Determination of the lethal dose 50% (LD_{50}) of cadmium chloride and the histopathological changes in male mice liver and kidneys

Talib Hussen Ali
Department of Biology / College of Education
University of Mosul

Received 05 / 01 / 2011 **Accepted** 06 / 04 / 2011

الخلاصة

تحديد الجرعة القاتلة (LD_{50}) للكادميوم تضمنت التجريع عل شكل كلوريد الكادميوم ($CdCl_2$) لمجموعة من الحيوانات وبجرعات متدرجة لتحديد الجرعة التي تقتل ٥٠% من فئران التجارب ضمن فترة زمنية محددة (٢٤-٤٨ ساعة). وحدد محتوى الكادميوم في كبد وكلية الفئران المعام لة وحيوانا ت السيطرة بواسطة جهاز الامتصاص الذري وتم قياس مستوى أنزيم الفوسفاتيز القاعدي في أنسجة الهدف . ثم جرعت ذكور الفئران بحقنة مساريقية 0.15 مل / كلوريد الكادميوم (والمساوية لقيمة LD_{50} والمحددة في الدراسة الحالية والمساوية لـ 5.98 ملغم /كغم من وزن الجسم) ولفترات قصيرة الأمد (٢٤ و٤٨ و ٧٢ ساعة) ولفترات طويلة الأمد (١ و٤ و ٨ أسابيع) ثم شرحت الفئران بعد ذلك.

أظهرت النتائج تأثير تجريع الكادميوم القصير وطويل الأمد في كبد وكلية الفئران والتي اعتمدت على فترة التجريع . و أظهر فحص جهاز الامتصاص الذري لمحتوى الكادميوم في كل من الكبد والكلية زيادة معنوية اعتمادا على فترة التعريض وتراوحت من ٩٨% - ١٤٥ % في الكبد و ١٠٠ %- ٣٠٠ % في الكلية عند فترات التجريع قصيرة الأمد أما فترات التجريع طويل الأمد فكانت الزيادة من ١٤٣ %- ١٧٣ % في الكبد و ٢٠٠ %-٣٧٠ % في الكلية . كذلك أظهرت هـ ذه الدراسة تأثيرات نسجية أمراضية في أنسجة الكبد والكلية . تسبب الكادميوم عند فترات التجريع طويل الأمد تلف نسجي وشملت اضمحلال الأنابيب الكلوية وموت الخلايا المبطنة لها والتهاب الأنسجة البينية وانتفاخ الكبيبة في الكلية . كذلك لوحظ تلف أنسجة الكبد والمتمثلة في الالتهابات غير المخصصة وموت الخلايا فيما كانت هذه التأثيرات اقل وضوحا في فترات التعريض قصير الأمد . تشير نتائج فحوصات االكيميوحيوية لتأثير االكادميوم السمي

الواضح على الكبد والكلية والذي انعكس على شكل تغيرات مثيرة لمستوى فعالية أنزيم الفوسفاتيز القاعدي (ALP) في أنسجة الكبد والكلية . حيث اظهر زيادة معنوية تراوحت من ١٠٠% إلى ١٣٠% لمستوى فعالية أأنزيم الفوسفاتيز القاعدي (ALP) في الكبد بينما مستوى فعالية أنزيم (ALP) في الكلية انخفض من ٩٥% إلى ٦٥% عند المقارنة بمستوياتها في حيوانات السيطرة في جميع فترات التعريض المشابهة.

ABSTRACT

The lethal dose 50 (LD_{50}) test involves the administration of cadmium in the form of $CdCl_2$ to group of animals at an increasing dose in order to determine the dose that kills 50 percent of the test mice within a set of time frame (24 and 48h).

The cadmium contents of liver and kidneys from dosed and control animals were determined by atomic absorption spectroscopy moreover, levels of alkaline phosphatase (ALP), in the target tissue were measured.. Male mice were dosed by 0.15ml cadmium (equivalent to the LD_{50} 5.98 mg/kg body weight) in the form of $CdCl_2$ via intraperitoneal injection for short-term treatment once per 24, 48 and 72h and, for long-term treatment once per week for 1, 4and 8 weeks then, sacrificed.

The results showed that the effect of short-term and long-term Cd-administration on hepatic and renal Cd accumulation in the Swiss mice was found to be time dependent fashion. Atomic absorption examination showed that the Cd-content in both liver and kidneys increased significantly time dependent by 98-145% in liver and 100-300% in kidney at short-term treatment whereas for long-term treatment this increasing were 143-173% in liver and 200-370% in kidney respectively.

This study also presents the histopathological effects in mice liver and kidney. Long-term Cd exposure produced damage to the entire kidney, including tubular degeneration, tubular cell apoptosis, interstitial inflammation and glomerular swelling and also resulted in liver injury including non-specific chronic inflammation and apoptosis, while it was less pronounced in short-term exposure.. Results of the biochemical profile indicated marked hepatic and renal toxic effect of cadmium reflected by the dramatic change in alkaline phosphatase (ALP) activity level, in the liver and kidney tissue. a significant increase (from100 to130%) in hepatic ALP activity, while the renal ALP activity level decreased by (from 95% to 65 %) as compared with those levels of the control in all relative periods of exposure.

KEY WORDS: Cadmium, Histopathological, LD_{50}, ALP.

INTRODUCTION

Cadmium(Cd) is an environmental pollutant commonly considered to be one of the most important toxic for human and animals, due to increasing level in the environment as a results of industrial and agricultural practices (1)). Cadmium is extremely hazardous to life and has been involved in historic poising episodes of human and animal population. It is a serious lethal occupational and environmental toxic, known for its high toxicity,which may affect living systems in various ways (2). The effective dose values (LD_{50}) of cadmium as documented in environmental health criteria for a single intraperitoneal dose of cadmium in the form of $CdCl_2$ for the control mice was ranging between 5,6.75 and 7mg/kg b.w (3,4,5).

Cadmium is deposited in various organs and tissue, cadmium toxicity has been known to results via ingestion of cadmium containing food (6). During chronic exposure, Cd induced toxicity in the liver and kidneys is dependent on renal and hepatic Cd concentration (7,8,). There were differences in animal susceptibility to Cd toxicity regarding concentration level and the reason for this differences is not known and remain to be elucidated (9). Cadmium has been shown evidence to cause histopathological changes in liver and kidney in small rodent (10). Most of the Cd that is absorbed after oral exposure accumulates in the liver and kidneys, where it induces injury including protein denaturation and formation of reactive oxygen species (ROS) and lipid peroxidation(11), which in turn depress hepatic and renal functions (12,13). Acute exposure to Cd produces hepatic injury,whereas chronic exposure results in renal injury (1).

Chronic Cd exposure produces damage to the entire kidney,including tubular degeneration, tubular cell apoptosis, interstitial inflammation and glomerular swelling (14). Chronic exposure to Cd results also in liver injury including non-specific chronic inflammation and apoptosis (8).

Enzyme are necessary for normal cellular metabolism including that of liver and the degenerative changes due to metal toxicity exhibit in the liver alter the level of number of enzymes. The morphological changes are associated with biochemical evidence including the change of alkaline phosphatase activity. ALP is a membrane bound enzyme found in liver and it is present in all membranes where active transport occurs. This enzyme is biomarker of hepatic damage(15).

Most studies investigate the heavy metals accumulation in plasma and soft tissues (brain) in mice and rats. The aim of the present to evaluate liver and kidney tissues cadmium (Cd) content in mice liver and kidneys after different treatment periods. In addition, to determine the toxicity of cadmium metal on liver and kidney architecture and the activity alkaline phosphatase (ALP), the marker enzyme of the degree of organ damage.

MATERIALS AND METHODS

Male Swiss mice (aged at least 2months) not less than average initial body weight of about 25g were used in this study. the animals were housed at laboratory conditions

(22±1 Cº) in plastic cages(one per cage) and allowed free access to drinking water and commercial rodent chow.

Determination of cadmium LD_{50}

The lethal dose of Cadmium50(LD_{50}) test involves the administration of cadmium via Intraperitoneal injection in the form of hydrous $CdCl_2$ and was determined using 30 male mice divided randomly into 5 groups (n =5), intraperitoneal injected (i.p) at increasing dose (1mg/kg, 3mg/kg, 5mg/kg, 7mg/kg and 9mg/kg respectively), in order to determine the dose that kills50 percent of the tested animals within a set time frame (24h and 48h LD_{50}), Several dose levels of cadmium were given until the lethal dose kills half of the test mice population. A sixth group of mice used as the control, received no treatment. This test also was allowed to proceed for 24 and 48hrs, then the lethal dose for 50% of the population (LD_{50}) was obtained using the computer software EPA probit analysis program version 1.5 of Finney[16].The 24h and 48h LD50 were graphed by plotting the log concentration against percentage mortality occurred at 24 and 48hrs respectively, using Microsoft Excel program.

Cadmium tissue content

For cadmium accumulation and Alkaline phosphatase activity experiments, thirty five mice, were randomly divided into the following experimental groups (5 animals each):

a) Control receiving only distilled water.
b) 0.15mg cadmium chloride $CdCl_2$, the intraperitoneal Cd-injection dose of 0.15 mg was derived from the LD_{50} calculated in this study, for a single intraperitoneal dose of cadmium in the form of $CdCl_2$ in mice was 5.98 mg/body weight). Mice before scarification were kept at the following designed experimental periods: 1- For short-term treatments, mice exposed to intraperitoneal Cd-injection once at 24, 48 and 72 hours. 2- For long –tem treatment mice exposed to intraperitoneal Cd-injection once at week 1, 4 and week 8.

Atomic absorption spectroscopy (AAS) was selected as the analytical technique to determine elemental cadmium content in liver and kidney from sacrificed animal, using the method described by Chapman [17] method. Briefly, 25 mg of liver, kidney were fixed for two hours in formaldehyde, and then heated in quarts beakers at 150 $^{\circ}$C to evaporate the excess formaldehyde. Samples were digested overnight in a fume hood with mixture of 3:1 pure nitric and sulpheric acids. The mixture was heated with perchloric acid for 2 hrs until the color became whitish, then

cooled for 10 minutes and diluted with deionized distilled water. The colorless solution was filtered before it is read by Pye Unicam SP9 atomic absorption spectrometer (Philips). The same deionizing water was used as a blank.

Light microscope preparation

For light microscopic preparation, small tissue samples from liver and kidney were dissected out and fixed in Bouin´s solution for 16h and subsequently placed in 70% ethanol. dehydrated in a graded ethanol series, cleared with xylene, infiltrated and embedded in paraffin wax. Thin section (5 µ) were cut and stained with hematoxylin - eosin for histological examination.

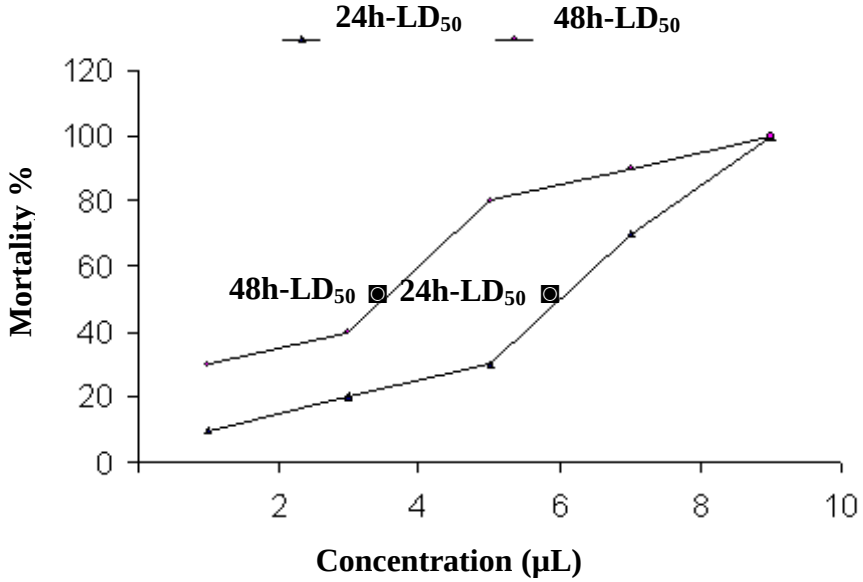
Level of Alkaline phosphatase (ALP)

Mice were sacrificed, liver and kidney specimens from each experimental group (short and long-term treatments) were homogenized. The homogenates were centrifuged at 300rmp for 10 minutes. The clear supernatants were used for the estimation of ALP activity. The colometeric method using commercial Kit (Biomaieux Ltd) was used. For ALP activity assay,the procedures outline in the respective Kit manuals was adopted. The color formation at the end was read at 510 nm using visible spectrophotometer. The enzyme activity was expressed in Unit / 100mg/ protein.

Statically analysis : using one-way analyses of variance.

RESULTS

The LD_{50} values of both 24 and 48hours of cadmium were 5.98 mg/kg and 3.59mg/kg respectively, no death was recorded among the control group (Fig1).



Fig(1): The lethal dose of Cadmium50 (LD_{50})

Atomic absorption spectroscopy revealed that cadmium accumulated in liver and kidneys as a consequences of Cd – toxicity.Tissue injury was indicated by the histopathological changes and change in alkaline phosphatase activity in both liver and kidneys were found to be positively correlated with exposure time.

Table1 and Table1 2. shows the results from AAS analyses of the liver and kidney of mice exposed to both short-term and long-term Cd exposure, the concentration of Cd for short-term Cd exposure, significantly increased ($p<0.05$) in both liver and kidney, and the percentage of the increase ranged from 98-145% in liver 100-300 % in kidney and by 143-175 in liver and by 200-3705% in kidney at long-term treatment in comparison to untreated control group. The results highlights that Cd precipitated in kidney more than that of liver. Moreover, Cd content in both liver and kidney increased gradually as period of exposure prolonged (72 hrs, 1,4 and 8weeks) respectively.

Table 1: Concentration (µg/g tissue) of cadmium in liver and kidney following short-term $CdCl_2$ exposure and associated controls.

Treatments	Organ	
	Liver (µg/g tissue)	Kidney (µg/g tissue)
Control	0.5177±0.0392	0.379±0.0.021
24h Cd	1.045 ±0.288*	0.79 ±0.053*
48h Cd	1.101 ±0.112*	1.315±0.0403*
72h Cd	1.27 ±0.0.12*	1.59 ±0.0.159*

*Significant at ($P \leq 0.05$)

Table 2: Concentration (µg/g tissue) of cadmium in liver and kidney following long-term $CdCl_2$ exposure and associated controls.

Treatments	Organ	
	Liver (µg/g tissue)	Kidney (µg/g tissue)
Control	0.5177±0.0392	0.379±0.0.021
1Week Cd	1.26 ±0.17*	1.21 ±0.063*
4Week Cd	1.35 ±0.078*	1.58 ±0.097*
8Week Cd	1.412±0.045*	1.87±0.036*

*Significant at ($P \leq 0.05$)

The impact of long-term Cd exposure on mice, reflected a higher rate of deterioration in the liver and kidneys histoarchitecture. Light microscope observation revealed that treatment with 5.98mg kg/B.W Cd induced morphological changes in the liver. These changes included

nuclear damage, a marked cytoplasmic vacuolization, liver cell necrosis and eventually followed by degenerative and cell death (fig.2b).

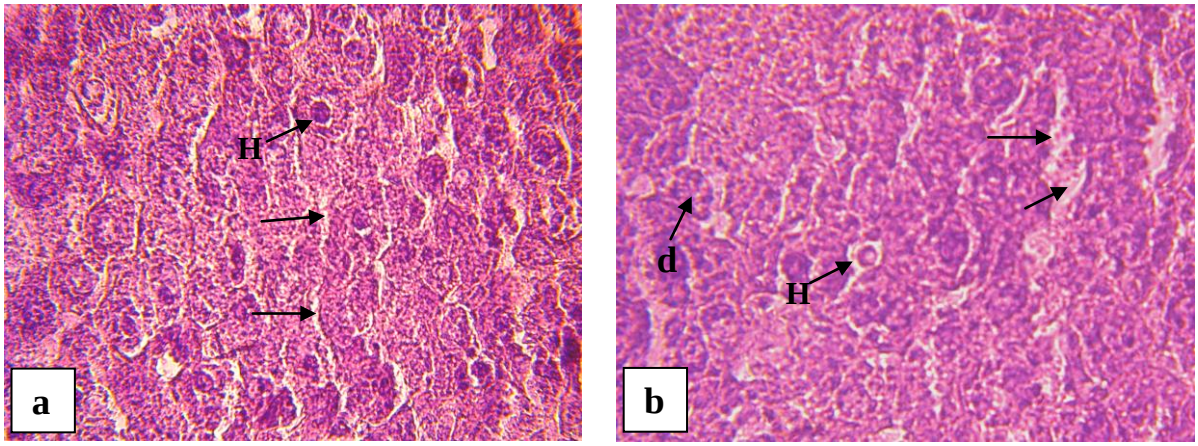


Fig (2).Transverse section of liver mice (a) control (b) treated with Cd (H – Hepatocyte, d – degeneration, arrows – Sinusoid)

Fig(2). (a) Shows the control mice kidney feature and (b) shows the various histological alterations in kidney architecture due to Cd treatment. The mice renal capsule is composed of glomerulus surrounded by Bowman's capsule (Fig.2a). The degenerative of tissues were observed after Cd treatment regardless of exposure period. The glomerulus started to shrink as a result, an increase in Bowman's capsule space and a decrease in both Bowman's capsule and glomerular size were noted. The glomerulus cells were found to go through atrophy, the renal tubules were found to lose their regular shape and the intertubular space decreased (Fig.3a). The above mentioned histopathological alteration were less pronounced in short-time exposure (figurers not shown in this study).

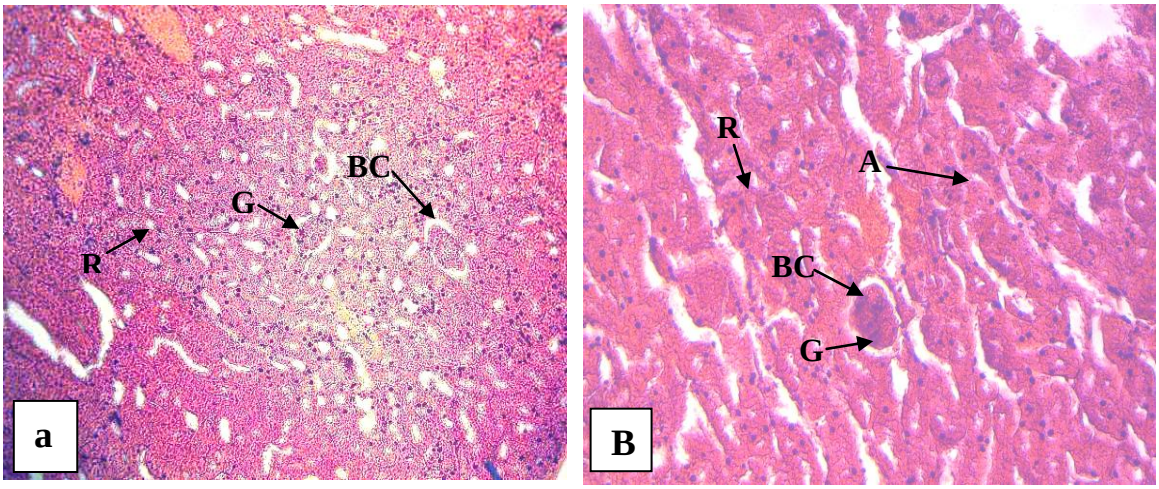


Fig (3) Transverse section of kidney Mice (a) control (b) treated with Cd (BC, Bowman's capsule, G-glomerulus, R-intratubular cavity, N-necrosisand, A, Atrophy)

The morphological changes are associated with biochemical evidence indicated by a change in alkaline phosphatase activity. (Table 3). Level of alkaline phosphatase (ALP) activity, in normal untreated control mice liver tissue, showed 4.32± 0.36. At median-lethal dose of $CdCl_2$, 72hrs and 8weeks treated mice, the level of ALP activity in the liver tissue increased significantly up to 46.71 ± 2.11 and 57.13 ± 4 respectively. while Level of alkaline phosphatase (ALP), in normal untreated control mice kidney tissue, the level of ALP activity showed 502.46 ± 11.7. At median-lethal dose of $CdCl_2$ treated mice for both periods of exposure, the level of ALP activity significantly decreased down to 173.24 ± 2.37 and 21.45 ± 3 respectively.

Table (3): Alkaline Phosphatase (ALP) level in Mice liver and kidney at short-term and long Cd Exposure.

Treatments	Organ	
	Liver Alkaline phosphatase (U/100mg/protein)	Kidney Alkaline phosphatase (U/100mg/protein)
Control	4.32± 0.36	502.46 ± 11.7*
72h Cd	46.71 ± 2.11*	173.24 ± 2.37
8Week Cd	57.13 ± 4*	21.45 ± 3*

*Significant at ($P \leq 0.05$)

DISCUSSION

Results of the current study (Fig.1) showed that the LD50 values for $CdCl_2$ via intraperitoneal injection was 5.98 and 3.9 mg/kg/ b.w for 24h exposure and 48h exposure respectively in male mice.

The designer of LD_{50} test in 1927 acknowledged its serious inadequacies intending it only for certain narrow medical purposes (18).Inadequacies was due to the continuous changes in different factors affected Cd toxicity so it has been well documented that species, age, weight, sex, temperature, pH, animal susceptibility, food in addition to method of by which chemical administrated have marked effect on LD_{50} results (19). Nevertheless, use of the LD_{50} test has become widespread as general measure of chemical toxicity and has been challenged for decades as both unreliable and informative criteria. Thereby it is useful to reconsider the repeat determination of the LD_{50} before carrying out laboratory experiments.

Liver and kidney are critical organs used to describe and document the effect of pollutants, during exposure, Cd induced toxicity in the liver and kidneys is dependent on hepatic and renal Cd concentrations (7). Based on results of this study and recent experiments(5,20), the selected dose of Cd (5.98 mg/kg b.w) appeared to be toxic to liver and kidneys.

Cadmium is transported in the blood and widely distributed in the body but accumulates primarily in the liver and kidneys(1). Hepatic and renal and toxicity may occurred if toxic Cd level is attained in these organs regardless to exposure periods (21).

Results in Fig.(1) of the present study demonstrates that, mice treated with Cadmium developed progressive liver alteration characterized by cytoplasmic vacuolation, necrosis, regenerative and eventually followed by cell death. The liver damage has been reported in other mammalian and non mammalian species, in addition necrosis strongly associated with oxidative stress (22,23). Beside direct damage of proteins, another underlying mechanism for toxicant induce cell injury may have influential factors which may have direct or indirect effect by generation bioactive molecules intracellularly (e.g. free radicals), causing functional and structural alteration at tissue levels due to the ability of oxidant substances to cause oxidative stress in the liver (24).

Many studies in experimental animals have demonstrated an association between morphological and functional changes in the kidneys. exposure to cadmium leads to pathological changes in the kidneys. The morphological changes are initially limited to tubular epithelial cell degeneration, but this is followed by cellular atrophy WHO(4). Roya and Bhattacharya[(26)] reported the accumulation metals in various organs and tissues and excreted through glomurlar filtration, but they did not explain the consequences of pathway impact. The present investigation highlights the effect of 5.98 mg/kg b.w. dose of cadmium chloride (Cd Cl_2) on kidney histopathology. Fig..(3b) showed that was a shrinkage in Bowman's capsule space followed by enlargement of glomerulus, which may be due an increase in the filtration rate as a mechanism to overcome the toxic effect (25,26).

The morphological changes are associated with biochemical evidence including the change of alkaline phosphatase activity. ALP is a membrane bound enzyme found in liver and it is present in all membranes where active transport occurs This enzyme is biomarker of hepatic damage, thus its bioassay can serve as a diagnostic tool for necrosis of the liver (15). Rich source of ALP are the bile canaliculi of liver and proximal tubules in the kidney. This enzyme is currently being used as markers of Cd-induced tissue damage, damage to any of the organs or tissues would lead to elevated activity of ALP. For example alkaline phosphatase (ALP) is released in both acute and chronic liver disorders (27,28).

Change in ALP activity induced by variety cadmium exposure periods are presented in Table 1and 2. Decrease or inhibition in ALP activity in kidneys, probably indicates effect on cell growth and proliferation and may be taken as index for cell damage and necrosis (29). In Cd treated mice liver, the activity of ALP was significantly increased

compared to their normal level of control group. Therefore the increase of the activity of the enzyme in liver, many be due to a leakage out of the enzyme from tissue, due to liver necrosis in heavy metal exposition (30,31). Results in Table1and 2 of both exposure periods, shows relative to the liver Cd-induced increases in the activity of ALP, it was considerably low and was almost inhibited in kidney. This contradictory possibly interpreted either that liver responds more readily to Cd-induce ALP biosynthesis and enhanced activity than the kidney or the kidney does not respond to Cd-induced ALP synthesis, rather it responds to change brought about by Cd with enhance the activity of kidney constitutive ALP. The later appears to be more plausible interpretation to Obi and Olabode[28] with similar comparison on rat bone and kidney study.

In conclusion, the present study showed that the exposure of mice to Cd caused significant physiological alteration reflected in elevation of liver ALP activity. Decrease ALP activity may be taken as index of hepatic parenchyma damage and hepatocytic necrosis (32). Inhibition of ALP reflects alteration in protein synthesis (33).

REFERENCES

1) Goering, PL., Waalkes, MP., Klaassen, CD. Toxicology of cadmium In: Goyer, R.A. Cherian, M.G (Eds). Toxicology of metals: Biochemical aspects. Springer, New York. pp.189-213. 1995.

2) Ostrowski SR, Wibur S, Chou CH, Pohl HR, Stevens YW, Allred PM, Roney N, Fay M and Tylenda CA. latent effects – carcinogenesis, neurotoxicology and development deficits in humans and animals. Toxicol. Ind. Heath. 15: 602-644.,1999.

3) Leber PA and Miya TS. Amechanism for cadmium and zinc – induced tolerance to cadmium toxicity: involvement of metallothionein. Toxicology and Applied Pharmacology. 37(3): 403-414, 1976.

4) WHO Cadmium (Environmental Health Criteria 134), Geneva..1992.

5) Bench G, Corzeh, M.M, Martinelli, et al. Cadmium concentration in the testes, sperm, and spermatids of mice subjected to long-term cadmium chloride exposure. Cytometery. 35:30 – 36, 1999.

6) Pascoe G.A, Blanchet R.J and Linde G. Food chain analysis of exposures and risks to wildlife at metals contaminated wetland. Arch. Environ. Contam. Toxicol. 30,306-318, 1996.

7) Liu, J., Habeebu, S.S., Klaassen, CD. Acute Cd MT injection is not a good model to study chronic Cd nephropathy; comparison of

chronic CdCl2 and CdMT exposure with acute CdMT injection rats. Toxicol. Appl. Pharmacol. 153 : 48-58, 1998.

8) Habeebu, SS., Liu, J., Liu, Y., Klaassen, CD. Metallothionein-null mice are more sensitive than wild-type mice to liver injury by repeated exposure to cadmium. Toxicol. Sci. 55: 223-232, 2000.

9) Wlostowski, T., Krasowska, A., Bonda, E. joint effects of dietary cadmium and polychlorinated biphenyls on metallothionein induction, lipid peroxidation and histopathology in the kidney and liver of bank voles. Ecotoxicol. Environ. Saf. 69:403-410, 2008.

10) Wlostowski, T., Krasowska, A., Laszkiewcz-Tiszczeullo, B. Dietary cadmium induces histopathological changes despite a sufficient metallothionein level in the liver and kidneys of bank vole (*Clethrionomys glareolus*). Comp. Biochem. Physiol. 126: 21-28, 2000.

11) Basun H, Forssel LG, Wetterberg L and Winblad B.. Metals and trace elements in plasma and cerebrospinal fluid in normal aging and Al zhermer's disease. J.Neural. Trans. Park Dis. 3: 231-258. 1991.

12) Nordberg., M., Nordberg, GF. Toxicological aspects of metallothionein. Cell.Mol. Biol. 46: 451-463,2000.

13) Thevenod F., Friedmann JM. Cadmium-mediated oxidative stress in kidney proximal tubule cell induced degeneration Na^{+}/pathway. FASEB J. 13 : 751-755, 1999.

14) Groten, JP., Koeman, JH., van-Nesselrooij, JH., Luten, JB., Fentener-van Vlissingen. JM., Steinhuis, WS., van-Bladeren, PJ., Comparison of renal toxicity after long-term oral administration of cadmium chloride and cadmium- metallothionein in rats. Fundam. Appl. Toxicol. 23: 544-552, 1994.

15) Coles E.H. Veterinary Clinical Pathology", 4thEdn., Saunders, Philadelphia, p. 486. 1989.

16) Finney D.J. Probit analysis. 3d ed. Cambridge Univ. Press, Cambridge UK.p.333. 1971.

17) Chapman HD, Partt PF. Methods of analysis for soil, plant and water. Univ. of Calif. Div AgricSc i.1961.

18) Trevan JW.. The error of determination of toxicity.Proc Roy Soc 101B, 483-514, 1927.

19) Wiehe WH. The effect of ambient temperature on the action of drugs. Ann. Rev. Pharmacol. 13:409-425, 1973.

20) World Health Organization. Environmental Health Criteria, Guide to Short-Term Tests for Detecting Mutagenic and Carcinogenic Chemicals. World Health Organization, Geneva 51:, p 100–114. 1985.

21) Wang, X P. and EC. Foulkes. Specificity of acute effects of cadmium on renal function. Toxicology 30:243-247, 1984.

22) Bondy g, Armstrong C, Coady L, Doucet J. Roberston P, Freeley M and Barker M. Toxicicity of the chlordane metabolite oxychlordane in female rats. Cinlcal and histopathological changes. Food. Chem. Toxicol. 41: 29-34. 2003.

23) Avei A, Kaámaz M and Durak I.. Peroxidation in muscle and liver tissues from fish in a contaminated river due to a petroleum refinery industry. Ecotoxicol. Environ.SAf. 60:101-105, 2005.

24) Williams GM and Iatropoulos, MJ. Nonlinearities in 2-Acetylaminofluorene Exposure Responses for Genotoxic and Epigenetic Effects Leading to Initiation of Carcinogenesis in Rat Liver Toxicol. Pathol. 30: 41 – 53, 2002.

25) Roya S and Bhattacharya S. Arsenic-induced histopathology and synthesis of stress proteins in liver and kidney of Channa punctatus. Ecotoxicology and Environmental Safety.65(2): 218-229, 2006.

26) De Sliva PE. Determination of lead in plasma and studies on its relationship to lead in erythrocytes Br.J. Ind. Med. 38: 209 – 217, 1981.

27) Kido, T; Kobayashi, E; Hayano, M; Nogawa, K; Tsuritani I; Nishijo, M; Tabata, M; Nakagawa H; Nuyts, GD; Debroe, ME Significance of elevated urinary human intestinal alkaline phosphatase in Japanese people exposed to environmental cadmium. Toxicol. Lett 80 (1-3): 49 – 54, 1995.

28) Obi FO and Olabode OI. Effects of cadmium exposure on bone and kidney alkaline phosphatase activity and acid phosphatase activity in testis and prostate gland in male Rat. J.Appl. Sci. Environ. Mang. 6(1):9-13,, 2002.

29) Al- Attar AM.. The influence of dietary Grapeseed oil on DMBA-induced liver enzymes disturbance in the Frog *Rana ridibunda*. Pakistan journal of nutrition.3(5):304-309, 2004.

30) Tandon S,K, Khandewal S, Mathur AK and Ashquin M. Preventive effects of nickel on cadmium hepatotoxicity and nephrotoxicity.Ann. Clin. Lab. Sci. 14: 390-396, 1984,

31) Novelli J and Barbosa LL. Different/combined effect of water contamination with cadmium and nickel on tissues of rats. Environ. Pollution. 103: 295-300, 1998.

32) Onikienko FA, Enzymatic changes fro early stages of intoxication with small doses of chloro-organic insecticides. Giginea.1. Fizid. Truda. Proizy. Toksilol. Klinika (Kietc.Gos.IZ.Med.Lit.UKhssr). 77. 1963.

33) Vema SR, Saxen M and Tonk IP. The influence of 1det 20 on the biochemical composition and enzymes in the liver *Clarics batraclus*. Environ. Poll. 33:245-255, 1984.