5. DISCUSSION

5.1. Oxidative stress, altered antioxidant and immune defense system upon *in vivo* exposure to lead acetate and sodium arsenite in macrophages of fish *Channa punctatus*

The presence of metal pollutant in fresh water is known to disturb the delicate balance of the aquatic ecosystem. Among the various toxic pollutants, heavy metals are particularly severe in their action due to tendency of bio-magnification in the food chain (Murugan *et al*., 2008). They readily tend to concentrate in different organs of fishes resulting in bioaccumulation and biomagnification of these metals to a toxic level even when the exposure is low (Adeniyi and Yusuf, 2007; Mertz, 1981; Charis and Abbasi, 2005).Fish has been reported to accumulate metals from water by diffusion via skin and gills as well as oral consumption (Nussey *et al*, 2000; Oguzie, 2003). Our results showed significant level of lead and arsenic accumulation in liver, gills, muscle, kidney and intestine when treated singly. These results significantly correlate with the altered antioxidant as well as the non-specific immune functions of the intestinal macrophages.

Heavy metals not only deteriorate the physico-chemical equilibrium of the aquatic body, but also disrupt the food web and, bring about morphological, physiological and cytogenetical changes in the aquatic inhabitants (Scalon *et al*., 2010). The functions of teleostean digestive tract include digestion, nutrients absorption, hormone secretion, immune protection and water and salt transfers for hydro mineral homeostasis. It regulates energy and material exchange between the environment and the internal medium (Khojasteh, 2012). Thus knowledge of the intestinal ultrastructure and morphology is essential for understanding the related functional mechanisms. Scanning Electron Microscopy (SEM) is an important tool that reveals information on the surface ultrastucture and morphological changes that may

occur in biological tissues. Lead and arsenic induced severe inflammatory damage in intestinal epithelium, disarrangement and fragmentation of mucosal foldings, as observed in the present study, causes impairment of tissue organs which in turn has an obvious effect on its defense system.

Phagocytes, upon stimulation with various agents, produce reactive oxygen species (ROS) through activation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (Forman and Torres, 2002). NADPH oxidase is a superoxide-producing enzyme consisting of the membrane (gp91PHOX and p22PHOX) and the cytosolic (p47PHOX, p67PHOX, and p40PHOX) components (DeLeo and Quinn, 1996; Babior, 1999). In addition, small G-proteins such as rac1, as well as kinases including PKC, regulate its activity (El-Benna *et al*., 2008). Under normal circumstances, activation of cells of nonspecific immunity may be beneficial to the host cell, particularly the reactive intermediates released during phagocytic respiratory burst activity possess bactericidal activity. Significant elevation in the respiratory burst activity upon lead and arsenic exposure, as observed in the present results, may suggest overactivation of the superoxide-producing enzyme NADPH oxidase and generation of large amount of ROS. Further, it could also be assumed that lead and arsenic in macrophage might have suppressed the activity of the regulatory proteins leading to uncontrolled enzyme activity which is destined to cell damage when treated singly.

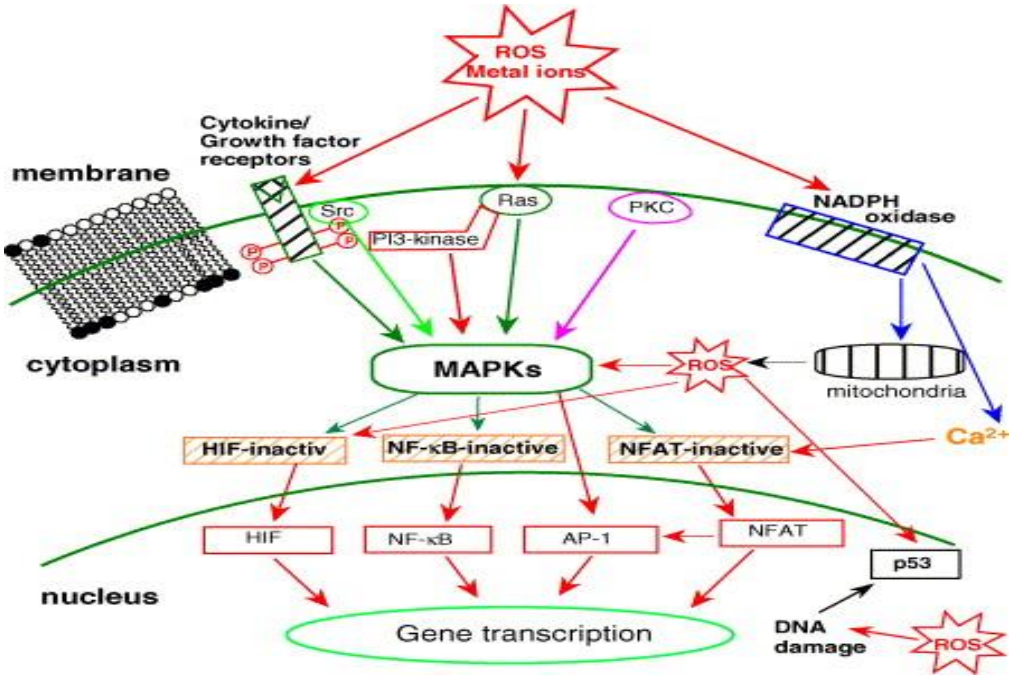

Figure 1: Mechanism showing how metal ions induce oxidative stress by altering MAPKs and other signaling molecules within cells of living organisms.

Heavy metal induced elevation in lipid peroxidation has been reported in the present work. When the animal's defenses are insufficient to neutralize ROS, oxidative damage may occur, and one of the most serious damages is formation of membrane lipid peroxides (Sancho *et al*., 2000). The integrity of plasma membrane is essential for cell viability, and as a consequence of overactivation of phagocytes, its fluidity seems to be affected in fish (Dimitrova *et al*., 1994). Thus it may be hypothesized that lead and arsenic may stimulate the peroxidation of lipids by acting as catalysts in the formation of oxygen radicals when treated singly.

The present study reported elevated level of protein carbonyls in both lead exposed and arsenic exposed fish when compared with the untreated group. Metals are well known to

oxidatively modify the amino acyl side chain of proteins via metal catalyzed oxidation (MCO) reactions. Thus, it could be assumed that lead and arsenic induced the formation of protein carbonyls resulting in damaged protein susceptible to degradation and may lose some or all of its function.

A significantly greater degree of DNA fragmentation was reported in both lead exposed and arsenic exposed fish as compared with the control group. Apoptosis includes a series of changes in cell volume, disintegration of DNA and other morphological changes. A greater percentage of DNA susceptible to fragmentation upon heavy metal exposure may indicate an alteration in integrity or morphology in target cell leading to apoptosis in due course.

Many aquatic organisms including freshwater teleost have been shown to possess defense pathways to protect them against damages induced by oxyradical production (Secombes, 1990). Both enzymatic and non-enzymatic processes contribute to reducing the impact of ROS in fishes (Zelikoff *et al*., 1991). Oxidative stress occurs when the critical balance between oxidants and antioxidants is disrupted due to the depletion of antioxidants or excessive accumulation of ROS, or both, leading to cellular damage (Santos *et al*., 2004). The superoxide dismutase- catalase (SOD–CAT) system provides the first defense against oxygen toxicity and represents a cellular defense mechanism to counteract toxicity of ROS. SOD catalyzes the dismutation of the superoxide anion radical to water and hydrogen peroxide, which is detoxified by the CAT activity (Dimitrova *et al*., 1994). Our results showed significant decrease in the activity of CAT and significant increase in SOD activity when treated singly with lead and arsenic. Low levels of catalase in lead treated and arsenic treated

fishes could be attributed to high production of superoxide anion radicals, which has been reported to inhibit catalase activity. The increase in SOD activity in lead treated and arsenic treated fishes may be due to increased generation of reactive oxygen species.

One of the most remarkable effects of lead and arsenic exposure on macrophage was a time-dependent decrease in GSH. GSH is the most well studied antioxidant molecule in fish. Heavy metal cations are characterized by an extremely high affinity to –SH residues (USPHS, 1997) resulting in decrease of GSH level. Sandhir *et al.*(1994) established that glutathione reductase, the enzyme responsible for recycling of glutathione from the oxidized form (glutathione disulfide; GSSG) to the reduced form (reduced glutathione; GSH) is deactivated by lead and arsenic when treated singly, resulting in low levels of GSH. The results of the present study clearly show depleted levels of GSH in both lead treated and arsenic treated group of fish as compared to control, and may contribute to the above facts. Antioxidant enzymes that have often been studied as oxidative stress biomarkers link detoxification of ROS with the metabolism of reduced glutathione (Stegeman *et al*., 1992; Viarengo and Nott, 1993). These include glutathione peroxidase (GPx), an enzyme removing hydrogen peroxide by the simultaneous oxidation of reduced GSH to its oxidized form glutathione disulfide (GSSG) and glutathione reductase (GR), an enzyme catalyzing the conversion of GSSG back to its reduced bioactive form thus maintaining GSH/GSSG equilibrium (Paskerova *et al*., 2012). Glutathione S- transferase (GST), an enzyme involved in the detoxification process and in protecting against peroxidative damage, is ubiquitous in the cytosol and microsomes of eukaryotes (Sreejai and Jaya, 2010). The results of the present study reveal significant depletion of GPx, GR and GST activity indicates an impaired detoxification mechanism of the fish upon low concentration of lead and arsenic exposure.

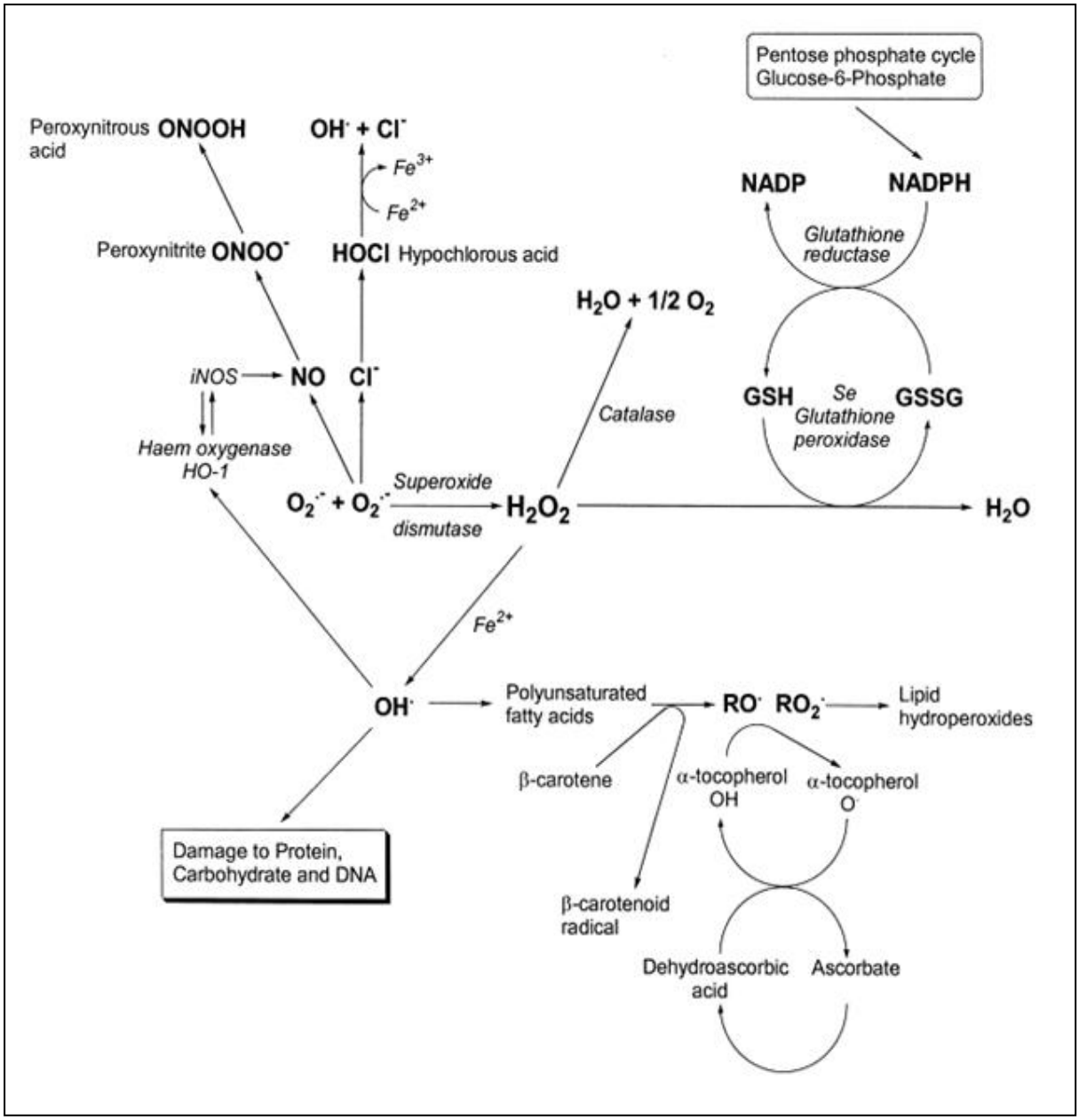

Figure 2: Mechanism showing the role of enzymatic and non- enzymatic antioxidant sysem in combating free radicals produced in living organisms.

Macrophages play a critical role in body's defense system by eliminating microorganisms from infected tissues. Macrophages recognize and engulf bacteria into phagosomes, which subsequently fuse with lysosomes. These phagosomes mature into phagolysosomes upon vesicle-mediated delivery of various antimicrobial effectors.

Reduction of phagocytic capacity by lead exposed and arsenic exposed group may suggest that the biological activity of alternate complement pathway is altered which is an important pathway in the defense mechanism of fish (Ellis, 2001), and the C3 component of it is likely to be inhibited, making the host cells prone to infection and diseases.

Prolonged exposure to lead and arsenic tends to inhibit the killing capacity of macrophages when treated singly as seen from the findings that show a significant increase in bacterial viability within the macrophages. This indicates that heavy metal exposed leucocytes are somehow less potent to kill the bacteria efficiently that allows pathogens to easily gain access to the host tissues.

Numerous substances generated during inflammation, like cytokines and chemokines, have the capacity to enhance the speed of macrophage and orient the movement in the direction of an increased concentration gradient of the agent. Lead and arsenic, when exposed singly on macrophages, may lead to altered expression of cell adhesion molecules, chemokine receptors etc. on the macrophage surface which further alters the shape and orientation of macrophages so that they migrate slowly and adhere and engulf bacteria poorly.

Adherence of antigen to the macrophage cell membrane is a vital step in phagocytosis. Phagocytosis first requires attachment of the bacteria to the surface of the phagocyte, which may involve hydrophobic interactions or sugar/lectin interactions (Secombes, 1996). However, this property of cell adherence is significantly inhibited upon lead exposure and arsenic exposure when treated singly as it is evident from a fall in absorbance at 570 nm with respect to control suggesting that these heavy metals may either

suppress enough production of lectins or may reduce its opsonising activity, which may have obvious effect on the host defense mechanism.

In recent years, NO has been shown to be a very important molecule in regulating immune functions as well as having a direct antimicrobial effect (Gunasegaran *et al*.,1993; Liew *et al*., 1990). Reactive nitrogen species (RNS), a family of antimicrobial molecules derived from nitric oxide (NO), are produced via the enzymatic activity of inducible nitric oxide synthase 2 (NOS2) (Fang,2004; Nathan and Shiloh, 2000). The present results illustrates that the amount of NO production by arsenic and lead exposed groups have been found to be strikingly less as compared to the control group. iNOS gene expression is dependent on tumor necrosis factor α (TNF- α) nuclear factor kappa B (NFκB) activation. Thus, modulation of NFκB activity affects the induction of iNOS. Heavy metals have a high affinity to bind to the haemoprotein subunit of the iNOS enzyme.

Myeloperoxidase (MPO) produced by macrophages is known to play an important role in cellular defenses against various bacterial infections. MPO can generate oxidants from hydrogen peroxide (H_2O_2) and a range of co-substrates, most notably hypochlorous acid (HOCl), which is strongly bactericidal and markedly increases the antibacterial potency of ROS, leading to subsequent increase in antimicrobial activity of macrophage. Insufficient release of MPO from lead treated and arsenic treated macrophages may indicate that its bactericidal potency has been suppressed resulting in poor host defense mechanism.

Macrophages play an essential role for the initiation and activation of the innate immune system, by recognizing and releasing a large number of cytokines such as tumor necrosis factor- a (TNF- α), interleukin-1 (IL-1) and IL-6 (Nathan, 1987).The upregulation of secreted inflammatory cytokines by macrophages occurs upon the selective recognition and interaction of the pathogen-associated molecular patterns (PAMPs) that are conserved in bacterial species by the pattern recognition receptors (PRRs) such as the Toll-like receptor (TLR) proteins. Levels of TNF-α and IL- 1β was found to be significantly less in lead treated and arsenic treated groups than that of control group. Cytokine expression becomes downregulated, probably by the action of the heavy metals at multiple levels viz., PAMP recognition, receptor dysfunction and/or by affecting the cell-signalling network rendering the fish to an immunocompromised and anti-inflammatory state.

As a conclusion, lead and arsenic has a deleterious effect on the immune functions of fish, specifically on its intestine and liver. The *in vivo* exposure to sub-lethal concentration of lead acetate and sodium arsenite caused alterations in the antioxidant defense system and induced oxidative stress in fish. The *in vivo* exposure to sub-lethal concentration of lead acetate and sodium arsenite also caused suppression of the immune parameters (phagocytic activity, intracellular killing, cell adhesion, chemotactic migration, nitric oxide release, myeloperoxidase release and cytokine release) measured in the intestinal and liver macrophage of *C. punctatus.* These results suggest that the alterations in the phagocytic activity and the oxygen dependent killing mechanisms as well as the antioxidant defense mechanisms can be used as potential biomarkers for risk assessment in aquatic ecosystem.

5.2. Oxidative stress, altered antioxidant and immune defense system upon *in vitro* exposure to lead acetate and sodium arsenite in macrophages of *Channa punctatus*

A disturbance in the balance between the prooxidants and antioxidants leading to detrimental biochemical and physiological effects is known as oxidative stress. This is a harmful condition in which increase in free radical production, and/or decrease in antioxidant levels can lead to potential cell damage. Freshwater fishes have evolved mechanisms to counteract the impact of ROS. These include various antioxidant defense enzymes such as superoxide dismutase, catalase and glutathione S- transferase (Saliu and Bawa- Allah, 2012). Aerobic organisms have developed a comprehensive antioxidant defense system, comprising both molecular and enzymatic defenses, against the dangers of oxygen radicals (Halliwell and Gutteridge 1999), thereby preventing excess oxidation and damage.

The observations from the present study reveal that the respiratory burst activity of both lead treated and arsenic treated group increased significantly as compared to that of the control group. This apparently, should promote intracellular killing. However, elevation in respiratory burst activity in lead treated and arsenic treated cells may be due to an overactivation of superoxide anion producing enzyme NADPH oxidase and subsequent generation of large amount of ROS that escapes the phagocytic milieu to cause macromolecular damage.

The results in the present study illustrate a pronounced effect on the protein of lead exposed and arsenic exposed cells rapidly converting them to protein carbonyls as compared to the control. Metals are known to directly induce the formation of protein carbonyls via

metal catalyzed oxidation (MCO) reactions, resulting in damaged protein susceptible to degradation that may lose some or all of its function.

The results of the present study show a significant increase in the degree of DNA fragmentation in lead exposed and arsenic exposed groups when compared with the control. A greater percentage of DNA susceptible to fragmentation upon heavy metal exposure may indicate an alteration in integrity or morphology in target cell leading to apoptosis in due course.

SOD is an essential metalloenzyme detoxifying superoxide radicals to hydrogen peroxide. CAT, on the other hand, which is a metal-containing enzyme, is the most efficient enzyme that promotes redox reaction converting hydrogen peroxide into water and oxygen. Increase in SOD may result in the enhanced formation of hydrogen peroxide. This combined with decrease in catalase activity could lead to increased hydrogen peroxide accumulation within the host cells and thus inducing oxidative stress.

Glutathione (GSH) is a key component in such metal scavenging due to the high affinity of metals to its thiol (-SH) group. Glutathione exists in reduced (GSH) and oxidized (GSSG) forms. In the reduced state, the thiol group of cysteine is able to donate a reducing electron directly to unstable molecules such as ROS. In donating an electron, GSH itself becomes reactive, but readily reacts with another reactive GSH to form GSSG (Jozefczaket al., 2012). However, upon heavy metal treatment, cells' antioxidant defense system becomes incapable to cope with the massive GSH-consuming effects (direct metal-GSH binding, GSH oxidation, PC synthesis), causing a reduction in free reduced GSH.

Macrophages are capable of ingesting and digesting exogenous antigens, such as whole microorganisms and insoluble particles, and endogenous matter, such as injured or dead host cells, cellular debris, and activated clotting factors. Upon heavy metal exposure, macrophages fail to undergo phagocytosis which is clearly evident from the results of the present study which show a significant decrease in the phagocytic index of both lead treated and arsenic treated groups when compared with the control.

Activated macrophages produce a number of reactive nitrogen intermediates including nitric oxide (NO) that have potent antimicrobial activity. Furthermore, myeloperoxidase and halide anions constitute a potent halogenating system capable of killing both bacteria and viruses. Heavy metals like lead and arsenic inhibits their activity, which is revealed from the present study, thereby rendering the host cells susceptible to diseases.

Macrophages play an essential role for the initiation and activation of the innate immune system, by recognizing and releasing a large number of cytokines such as tumor necrosis factor- a (TNF- α), interleukin-1 (IL-1) and IL-6 (Nathan, 1987). Phagocytosis is mediated largely by white blood cells such as neutrophils and macrophages, cells which engulf and kill the foreign body, and that concurrently coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines (Aderem and Underhill, 1999). The present results illustrate a significant fall in the cytokine levels (TNF-α and IL-1) thus, rendering the fish to an immunocompromised and anti-inflammatory state.

5.3. Oxidative stress, altered antioxidant and immune defense system upon simultaneous exposure to lead acetate and sodium arsenite *in vivo* in macrophages of *Channa punctatus*

Heavy metals are accumulated in fish body in different amount. These variations result from different affinity of metals to fish tissues, different uptake, deposition and excretion rates. Most of them accumulate mainly in liver, kidney and gills. Fish muscles usually contain the lowest levels of metals as compared to other tissues.. Accumulation of metals in various organs of fish may cause structural lesions and functional disturbances (Jezierska and Witeska, 2006).The present results showed significant level of both lead and arsenic accumulation in liver, gills, muscle, kidney and intestine when treated simultaneously with lead and arsenic as compared to the control. These results significantly correlate with the altered antioxidant as well as the non-specific immune functions of the macrophages, and also contribute to the above facts.

The mucosa of teleosts provides a protective physical barrier that is important in terms of both osmoregulation and pathogen defense. Simultaneous exposure to lead and arsenic induces severe inflammatory damage in intestinal epithelium, disarrangement and fragmentation of mucosal foldings, as observed in the present study, causing impairment of tissue organs which in turn has deleterious effects on its defense system.

Oxidative stress is an imbalance between the production of reactive oxygen species (ROS) and the cell's ability to reduce ROS, detoxify reactive intermediates and repair damage that may occur in cellular molecules. This imbalance may occur as a result of increased ROS production, a decrease in defense mechanisms or both. ROS are produced endogenously within the cell, however many environmental parameters including exposure to

heavy metals are known to induce oxidative stress (Almroth, 2008). The ROS can rapidly attack all types of biomolecules such as nucleic acids, proteins, lipids, and amino acids (De Vos and Schat, 1991; Mehta *et al*., 1992; Luna *et al*., 1994), leading to irreparable metabolic dysfunction and cell death. Elevation in respiratory burst activity may indicate that simultaneous treatment of lead and arsenic potentiates the activity of superoxide-producing enzyme NADPH oxidase generating large amount of ROS thus, exerting a variety of damaging effects.

The level of malonaldialdehyde (MDA) in the tissue is considered a measure of lipid peroxidation status. Lipid peroxidation is linked to the production of O^{-2}. Metal-mediated formation of free radicals causes augmentation of lipid peroxidation. Lipid peroxides, formed by the attack of radicals on polyunsaturated fatty acid residues of phospholipids, can further react with redox metals, finally producing mutagenic and carcinogenic MDA (Reyes *et al*., 2013). The results of the present study clearly show that simultaneous intoxication of fishes with lead and arsenic may enhance formation of MDA when treated with that of the single metal treated groups.

Oxidative stress can cause a range of reversible (glutathionylation) and irreversible (carbonylation) modifications of protein amino acid side chains(Ghezzi and Bonetto, 2003), which affect the response of numerouscell signalling pathways (Tan *et al*., 1995; Tan *et al*., 2001; Persad*et al*., 1997; Persad *et al*., 1998; Ward *et al*., 2000; Mahadev *et al*.,2001; Humphries *et al*., 2002; Kwon *et al*., 2004; Goldstein *et al*., 2005).The present results prominently show that oxidative stress induced by simultaneous exposure to lead and arsenic enhances protein carbonylation in macrophages of *Channa punctatus*.

DNA damage has been proposed as a useful parameter for assessing the genotoxic properties of environmental pollutants (Kohn, 1983). Many of these pollutants are chemical carcinogens and mutagens with the capacity to cause various types of DNA damage. In the present study, it has been observed that lead and arsenic increased the extent of DNA fragmentation when exposed simultaneously as compared to the control and the single metal treated group. These results may be regarded as an indicator of increased ROS production upon interaction of lead and arsenic within host cells.

Superoxide anions and hydrogen peroxide (H_2O_2) are produced inadvertently in the cytoplasm primarily when oxygen collides with various redox enzymes (Imlay, 2009). Under normal conditions, superoxide anions can undergo spontaneous dismutation in a pH- and concentration-dependent reaction to yield H_2O_2 and O_2^-, the reaction being catalysed by superoxide dismutases. Further, H_2O_2 is detoxified by the enzyme catalase. Both O_2^- and H_2O_2 can damage a variety of biomolecules (Anjem *et al*., 2009; Imlay, 2009). The results of the present study showed significant decrease in the activity of CAT, in contrast to SOD activity that significantly increased upon simultaneous treatment of fishes with lead and arsenic. The heme groups of catalase lie deeply buried and are accessible to solvent by way of a narrow channel lined with hydrophobic residues (Reid *et al*., 1981). O_2^-, overproduced upon heavy metal intoxication, is small enough to gain access to the heme group of catalase and would convert the resting enzyme to the ferro-oxy state**,** rendering the enzyme in an inactive form (Chance, 1949). On the other hand, increased activity of SOD may lead to uncontrolled production of free radicals like H_2O_2 thus exerting cellular toxicity.

Glutathione related enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR) function either directly or indirectly as antioxidant and glutathione-S

transferase (GST) plays an important role in metabolic detoxification.GR catalyzes the formation of GSSG (oxidized form) to GSH (reduced form) thus maintaining the GSSG/GSH ratio in cells. The present study revealed that simultaneous exposure to lead and arsenic decreased the glutathione related enzyme activity and the antioxidant molecule GSH significantly.

Phagocytosis occurs when bacteria have adhered to the surface of the phagocyte. It involves recognition and attachment of a foreign particle, engulfment and digestion. A particle attached to the surface membrane initiates the ingestion phase by activating an actin-myosin contractile system which extends pseudopods around it. As adjacent receptors attach to the surface of the foreign particle, the plasma membrane is pulled around the particle until it is completely enclosed in a vacuole (phagosome). Then, cytoplasmic granules fuse with the phagosome and discharge their contents around the micro-organism, which is subjected to a considerable battery of microbicidal mechanisms. Upon simultaneous exposure of lead and arsenic, macrophages fail to perform its normal functions, probably by deactivating the actin-myosin contractile system, thereby allowing foreign particles or pathogens to gain access to the body which is clearly depicted in the results of the present study.

Professional phagocytes have evolved and are known to internalize and kill as many antigens as possible. The present study shows that macrophages upon simultaneous treatment of lead and arsenic fails to kill pathogens efficiently when compared with that of the control group. The present study clearly illustrates a greater percentage of viable bacteria within the multi- metal treated cells as compared to the control and the single metal treated groups which suggest that both lead and arsenic limits its intracellular killing activity after internalization of the pathogen.

Chemotaxis is the process by which phagocytic cells are attracted by various molecules and migrate to the sites of inflammation, tissue damage or immune reactions. Activated macrophages display cytoskeletal rearrangement and subsequent chemotaxis. It has been shown that chemotactic activation is mediated by a seven-transmembrane-spanning receptor coupled to heterotrimeric G protein, resulting in transduction of signals to the interior of cells and phosphorylation of multiple proteins (Jacobs *et al*., 1995; Haribabu *et al*., 1999). A central role of phosphatidyl inositol 3-kinase (PI3K) in innate immunity is to respond to chemoattractants (Fruman and Cantley, 2002; Stephens *et al*., 2002). The activation of mitogen-activated protein kinase (MAPK) seems to be another key component in signal transduction associated with cell migration (English *et al*., 1999). The results in the present study show a significant decrease in chemotactic migration of macrophages upon simultaneous exposure to lead and arsenic as compared to the control and the singly metal treated groups. Inhibition of macrophage migration probably involves lead and arsenic induced inhibition of PI3K activation with consequent reduction of phosphorylation of Akt and ERK1/2 thereby resulting in poor defense mechanism.

The results of the present study show a significant decrease in NO release upon simultaneous exposure to lead and arsenic on macrophages as compared to control and the single metal treated group. Inhibitory influence of toxic heavy metals like lead and arsenic might be exerted on the catalytic site of NOS by direct binding or by interference with electron transfer during catalysis, thus, suggesting the possibility of iNOS being sensitive to lead and arsenic. Huang *et al*., (2002) established that in the absence or too low a level of NO, IL-12 signaling in NK cells is blocked, thus reducing the IFN-γ release of NK cells (Diefenbach *et al*., 1999) and rendering the innate defense ineffective against invading pathogens. However, when NO escapes from the phagocytic milieu due to morphological

damage to macrophages exposed to xenobiotic agents like lead and arsenic, it reacts with singlet oxygen species to form peroxynitrite radicals that cause DNA fragmentation in the cells. Increase in DNA fragmentation, a marker of apoptosis, corroborates with previous studies that show heavy metal induced reactive nitrogen species induce the caspase cascades, probably through p53 mediated mechanism.

Myeloperoxidase (MPO) is a highly basic heme enzyme that is released extracellularly by activated neutrophils, monocytes and macrophages. MPO catalyses the oxidation of halide (Cl^-, Br^-) and pseudohalide (thiocyanate ion, SCN^-) ions by H_2O_2 (hydrogen peroxide) to the corresponding hypohalous acids: HOCl (hypochlorous acid), HOBr (hypobromous acid) and HOSCN (hypothiocyanous acid; cyanosulfenic acid) (Davies *et al*., 2008; Klebanoff, 2005; Kettle and Winterbourn, 2007). Upon simultaneous treatment of lead and arsenic the enzyme poorly performs its activity as it is clearly evident from the present study which depicts a significant fall in percentage of enzyme release when compared to control and single metal treated group.

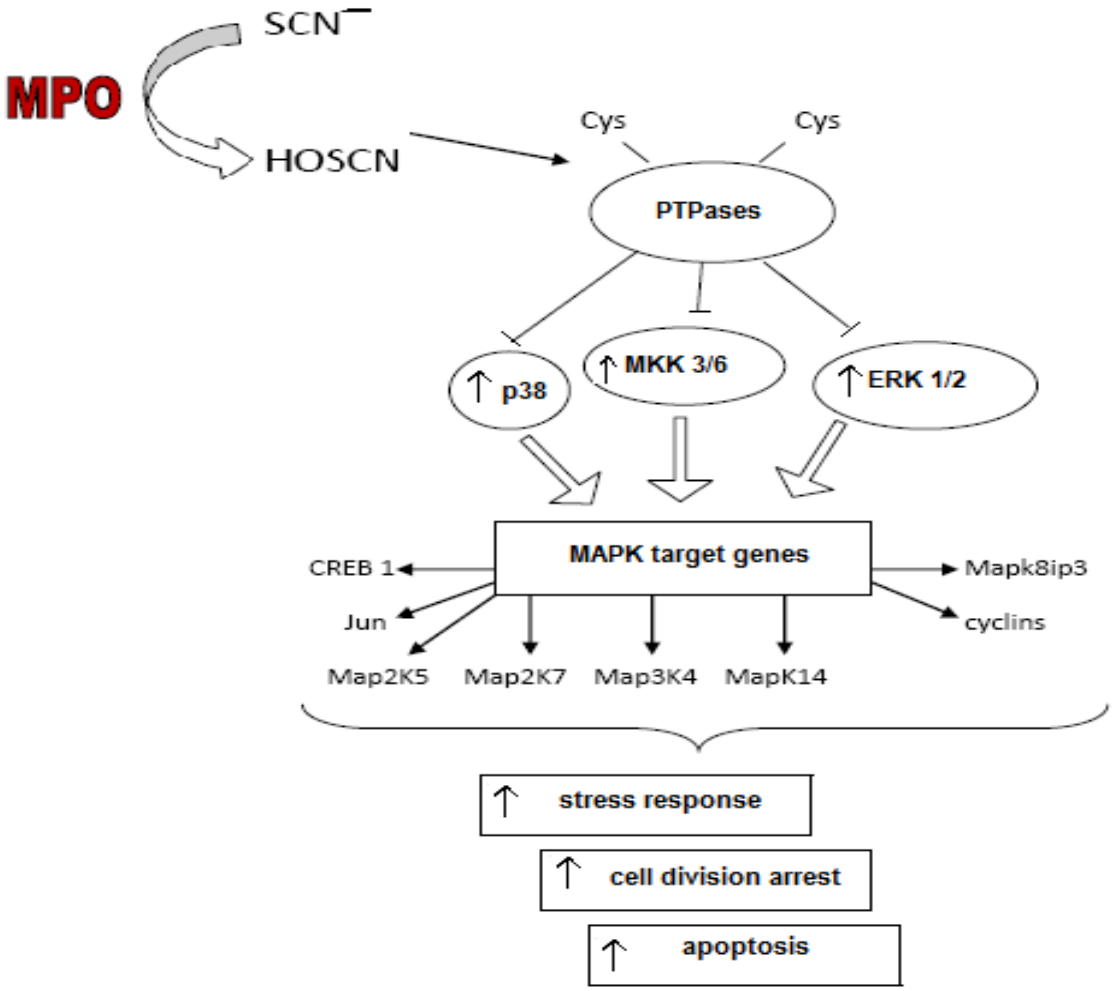

Figure 3: A flowchart showing how the activity PTPase is stimulated upon MPO catalysed SCN^- ion and thereby altering the functions of MAPK target genes leading to stress response.

Macrophages play an essential role for the initiation and activation of the innate immune system, by recognizing and releasing a large number of cytokines such as tumor necrosis factor- a (TNF- α), interleukin-1 (IL-1) and IL-6 (Nathan, 1987). The upregulation of secreted inflammatory cytokines by macrophages occurs upon the selective recognition and interaction of the pathogen-associated molecular patterns (PAMPs) that are conserved in bacterial species by the pattern recognition receptors (PRRs) such as the Toll-like receptor (TLR) proteins. Levels of TNF-α and IL- 1β was found to be significantly less in multi-

metal treated groups than that of control group and single metal treated group. Cytokine expression becomes downregulated, probably by the action of both the heavy metals at multiple levels viz., PAMP recognition, receptor dysfunction and/or by affecting the cell-signalling network rendering the fish to an immunocompromised and anti-inflammatory state. The following figure summarises the effects of lead and arsenic exposure in the fsh macrophages:

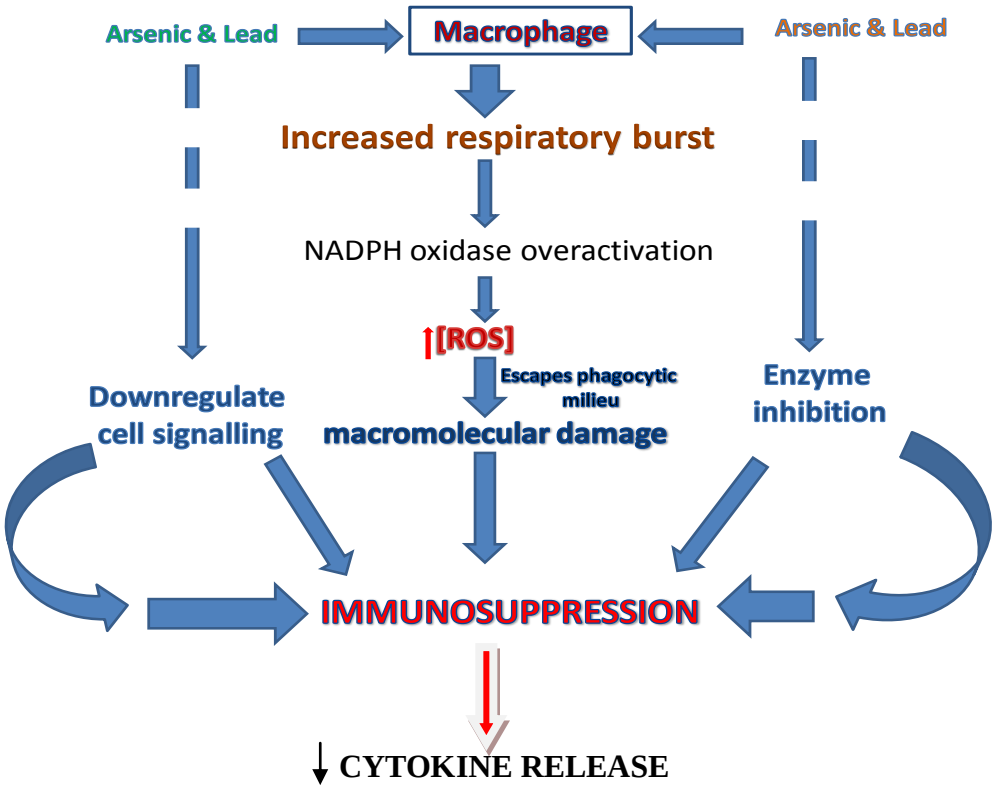

Figure 4: An overview of the general mechanism of action of lead and arsenic exposure in fish macrophages

5.4. Synergstic effects on CAT and NO release upon simultaneous exposure to lead acetate and sodium arsenite *in vivo* in macrophages of *Channa punctatus*

Interactivity is an important parameter for mixed wastes that is often overlooked during

hazard assessments. Since interactivity may increase the potential toxicity of a waste it should be considered in assessing the risks associated with mixtures of chronic toxicants. Unfortunately, there is limited data on the interactivity of many compounds. Interactivity can be synergistic, additive, antagonistic or potentiative. Synergistic interactions are those where the toxicity of a constituent increases the potential of another to give a combined effect.

Lead, arsenic, cadmium and chromium are frequently found together in soils of hazardous waste sites and contaminated water bodies where they co-occur. No adequate epidemiological or toxicological studies are available for the quaternary mixture. A drinking water study of a mixture of lead, cadmium and chromium in diethylnitrosamine- initiated rats gave no evidence of promoting activity of the mixture. Results of an intermediate – duration dietary study of toxicity and interactions for lead, arsenic and cadmium in rats indicated the effects of the trinary mixtures, suggesting that components based approaches that focus on interactions for the binary mixture might be useful in predicting the toxicity of the mixture.

Whether actual chemical transformations are responsible for the heightened toxicity is subject to further investigation. From the study on nitric oxide release from intestinal macrophages of fish exposed to increasing concentrations of arsenic and lead (singly and in combinations) as well as control, it was found that arsenic and lead exert a synergistic effect on each other as shown in the isobologram. Interactivity can be synergistic, additive, antagonistic or potentiative.

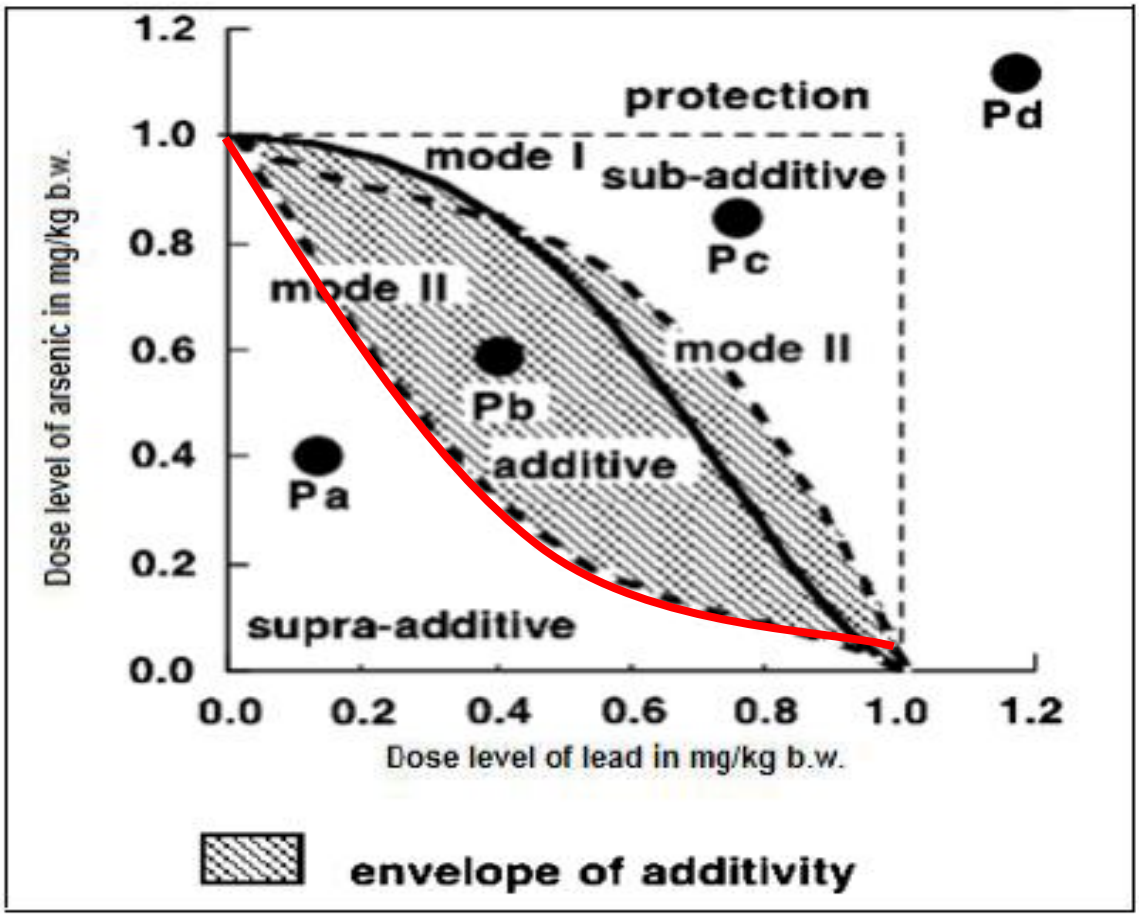

Figure 5: A model isobologram showing the envelope of additivity. The red line marks synergy between the metals lead and arsenic

Synergistic interactions are those where the toxicity of a constituent increases the potential of another to give a combined effect on various systems like

i) Innate immune function- Nitric oxide (NO)

ii) Antioxidant status- Catalase (CAT)

Multi-metal synergism has been proved by applying a multivariate ANOVA to the experimental results of NO release and constructing an isobologram running an ordinary least squares regression between effects (NO release) and dose levels of metals (single and multi-

metal) in log-linear form. In the isobologram, on plotting the concentrations of arsenic against the concentrations of lead at which effect (in this case NO release and/or catalase activity) remains constant a convex line showing synergism is demonstrated. Here, shortfall of NO release for treated group values (both single and multi-metal) from the mean NO release of the control group is supposed to reflect the impact of heavy metal exposure. The figure given above portrays that a simultaneous exposure to lead and arsenic has a synergistic effect as compared to the effects of independent exposure to them. Lead and arsenic are present as a fraction of their minimal effective concentrations. The exact mechanism of this multi-metal interaction on fish immune functions is yet to be elucidated.

It can therefore be suggested that exposure to lead and arsenic not only inhibit normal functional activities of fish macrophages, but also that, a simultaneous exposure to them, has a synergistic effect. The studies reported herein indicate that further issues await examination in determining the precise mechanism of lead and arsenic induced synergistic oxidative stress, altered antioxidant system and immunotoxicity.