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Heavy metal induced oxidative stress & its possible reversal by chelation therapy

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Exposure to heavy metals is a common phenomenon due to their environmental pervasiveness. Metal intoxication particularly neurotoxicity, genotoxicity, or carcinogenicity is widely known. This review summarizes our current understanding about the mechanism by which metalloids or heavy metals (particularly arsenic, lead, cadmium and mercury) induce their toxic effects. The unifying factor in determining toxicity and carcinogenicity for all these metals is the generation of reactive oxygen and nitrogen species. The toxic manifestations of these metals are caused primarily due to imbalance between pro-oxidant and antioxidant homeostasis which is termed as oxidative stress. Besides these metals have high affinity for thiol groups containing enzymes and proteins, which are responsible for normal cellular defense mechanism. Long term exposure to these metals could lead to apoptosis. Signaling components affected by metals include growth factor receptors, G-proteins, MAP kinases and transcription factors. Chelation therapy with chelating agents like calcium disodium ethylenediamine tetra acetic acid ($CaNa_2EDTA$), British Anti Lewisite (BAL), sodium 2,3- dimercaptopropane 1-sulfonate (DMPS), meso 2,3-dimercaptosuccinic acid (DMSA) *etc*., is considered to be the best known treatment against metal poisoning. Despite many years of research we are still far away from effective treatment against toxicity caused due to exposure to heavy metals/metalloids. The treatment with these chelating agents is compromised with number of serious side-effects. Studies show that supplementation of antioxidants along-with a chelating agent prove to be a better treatment regimen than monotherapy with chelating agents. This review attempts a comprehensive account of recent developments in the research on heavy metal poisoning particularly the role of oxidative stress/ free radicals in the toxic manifestation, an update about the recent strategies for the treatment with chelating agents and a possible beneficial role of antioxidants supplementation to achieve the optimum effects. We have selected only arsenic, lead, mercury and cadmium for this article keeping in view current concerns and literature available.

Key words Antioxidants supplementation - apoptosis - chelation therapy - combination therapy - heavy metal toxicity - oxidative stress

Although, many studies have reported the toxic and carcinogenic effects of metals in human and animals, it is also well known that these metals form a crucial part in normal biological functioning of cells. Several essential transition metals like copper, zinc, iron and manganese participate in controlling various metabolic and signaling pathways. However, their coordination chemistry and redox properties have provided them with an added advantage that these metals could escape out of the control mechanism such

as transport, homeostasis, compartmentalization and binding to designated cell constituents. They interact with protein sites other than those which are tailor-made for them by displacing other metals from their natural binding sites. Although, this process does not occur on a regular basis but such an action by metals could lead to malfunctioning of cells and eventually toxicity.

Metal induced toxicity is very well reported in the literature[1]. One of the major mechanisms behind heavy metal toxicity has been attributed to oxidative stress. A growing amount of data provide evidence that metals are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules[1]. One of the best evidence supporting this hypothesis is provided by the wide spectrum of nucleobase products typical for the oxygen attack on DNA in cultured cells and animals[2].

In-depth studies in the past few decades have shown metals like iron, copper, cadmium, mercury, nickel, lead and arsenic possess the ability to generate reactive radicals, resulting in cellular damage like depletion of enzyme activities, damage to lipid bilayer and DNA[3]. These reactive radical species include a wide variety of oxygen-, carbon-, sulfur- and nitrogen- radicals, originating not only from superoxide radical, hydrogen peroxide, and lipid peroxides but also in chelates of amino-acids, peptides, and proteins complexed with the toxic metals. These metals generate reactive species, which in turn may cause neurotoxicity, hepatotoxicity and nephrotoxicity in humans and animals[2, 3].

This review paper provide an overview of the current knowledge of toxic effects of metal induced oxidative stress and also suggest the possible measures which could reduce the toxic effects of metals in terms of reducing the concentration of toxic metal and achieve physiological recoveries. Since the list of metals is very long that are known to cause oxidative damage, we have confined our review to toxic effects of lead, arsenic, cadmium and mercury.

Lead

Lead (Pb) is not number one metal of the periodic table but its usage has made it number one. This metal is used since 5000 yr initiated. Lead became popular because of its dense, ductile, malleable and corrosion resistant properties[4]. These properties have made lead useful in building materials, pigments to glaze ceramics, water pipes and glass, paints and protective coatings and acid storage batteries and gasoline additives. Due to its wide applications and usage, exposure of humans to lead and its derivatives in day-to-day life is unavoidable. Lead poisoning is one of the oldest and the most widely studied occupational and environmental hazards[5].

Lead is known to induce a broad range of physiological, biochemical, and behavioural dysfunctions in laboratory animals and humans[5-7], including central and peripheral nervous systems[8], haemopoietic system[9], cardiovascular system[10], kidneys[11], liver[12], and male[13], and female reproductive systems[14]. Lead, however, was reported to have no pro-oxidant catalytic activity with respect to lipid peroxidation (LPO). Yiin and Lin[15] demonstrated a significant enhancement of malondialdehyde (MDA) when lead was incubated with linoic, linolenic and arachidonic acid. These initial studies for the first time and subsequent studies demonstrated that lead exposed animals showed increased lipid peroxidation or decrease in antioxidant defence mechanism[16,17]. A number of researchers have also shown enhanced rate of lipid peroxidation in brain of lead exposed rats[15-17]. They further went to show that the level of lipid peroxidation was directly proportional to lead concentrations in brain regions[18-20]. Similar effects were shown by Sandhir and Gill[21] in liver of lead exposed rats. Although the mechanism by which lead induces oxidative stress is not fully understood, a large number of evidences indicate that multiple mechanisms may be involved.

One of the prime targets to lead toxicity is the heme synthesis pathway. Lead affects this system by: (*i*) inhibiting the heme and haemoglobin synthesis; and (*ii*) changing the RBC morphology and survival; A schematic presentation of the effects of lead on heme synthesis is shown in Fig. 1. In this pathway, δ-aminolevulinic acid dehydratase (ALAD), a cytosolic sulfhydryl enzyme is the most sensitive enzyme to lead insult. It is reported that low blood lead levels (about 15 µg/dl) is sufficient to inhibit the activity of this enzyme[22]. Apart from this, lead also decreases the activity of ferrochelatase, the last step of heme synthesis. Failure of normal functioning of ALAD to convert 2 molecules of ALA into prophobilinogen decreases heme formation. This in turn stimulates ALA synthetase, the first enzyme of heme biosynthesis by negative feedback inhibition. As a result of this there is an increased accumulation of ALA and decreased formation of porphobilinogen resulting in the circulation

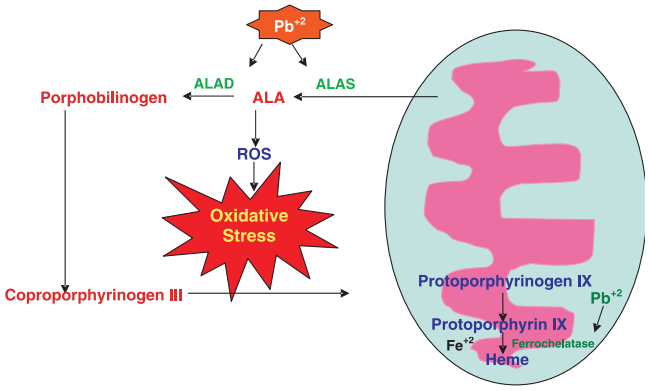

Fig. 1. Effect of lead on heme biosynthesis.

of ALA in blood and excretion in urine[23,24]. A number of studies have shown that accumulation of ALA induces ROS generation[25,26]. Bechara *et al*[27] in an introducing studies suggested the steps for ALA mediated ROS generation. It was suggested that first ALA enol form is generated by tautomerization. Secondly, ALA enol acts as an electron donor to molecular oxygen, together with an electron transfer from oxy Hb to oxygen resulting in methyl Hb, ALA radical, and H_2O_2 generation[27]. H_2O_2 and $O_2^{\bullet-}$, which are now present as a result of both ALA and ALA/oxyhemoglobin coupled autoxidation, can interact and generate $HO^{\cdot}$ radicals, which have the highest reactivity among ROS.

The hydroxyl radical formed in Haber Weiss reaction can react with cysteine-containing proteins to form thiyl radicals. These thiyl radicals may react with reducing agents like GSH in cells to form an intermediate that can react with molecular oxygen to form a glutathionylated protein and superoxide ion (Fig. 2).

Besides oxyhemoglobin, methemoglobin and other ferric and ferrous complexes have also been shown to trigger ALA oxidation[28]. Accumulation of ALA is now a well-accepted source of ROS and oxidative damage in the pathophysiology of lead intoxication. Fuchs *et al*[29] also provided evidence for the genotoxic effects of ALA. They demonstrated that the final oxidation product of ALA, *i.e.*, 4, 5-dioxovaleric acid, is an effective alkylating agent of the guanine moieties within both nucleoside and isolated DNA. They reported an increased levels of 8-oxo-7, 8-dihydro-29-deoxyguanosine and 5-hydroxy-29-deoxycytidine in DNA of rats chronically treated with ALA[29]. Inhibition of ferrochelatase to incorporate iron into protoporphyrin ring, leads to binding of zinc to protoporphyrin and form zinc protoporphyrin[30] (ZPP). The presence of ZPP is also used as an indicator for lead poisoning.

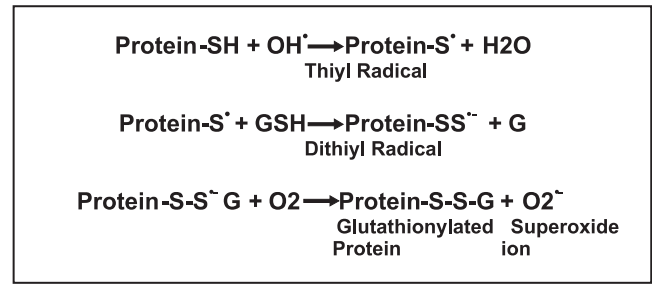

Fig. 2. Reaction of hydroxyl radical with sulfhydryl group containing protein and generation of superoxide ion.

Lead poisoning is a potential factor in brain damage, mental impairment and severe behavioural problems, as well as neuromuscular weakness, and coma[31]. Many authors attribute the neurological symptoms of lead poisoning to the ability of 5-aminolevulinic acid (ALA) to inhibit either the K^+-stimulated release of γ-aminobutyric acid (GABA) from preloaded rat brain synaptosomes or the binding of GABA to synaptic membranes[32]. Moreover, the developing organism presents a 5-fold greater absorption of lead and lacks a functional blood brain barrier[33]. Perinatal exposure to low levels of lead has been involved in behavioral and neurochemical alterations detected in both suckling and adult rats[34]. We also recently reported that lead causes neurological and behavioral changes in rats chronically exposed to lead acetate in drinking water. It was observed that lead increase ROS levels along with elevated intracellular Ca^{2+} which in turn causes a fall in the mitochondrial potential and lead to apoptosis via the cytochrome c release[35]. There was an excessive production of nNOS and MAO, depletion of GABA, 5HT and AchE, which are important neurotransmitters that control neurobehavioral changes[35]. Xu *et al*[36] too showed that lead could induce DNA damage and apoptosis in PC 12 cells, accompanied by an up regulation of Bax and down regulation of Bcl_2. Additionally, the expression of p53 increased, and caspase-3 was activated. Fox *et al*[34] based on observation by confocal microscopy, histological, and biochemical studies that elevated Ca^{2+} and/or Pb^{2+} were localized to photoreceptors and produced rod-selective apoptosis. Ca^{2+} and/or Pb^{2+} induced mitochondrial depolarization, swelling, and cytochrome c release. Subsequently caspase-9 and caspase-3 were sequentially activated. The effects of Ca^{2+} and Pb^{2+} were additive and completely blocked by the mitochondrial permeability transition pore (PTP) inhibitor cyclosporin A, whereas the calcineurin inhibitor FK506 had no effect. The caspase inhibitors carbobenzoxy-Leu-Glu-His-Asp-CH_2F and carbobenzoxy-Asp-Glu-Val-Asp-

CH_2F, but not carbobenzoxy-Ile-Glu-Thr-Asp-CH_2F, differentially blocked post-mitochondrial events. The levels of reduced and oxidized glutathione and pyridine nucleotides in rods were unchanged. The results demonstrate that rod mitochondria are the target site for Ca^{2+} and Pb^{2+}. Moreover, they also suggested that Ca^{2+} and Pb^{2+} bind to the internal metal (Me^{2+}) binding site of the PTP and subsequently opening PTP, which initiates the cytochrome c-caspase cascade of apoptosis in rods.

Another mechanism for lead-induced oxidative stress is on the antioxidant defense systems of cells. Several studies have shown that lead alters the activity of antioxidant enzymes like superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glucose 6-phosphate dehydrogenase (G6PD) and antioxidant molecules like GSH in animals[38] and human beings[39-41]. Although these findings suggest a possible involvement of oxidative stress in the pathophysiology of lead toxicity, it is not clear whether these alterations are the cause of the oxidative damage or a consequence of it[42]. Apart from ALAD, (G6PD), an thiol containing first enzyme of the pentose phosphate pathway, that provides extra mitochondrial NADPH to the cells through the oxidation of glucose-6-phosphate to 6-phosphogluconate, which in turn provide the NADPH to maintain constant levels of GSH to GR, mediates the conversion of GSSG to GSH. G6PD is particularly very crucial for the RBCs as they lack mitochondria. G6PD activity has been shown to measure in RBCs of lead treated rats[43] as well as RBCs of lead-exposed workers[44]. The SH groups of G6PD also play a crucial role in maintaining the enzymes tertiary structure[44]. Although, formation of lead-sulfhydryl complex was suggested as a plausible mechanism[44,45] but Lachant *et al*[46] provided evidence for lead-SH interactions between lead and G6PD by preventing the loss of G6PD activity when incubating the cells with thiol reagents (GSH and 2-mercaptoethanol) prior to incubation with lead. The same group suggested another mechanism for G6PD inhibition by lead via kinetic studies where lead is indicated as being a non-competitive inhibitor of both glucose-6-phosphate and NADP for G6PD. The authors concluded that inhibition of the pentose phosphate pathway might then render the lead-treated RBC more susceptible to oxidative damage[46]. However, the scenario in the *in vivo* system is much more complex for the effect of lead on G6PD. The important regulation of the pathway is $NADP^+$/NADPH ratio, which is known to change in favour of oxidized form under stress conditions. Gurer *et al*[43] reported an increase in G6PD activity in RBC of lead treated rats which was confirmed by few other studies[47,48]. However, contradicting results were also reported. Howard[49], Rausa[50] and Calderon-Salinas *et al*[51] showed a decreased G6PD activity whereas Rogers *et al*[52] showed no change in G6PD levels after lead intoxication. Hence, the available data suggests that lead exposure could increase or decrease G6PD activity depending on the concentration, duration and magnitude of oxidative stress after lead poisoning.

Wang *et al*[53] demonstrated that BALB/c dams which were exposed to 600 ppm of lead-acetate in drinking water during pregnancy and lactation showed elevated signs of plasma and brain lead and 5-aminolevulinic acid (ALA) concentrations of weaned pups. They also showed that activities of superoxide dismutase, glutathione peroxidase (GPx) and glutathione reductase (GR) decreased significantly in hypothalamus, corpora quadrigemina and corpus striatum.

The heavy metals, lead, mercury and cadmium, all have electron-sharing affinities that can result in the formation of covalent attachments mainly between heavy metal and sulphydryl groups of proteins. The tripeptide, glutathione (GSH), is found in mammalian tissues at millimolar concentrations and, therefore, accounts for more than 90 per cent of the total non-protein sulphur[54]. The intracellular levels of oxidized glutathione (GSSG) increase from metabolism of H_2O_2 by glutathione peroxidase and decrease from export of GSSG from the cell and from glutathione reductase and NADPH-mediated reconversion of GSSG to GSH[55]. GSH/GSSG ratios in normal mouse liver tissues range from 50 to 200[56]. Because of the low concentrations of GSSG relative to GSH, small increases in the oxidation of GSH to GSSG results in increase ROS and H_2O_2 production. Increase in GSSG will promote oxidation of protein cysteinyl thiols, shifting the equilibrium of thiol-disulfide exchange significantly in the direction of mixed disulfide formation and, changes protein conformation. Reduction of mixed disulfides, and reversion to the original protein conformation, is enzyme mediated by thiol reductants such as thioredoxin, glutaredoxin, and protein-disulfide isomerases[56,57]. Lead is known to deplete GSH level which result in the excess formation of GSH from cysteine via the γ-glutamyl cycle but GSH is usually not effectively supplied, if depletion continues because of chronic metal exposure. Several enzymes in antioxidant defense systems may protect the imbalance

between pro-oxidant and antioxidant but unfortunately, most of the enzymes contain sulfhydryl groups at their active site hence become inactive due to direct binding of lead to sulfhydryl group[58]. Zinc, which serves as a cofactor for most of the enzymes, is also replaced by lead, which is another factor behind the inactivation of enzymes.

The antioxidant enzymes SOD, catalase and GPx are potential targets of lead. Selenium is essential for GPx activity, and lead forms a complex with selenium, thereby decreases its activity[59]. Inhibition of heme synthesis by lead is well reported and since CAT is a heme-containing enzyme, its activity decreases[60]. SOD requires copper and zinc for its activity. Copper ions play functional role in the reaction by undergoing alternate oxidation whereas zinc ions seem to stabilize the enzyme[61]. Both the metal ions are replaced by lead, which decreases the activity of SOD.

Overall, these inhibitory effects of lead on various enzymes would probably result in impaired antioxidant defences by cells and render cells more vulnerable to oxidative attacks (Fig. 3).

Arsenic

Arsenic is the 33rd element of the Periodic table of elements with the most common oxidation numbers of +5, +3, and -3. Arsenic has the capability to form both inorganic and organic compounds in the environment and human body. One of the most common sources of arsenic contamination is drinking water, where concentrations could range from 0.01 mg/l to 4 mg/l[62]. There are numerous geographical locations across the world where high levels of arsenic in the ground waters has caused great concern, especially in the Indo-Bangladesh region where over a million people are reported to be suffering from arsenic poisoning. This kind of slow, low level, inevitable poisoning has caused serious concerns about the health of all living species in such areas. Inorganic arsenic exists mainly in 2 forms arsenite (As^{III}) and arsenate (As^{V}). While arsenite has a tendency to readily react with the sulfhydryl groups of proteins and this turn inhibit biochemical pathways, arsenate acts as a phosphate analogue and interferes with phosphorylation reactions[63]. Most of the absorbed arsenate is reduced to arsenite in blood; the toxic effects manifested by both the molecules are quite similar. However, the trivalent species (arsenite) is considered to be the biologically active form and the major source to arsenic toxicity. Apart from possessing the property for biochemical toxicity, arsenic is also well documented for its carcinogenic effects. Exposure to arsenic is linked with a risk of developing tumors of the lung, skin, liver, bladder, and kidney[64]. However, arsenic is neither classified as an initiator nor a promoter of carcinogenic agents. It probably does not act as a classical carcinogen, but rather enhances the carcinogenic action of other carcinogens[65]. Arsenic exposure is also known to cause alterations in neurotransmitters level[66]. Besides being carcinogenic, arsenic compounds have been used as medicine to treat acute promyelotic leukemia (APL)[67]. The inorganic arsenics can be either methylated (monomethylarsonic acid, MMA) or dimethylarsinic acid (DMA) *in vivo*. Recent *in vivo* studies have also indicated that methylated forms of arsenic may also serve as co-carcinogens or tumor promoters[67].

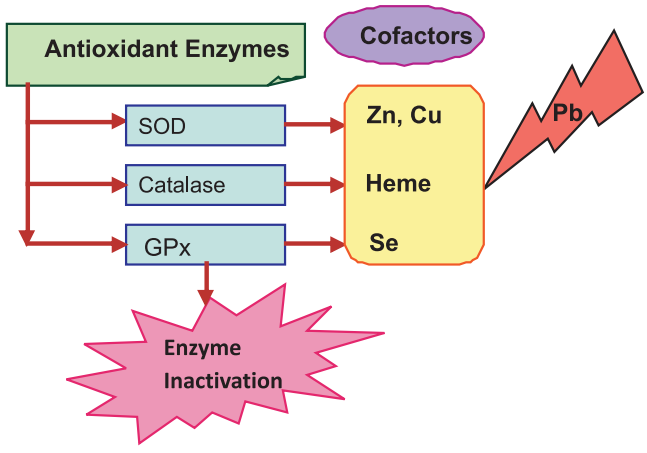

Fig. 3. Effect of lead on various antioxidant enzymes and their cofactors leading to inactivation of enzyme activity.

Arsenic and oxidative stress

Arsenic is one of the most extensively studied metals that induce ROS generation and result in oxidative stress[68]. Shi *et al*[68] provided evidence that arsenic generates free radicals that leading to cell damage and death through the activation of oxidative sensitive signaling pathways. Arsenic is known not only to produce ROS but also superoxide ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), the peroxyl radical ($ROO^{\bullet}$), nitric oxide ($NO^{\bullet}$)[69], hydrogen peroxide (H_2O_2), dimethylarsinic peroxyl radicals $(CH_3)_2AsOO^{\bullet}$ and also the dimethylarsinic radical $(CH_3)_2As^{\bullet}$[70]. However, the exact mechanism responsible for the generation of these reactive species is not yet clear, but some studies proposed the formation of intermediary arsine species[71].

Iwama *et al*[72] showed that when U937 cells were exposed to arsenic at a concentration of 1-10 µM there was generation of detectable levels of super-oxide. Similar studies in different cell types like, human-hamster hybrid cells[70] and human vascular smooth muscle cells (VSMC)[74] have shown the generation of $O_2^{\bullet-}$ radicals during arsenic treatments. EPR spin trapping with DMPO and ERP spectroscopy too have detected superoxide and hydrogen peroxide levels in human keratinocytes cell line[75] and vascular endothelial cells[76].

The induction of H_2O_2 too has been observed in HEL30 cells[77], NB4 cells[78], and CHOK1 cells. Cantoni and co workers[79] demonstrated that CHO cells that were H_2O_2 resistant also conferred resistance to arsenite insult providing evidence that arsenic mediated toxicity is mediated through H_2O_2. It is also suggested that arsenite promotes the production of $^{\bullet}OH$ from H_2O_2 in CHO-K1 cells[80]. These results indicate that $O_2^{\bullet-}$ is likely the primary species induced by arsenic in various types of cells, and the formation of $O_2^{\bullet-}$ leads to a cascade of other ROS species such as H_2O_2 and $^{\bullet}OH$ by $O_2^{\bullet-}$ dismutation and Fenton reaction.

The above reports have demonstrated that arsenic exposure results in the generation of ROS in various cellular systems (Fig. 4). However, the source or mechanism of ROS formation remains elucidative. A number of hypothesis and results have suggested that mitochondria could be one of the major sources of ROS production. Corsini *et al*[81] showed that addition of rotenone, a complex I inhibitor of the mitochondrial respiratory chain, could completely abrogate the generation of cellular ROS induced by arsenite in HEL 30 cells. Apart from this, ubiquinone site in another place, which is susceptible to arsenite, induced ROS

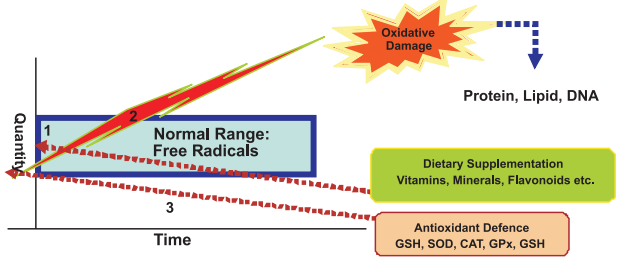

Fig. 4. Diagram showing relation between quantity of free radicals and time. **1**; shows normal range of free radicals with the passage of time, **2**; shows overproduction of free radicals which leads to oxidative damage of biomolecules, **3**; shows lower concentration of free radicals which is maintained either through dietary supplementation or antioxidant defense system of the body.

generation[68]. Samikkannu *et al*[82] recently showed that arsenite can inhibit pyruvate dehydrogenase (PDH) activity by binding to the vicinal dithiols in both the pure enzyme and tissue extract. There are three other sources in the mitochondria that have been proposed as sources of ROS generations, firstly, the intermediary arsine species that may be formed[83]. Radical species analysis using EPR techniques have detected appearance of $(CH_3)_2AsOO^{\bullet}$, as a product of dimethylarsine and molecular oxygen reactions. This dimethylarsenic peroxyl radical is assumed to play a major role in DNA damage and may produce superoxide anion during the process[81,83]. Secondly, methylated arsenic species can release redox-active iron from ferritin and this free iron could play a role in generating reactive oxygen species by promoting conversion of $O_2^{\bullet-}$ and H_2O_2 into the highly reactive $^{\bullet}OH$ radical through the Haber–Weiss reaction[84]. Thirdly, ROS may also be formed during the oxidation of arsenite to arsenate[85].

Arsenic is known not only to generate reactive oxygen species (ROS) but reactive nitrogen species (RNS) through the damage of lipid membranes and DNA[63]. Arsenic is also the most well studied heavy metal in the area of NO production in biological systems. However, NO production induced by arsenic is currently controversial[68]. $NO^{\bullet}$ is a messenger molecule that plays an important role in the immune response, neurotransmission and vasodilatation. Several conflicting reports concerning arsenic-induced production of $NO^{\bullet}$ have been published. Pi *et al*[69] reported that prolonged exposure to arsenic impairs production of endothelial NO in human blood. On the other hand, porcine aortic endothelial cells did not show any increase in NO production on arsenite exposure[83]. Similar results too were obtained with hepatocytes and human liver cells[84]. Lynn *et al*[71] have shown increase in the nitrite levels in CHO-K1 cells. This increase in nitrite levels suggested NO production. Increased NO production also has been observed in C3H10T1/2 cells[88]. It appears that the stimulation of NO production by arsenite is through activation of endogenous NO synthase. Free radicals could also be generated by flavin enzymes such as NAD(P)H oxidase and NO synthase with arsenic exposure. In cultured cells, arsenic is shown to up regulate NAD(P)H oxidase gene expression of $p22^{phox}$ and translocation of Rac1[89], thus enhancing $O_2^{\bullet-}$ production. Although arsenic is known to generate ROS but reports also suggest that mono-methylarsonous which is produced from arsenic covalently binds to the

reactive thiols of endothelial NO synthase, resulting in its enzyme activity[90] (Fig. 5).

It is well known that ROS play a significant role is altering the signal transduction pathway and transcription factor regulation. Numerous reports have indicated that arsenic affects transcriptional factors either by activation or inactivation of various signal transduction cascades. In Fig. 5, we have tried to show some of the effects of arsenic (III) on alteration of signal transduction pathways. Arsenic-mediated activation of MAPK signalling through the EGFR/MEK, EGFR/Ras/MEK or Src/EGFR cascade has been reported in number of cell lines[91,92].

Oxidative stress is an imbalance between free radical generation and the antioxidant defense system. Many reports evidenced a decrease in the levels of antioxidants after arsenic exposure. Decreased antioxidant levels in plasma from individuals exposed to arsenic in Taiwan have been reported by Wu *et al*[93]. They showed that there was a significant inverse correlation between plasma antioxidant capacity and arsenic concentration in whole blood. Several papers have reported decreased levels of GSH after exposure to arsenic[94,95]. GSH, a tripeptide, plays an important role in maintaining cellular redox status and its level is considered a significant marker of oxidative stress.

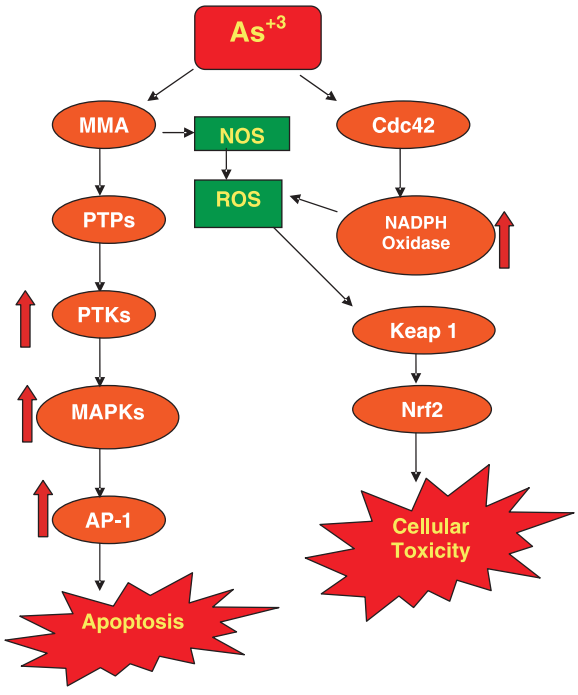

Fig. 5. Arsenic induced ROS generation and its impact on cellular pathways

Following three pathways may decrease cellular levels of GSH (*i*) GSH possibly acts as an electron donor for the reduction of pentavalent to trivalent arsenicals (*ii*) arsenite has high affinity to GSH and (*iii*) oxidation of GSH by arsenic-induced generation of free radicals. Taken together, exposure to arsenite is likely to cause depletion of GSH level. We too have shown that arsenic exposure not only decreased GSH levels but also reduces the levels of glutathione reductase (GR). We also showed that reduced GR levels leads to an increase in GSSG levels which contribute in elevation of arsenic toxicity in guinea pigs[96].

Generation of reactive oxygen species, alterations in the signal cascade and an imbalance in antioxidant levels, in turn triggers cellular apoptosis in cells. The action of arsenic-induced apoptosis is complex. H_2O_2 is apparently involved in the induction of apoptosis by arsenite[89]. H_2O_2 may play a role as a mediator to induce apoptosis through release of cytochrome c to cytosol, activation of CPP32 protease, and PARP degradation[68]. Reports have shown that generation of free radicals triggered apoptosis in various cell lines like NB4 cells[78] and CHO-K1 cells[97] when exposed to arsenite. The resulting oxidative stress may also affect the levels and functions of redox-sensitive signaling molecules, such as AP-1, NF-κB, and p53, derange the cell signaling and gene expression systems, and/or induce apoptosis. Both AP-1 and NF-κB are considered stress response transcription factors that govern the expression of a variety of pro-inflammatory and cytotoxic genes[98]. p53 gene is an important tumor-suppressor gene whose protein product plays an important role in cell cycle control, apoptosis, and control of DNA repair. Both NF-κB and AP-1 are modulated in various cells exposed to arsenic. Arsenite has shown to alter AP-1 and NF-κB in BEAS-2B cells[99], HEL30 cells[81], human MDA-MB-435 breast cancer and rat H4IIE hepatoma cells[100].

On one hand, arsenic causes oxidative stress, as determined by 8-OHdG formation[101], lipid peroxide production through reactive oxygen species generation, reduction of glutathione (GSH) content[97], and increased levels of antioxidant proteins such as heme oxygenase-1 (HO-1), A170, and peroxiredoxin 1 (PrxI)[102]. On the other hand, arsenic-mediated cytotoxicity is thought to be due to high accumulation of this metalloid in the cells. Thus, it is likely that mammals, including humans, would possess some transcription factor(s) regulating proteins that play a critical role in the cellular defense against oxidative stress and the cellular accumulation

of arsenic. Nuclear factor-erythroid 2-related factor 2 (Nrf2) is a basic-leucine zipper transcription factor that activates the antioxidant responsive element (ARE) and electrophilic responsive element (EpRE), thereby upregulating the expression of a variety of downstream genes[104]. Normally, Nrf2 is bound to an inactive complex Kelch -like ECH associated protein (Keap 1)[103,104]. Once, Keap 1 is modified with radicals, Nrf2 is dissociated from the complex and translocates from the cytosol to the nucleus and binds to the promoter region and stimulate gene expression of proteins like antioxidant proteins, Phase II xenobiotics - metabolizing enzymes and Phase III transporters proteins.

Cadmium

Cadmium is the 48th element and a member of group 12 in the Periodic table of elements. The most common oxidation number of cadmium is +2. About 13,000 tons of cadmium is produced yearly worldwide, mainly for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings and alloys. The toxicity of cadmium relates to smelting where the main route of exposure is through the lungs. Soluble cadmium salts accumulate and result in toxicity to the kidney, liver, lungs, brain, testes, heart, and central nervous system. Cadmium is listed by the US Environmental Protection Agency as one of 126 priority pollutants. The most dangerous characteristic of cadmium is that it accumulates throughout a lifetime. Cadmium accumulates mostly in the liver and kidney and has a long biological half-life of 17 to 30 yr in humans[105]. Cadmium can cause osteoporosis, anemia, non-hypertrophic emphysema, irreversible renal tubular injury, eosinophilia, anosmia and chronic rhinitis. Cadmium is a potent human carcinogen and has been associated with cancers of the lung, prostate, pancreas, and kidney. Because of its carcinogenic properties, cadmium has been classified as a #1 category human carcinogen by the International Agency for Research on Cancer of USA[106].

Cadmium, unlike other heavy metals is unable to generate free radicals by itself, however, reports have indicated superoxide radical, hydroxyl radical and nitric oxide radicals could be generated indirectly[107]. Watanabe *et al*[108] showed generation of non-radical hydrogen peroxide which by itself became a significant source of free radicals via the Fenton chemistry. Cadmium could replace iron and copper from a number of cytoplasmic and membrane proteins like ferritin, which in turn would release and increase the concentration of unbound iron or copper ions. These free ions participate in causing oxidative stress via the Fenton reactions[109,110]. Recently, Watjen and Beyersmann[111] showed evidence in support of the proposed mechanism. They showed that copper and iron ions displaced by cadmium, were able to catalyze the breakdown of hydrogen peroxide via the Fenton reaction[111].

Casalino *et al*[112] proposed that cadmium binds to the imidazole group of the His-74 in SOD which is vital for the breakdown of hydrogen peroxide, thus causing its toxic effects. Cadmium inhibition of liver mitochondrial MnSOD activity was completely removed by Mn(II) ions, suggesting that the reduced effectiveness of this enzyme is probably due to the substitution of cadmium for manganese. These authors also observed antioxidant capacity of Mn(II) ions, since they were able to normalize the increased TBARS levels occurring when liver mitochondria were exposed to cadmium.

Numerous reports in animal model have depicted that cadmium intoxication significantly increased the malondialdehyde (MDA) and glutathione peroxidase (GSH-Px)[113-114]. Free radicals generated by cadmium were scavenged by GSH directly or via the GSH peroxidase/GSH system. Acute intoxication of animals with cadmium has shown increased activity of antioxidant defense enzymes like copper-zinc containing superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase[115].

Apart from oxidative stress mediated toxicity, cadmium is also known to cause its deleterious effect by deactivating DNA repair activity[116]. Although, there are a number of mechanism that exists to prevent DNA mismatch like direct damage reversal, base excision repair, nucleotide excision repair, double stand break repair and mismatch repair (MMR) but cadmium inhibits only MMR mode of repair. Jin *et al*[117] showed that cadmium-induced inhibition of MMR in human extracts leaves about 20-50 per cent of DNA mismatch unrepaired[117]. Inhibition of MMR leads to the propagation of cellular errors, thus the toxic effects of cadmium can be amplified in cells by creating mutations in genes that induce further faulty functions. Studies have also shown that the number of cells with DNA single strand breaks and the levels of cellular DNA damage was significantly higher in

cadmium exposed animals. Interaction of cadmium with essential nutrients has been summarised in Fig. 6.

Reports have shown that antioxidants like vitamin C and Vitamin E have shown protection against cadmium induced toxicity in different animal models[115,118]. Supplementation of these natural antioxidants reduced ROS levels, lipid peroxidation, haematological values and enzymatic and non-enzymatic components of antioxidant defence system. Contrast to these reports, Cosic *et al*[119] showed that presence of antioxidants like cysteine, glutathione and ascorbate induced more DNA damage in *in vitro* experiments. This DNA damage was considered to be due to the generation of reactive species. They also suggested that cadmium binds covalently with DNA and forms intrastrand bifunctional AT adducts. These results are in agreement with the cadmium displacement theory and deleterious effects of transition metal ion induced pro-oxidant effects of ascorbate[120,121]. The protective role of melatonin, an effective antioxidant and free radical scavenger, against cadmium was studied[121]. Melatonin slightly, but not significantly, reduced cadmium-induced lipid peroxidation in the testes. It is concluded that cadmium toxicity, at least with respect to the resulting lipid peroxidation, is reduced by the administration of melatonin.

Mercury

Mercury is the 80th element of the Periodic table of elements. Mercury is unique in that it is found in nature in several chemical and physical forms. At room temperature, elemental (or metallic) mercury exists as a liquid with a high vapor pressure and consequently is released into the environment as mercury vapor.

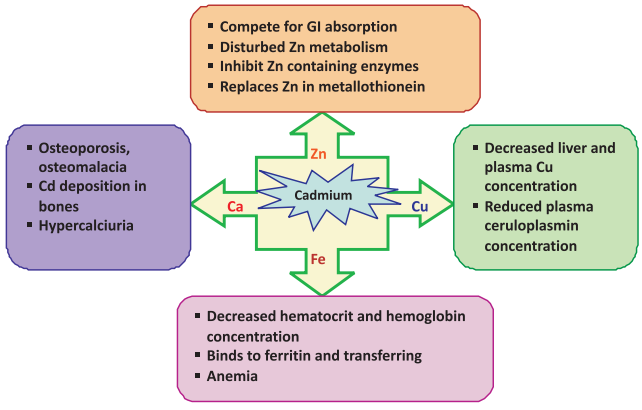

Fig. 6. Interaction of cadmium with essential nutrients by which it causes its toxic effects.

Mercury also exists as a cation with an oxidation state of +1 (mercurous) or 2+ (mercuric). Of the organic forms of mercury, methyl mercury is the most frequently encountered compound in the environment. It is formed mainly as the result of methylation of inorganic (mercuric) forms of mercury by microorganisms in soil and water. In the environment, humans and animals are exposed to numerous chemical forms of mercury, including elemental mercury vapor (Hg), inorganic mercurous (Hg (I)), mercuric (Hg (II)) and organic mercuric compounds[122]. Environmental mercury is ubiquitous and consequently it is practically impossible for humans to avoid exposure to some form of mercury. All forms have toxic effects in a number of organs, especially in the kidneys[123]. Elemental, inorganic, and organic forms of mercury exhibit toxicologic characteristics including neurotoxicity, nephrotoxicity, and gastrointestinal toxicity with ulceration and hemorrhage. However, organic mercury has a lesser insult on the kidneys. Pars recta of the proximal tubules of the nephrons are the most susceptible region for the toxic effects of mercury[123]. Mercurous and mercuric ions impart their toxicological effects mainly through molecular interactions for instance mercuric ions have a greater affinity to bind to reduced sulfur especially in the thiol containing molecules like GSH, cysteine, and metallothionein (MT)[124]. However, the binding affinity of mercury to oxygen and nitrogen atoms is relatively very low when compared to sulfur[63]. Therefore, toxic effects in the kidneys are mainly governed by the biological interactions between MT, GSH and albumin[125]. Once inorganic mercuric ions gain entry into proximal tubular cells, it appears that they distribute throughout all intracellular pools[126,127]. The cytosolic fraction was found to contain the greatest content of mercury. Interestingly, the relative specific content of mercury was shown to increase to the greatest extent in the lysosomal fraction when rats were made proteinuric with an aminoglycoside or when rats were treated chronically with mercuric chloride[128]. Although the current model of mercury induced nephrotoxicity revolve around the conjugation of mercury ions with GSH and cysteine, other thiols especially homocysteine and NAC too play a vital role in handling mercury in the kidneys[129,130].

One of the major molecules that help in scavenging and reducing the toxic effects of mercury is metallothionein, a small, low molecular weight (6-7 kDa) protein, rich is sulfhydryl groups[131]. MT induction is not only seen with Hg but various other metals like

Cd, Zn and Cu. Zalups and Cherian[132] demonstrated that a single, daily non toxic dose of mercury chloride could double the levels of MT in the renal cortex of rats. It is not just mercury chloride but even mercury vapours have shown to elevate the levels of MT[133].

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. Lund *et al*[134] demonstrated that administration of mercury resulted in GSH depletion, lipid peroxidation and also increased the formation of H_2O_2 in the kidneys of rats. Lund and coworkers[135] further demonstrated that it was the mitochondria of the rat kidney which were responsible for oxidative stress. In the in vitro experiment they showed that when mitochondria was supplemented with the respiratory chain substrate (succinate or malate) and blocker of complex I (rotenone) or complex III (antimycin A), there was a 4-fold increase in the H_2O_2 formation with inhibition of complex III and a 2 fold increase with complex I inhibition[135].

Mahboob *et al*[136] showed that when CD-1 mice were exposed to mercuric chloride, there were alterations in the lipid peroxidation (LPO), glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD) and GSH levels in different organs apart from kidneys[137]. Toxic effects of mercury have also been observed in oligodendrocytes, astrocytes, cerebral cortical and cerebellar granular neurons obtained from embryonic and neonatal rat brains[137].

Toxic insult of mercury also induces a number of stress proteins[138,139]. These large groups of proteins include heat shock proteins (HSPs) and glucose regulated proteins (GRPs). Papaconstantinou *et al*[138] showed an enhanced *de novo* synthesis of several stress proteins when chick embryos were exposed to mercury. Goering *et al*[139] too evaluated the differential expression of 4 HSPs in renal cortex and medulla of rats exposed to mercuric chloride. It has also been demonstrated that there is a time and dose dependent accumulation of HSP72 and GRP94 stress proteins on mercury (II) exposure[136]. While the accumulation of HSP72 was localized in the cortex, the GRP94 was accumulated in the medulla. In whole kidney, Hg (II) induced a time- and dose-related accumulation of hsp72 and grp94. Accumulation of hsp72 was predominantly localized in the cortex and not the medulla, while grp94 accumulated primarily in the medulla but not the cortex. The high, constitutive expression of hsp73 did not change as a result of Hg (II) exposure, and it was equally localized in both the cortex and medulla. Hsp90 was not detected in kidneys of control or Hg-treated rats[63].

Treatment for heavy metal poisoning

Chelation Therapy:

The term chelation comes from the Greek word "chelate" which means claw (Fig. 7). 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. The first experimental use of a chelator against metal poisoning was Kety and Letonoff's[140] attempt to use citrate as an antidote towards acute lead intoxication in 1941. This experiment signaled a new way of thinking in the treatment of acute and chronic metal intoxication. In most studies with chelating agents to treat cases of metal intoxication, focus has been primarily on the mobilization (mainly due to renal excretion) of toxic metal. As the important end point of chelation should be reduction of metal

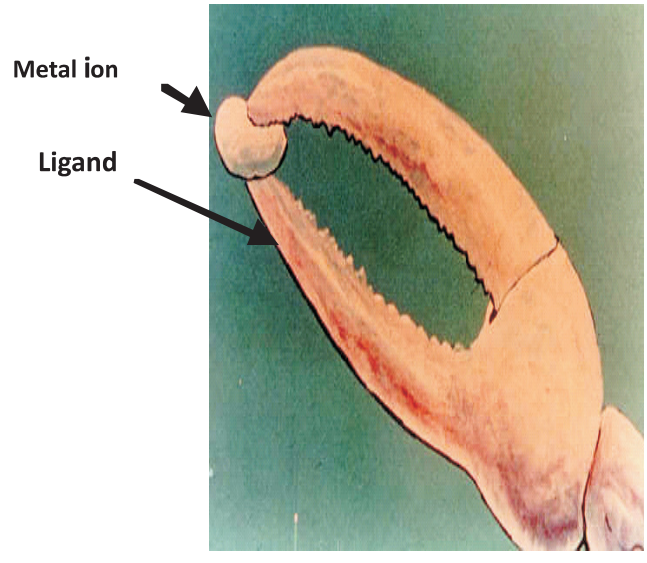

Fig. 7. Binding of ligand with metal ion gives a claw like structure know as chelate.

toxicity. Thus, a chelating agent forming a stable complex with a toxic metal may shield the metal ion from biological targets, thereby reducing the toxicity, even at times after administration where mobilization has not yet occurred, or it may expose the metal to the biological environment and prevent the metal from being scavenged by biological protective mechanisms and thereby increase the toxicity of the metal[141].

During the Second World War, 2,3-dimercaptopropanol (BAL) was developed as an experimental antidote against arsenic based war gases[142,143]. However, BAL is far from being an ideal chelator due to its high toxicity and the high frequency of various side effects. Increased brain deposition due to BAL administration has been reported for arsenite and organic mercury compounds, and BAL increased the toxicity of cadmium and lead in animal experiments[144].

The characteristics of an ideal chelator include: (*i*) greater affinity for the toxic metal; (*ii*) low toxicity; (*iii*) ability to penetrate cell membrane; (*iv*) rapid elimination of metal; and (*v*) higher water solubility.

Few conventional chelators

Calcium disodium ethylene diamine tetra acetic acid ($CaNa_2EDTA$)

$CaNa_2EDTA$ is a derivative of ethylene diamine tetra acetic acid (EDTA); a synthetic polyaminocarboxylic acid and since 1950s has been one of the main stays for the treatment of childhood lead poisoning[145]. Calcium salt of EDTA has been successfully utilized as a diagnostic agent for the assessment of body stores of lead. It has the LD_{50} value of16.4 mmol/kg in mouse. In addition to urinary excretion of lead $CaNa_2EDTA$ is responsible for the excretion and depletion of essential metals like Zn, Cu, Fe, Co and Mn because of its relative lack of specificity. Treatment with $CaNa_2EDTA$ resulted in rapid decrease in plasma zinc concentrations. According to a study done by Slechta *et al*[146], the rise in brain lead content in response to a single injection of 150 mg/kg of $CaNa_2EDTA$ was observed in rats exposed to 25 and 50 ppm of lead acetate. $CaNa_2EDTA$ cannot pass through cellular membranes and therefore its use is restricted to removing metal ions from their complexes in the extra cellular fluid. Another drawback with the EDTA treatment reported recently was redistribution of lead from the hard tissue deposits to soft organs[35,145,146]. Calcium salt of EDTA has the major toxic effects on the renal system causing the necrosis of tubular cells. Severe hydropic degeneration of proximal tubule cells has also been reported. These lesions along with some alterations in the urine like hematuria, proteinuria and elevated BUN are generally reversible when the treatment ceases. Thus $CaNa_2EDTA$ could not be regarded as a drug of choice against lead poisoning.

British Anti Lewisite (BAL)

2, 3-dimercaprol (BAL) is a traditional chelating agent that has been used clinically in arsenic poisoning since 1949. It is an oily, clear, colorless liquid with a pungent, unpleasant smell typical of mercaptans and having short half life. In humans and experimental models, the antidotal efficacy of BAL has been shown to be most effective when administered immediately after the exposure. Because of its lipophilic nature it is distributed both extra-cellular and intra-cellular sites. BAL is unstable and easily oxidized and therefore difficult to store, so require ready to use preparation. Beside rapid mobilization of arsenic from the body, it causes a significant increase in brain arsenic[143]. Due to its oily nature, administration of BAL requires deep intra-muscular injection that is extremely painful and allergic. Other side effects include vomiting, headache, lachrymation, rhinorrhea and salivation, profuse sweating, intense pain in the chest and abdomen and anxiety.

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[147]. DMSA has been tried successfully in animal as well as in cases of human arsenic poisoning[148]. 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[149]. Animal studies suggest that DMSA is an effective chelator of soft tissue but it is unable to chelate lead from bones[147]. We have characterized earlier that 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[150]. 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.

New chelating agents

Recently some mono and diesters of DMSA especially the higher analogues have been developed and tried against cases of experimental heavy metal poisoning. Mono and dimethyl esters of DMSA that have been studied experimentally with the aim of enhancing tissue uptake of chelating agents. In order to make the compounds more lipophilic, the carbon chain length of the parent DMSA was increased by controlled esterification with the corresponding alcohol (methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl, isopentyl and hexyl; Fig. 8). Walker *et al*[151] studied the effects of seven different monoalkyl esters of DMSA on the mobilization of lead in mice and observed that after a single parenteral dose of the chelator DMSA there was a 52 per cent reduction in the lead concentrations while with the monoesters the reduction varied from 54 to 75 per cent. Important esters of DMSA are as below:

Monoisoamyl DMSA (MiADMSA)

Monoisoamyl ester of DMSA (MiADMSA; a C_5 branched chain alkyl monoester of DMSA) has been found to be the most effective[152,153]. Mehta and Flora[154] reported for the first time the comparison of different chelating agents (3 amino and 4 thiol chelators) on their role on metal redistribution, hepatotoxicity and oxidative stress in chelating agents induced metallothionein in rats. Mehta *et al*[155] 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[154]. These drawbacks open the search for new treatment which 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.

Monomethyl DMSA (MmDMSA) and monocyclohexyl DMSA (MchDMSA)

MmDMSA has a straight and branched chain methyl group while MchDMSA has a cyclic carbon chain. Thus they can have better lipophilicity characteristic and might penetrate cells more readily that extra-cellularly acting chelating agent like DMSA. Both these chelating agents are orally active. Jones *et al*[156] in their *in vivo* study on male albino mice exposed to cadmium for seven days observed that administration of MmDMSA and MchDMSA produced significant reductions in whole body cadmium levels. Further, no redistribution of cadmium in brain was observed. The *in vivo* evaluation of these monoesters derived from higher alcohols (C_3 - C_6 monoesters) proved to have better efficacy as compared to the monoesters derived from lower ones (C1 - C2 monoesters)[156]. Their oral administration improves their advantage in the clinical treatment of heavy metal toxicity however, extensive studies are required to reach at a final conclusion.

Role of antioxidants in the treatment of metal poisoning

Antioxidants (AOX) are substances, which inhibit or delay oxidation of a substrate while present in minute amounts. The most important source of AOX is provided by nutrition[157]. Antioxidant molecules are thought to play a crucial role in counteracting

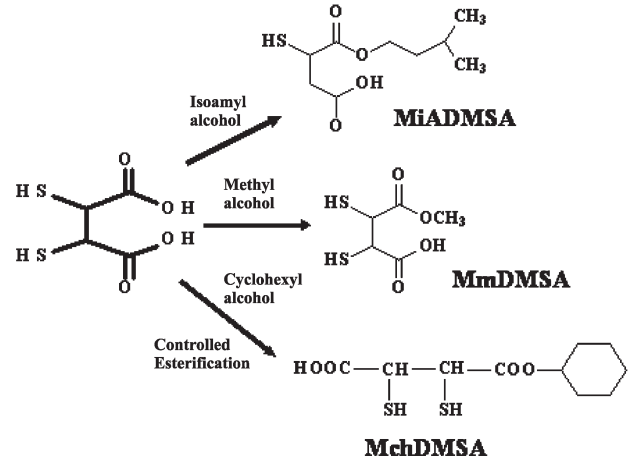

Fig. 8. Synthesis of monoesters of DMSA by controlled esterification process.

free radical induced damage to macromolecules and has been found to heel the free radical mediated cell damage. Nutritional antioxidants act through different mechanisms and in different compartments, but are mainly free radical scavengers: (*i*) they directly neutralize free radicals, (*ii*) they reduce the peroxide concentrations and repair oxide membranes, (*iii*) they quench iron to decrease ROS production, (*iv*) via lipid metabolism, short-chain free fatty acids and cholesteryl esters neutralize ROS[157]. Ramanathan *et al*[158] evaluated the molecular changes during arsenic exposure and possible therapeutic efficacy of antioxidants like Vitamin C and Vitamin E on arsenic induced apoptosis in rats. They reported that administration of Vitamin C and Vitamin E along with arsenic significantly reduced the extent of apoptosis. Apart from the free radical scavenging property, antioxidants are known to regulate the expression of number of genes and signal regulatory pathways and thereby may prevent the incidence of cell death[159]. Structures of various antioxidants are presented in Fig. 9.

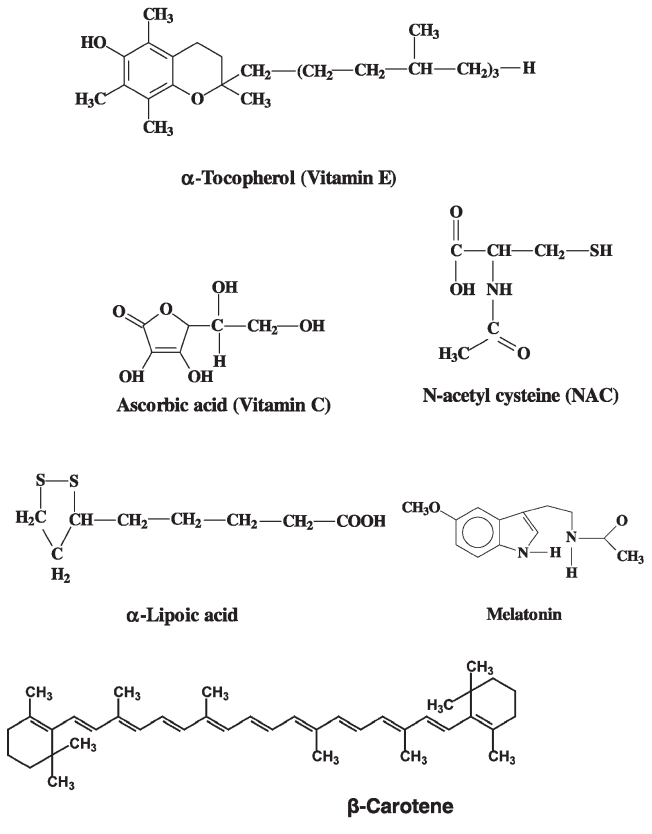

Fig. 9. Structures of potent antioxidant molecules.

Vitamins (E and C)

Vitamin E (α-tocopherol) is a fat-soluble vitamin known to be one of the most potent endogenous antioxidants. α-tocopherol is a term that encompasses a group of potent, lipid soluble, chain-breaking antioxidants that prevents the propagation of free radical reactions. Vitamin C is a water-soluble antioxidant occurring in the organism as an ascorbic anion. It also acts as a scavenger of free radicals and plays an important role in regeneration of α-tocopherol[159]. Supplementation of ascorbic acid and α-tocopherol has been known to alter the extent of DNA damage by reducing TNF-α level and inhibiting the activation of caspase cascade in arsenic intoxicated animals[158]. These studies strongly believed that vitamins supplementation perspective, though observed in animal model, will have sustainable curative value among the already afflicted populations, neutralizing impact on freshly emerging metal poisoning scenario and possible proactive protection to those potentially susceptible to heavy metal exposure. Our group has also reported beneficial effects of vitamins supplementation during arsenic intoxication[95]. *In vivo* and *in vitro* antioxidant effect of vitamin-E on the oxidative effects of lead intoxication in rat erythrocytes suggests that simultaneous supplementation of vitamin-E to lead treated erythrocytes prevent the inhibition of δ-aminolevulinic dehydratase activity and lipid oxidation[160]. Vitamin-E could be useful in order to protect membrane-lipids and, notably, to prevent protein oxidation produced by lead intoxication. The protective action and the synergistic action of both vitamins (C and E) against lead-induced genotoxicity are discussed by Mishra and Acharya[161]. A study found that the combination of vitamin C and thiamine was effective in reducing lead levels in blood, liver, and kidney. In addition, both lead-induced inhibition in the activity of blood δ-ALAD and elevation in the level of blood zinc protoporphyrin were reversed by such combination[162]. Early reports found that vitamin C might act as a possible chelator of lead, with similar potency to that of EDTA[163]. A cross-sectional study analyzed 4213 young and 15365 adult Americans with mean blood lead level of 2.5-3.5 mg/dl, respectively, and showed an inverse relationship between serum vitamin C and BLL[164]. In another study of 85 volunteers who consumed a lead-containing drink, vitamin C supplementation produced small reductions in lead retention[165]. However, a recent report stated that rats treated with ascorbic acid did not reduce lead burden in the liver, kidney, brain, and blood[166]. Although it is biologically plausible that vitamin C may

affect lead absorption and excretion, the effect is more obvious in low-exposed subjects with higher vitamin C supplementation. Vitamin E alone or in combination with conventional chelator, $CaNa_2EDTA$, was found to decrease the lead-induced lipid peroxide levels of liver and brain in rats[166].

β-Carotene

β-Carotene is a member of a family of molecules known as the carotenoids having basic structure made up of isoprene units. β-Carotene, a precursor of retinol (vitamin A), is the lipid-soluble antioxidant with properties somewhat analogous to that of vitamin E[159]. The long chains of conjugated double bonds (alternating single and double bonds) provide specific colors to carotene are also responsible for good anti-oxidative property. It can mop up oxygen free radicals and dissipate their energy. A significant reverse dose-response relationship with arsenic-related ischemic heart disease was observed for serum level of α- and β-carotene. Multivariate analysis showed a synergistic interaction on arsenic-related ischemic heart disease between duration of consuming artesian well water and low serum carotene level[167]. β-Carotene was found to be beneficial in recovering the activities of glutathione S-transferase, ACP, ALP and AChE in cadmium chloride intoxicated animals. In addition to that hematological variables also responded favorably in β-Carotene supplemented animals[168].

N-Acetylcysteine (NAC)

NAC a synthetic precursor of reduced glutathione (GSH) is a thiol-containing compound, which stimulates the intracellular synthesis of GSH, enhances glutathione-S-transferase activity, and acts solely as a scavenger of free radicals. It reduces liver injury caused by paracetamol over dosage in human[169] and attenuates liver injury and prevents liver and plasma glutathione (GSH) depletion in mice[170]. A study conducted by Santra *et al*[171] showed that treatment with NAC in arsenic intoxicated mice could deplete cellular stores of the GSH and is an effective intervention against oxidative stress developed due to arsenic exposure. Hepatoprotection by NAC could be due to effective detoxification of electrophiles generated by arsenic as well as its rapid elimination/excretion from the body. Efficacy of NAC as a potent antioxidant has also been reported in cadmium intoxication and it has been reported that simultaneous supplementation of NAC could protect Cd-induced nephrotoxicity and it can also act as a therapeutic agent against Cd intoxication[172]. One of the first report by Pande *et al*[173] suggested that NAC could be used both as preventive and therapeutic agent along with MiADMSA or DMSA in the prevention and treatment of lead poisoning. Combined administration of NAC along with DMSA post arsenic exposure lead to a significant turnover in variables indicative of oxidative stress and removal of arsenic from soft organs[174].

α-Lipoic acid

α-Lipoic Acid (LA) is an endogenous thiol antioxidant, which possesses powerful potential to quench reactive oxygen species, regenerate GSH and to chelate metals such as iron, copper, mercury and cadmium. LA is also known to mediate free-radical damage in biological systems[159]. LA is readily available from the diet, absorbed through the gut and easily passes through the blood-brain barrier. Exogenous supplementation with lipoic acid has been reported to increase unbound lipoic acid levels, which can act as a potent antioxidant and reduce oxidative stress both *in vitro* and *in vivo*[175]. Inside cells and tissues, lipoic acid is reduced to dihydrolipoic acid which is more potent antioxidant and its co-administration with succimer has been known to reduce lead induced toxic effects[176]. LA and its reduced form, dihydrolipoic acid (DHLA) are capable of quenching reactive oxygen and nitrogen species such as hydroxyl radicals, peroxyl radicals, superoxide, hypochlorous acid and peroxynitrite and chelating metals such as Cd^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} [176]. LA supplementation can change the tissue redox state directly by scavenging the free radicals and indirectly by bolstering the antioxidants and antioxidant enzymes. *In vitro* studies revealed that, among the mono and dithiols (glutathione, cysteine, dithiothreitol, and lipoic acid), lipoic acid was the most potent scavenger of free radicals produced during cadmium-induced hepatotoxicity[177]. It contributes its thiol groups to detoxify the divalent metal and subsequently ameliorates the cell membrane integrity[178]. Antidotal property of LA against Cd induced hepatotoxicity has also been reported[177]. LA serves as a protective tool against Cd-induced membrane damage and cell dysfunction in hepatocytes.

Melatonin

Melatonin (N-acetyl- 5 - methoxy tryptamine), a hormone produced by the pineal gland is a potent scavenger of reactive oxygen species and free radicals. Melatonin prevents the reduction of membrane fluidity caused by lipid per oxidation and thereby helps in

scavenging free radicals[179]. Pieri *et al*[180] suggested that melatonin is superior to all other free radical scavengers like vitamin E, vitamin C, GSH, and so forth, in neutralizing peroxyl radicals. Melatonin has been shown to be five times superior to glutathione in scavenging free hydroxyl radicals. Both methoxy group at position 5 of the indole nucleus and the acetyl group of the side chain of melatonin are essential to scavenge free hydroxyl radical[181]. Melatonin donates an electron to scavenge OH and becomes indolyl cation radical that in turn neutralizes superoxide radical[181]. Protective effects of melatonin against metal-induced oxidative damage have been reported in studies done mostly *in vivo* and *in vitro*[182-185]. A study conducted by Pal and Chatterjee[186] suggested that melatonin supplementation in arsenic-treated rats reduces free radical–mediated cytotoxicity and thereby helps in the restoration of normal cellular antioxidant status. The antioxidant effect of melatonin has been claimed as a protective factor towards carcinogenesis, neurodegeneration and aging[187]. A study by Kim et al[188] suggested that immunotoxicity induced by lead was significantly restored or prevented by melatonin (MLT). Splenic T and B cells were significantly increased by MLT treatment when compared with the treatment of Pb alone. The natural killer cell, phagocytic activity and the number of peripheral leukocytes were significantly enhanced in Pb plus MLT-treated mice when compared with the treatment of Pb alone[188]. The antioxidative effect of melatonin has also been reported by its ability to protect haematopoietic cells from the damaging effects of exposure to lead[189]. The protective effect of melatonin against lead-induced toxicity is attributed mainly to its lipophilic and hydrophilic nature[190] as well as to localize mainly in a superficial position in the lipid bilayer near the polar heads of membrane phospholipids[191]. Since membrane functions and structure are influenced by proteins in membranes, and lead is known to damage thiol proteins[192], it is possible that the protective action of melatonin to membrane damage induced by lead may be related partially to the ability of the indole group present in melatonin to prevent protein damage[193,194]. It has also been reported that melatonin stimulates superoxide dismutase mRNA levels in several tissues[194].

Additionally, melatonin reportedly stimulates several antioxidative enzymes, including glutathione reductase, glutathione peroxidase and superoxide dismutase, promoting quick disposal of H_2O_2 from rat brain cortical cells[195] also enhances the production of enzymes that are involved in the synthesis of glutathione[196] also prevents the reduction of membrane fluidity caused by lipid per oxidation, and thereby, helps in scavenging free radicals[197]. Chwelatiuk *et al*[198] reported that 8-week melatonin co-treatment with orally administered cadmium chloride decreased renal, hepatic and intestinal cadmium concentrations. It has been reported by Cano *et al*[199] that Cd modifies expression of two major clock genes, period (Per) 1 and Per 2, in the hypothalamic–pituitary unit while melatonin administration counteracted most of the effects of Cd and augmented hypothalamic Per 2, and adenohypophysial Per 1 and Per 2 gene expression. Immunotoxicity induced by Cd has also been reported to be significantly prevented by melatonin supplementation[187]. Melatonin supplementation is known to increase Hemagglutination (HA) titer, NK cell and phagocytic activity used for evaluation of non-specific immunocompetence and number of peripheral leukocytes[187].

Combination therapy

This is a new trend in chelation therapy that is to use two chelators, which act differently. The idea of using combined treatment is based on the assumption that various chelating agents are likely to mobilise toxic metals from different tissue compartments and therefore better results could be expected[146,200,201]. We reported observed that combined administration of DMSA and $CaNa_2EDTA$ against chronic lead poisoning lead to a more pronounced elimination of lead and better recoveries in altered lead sensitive biochemical variables beside no redistribution of lead to any other organ was noticed[147,220]. Co-administration of DMSA and MiADMSA at lower dose (0.15 mmol/kg) was most effective not only in reducing arsenic-induced oxidative stress but also in depleting arsenic from blood and soft tissues compared to other treatments. This combination was also able to repair DNA damage caused following arsenic exposure. We thus recommend combined administration of DMSA and MiADMSA for achieving optimum effects of chelation therapy[202].

Beside the use of the two different chelators for the combined therapy, number of studies have been reported where a co-administration of a dietary nutrients like a vitamins *e.g.*, thiamine[202,203], an essential metal *viz*., zinc[202,204,205] or an amino acid like methionine[206] with a chelating agent lead to many beneficial effects like providing better clinical recoveries as well

as mobilization of lead. We recently reported that combined administration of n-acetylcysteine and succimer led to a rapid mobilization of arsenic and lead, while, administration of α-lipoic acid, quercetin and DMSA provided a more pronounced recovery in lead induced altered biochemical variables indicative of oxidative stress[207,208]. We also reported that co-administration of naturally occurring vitamins like vitamin E or vitamin C during administration of a thiol chelator like DMSA or MiADMSA may be more beneficial in the restoration of altered biochemical variables (particularly the effects on heme biosynthesis and oxidative injury) although it has only limited role in depleting arsenic burden. It is evident from above that combination therapy is a new and a better approach to treat cases of metal poisoning. As only few experimental evidences are available and there is a need for in depth investigation in this area. It is thus proposed to investigate the effects of combination therapy particularly against arsenic poisoning, where a strong chelating agent is administered along-with another structurally different chelating agent, or a vitamin/ antioxidant/essential metal or an amino acid[147,209,210]. A study evaluating chronic arsenic intoxication (100 ppm in water for 12 wk) in rats evaluated the ability of NAC and a chelating agent, DMSA, to preserve hepatic and brain glutathione levels and to normalize erythrocyte enzyme levels[174]. Combined administration of vitamin C with DMSA and vitamin E with MiADMSA was found to have more pronounced depletion of brain arsenic and useful in the restoration of altered biochemical variables particularly the effects on heme biosynthesis and oxidative injury[94]. Vitamin E administration with MiADMSA was found to be beneficial in reducing body lead burden whereas co-administration of vitamin C was beneficial in reducing oxidative stress condition[209,210].

Use of herbal products could be a better option to meet the objective of finding a suitable treatment for arsenic poisoning. We studied few plant products and reported that extracts of *Centella asiatica, Hippophae rhamnoides* L., and *Moringa oleifera*[18,211-213] provided excellent protection to the altered biochemical parameters suggesting oxidative stress, organ damage, porphyrin metabolism *etc.*, but had little or no effect in depleting body arsenic burden except *Moringa oleifera*. It was suggested that these herbal extracts could be used as a complementary agent in providing better clinical recoveries when given along with a known thiol chelator[214].

Conclusion

The above discussion provides an insight into the role of reactive species in metal-induced toxicity. The "direct" damage may involve conformational changes of bio-molecules or alter specific binding sites, as in case of lead poisoning. On the other hand, "indirect" damage is a consequence of metal driven formation of reactive oxygen/nitrogen species involving superoxide, hydroxyl radicals or nitric oxide, hydrogen peroxide and/or endogenous oxidants. Apart from ROS induced oxidative stress, binding of these heavy metals to proteins rich -SH groups aggravates cellular toxicity. Although, there are number of chelating drugs which have been tried as treatment for metal poisoning but they are known to be compromised with side effects particularly their binding to essential metals within the system which significantly reduce their efficacy. These facts led to few novel strategies/approaches for treating cases of metal poisoning like including administration of antioxidants, either individually or in combination with chelating agents[215-220]. Recently we have also reported that interaction of nonmetal (fluoride) with metalloid (arsenic) also lead to some antagonistic effects[221,222]. Co-administration of antioxidant (natural or synthetic) or with another chelating agent has shown to improve removal of toxic metals from the system as well as better and faster clinical recoveries in animal models[223]. However, we still lack in-depth clinical studies with pre-existing or newer chelating agents in order to understand the mechanism underlying the beneficial effects of antioxidants and to explore optimal dosage and duration of treatment in order to increase clinical recoveries in case of humans.

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