RESEARCH PLAN PROPOSAL

CO-EXPOSURE TO LEAD, ARSENIC AND MERCURY ON OXIDATIVE STRESS VARIABLES AND THEIR RESPONSE TO CHELATION IN RATS

For registration to the degree of

Doctor of Philosophy

IN THE FACULTY OF SCIENCE

THE IIS UNIVERSITY, JAIPUR

Submitted by

ShrutiAgrawal

IISU/2013/41490

Under the Supervision of

Department of Life Sciences
May,2013

ACKNOWLEDGEMENT

First of all, I would like to thank almighty **'GOD'** for everything and for giving such a place and people to work with and to make this work possible.

It is my immense pleasure to acknowledge boundless motivation, affection and support; I received from my guide, seniors, family and friends.

I would like to express deep sense of gratitude to **Dr. SJS Flora** , **Scientist 'G'** at **DRDE(Gwalior)** for his ideas and suggestions during my work.He inspired me to put in best of my efforts in the work. I have got his encouragement and support at all levels. He was very generous in sharing both his time and the resources of his organization for successful completion of my Project.

I am extremely thankful to **Prof. PRADEEP BHATTANAGER** (Dean, Faculty of Life Sciences), IIS UNIVERSITY, who also gave me his kind suggestions and made me able to produce this report today.

CONTENTS

2. Review of literature
3. Objective of research
4. Proposed methodology for work
5. Experimental Design
6. Plan of work
7. Significance of study

8.References

INTRODUCTION

Metals and metal compounds are natural constituents of all ecosystems, moving between atmosphere, hydrosphere, lithosphere, and biosphere. Metal compounds are increasingly introduced in the environment and could finally accumulate in a/biotic systems. Exposure to heavy metals is potentially harmful especially for those metal compounds,which do not have any physiological role in the metabolism of cells. A heavy metalis a member of an ill-defined subset of elements that exhibit metallic properties, which would mainly include the transition metals, some metalloids, lanthanides, and actinides**(A dictionary of chemistry,2000)**.Heavy metals have a high atomic weight and a density much greater (at least 5 times) than water. In modern times, anthropogenic sources of heavy metals, i.e. pollution, have been introduced to the ecosystem. Waste-derived fuels are especially prone to contain heavy metals so they should be a central concern in a consideration of their use. There are more than 20 heavy metals, but lead (Pb),Mercury (Hg), and Arsenic (As) are of special concern.They are highly toxic can cause damaging effects even at very low concentrations. They tend to accumulate in the food chain and in the body and can be stored in soft (e.g., liver,kidney) and hard tissues (e.g., bone). Being metals, they often exist in a positivelychargedform and can bind on to negatively-charged organic molecules to formcomplexes. If heavy metals enter and accumulate in body tissue faster than thebody's detoxification pathways can dispose of them, a gradual buildup of thesetoxins will occur.

Any toxic metal may be called heavy metal, irrespective of their atomic mass or density**(Singh,2007).**These metals are a cause of environmental pollution from sources such as leaded petrol, industrial effluents, and leaching of metal ions from the soil into lakes and rivers by acid rain.**(A dictionary of chemistry,2000)**

Any metal (or metalloid) species may be considered a "contaminant" if it occurs where it is unwanted, or in a form or concentration that causes a detrimental human or environmental effect.

Heavy metals disrupt metabolic functions in two ways:

1. They accumulate and thereby disrupt function in vital organs and glands such as the heart, brain, kidneys, bone, liver, etc.

2. They displace the vital nutritional minerals from their original place, thereby, hindering their biological function. It is, however, impossible to live in an environment free of heavy metals. There are many ways by which these toxins can be introduced into the body such as consumption of foods, beverages, skin exposure, and the inhaled air.

Humans are subjected to a range of chemical exposures from the environment. Chemicals in air, water, soil and food, occupational exposures and lifestyle factors, all contribute to a complex exposure situation in our daily life. (**Chopra *et al*.,2002**) It has long been known that toxicity can be modified by simultaneous or sequential exposure to multiple agents in the environment. For some combined or mixed exposures the health effects may increase more than what would be expected from simply adding the effects of the individual components, therefore there is a concern that several less studied complex exposures may have a large impact on our health as a result of combined or mixed effects.**(Mahaffey *et al*.,1981)**

When toxicity is caused due to exposure of multiple metals at the same time is termed as multi-metal toxicity. Among the most common exposure is that of Arsenic, Mercury and Lead in combination**.**

REVIEW OF LITERATURE

Arsenic (As), Mercury (Hg), and lead (Pb) are commonly used in numerous industries to the extent that they have now generated a pollution problem. Numerous studies have reported high levels of these metals near smelter areas **(Carrizales *et al*.,2002)**. Acute exposure to As, Hg, and Pb produces a variety of toxic effects in several target organ systems; however, most individuals are chronically exposed to low levels of a mixture of these metals **(Goyer and Cherian,1995)**. These three metals/metalloids share several common mechanisms underlying their toxicities, including production of oxidative stress, reaction with sulfhydryl groups, and interference with essential metals. In addition, stress proteins and antioxidant enzymes have been proposed to provide common cellular protective mechanisms against the element-induced toxicities when they occur on an individual basis **(Bae,2001)**. Furthermore, these metals have been listed in the top ten hazardous substances and proposed as one of the mixtures for interaction profile studies by the Agency for Toxic Substances and Disease Registry (ATSDR). As and Hg have been classified as carcinogens and Pb as a possible carcinogen by the International Agency in Research of Cancer (IARC). **(Bae,2001).**

As, Hg ,andPb induce the generation of reactive oxygen species (ROS), which can damage DNA, lipids, and proteins. As generates ROS in the form of superoxide (O2•−), singlet oxygen (O_2), Peroxylradical(ROO•), nitric oxide (NO•), hydrogen peroxide ($H2O_2$), dimethylarsinicPeroxyl radicals ([(CH3)2AsOO•]), and the dimethylarsinic radical [(CH3)2As•] **(Wang *et al*.,2008).** Hg generates ROS in the form of superoxide (O2•−), hydrogen peroxide ($H2O_2$), hydroxyl radical (HO•), and lipid radicals (L•).Pb ROS-generating mechanism is mediated by delta-aminolevulinic acid dehydratase (*δ*-ALAD) inhibition, which provokes the accumulation of delta-aminolevulinic acid (*δ*-ALA). *Δ-ALA* is rapidly oxidized to generate free radicals such as superoxide (O2•−), hydroxyl radicals (HO•), and hydrogen peroxide (H_2O_2).There are several *in vivo* and *in vitro* reports suggesting when experimental animals were exposed to mercury (organic or inorganic) there was an induction of oxidative stress mainly because of the depletion of the naturally occurring thiols, especially GSH.(**Vallee *et al*.,1972)**demonstrated that administration of mercury resulted in GSH depletion, lipid peroxidation and also increased the formation ofH_2O_2in the kidneys of rats. Lund and co-workers further demonstrated that it was the mitochondria of the rat kidney which were responsible for oxidative stress.

Interactive effects of lead (Pb), cadmium (Cd) and arsenic (As) were evaluated in 30-, 90-, and 180-day factorial design drinking water studies in rats designed to test the hypothesis that ingestion of such mixtures at individual component Lowest-Observed-Effect-Levels (LOELs) results in increased levels of the pro-oxidant delta aminolevulinic acid (ALA), iron, and copper **(Wang *et al*., 2004)**. LOEL levels of Pb, Cd, and as mixtures resulted in the increased presence of mediators of oxidative stress such as ALA, copper, and iron. ALA increases were followed by statistically significant increases in kidney copper in the 90- and 180-day studies. Statistical evidence of interaction was identified for six biologically relevant variables: blood delta aminolevulinic acid dehydratase (ALAD), kidney ALAD, urinary ALA, urinary iron, kidney iron, and kidney copper.

TREATMENT FOR HEAVY METAL POISOINING:CHELATION TREATMENT

The term chelation comes from the Greek word "chelate" which means claw. Extensive experience demonstrates that acute and chronic human intoxications with a wide range of metals can be treated with considerable efficiency by the administration of a relevant chelating agent. Development of effective chelating agent is based on combinations of chemical considerations and whole animal experimentation on the toxicokinetics and toxicodynamics of metal and chelating agents, followed by clinical experience, with regard to monitoring metal excretion and status of tissue damage.

Meso 2, 3-dimercaptosuccinic acid (DMSA)

It is a chemical derivative of dimercaprol. It contains two sulfhydryl (-SH) groups and has been shown to be an effective chelator of toxic metal mainly lead and arsenic. Few major advantages of DMSA include its low toxicity, oral administration and no redistribution of metal from one organ to another. DMSA has been tried successfully in animal as well as in cases of human arsenic poisoning (**Flora *et al.,* 1997**). In an interesting perspective, double blind, randomised controlled trial study conducted on few selected patients from arsenic affected West Bengal (India) regions with oral administration of DMSA suggested that it was not effective in producing any clinical or biochemical benefits **(Gupta *et al.,* 1998)**. Animal studies suggest that DMSA is an effective chelator of soft tissue but it is unable to chelate lead from bones (**Flora *et al.,*2008**).Oxidative damage caused by lead may be implicated in the induction of the cell apoptosis. DMSA for being an antioxidant and a strong lead chelator has been shown to deplete significantly lead from hippocampus leading to recovery in the oxidative stress and apoptosis induced by lead **(Wang *et al*., 2004)**. DMSA is not known to

cause elevations in the excretion of calcium,zinc or iron, although zinc excretion has increased to 1.8 times base line during treatment. Renal toxicity has also been related to excretion of large amount of chelated metals that pass through the renal tubules in a relatively short period during therapy. One of the major drawback with the use of DMSA is that it is basically a soft tissue lead and arsenic mobilizer and thus unable to remove these metals from hard tissues and intracellular sites. Thus, its use particularly in chronic cases of heavy metal poisoning is limited and further investigation in this area is needed before approving this treatment protocol.

Monoisoamyl DMSA (MiADMSA)

Monoisoamyl ester of DMSA (MiADMSA);a C5 branched chain alkyl monoester of DMSA)has been found to be the most effective(**Flora *et al.*,2002**). **(Mehta and Flora,2001)**reported for the first time the comparison of different chelating agents (3 amino and thiolchelators) on their role on metal redistribution, hepatotoxicity and oxidative stress in chelating agents induced metallothionein in rats.**(Mehta *et al.*,2001*)***have suggested that MiADMSA had no effect on length of gestation, litter-size, sex ratio, viability and lactation. MiADMSA also potentate the synthesis of MT in liver and kidneys and GSH levels in liver and brain and also significantly reduced the GSSG levels in tissues. MiADMSA was found to be safe in adult rats followed by young and old rats. These metal chelators are given to increase the excretion of arsenic but unfortunately the uses of these chelators are comprised by number of drawbacks. These drawbacks open the search for new treatmentwhich has no side effects and maximum clinical recovery in terms of altered biochemical variables because the total elimination of metals from the environment is not feasible.

OBJECTIVES OF RESEARCH

1. To study the effects of multi-metal exposure on biochemical markers indicative of oxidative stress and antioxidant status in animal model.

2. To study the toxic effect of multi-metal exposure on heme-biosynthetic pathway and other haematological variables.

3. To understand the toxicokinetic and type of interaction (antagonistic/synergistic) between metals when co-administered *in vivo*.

4. To study the effect of chelation in reducing toxic metal levels during multi-metal Exposure.

PROPOSED METHODOLOGY OF THE WORK

Animals and treatment:

Male wistar rats of approximate weight (100-200) gm will be used as the animal model. The animals will be exposed to Arsenic, Lead and Mercury as in drinking water for subchronic and chronic exposure of 90 and 180 days respectively. Further the animals would also be exposed to multiple course of chelation therapy (DMSA, MiADMSA) in order to study the role of chelator to study the toxicity caused due to it.

EXPERIMENTAL DESIGN

Group. No	Group Treatment
Group-1	**Control**
Group-2	**Lead(Pb)**
Group-3	**Mercury(Hg)**
Group-4	**Arsenic(As)**
Group-5	**Lead(Pb)+Mercury(Hg)**
Group-6	**Lead(Pb) +Arsenic(As)**
Group-7	**Mercury(Hg) + Arsenic(As)**
Group-8	**Lead+Arsenic + Mercury**
Group-9	**Multimetal+chelator(DMSA)**
Group-10	**Multimetal+Chelator(MiADMSA)**

Multimetal-Combination of Lead, Arsenic and Mercury

PLAN OF WORK

Male Wistar rats (100-120 gms) are taken and divided into 10 groups of 15 animals in each group.

Exposure of metals individually (Group 2-4) and in combination (Group 5-8) for 90 and 180 days at different dose levels.

Chelator (DMSA and miADMSA) will be given for group 9 and 10.

Animals will be sacrificed after the completion of sub chronic and chronic exposure.

Biochemical parameters will be studied in Blood, Liver, kidney, and Brain.

Metal estimation in Blood, Liver, Kidney and Brain.

Biochemical assays:

After the above treatment, the animals will be sacrificed. Blood, liver, kidneys and brain will be removed for the following biochemical and metal analyses. The methods to be adopted for the estimation of these variables are given in the parenthesis-

Blood– δ-aminolevulinic acid dehydratase (ALAD) (Berlin and Schaller, 1974),

Glutathione (GSH) (Ellman, 1959 modified by Jollow*et al*., 1974),

ROS (Socci*et al.*, 1999),

Superoxidedismutase (SOD) (Kakkar*et al.*, 1983),

RBC and WBC counts

Haemoglobin and packed cell volume.

Liver–Reduced and oxidised Glutathione (GSH and GSSG) (Hissin and Hilf, 1973),

Thiobarbituric acid reactive substances (TBARS) (Onkawa and Onishi, 1979),

Catalase (Aebi, 1984),

SOD.(Kakkar*et al.*, 1983),

Brain– Acetylcholine esterase (AChE) (Ellman*et al.*, 1961),

Monoamineoxidase (MAO) (Wurtman and Axelrod, 1969),

Glutathione peroxidase (GPx) (Flohe and Gunzler, 1984),

Glutathione S-transferase (GST) (Habig*et al.*, 1974),

5-hydroxytryptamine (5-HT),

Norepinephrine (NE), Dopamine (DA) (Sadavongvivid, 1970),

SOD, ROS, GSH, GSSG,

Metal estimation: Liver, brain and blood –As (Parker et al 1967)

SIGNIFICANCE OF THE STUDY:

By doing these experiments the toxicity due to multi-metal exposure could be evaluated like oxidative stress and alterations in the heme biosynthetic pathway.Thetoxicokinetic interaction between the metals when administered concurrently could be studied .By using DMSA and MiADMSA to remove heavy metal toxicity, we will be able to:

1) Deduce the effectiveness of this chelator on multi-metal removal.

2) See how it affects the biochemical parameters of the main target organs like, blood, liver and brain in terms of removing the oxidative stress caused by the metals.

3)Check if administration of MiADMSA and DMSA at a particular selected dose, is safe for use.

REFERENCES

1. A dictionary of chemistry. Oxford university press. Oxford reference. Oxford University Press. 2000
2. Aebi, H. (1984) Catalase invitro. *Methods in Enzymol.*, **105**, 121-126.

3. Bae D.S, Gennings.C, Carter.W.H Jr., Campain.(2000) Toxicological interactions among arsenic, cadmium, chromium, and lead in human keratinocytes.ToxicologicalSciences,**63,** 132–142.

4. Berlin, A. and Schaller, K.H.(1974) European standardized method for the determination of -aminolevulinic acid dehydratase activity in blood. *Z. Klin. Chem.Klin. Biochem*.**12**, 389-390.

5. Burbure.C, Buchet, A. Leroyer et al.(2006) Renal and neurologic effects of cadmium, lead, mercury, and arsenic in children: evidence of early effects and multiple interactions at environmental exposure levels. *Environmental Health Perspectives*,**114**,584–590.

6. Carrizales .L,Razo .I, T´ellez-Hern´andez et al.(2006) Exposure to arsenic and lead of children living near a copper-smelter in San Luis Potosi, Mexico: importance of soil contamination for exposure of children. *Environmental Research*. 101,1–10.

7. Chopra A, Doiphode W.(2002) Ayurvedic medicine: Core concept, therapeutic principles, and current relevance. *MedClin North Am.***86**, 75–89.

8. Cory-Slechta DA, Schaumburg HH.(2000) Lead, inorganic. In: Spencer P.S., Schaumburg, H.H., Rudolph, A.C., editors. Experimental and clinical neurotoxicology. *Oxford University Press* 708–720.

9. Casalino E, Calzaretti G, Sblano C, Landriscina C. Molecular inhibitory mechanisms of antioxidant enzymes in rat liver and kidney by cadmium. *Toxicology*, 2002, 30, 37-50.

10. Ellman, G.L.(1959): Tissue sulphydryl groups. *Arch Biochem*. **82**, 70-77.

11. Flohé L, Günzler WA.(1984): Assays of glutathione peroxidase. *Methods Enzymol.***105**, 114-21.

12. Flora SJ, Mehta A, Rao PV, Kannan GM, Bhaskar AS, Dube SN, Pant BP.(2004) Therapeutic potential of monoisoamyl and monomethyl esters of meso 2,3-dimercaptosuccinic acid in gallium arsenide intoxicated rats. *Toxicology.***195**,127-46.

13. Flora SJS, Dubey R, Kannan GM, Chauhan RS, Pant BP, Jaiswal DK.(2002) (DMSA) and monoisoamyl DMSA effect on gallium arsenide induced pathological liver injury in rats. *ToxicolLett*.***132,*** 9-17.

14. Flora SJS, Dubey R, Kannan GM, Chauhan RS, Pant BP, Jaiswal DK.(2002)meso 2, 3-dimercaptosuccinic acid (DMSA) and monoisoamyl DMSA effect on gallium arsenide induced pathological liver injury in rats. *ToxicolLett*. ***132,***9-17.

15. Flora SJS, Mehta A, Rao PVL, Kannan GM, Bhaskar ASB, Dube SN, *et al*.(2004) Therapeutic potential of monoisoamyl and monomethyl esters of meso 2,3-dimercaptosuccinic acid in gallium arsenide intoxicated rat. *Toxicology.* ***195,*** 127-46.

16. Flora SJS, Mittal M, Mehta A.(2008) Heavy metal induced oxidative stress and its possible reversal by chelation therapy. *Ind J Med Res*.**128**, 221-243.

17. Flora SJS, Pant BP, Tripathi N, Kannan GM, Jaiswal DK.(1997) Distribution of arsenic by diesters of Meso 2, 3- dimercaptosuccinic acid during sub-chronic intoxication in rats. *J Occup Health*. ***39,*** 119-23.

18. Florea AM, Busselberg D,(2005) Toxic effects of metals:modulation of intracellular calcium Homeostasis. *Matwiss.u.Werkstofftech*. **36**, 1–4.

19. Florea AM,(2005) Toxicity of Alkylated Derivatives of Arsenic, Antimony and Tin: Cellular Uptake, Cytotoxicity, Geotaxis Effects, Perturbation of Ca2+ Homeostasis and Cell Death.

20. George L. Ellman, K. Diane Courtney, Valentino Andres, Jr and Robert M. Featherstone (1961): A new and rapid colorimetric determination of Acetylcholinesterase activity. *Biochemical Pharmacology.***7**, 88-95

21. Goyer RA, Cherian MG, Renal effects of metals. In: Goyer, R.A., Klaassen, C.D., Waalkes, M.P(1995), Metal Toxicology. Academic Press, *San Diego*, 389–412.

22. GuhaMazumder DN, Das Gupta J, Santra A.(1998) Chronic arsenic toxicity in West Bengal-The worst calamity in the world. *J Indian Med Assoc,**96,*** 4-7.

23. Habig, W. H., Palist, M. J. and Jakoby, W. B. (1974) *J. Biol. Chem*., **249**, 7130.

24. Hermes-Lima.M, ValleV.G.R,Vercesi A.E, and Bechara E.J.H.(1991) Damage to rat liver mitochondria promoted by *δ*-aminolevulinic acid-generated reactive oxygen species: Connections with acute intermittent porphyries and lead poisoning. *Biochimica et Biophysical Acta*, **1056**, 57–63.

25. Hissin, P.J, Hilf,R.(1976) A fluorometric method for determination of oxidised and reduced glutathione in tissues. *Analytical Biochemistry*, **74**,214–226.

26. IARC, International Agency for Research on Cancer, Beryllium, cadmium, mercury, and exposures in the glass manufacturing industry.(1993) In: International Agency for Research on Cancer Monographs on the Evaluation of Carcinogenic Risks to Humans, *IARC Scientific Publications, Lyon,* **58**, 119–237.

27. Farant J.P.and Wigfield D.C. (1982). Bio monitoring lead exposure with *δ*-aminolevulinatedehydratase (ALA-D) activity ratios. *International Archives of Occupational and EnvironmentalHealth*, **51**, 15–24.

28. JollowD.J ., Mitchell J.R , Zamppaglione Z. and Gillette J.R.(1974): Bromobenzene induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolites. *Pharmacol.*, **11**, 151-155.

29. Kakkar, P., B. Das and P.N. Viswanathan, (1984). A modified spectrophotometric assay of superoxide dismutase. *Indian J. Biochem. Biophys*. **21**,130-132.

30. Mahaffey, K.R., Capar, S.G., Gladen, B., Fowler, B.A.(1981). Concurrent exposure to lead, cadmium and arsenic: effects on toxicity and tissue metal concentrations in the rat. *J. Lab. Clin. Med.* **98**, 463–481

31. Mehta A, Flora SJS. (2001)Possible Role of metal redistribution, hepatotoxicity and oxidative stress in chelating agents induced hepatic and renal metallothionein in rats. *Food ChemToxicol*. ***39,*** 1029-38.

32. Onkawa, H., Ohishi, N., Yagi, K.(1979) Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal. Biochem*. **95**, 351–358.

33. Parker, M. M., Humoller, F.L., and Mahler, D. J.(1967) Determination of copper and zinc in biological material. *Clin. Chem*. **13**, 40 – 48

34. Quintanilla-Vega B, D. Hoover, W. Bal, E. K. Silbergeld, M.P. Waalkes, and L. D. Anderson.(2000) Lead effects on protamine-DNA binding, *American Journal of Industrial Medicine*. **38**, 324–329.

35. Singh MR.(2007). Impurities-heavy metals: IR perspective.

36. Socci, D.J., K.B. Bjugstad, H.C. Jones, J.V. Pattisapu and G.W. Arendash(1999): Evidence that the H-Tx rat model. *Exp. Neurol*. **155**, 109-117.

37. Vallee B.L and Ulmer D.D (1972) Biochemical effects of mercury, cadmium, and lead.*Annual Review of Biochemistry*. **41**, 91–128.

38. Wang, G., Chen, X., Lipsky, M., Whittaker, M.H., Fowler, B.A.(2004). Interactions of lead, cadmium and arsenic in rat kidneys. Society of Toxicology Meeting Abstract. Toxicologist, **78**. *Interscience*, 238. Abstract No 1159.

39. Wang.G and Fowler B.A,(2008) Roles of biomarkers in evaluating interactions among mixtures of lead, cadmium and arsenic, *Toxicology and Applied Pharmacology*. **233**, 92–99.

40. Wurtman R J, Axelrod J. (1963): A sensitive and specific assay for the estimation of Monoamineoxidase. *BiochemPharmacol.***12**, 1439–1441.