic Environmental Pollutants. Ecotoxicol. Environ. Saf., 64: 178-189.

Viarengo A, Burlando B, Cavaletto M, Marachi B, Ponzano E & Blasco J. 1999. Role of Metallothionein against Oxidative Stress in the Mussel, *Mytius galloprovincialis*. Am. J Physiol. (Regul Integr Comp Physiol), 277: 1612-1619.

Vinodhini R & Narayanan M 2008. Effect Of Heavy Metals Induced Toxicity on Metabolic Biomarkers in Common Carp (*Cyprinus carpio L*.). Maejo Int. J Sci. Technol., 2(01): 192-200.

Watanabe M, Henmi K, Ogawa K & Suzuki T 2003. Cadmium Dependent Generation of Reactive Oxygen Species and Mitochondria DNA Breaks in Photosynthetic and Non-Photosynthetic Strains of Euglena Gracilis. Comp. Biochem. Physiol. C: Toxicol. Pharmacol., 134: 227-234.

Xiao P, Jia XD, Zhong WJ, Jin XP & Nordberg G 2002. Restorative Effects of Zinc and Selenium on Cadmium Induced Kidney Oxidative Damage on Rats. Biochem Environ Sci., 15: 67-74.

Xiaoyan L, Hainan C & Paul NE 2004. Metallothionein Protects Islets from Hypoxia and Extends Islets Graft Survival by Scavenging Most Kinds of Reactive Oxygen Species. J. Biol. Chem., 279(1): 765-771.

Yu-Mei Z, Xue-Zhong L, Hao L, Li M & Zong-Ping L 2009. Lipid Perioxidation and Ultrastructural Modifications in Brain after Perinatal Exposure to Lead and / or Cadmium in Rats Pup. Biol Environ Sci., 22