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Guest Editors: Ana-Maria Florea, Dietrich Büsselberg, and David Carpenter

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Editorial Board

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Editorial

Metals and Disease

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Metals are a part of our daily life. They are used for construction and for countless products (e.g., cars, mobile phones, or computers). Without the use of metals, human society would have not been developed to its current stage. While the raw materials are provided by our planet, humans learned to extract metals from mineral ore and to refine them.

As a consequence to their wide use in the modern world, we are all exposed to metals and metal compounds to a degree that was rare before the industrial age. Exposing the cell(s) of living organisms to elements which were hardly available during genesis of life causes multiple effects on those cells and ultimately human health will be affected.

Some metals serve critical functions in the human body. Iron is a necessary component of hemoglobin in red blood cells. Copper, manganese, zinc, cobalt, chromium, molybdenum, and selenium are all required as enzyme cofactors or prosthetic groups, and human disease results if the diet is deficient in the metals. However, exposure of humans to excessive levels of "physiologic" metals are associated with disease. Other metals, such as lead, aluminum, and arsenic, have no known beneficial effects in the human body and have only toxic actions. (This issue does not describe the positive health effects of some essential metals which are crucial to maintain normal body function.)

This special issue presents the latest scientific insights into how metals influence and change cell and body function. One of the contributions of this special issue gives "a global primary health care perspective" linking the risks of metal exposure of humans to the contamination of the environment. The authors highlight the concern that primary care workers possibly underestimate occupational and environmental exposures to chemicals in clinical evaluations. The paper summarizes worldwide studies which explore the relationship between metal exposures and adverse health effects. Finally it suggests some guidelines to evaluate basic occupational and environmental exposure.

Other two papers emphasize health effects of metals on the cardiovascular and the nervous system. Clearly both systems have a major impact on human health. The papers demonstrate that the functions of both systems are clearly impaired by metals. The paper on the cardiovascular system proposes a mechanism of action for interactions between genetic, nutritional, and environmental factors, while the paper on the nervous system emphasizes the multiple sites of action of a single metal at the pre- and postsynaptic terminal as well as the targets for effects which impair synaptic transmission and, therefore, learning and memory function. Not only does a single metal have many sites of action (and the most sensitive has to be defined), but also different metals might both share some targets and act at different targets. Therefore, the environmental exposure might result in additive effects. The authors conclude that the multiple effects of metals may occur simultaneously and are dependent on the specific metal species, concentrations, and the cell type involved.

Another paper in this special issue is somehow unique in this volume since it not only describes the negative health effects of metals but also illustrates that under certain specific circumstances metals might have beneficial effects and could

be used to fight specific diseases (e.g., cancers). The authors conclude that metals could actually be both risk factors as well as healing agents for specific forms of breast cancer.

The diversity of molecular mechanisms modulated by exposure to arsenic and its relation to acute- and chronic-toxicity as well as in regard to cancer development is discussed in one of the papers of this special issue.

Two papers of this issue are research investigations dealing with exposure to lead or aluminum, respectively. The investigation on the early effects of long-term exposure to lead shows that mainly motor performance parameters are an early neurotoxic indicator of lead toxicity while the subjects exhibited a slowed poststrain resetting behavior of the vegetative nervous system, which correlated with the individual blood lead level.

The exposure to low concentrations of aluminum chloride on thymocytes and lymphocytes results in a rapid and dose-dependent injury of these cells illustrating that aluminum has cytotoxic effects on cells of the immune system.

Finally, there is a paper that takes a different point of view and gives some important general information for physicians on how they could recognize and prevent overexposure to methylmercury from fish and seafood consumption.

Overall, every single person gets in contact with metals in daily life. Some of these metals have considerable health effects. While most of these effects have negative consequences resulting in a large variety of different symptoms (which could be minor or severe); some of the biological properties of these metals and their compounds could be used to treat life-threatening diseases (e.g., cancer).

To understand the negative and positive effects of metals and metal compounds on human health and disease, more research is needed. Additionally the public should be educated and sensitized in order to minimize the environmental contamination and prevent metal-induced intoxications.

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Review Article

Metal Toxicity at the Synapse: Presynaptic, Postsynaptic, and Long-Term Effects

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Metal neurotoxicity is a global health concern. This paper summarizes the evidence for metal interactions with synaptic transmission and synaptic plasticity. *Presynaptically* metal ions modulate neurotransmitter release through their interaction with synaptic vesicles, ion channels, and the metabolism of neurotransmitters (NT). Many metals (e.g., Pb^{2+}, Cd^{2+}, and Hg^{+}) also interact with intracellular signaling pathways. *Postsynaptically*, processes associated with the binding of NT to their receptors, activation of channels, and degradation of NT are altered by metals. Zn^{2+}, Pb^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+}, Co^{2+}, Li^{3+}, Hg^{+}, and methylmercury modulate NMDA, AMPA/kainate, and/or GABA receptors activity. Al^{3+}, Pb^{2+}, Cd^{2+}, and As_2O_3 also impair *synaptic plasticity* by targeting molecules such as CaM, PKC, and NOS as well as the transcription machinery involved in the maintenance of synaptic plasticity. The multiple effects of metals might occur simultaneously and are based on the specific metal species, metal concentrations, and the types of neurons involved.

1. Introduction

Metals and their compounds are distributed in ecosystems as a result of natural processes as well as anthropogenic activities. Metals are used in their elementary form as well as in compounds for various human needs. Therefore, a number of these metals enter our environment as a consequence of their widespread use in preservatives, biocides, and paints [1]. They are taken up by organisms through inhalation or by ingestion of food and water contaminated with these metals. For living systems, metals can be divided in those which are essential for life, such as cobalt (Co), copper (Cu), zinc (Zn), manganese (Mn), and iron (Fe); which are potentially toxic only at higher concentration, and those which have no known biological function, which can be toxic at all concentrations such as cadmium (Cd), chromium (Cr), mercury (Hg), and *lead* (Pb) [13] (for all abbreviations used in the review please refer to the abbreviations section; to facilitate reading, the names of the specific metals discussed are given in *italics*).

Since the uptake mechanisms of the body are not able to distinguish between "physiologically required" and harmful metals, the toxic metals absorbed consequently might interact with the functions of the central nervous system (CNS), liver, kidneys, and hematopoietic system, thus presenting a significant health hazard. In this review, we will examine the effects of these metals in the CNS, specifically at the synapse.

The human brain has about 10^{11} neurons, which interconnect and "communicate" with each other through synapses. It is estimated that each neuron has approximately 7000 synapses. At the presynaptic side of the synapse the incoming electrical signal, in from of action potentials, is transformed to a chemical signal in the form of neurotransmitter release. Synaptic transmission depends on the timely opening of membrane channels, the precise functioning of intracellular signaling pathways, and metabolic pathways involved in the synthesis and the release of neurotransmitters. Postsynaptically the binding of neurotransmitters changes the membrane potential, resulting in a hyper- or depolarization of the neuron and in the generation of an action potential when the threshold potential is reached. These are crucial process and the basis of all higher cognitive functions including learning and memory.

Therefore, we highlight the mechanisms by which metals and their compounds interfere with the processes of synaptic transmission and synaptic plasticity. This review covers the effects of metals on signal transmission from the presynaptic to the postsynaptic membrane, as well as the effects on synaptic plasticity with an emphasis on learning and memory, since subtle alterations in synaptic transmission due to the interaction of metals may have profound toxic effects in the CNS [14].

Some metals, which have already been shown to alter synaptic transmission, are discussed in this review. The metals are listed in an alphabetical order below with a short description of their neurotoxic effects, to show their relevance to this study (for more details regarding the neurotoxicity of these metals see [15]).

Aluminum was found present in high concentrations in brains of patients with Alzheimer's disease, Parkinson's disease, and dialysis encephalopathy and could contribute to neurogenerative disorders [21]. In animals the administration of *aluminum* salts results in neurofibrillary degeneration, a condition similar to the encephalopathy in Alzheimer's disease [3].

Arsenic, one of the oldest known poisons, due to its cholera-like symptoms, became a favorite poisoning agent and earned the title the "Poison of Kings" [15]. An acute ingestion of *arsenic* affects many systems of the body including gastrointestinal, cardiovascular, respiratory, and the nervous system. Even today, chronic low-dose exposure to *arsenic* is very common in countries like Bangladesh, India, Taiwan, and other parts of South East Asia due to contamination of groundwater by *arsenic*. It is a major cause of infant mortality in Bangladesh [8]. Chronic manifestations of *arsenic* poisoning are pigmentation changes, gastrointestinal problems, anemia, liver disease, black foot disease, and Mees' lines on the nails. Central neuropathy due to *Arsenic* poisoning usually manifests as impairment of learning, short-term memory and concentration. However, peripheral neuropathy is more frequently observed and this might last for several years. It manifests as a rapid and severe ascending weakness and sometimes these patients require mechanical ventilation [8, 15].

Cadmium and *manganese* also have neurotoxic effects, where *cadmium* damages cells of the cerebellar cortices of young rats as well as rabbits and chronic *manganese* poisoning causes extrapyramidal symptoms much like those of Wilson's disease and Parkinsonism [9]. Moreover, increased total *cadmium* levels in human hair were associated with mental retardation and impairment in visual motor abilities [4]. Similar toxicities also occur in humans.

Lead, whose mechanisms of neurotoxicity have been extensively studied, was discovered more than 5000 years ago and was used in the ancient world for lead water piping, as utensils, to sweeten food and wine, and as a constituent of eye paints [2]. It was discovered that acute exposure to *Pb* could cause lead colic and mental disturbances and even chronic exposure to low concentrations of lead in children caused several cognitive and behavioral disturbances. Since *Pb* crosses the placenta, prenatal exposure to lead can have especially severe consequences [4, 5, 15].

Exposure to dietary *methylmercury* leads to Minamata disease, which manifested in patients as paresthesias followed by irreversible impairment of vision, hearing, speech, gait, and ultimately leads to death. In addition, cognitive impairment ensued with prenatal exposure to *methylmercury* [4].

Organo-tins are industrially produced in large quantities for applications as PVC stabilizers, glass coverings, silicone, wood preserver additives, and antifouling paints. Moreover, considerable amounts of *organo-tins* are released in the environment causing large concern about their impact on human health. Due to their lipophilicity *organo-tins* are taken up by humans and distributed in different tissues. In mammalian organs such as brain, liver, and kidneys, *organo-tins* are biotransformed and this process may increase their toxicity [7]. Specifically *alkyl-tins* have been shown to cause neurotoxicity [15].

Even though metals are well known for their various toxicities, they are also used as therapeutic agents. *Lithium* salts have been used in the treatment and prophylaxis of bipolar affective disorder [10, 16]. *Arsenic* in the form of *arsenic trioxide* is used for the treatment of leishmaniasis, leukemia, and trypanosomiasis [8, 15]. The specific toxicities of some metals are actually being used to man's benefit, especially for the treatment of cancers. *Cisplatin* (*cis-diammine-dichloro-platin = CDDP*) is used as an anticancer drug and testicular cancer, endometrial cancer, prostatic tumors, bladder carcinoma, and small cell bronchial carcinoma [17] are successfully treated with this drug.

With the wide description of harmful effects of metals as well as their irreplaceability in modern life and medicine, it becomes essential to demarcate the level at which metals become toxic. This includes concentrations of metals as well as their targets of actions. Recognizing the targets sites at which metals interact can serve as a stepping-stone for the development of therapeutic agents to counteract metal toxicity as well as the side effects of anticancer drugs such as *arsenic* and *cisplatin* compounds.

This paper aims to review the literature available of the mechanisms of actions of metals at targets presynaptically, postsynaptically, and on long-term potentiation (LTP) and summarizes the findings in a logical and easily comprehensible manner. In the first part the toxic effects of organic and inorganic metals on the ***pre**sy*napticpart will be described (Section 2), followed by a review of their ***post***synaptic actions (Section 3), and the review finally looks at the impairment of ***synaptic plasticity*** (Section 4) before concluding remarks are made (Section 5).

2. Presynaptic Targets of Toxic Metals

Presynaptically, the action potential, which is an electrical signal, is transduced to a chemical signal in the form of neurotransmitter release. Generally, the action potential induces a membrane depolarization, which opens voltage gated calcium channels allowing the influx of Ca^{2+}. Ca^{2+} activates calmodulin (CaM) and therefore CaM kinases (CamK) are activated, which leads to the phosphorylation

of synaptic vesicle associated proteins and the conversion of the reserve pool of synaptic vesicles to a readily releasable pool of vesicles. Ca^{2+} also binds synaptotagmin, a calcium sensor protein in the vesicle membrane and triggers neurotransmitter vesicle fusion and the release of neurotransmitter [18] (Figure 1).

Metals interact with specific targets in these pathways and the same metals might even interact with various targets simultaneously. For instance, *aluminum* blocks voltage gated calcium channels, decreases the biological activity of CaM, and also inhibits Ca^{2+} ATPase [19, 20]. In addition, if a metal interacts with an upstream target of a pathway, it may influence all the processes succeeding it. For example, *cadmium* reduces voltage activated calcium channel currents, therefore, it can influence the intracellular calcium concentration and consequently the activation of CaM and calcium-dependent intracellular signaling pathways [6, 11]. Notably, *cadmium* caused a decrease in release of excitatory neurotransmitters glutamate and aspartate while it caused an increase in the release of inhibitory neurotransmitters GABA and glycine [12].

The upcoming sections (Sections 2.1 to 2.6) highlight the literature relating to the toxic effects of metals on presynaptic targets including voltage-activated ion channels (Section 2.1), signaling cascades (Section 2.2), transporters (Section 2.3), synaptic vesicle associated proteins (Section 2.4), neurotransmitters (Section 2.5), and neurofilaments and microtubules (Section 2.6). For ease of access, wherever possible metals are described in alphabetical order in each Section.

2.1. Voltage-Activated Channels

2.1.1. Voltage-Activated Calcium Channels. Voltage activated calcium channels open by a depolarization. They are subdivided into high- and low-voltage activated channels. The high-voltage activated channels, which have to be depolarized to more positive voltages than −30 mV for activation, include the L-type, P/Q-type, N-type, and R-type, where the L-type has a "long-lasting" current. The other types are divided on the basis of their inactivation and their susceptibility to various peptide toxins. There are also low-voltage activated channels which are mainly composed of the T-type channels which have a small, fast inactivating and therefore transient current [22].

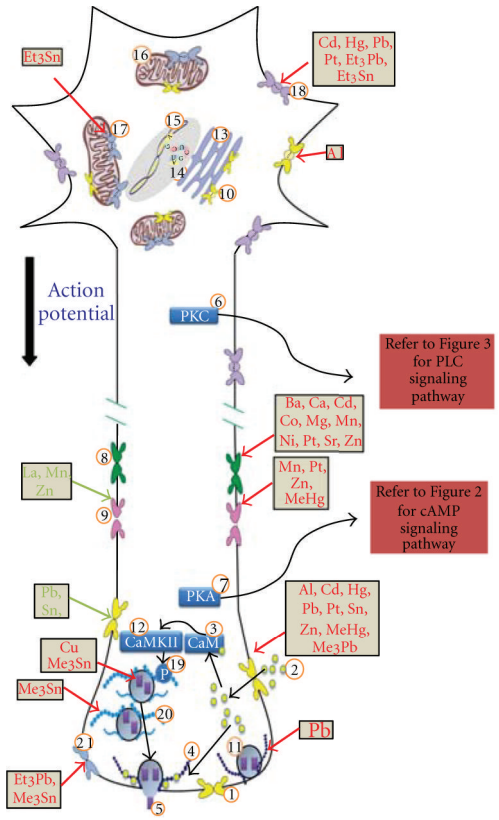
Aluminum (Al^{3+}) blocked N- and L-type voltage activated calcium channels in cultured rat dorsal root ganglions, with a threshold concentration of 20 μM and a Hill's coefficient of 3 (Table 1). It also required an open channel for its actions thereby indicating that the possible site of action of Al^{3+} was inside the channel. The current-voltage relation was shifted to depolarizing voltages in the presence of Al^{3+} [19]. *Aluminum* also blocked voltage-activated calcium channels *in vivo* in rats when given 10 mg per kg body weight per day intraperitoneally for 4 weeks. Inhibition was nearly 85% in the corpus striatum, 58% in the cerebral cortex, and 46% in the hippocampus [20].

Cadmium (Cd^{2+}) effectively reduced voltage-activated calcium channel currents, which were high threshold and fast inactivating types in cultured chick dorsal root ganglion cells, at concentrations of 20 μM. This block was released at hyperpolarizing voltages, which may be due to shifts in gating and permeability of the channels. When the membrane potential was hyperpolarized, the channels conducted transiently, as Cd^{2+} exited the channels, but closed again thereafter. The channels can close with Cd^{2+} in the channel pore, therefore implying that Cd^{2+} does not affect the closing mechanisms of the channels [6]. Similar results were obtained in squid giant fiber neurons. In addition, a kinetic model was created and the binding site for Cd^{2+} was determined to be near the outer end of the pore, and the entry of Cd^{2+} into the pore was voltage independent while its exit was voltage dependent [11].

Lead (Pb^{2+}) is a potent blocker of voltage-activated calcium channels in invertebrate *Aplysia* neurons as well as in mammalian neurons [19, 23, 25, 27, 28, 30]. There is no change in the voltage dependence of activation or inactivation of the channels in mammalian neurons, which suggests an external binding site for Pb^{2+} [25, 27, 28]. In mammals Pb^{2+} blocked N-, L- and T-type voltage activated calcium channels [19, 27, 28, 30]. The block of L- and T-type channels was concentration dependent and reversible in N1E-115 mouse neuroblastoma cells [30]. The concentration for 50% inhibition (IC_{50}) of L-type channels was 30 nM, and for N-type channels it was 80 nM free Pb^{2+} where 10 mM Ba^{2+} was used as the charge carrier in cultured E-18 rat hippocampal neurons [28]. Some contradictory data were obtained in a study of mouse N1E-115 neuroblastoma cells, where in five of the fifteen cells studied, 2.3 μM Pb^{2+} enhanced L-type calcium channel currents and also enhanced the inactivation of L-type channels at holding potentials of −60 to −40 mV [30]. A study on human neuroblastoma cells SH-SY5Y determined that *lead acetate* at concentrations of 1 to 30 μM blocked voltage-activated calcium channels, both N- and L-types in a concentration-dependent and reversible way. More importantly, the concentrations used in the study were inclusive of the blood level concentrations at which children present with neuropsychological disorders (between 1.5–2.5 μM) [29].

Mercury (Hg^{2+}) blocked voltage-activated calcium channels with an IC_{50} of 1.1 μM *in vitro* in rat pup dorsal root ganglion cells, and it required a partially open channel for its block [24]. *Mercury* (Hg^{2+}) blocked neuronal N- and R-type calcium channels transiently expressed in human embryonic kidney 293 cells with an IC_{50} of 2.2 and 0.7 μM. This effect was partially reversible in N-type but not in R-type channels [26]. *Mercury* also blocked T-type calcium channel currents in the concentration range of 0.5–2 μM in cultured rat dorsal root ganglion cells. In addition the current-voltage relation was shifted to positive voltages implying that *mercury* may have an effect on channel gating [40].

Platinum in the form of *cis-diammine-dichloro-platin (CDDP)* reduced voltage-activated calcium channel currents in dorsal root ganglion cells of rats *in vitro*. *CDDP* reduced peak *calcium* current with an IC_{50} of 23.9 ± 4.5 μM and sustained current with an IC_{50} of 38.8 ± 6.1 μM in small



No.	Symbol	Target	No.	Symbol	Target	No.	Symbol	Target
1		Voltage-gated calcium channels	8		Voltage-gated sodium channels	15		DNA
2		Calcium ions	9		Voltage-gated potassium channels	16		Mitochondria
3	CaM	Calmodulin	10	ATPase	Ca^{2+} ATPase	17	ATPase	Mitochondrial ATPase
4		Synaptotagmin	11		Neurotransmitter vesicle	18	ATPase	$Na^{+}K^{+}$ ATPase
5		Neurotransmitter	12	CaMKII	Calmodulin kinase	19	P	Phosphate
6	PKC	PKC	13		Endoplasmic reticulum	20		Synapsin
7	PKA	PKA	14		mRNA	21		Transporter for neurotransmitter reuptake

Figure 1: Presynaptic targets of neurotoxic metals. Events at the synapse from the arrival of the action potential which results in the membrane depolarization-induced opening of voltage-activated calcium channels and the entry of calcium which activates CaM, which activates CaM kinases and causes the phosphorylation of synaptic vesicle-associated proteins and an increase in readily releasable neurotransmitter vesicles. Calcium also binds synaptotagmin and causes exocytosis of neurotransmitter from the vesicles. Shown in boxes are the metals and the targets at which they act in the synaptic transduction pathway. A table at the end indicates the symbols and what they indicate. Green indicates activation or upregulation while red indicates inhibition or downregulation. Please refer to the section of Abbreviations and metals.

Table 1: Effects of metals on voltage-activated ion channels (↑—activation/upregulation, ↓—inhibition/downregulation).

Target		Voltage-gated channels									
		Calcium channels					Sodium channels			Potassium channels	
		L	N	T	R	All[ii]	Tetrodotoxin sensitive	Tetrodotoxin resistant	All[i]		
Al	Effect	↓	↓			↓					
	Conc	20 μM	20 μM			50 μM					
	Ref	[1]	[1]			[2]					
Cd	Effect		↓			↓	↓	↓			
	Conc		20 μM			2.2, 125 μM	5 mM	0.2 mM			
	Ref		[3]			[4], [5]	[6]	[6]			
Co	Effect					↓					
	Conc					500 μM					
	Ref					[7]					
Hg	Effect		↓	↓	↓	↓					
	Conc		2.2 μM	0.5–2 μM	0.7 μM	1.1 μM					
	Ref		[8]	[9]	[8]	[10]					
La	Effect									↑	
	Conc									10 μM	
	Ref									[11]	
Mn	Effect									↓	↑
	Conc									10 mM	1 mM
	Ref									[12]	[12]
Pb	Effect	↓	↓	↓		↓					
	Conc	30 nM[i], 0.7, 0.64, 0.1 μM	80 nM[i], 0.64, 0.1 μM	1.3 μM, 6 μM, 6 μM		1, 1, 0.6, (1–30) μM					
	Ref	[13], [14], [15], [1]	[13, 15] [1], [5], [1]	[14], [15], [4]		[16], [15], [4], [17]					
Pt	Effect					↓			↓	↓	
	Conc					23.9 μM			10 μM	10 μM, 100 μM	
	Ref					[18]			[18]	[18], [18]	
Sn	Effect					↓	↑				
	Conc					50 μM	50 μM				
	Ref					[19]	[20]				
Zn	Effect	↓	↓	↓		↓		↓	↓		↑
	Conc	5,69 μM	5,69 μM	20 μM		2 mM, 69 μM		2 mM	50 μM		30 μM
	Ref	[1, 21]	[1, 21]	[21]		[10, 16]		[6]	[6]		[22]

[i] Extent of block and EC_{50} differ based on concentration of charge carrier used.
[ii] Paper does not describe which sub-type is affected.

neurons with a diameter of $\leq 20\,\mu$m. Surprisingly, in large neurons with a cross-sectional diameter of $\geq 25\,\mu$m, the peak calcium current was only reduced by 14.1 ± 2.3% even with a concentration of 100 μM *CDDP*. It is unlikely that the voltage-activated calcium channel currents were blocked directly since the small and large cells were unequally affected and the Hill's coefficient was not 1. *CDDP* probably decreases voltage-activated calcium channel currents by acting through an intracellular pathway more prominent in small neurons, possibly through IP_3 receptor activation as described later [17].

Tin (Sn^{2+}) used as *stannous chloride ($SnCl_2$)* decreased voltage-activated calcium channel currents *in vitro* in rat dorsal root ganglion cells in a concentration-dependent manner with a threshold of 1 μM. These effects were found to be irreversible [41]. However, contradictory results were obtained in a study of motor nerve terminals of frog, where nerve muscle preparations were exposed to 50 μM $SnCl_2$, which caused an increased inward Ca^{2+} current [34].

Zinc (Zn^{2+}) blocked voltage-activated calcium channels in cultured rat dorsal root ganglion cells [19, 24, 31]. The IC_{50} for this effect on N- and L-type channels was 69 μM Zn^{2+} while the Hill's coefficient was 1. T-type currents were more sensitive, and the block was partly reversible in 50% of the neurons [31]. *Zinc* did not require an open channel for this blocking effect [24]. The current voltage relationship shifted to more depolarizing voltages in the presence of Zn^{2+}, implying that the mechanism of action of Zn^{2+} may involve the screening of charges in the vicinity of the channels [19].

Methylmercury (MeHg) caused an increase in calcium influx and therefore $[Ca^{2+}]_i$ through nifedipine and ω-conotoxin sensitive mechanisms, that is, through either, L-, N-, or Q-type calcium channels [36]. However, *methylmercury* caused an irreversible time and concentration dependent block of calcium channel currents at concentrations between 0.25 and 1 μM *in vitro* in rat cerebellar granule neurons. The block did not require depolarization, indicating that it did not require an open channel. Increasing the frequency of stimulation of cells increased the magnitude of block at 0.25 μM and 0.5 μM but not at 1 μM, which may suggest the presence of other counteracting effects. None of the calcium channel antagonists used—ω-conotoxin GVIA, ω-conotoxin MVIIC, ω-agatoxin IVA, calcicludine, and nimodipine, were able to decrease the *MeHg*-induced block of calcium channel currents [38]. *MeHg* blocked N-, R-, and L-type voltage-activated calcium channels [26, 39]. *MeHg* blocked human neuronal N- and R- type calcium channel currents transiently expressed in human embryonic kidney 293 cells with an IC_{50} of 1.3 μM and 1.1 μM respectively (Table 4). This block was determined to be irreversible [26].

Trimethyl lead blocked voltage-activated calcium channels with a threshold concentration of 0.5 μM *in vitro* in rat dorsal root ganglion cells. This block was irreversible and concentration dependent but not voltage dependent. It required an open channel and the IC_{50} was between 1–5 μM [33].

2.1.2. Voltage-Activated Sodium Channels. There are 9 subtypes of voltage-activated sodium channels Na_v 1.1–Na_v 1.9 distinguished by their sensitivity to tetrodotoxin and their rate of inactivation. Na_v 1.8 and Na_v 1.9 have relatively slower inactivation [35]. Na_v 1.1, Na_v 1.2, Na_v 1.3, and Na_v 1.7 are expressed in neurons and are highly sensitive to tetrodotoxin. Na_v 1.5, Na_v 1.8, and Na_v 1.9 are relatively tetrodotoxin resistant and are found in heart and dorsal root ganglion neurons. Na_v 1.4 and Na_v 1.6 are mostly expressed in skeletal muscle and the CNS, respectively [37].

Zinc (Zn^{2+}) and *cadmium (Cd^{2+})* reduced both tetrodotoxin-sensitive and tetrodotoxin-insensitive voltage-activated sodium channel currents in voltage clamp experiments in neuronal, cardiac, and skeletal muscle cells [42]. Tetrodotoxin-resistant channels were more sensitive to Zn^{2+} and Cd^{2+} with IC_{50} of the block being 50 μM and 0.2 mM, respectively; tetrodotoxin-sensitive channels were less resistant with IC_{50} of the block being 2 mM and 5 mM for Zn^{2+} and Cd^{2+}, respectively [42] (compare effects in Table 1). It was suggested that the site of action of Zn^{2+} contains cysteine sulfhydryl groups in or near the saxitoxin binding site since Zn^{2+} was able to relieve the saxitoxin-induced block of the channel in a competitive manner, and the blocking action of *zinc* was inhibited by sulfhydryl-specific alkylating reagents. These experiments were done in voltage-activated sodium channels taken from the hearts of dogs or calves [32].

$SnCl_2$ had an effect on voltage-activated sodium channel currents of the mollusk *Lymnaea stagnalis in vitro* where it shifted the current voltage curve to the left. $SnCl_2$ increased voltage-activated sodium channel currents at a concentration of 10 μM, but caused a depression in current at concentrations above 25 μM. Organic tin in the form of $(CH_3)_3SnCl$ decreased significantly the Na current only at high concentrations above 100 μM. Additionally the current voltage curve was shifted to the left. These effects were time dependent and irreversible [43].

Cobalt, *manganese*, *nickel*, *calcium*, *magnesium*, *strontium,* and *barium* in divalent cation form blocked both tetrodotoxin-sensitive and tetrodotoxin-insensitive channels in channels incorporated into planar bilayers in the presence of batrachotoxin. The block was voltage dependent and the sequence of affinity to block was $Co^{2+} \cong Ni^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+} > Sr^{2+} > Ba^{2+}$. The suggested mechanisms of block included a specific divalent cation binding site and surface charge screening [44]. Also 10 μM of the anticancer drug *CDDP* reduced voltage-activated sodium channel currents by 9.2%± 7.2% in rat dorsal root ganglions *in vitro* [17].

2.1.3. Voltage-Activated Potassium Channels. The family of voltage-activated potassium channel includes K_v1–6, K_v 8, and K_v 9, where the principal subunit of the channels contains 6 transmembrane domains. All these channels are expressed in brain tissue [45]. Whole cell patch-clamp measurements of transient voltage-dependent potassium currents in rat suprachiasmatic nucleus neurons showed that Zn^{2+} potentiated current when activated from a holding potential of −60 mV (approximately the resting membrane potential). This potentiation was voltage dependent and arose from a shift of the inactivation current to more positive voltages. Zn^{2+} (30 μM) shifted the half-inactivation voltage by 20 mV from −80 mV to −60 mV [46]. Kuo and

Chen showed that at hyperpolarized voltages Zn^{2+} inhibited voltage-dependent transient K^+ currents which can be accounted for by the selective binding of Zn^{2+} to closed K^+-channels with a dissociation constant of approximately 3 μM, which kept the channels closed and slowed the activation of the current [47].

Whole cell patch clamp studies in central neurons of Drosophila third instar larvae showed that millimolar Ca^{2+} and Mg^{2+} concentrations and micromolar concentrations of Zn^{2+} increased the peak inactivation current and shifted the steady-state inactivation curve of voltage gated potassium channels to more positive voltages, but had no effect on the voltage dependence of activation. A micromolar concentration Cd^{2+} had the same effect; however, millimolar concentrations of Cd^{2+} had an effect on both steady state inactivation and activation curves, where the midpoint of the activation curve was shifted more positively. The potency of effect on the inactivation current in terms of amount of shift of steady state inactivation curves was Zn^{2+} (2 mM) $>Cd^{2+}$ (2 mM) $>Ca^{2+}$ (20 mM) $>Mg^{2+}$ (20 mM). The mechanism of action was most likely through specific binding to the channels at extracellular sites [48].

10 μM *cisplatin* in the form of *CDDP* reduced voltage-gated potassium channel currents by 20.9 ± 4.8% in small dorsal root ganglion neurons while 100 μM *CDDP* reduced the peak current by 12.8 ± 3.4% [17]. Micromolar concentrations of *lanthanum* (La^{3+}) enhanced outward voltage-gated potassium channel currents evoked by depolarization steps from −50 mV in rat cerebellar granule neurons. 10 μM La^{3+} shifted the steady state inactivation curve by approximately 40 mV in the depolarizing direction and increased the slope factor slightly [49].

Mayer and Sugiyama showed that fast activating transient potassium channel currents were reduced by 10 mM *manganese* (Mn^{2+}) in cultured rat sensory neurons. This reduction was due to a depolarizing shift of the activation curve and a slight reduction in maximum conductance. At the same concentration, steady state inactivation curves were also shifted to depolarizing voltages. The positive shift of steady state inactivation and activation curves were obtained for other metals as well, where the potency of shift was $Cd^{2+} > Mn^{2+} = Co^{2+} > Ca^{2+} > Mg^{2+}$. Lower concentrations of Mn^{2+} (1 mM), however, increased the amplitude of fast inactivating transient potassium channel currents at prepulse potentials from −50 to −70 mV, which was due to a shift of the inactivation curve with no significant shift in the activation curve. These effects may have been due to binding to a specific site within the channel or to phospholipids in close proximity of the gating apparatus [50].

Organic metals also affect voltage-gated potassium channels. *Methylmercury (MeHg)* blocked voltage-gated potassium channels irreversibly, with an IC_{50} of 2.2 ± 0.3 μM in a concentration-dependent manner. The Hill's coefficient for this block was ∼1 [51].

2.2. Signaling Cascades

2.2.1. The cAMP System. G-protein-coupled receptors (GPCR) are coupled to G_s or $G_{i/o}$, where G_s acts as a stimulator of adenylate cyclase and the Gα subunit of $G_{i/o}$ acts as an inhibitor of PKA (Figure 1). PKA phosphorylates Ca^{2+}-channels, thereby enhancing the influx of Ca^{2+} and this increases the release of neurotransmitters [52]. Also PKA phosphorylates SNAP-25 and this leads to a larger pool of readily releasable vesicles [52]. The cAMP-system appears to enhance the release of neurotransmitter in response to a stimulus (Figure 2).

In vitro and *in vivo* exposure to *lead acetate* decreased cAMP-dependent synaptic vesicle protein phosphorylation in rat brain which is most likely a contributing mechanism of *lead* toxicity [53].

Gs. Rodrigues and colleagues determined the effect of *lead acetate* on rat cerebral cortex membranes using 5′ Guanylylimidodiphosphate (Gpp(NH)p). Gpp(NH)p is a nucleotide phosphorylase-resistant GTP analogue, which is known to stimulate adenylate cyclase by saturating Gs. On preincubation of membranes with *lead acetate*, the stimulatory effect of Gpp(NH)p on the adenylate cyclase activity was inhibited [10].

Adenylate Cyclase. The same group [10] also determined the effects of *lead* acetate on adenylate cyclase activity in the cerebral cortex membranes and found that *lead* caused a concentration-dependent inhibition of adenylate cyclase activity with an IC_{50} of 2.5 ± 0.1 μM (Table 2) [10].

In another series of experiments, Ewers and Erbe [54] determined the effects of *lead, cadmium, and mercury* on adenylate cyclase of the cerebrum, cerebellum, and the brain stem, *in vitro* and *in vivo*. Adenylate cyclase activity was determined in terms of the number of moles of cAMP formed. Concentrations between 0.1 and 30 μM *lead nitrate*, *cadmium nitrate,* or *mercury nitrate* inhibited adenylate cyclase activity *in vitro* in homogenates of the cerebrum, brain stem, and the cerebellum. *In vivo* studies were performed on rats, which received *lead acetate* dissolved in sterile demineralized water, cAMP formation was determined 1 hour, 4 hours, and 24 hours after treatment. In the cerebellum, and brainstem, adenylate cyclase activity increased after one hour by about 25% but was unaffected in the cerebrum. After four hours, adenylate cyclase declined by 29%, 33%, and 21% in the cerebrum, cerebellum, and brainstem respectfully. By 24 hours adenylate cyclase activity had returned to normal in the cerebrum and brainstem but not in the cerebellum [54]. These differences in the effects of *lead* acetate on adenylate cyclase in different parts of the brain may be an indicator of the varied effects of *lead* on different isoforms of adenylate cyclase.

Zinc (Zn^{2+}) inhibited adenylate cyclase with an IC_{50} of 1-2 μM and a Hill's coefficient of 1.33, which was not competitive with Mg^{2+} or Mg^{2+}ATPase [82]. Both the CI and the CII domains of adenylate cyclase bind Zn^{2+} with high affinity which is correlated with Zn^{2+} inhibition of enzyme activity [83].

2.2.2. The PLC System. The PLC system consists of GPCRs coupled to Gq, which activate DAG and IP_3 through PLC.

Table 2: Effects of metals on presynaptic signaling pathways (↑—activation/upregulation, ↓—inhibition/downregulation).

Target		Pathways						
		PKC		Adenylate cyclase	Phosphodiesterase	CaM	IP3	Intracellular calcium
Al	Effect	↓	↑			↓		↑
	Conc	0–100 μM				0–1000 microM		0–1000 μM
	Ref	[2]	[23]			[2]		[2]
As	Effect							↑
	Conc							1 μM
	Ref							[24]
Cd	Effect			↓	↓			↑
	Conc			0.4, 0.9, 1.4[(i)] μM				0.1–1 mM
	Ref			[25], [25], [25]	[25]			[26]
Hg	Effect			↓	↓			
	Conc			0.8, 0.5, 0.9[(i)] μM				
	Ref			[25], [25], [25]	[25]			
Ni	Effect	↓						
	Conc	30 μM						
	Ref	[27]						
Pb	Effect		NC	↓	↓			
	Conc		1500–10000 ppm	2.5, 8.6,1.9, 8.0[(i)]μM				
	Ref		[25]	[28], [25], [25], [25]	[25]			
Pt	Effect						↑	↑
	Conc						1 nM–10 μM	1 nM–10 μM
	Ref						[29]	[29]
Zn	Effect			↓				
	Conc			1-2 μM				
	Ref			[30]				

[(i)] The three different concentrations indicate actions in different areas of the brain—cerebrum, cerebellum, and brainstem.

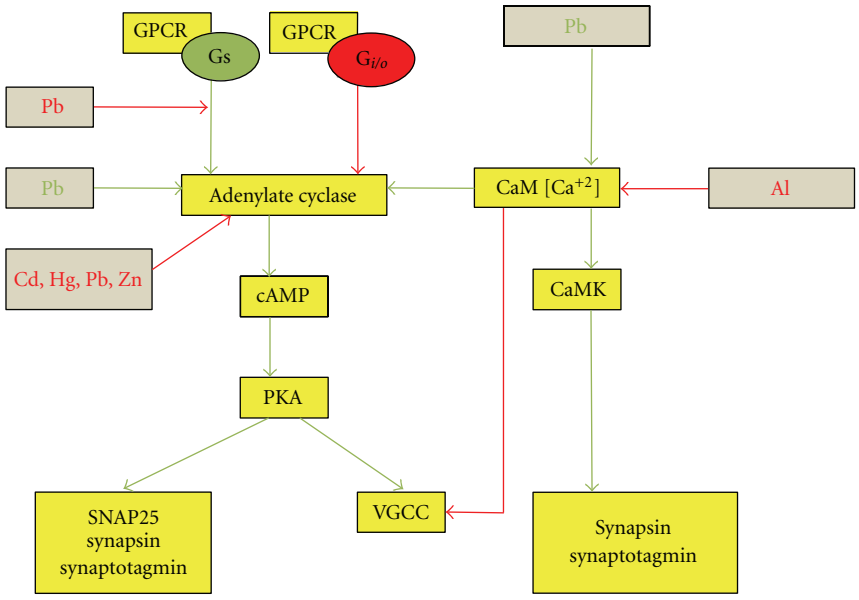


Figure 2: Effects of Metals on the cAMP Signaling Pathway at the Presynaptic Terminal (green: activation/increase, red: inhibition/decrease). GPCR's are coupled to G_s or $G_{i/o}$, where G_s stimulates adenylate cyclase and $G_{i/o}$ inhibits PKA. PKA phosphorylates Ca^{2+}-channels, thereby enhancing the influx of Ca^{2+} and this increases the release of neurotransmitters. PKA phosphorylates SNAP-25 and this leads to a larger pool of readily releasable vesicles. The cAMP-system appears to enhance the release of neurotransmitter in response to a stimulus. Metals act at different points in this pathway either enhancing certain processes or inhibiting some. A green color indicates an activation or an increase, and a red color indicates an inhibition or a decrease.

IP_3 causes an increase of intracellular calcium ($[Ca^{2+}]_i$) and the activation of DOC2 and synaptotagmin which leads to increased evoked release and readily releasable pool size. DAG through PKC causes an activation of voltage-gated calcium channels. PKC phosphorylates Munc 18, which negatively regulates syntaxin and synaptic vesicle fusion [52, 84]. PKC activation eventually leads to an increase of spontaneous and evoked neurotransmitter release and more readily releasable pool of vesicles (Figure 3) [52].

PKC. Metals that inhibit PKC include *lead, aluminum,* and *nickel. Pb^{2+}* inhibits PKC enzymes through interactions with its catalytic domains [85]. The effect of *aluminum* on PKC is debated: Julka and Gill demonstrated that *aluminum* lactate given to male albino rats for four weeks, caused an inhibition of PKC at all concentrations used (up to 100 μM). This was shown both *in vivo* and *in vitro.* The largest inhibition was observed in the cerebral cortex (47.73%) followed by the hippocampus (45.95%) and the corpus striatum (38.74%) [20]. However, contrasting findings were determined by Johnson and coworkers who showed that *aluminum* sulfate, when given orally for a period of 4 months to male Sprague-Dawley rats, showed an increase in PKC specific activity by 60% and total activity by 70% in the soluble fraction of cerebral cortex homogenates [69]. The different effects of *aluminum* could be attributed to the mode of intake reflecting differences in concentration of *aluminum* absorbed and its distribution to the brain or the duration of exposure.

Microarray analysis in neuronal PC12 cells indicated that exposure to *Nickel* (Ni^{2+}) caused a decline in the transcription of two isoforms of PKC- prkcc, prkz, and two regulatory binding proteins prkcbp1 and prkcdbp, and also caused temporary upregulation and downregulation of prkcq at 24 hours and 72 hours, respectively [86]. These effects are important in terms of the events at the synapse because PKC activates voltage-activated calcium channels, and increases the secretion of neurotransmitter through effects on proteins involved in neurotransmitter exocytosis-Munc-18, and SNAP25 (a SNARE protein) [87].

IP_3. Increase of $[Ca^{2+}]_i$ in human cervix adenocarcinoma cells by *cisplatin* (0.001–10 μM) was dependent on extracellular Ca^{2+} and was blocked by an IP_3 receptor blocker. The types 1–3 IP_3 receptors were at the cellular membrane of these cells, which suggests a possible mechanism of *cisplatin*-induced calcium entry through IP_3 receptor activation. This was supported by the observation that the same results were not obtained in human osteosarcoma cells, which in addition did not show the presence of types 1–3 IP_3 receptors at cell membrane [88]. *Arsenic trioxide (As_2O_3)* similarly caused an increase in intracellular calcium which was dependent on calcium release from the intracellular calcium stores through the activation of IP_3receptors [88]. *MeHg* also causes an increase in intracellular calcium, which may be due to release from intracellular stores through inositol phosphate. *MeHg* doubled intracellular inositol phosphate levels at concentrations above 3 μM *in vitro* in rat cerebellar granule neurons [89].

2.2.3. Intracellular Calcium ($[Ca^{2+}]_i$). Aluminum (Al^{3+}) caused an increase of $[Ca^{2+}]_i$ in rat synaptosomes, which

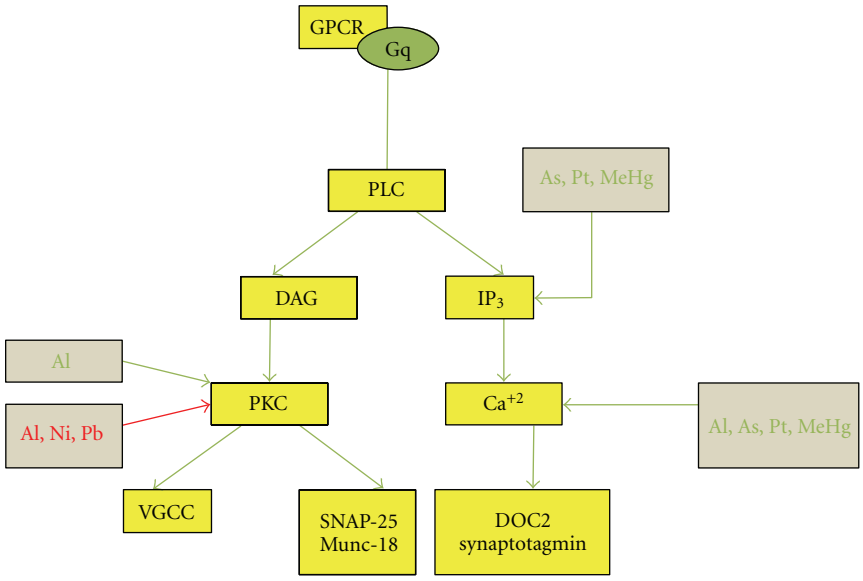


Figure 3: Effects of metals on the PLC signaling pathway at presynaptic terminal (green: activation/increase, red: inhibition/decrease). The PLC system consists of GPCRs coupled to Gq, which activate PLC, which activates DAG and IP_3. IP_3 increases intracellular calcium ($[Ca^{2+}]_i$) and activates DOC2 and synaptotagmin which leads to increased evoked release and readily releasable pool size. DAG activates PKC, which activates voltage-gated calcium channels. PKC phosphorylates Munc 18, which negatively regulates vesicle fusion and syntaxin. PKC activation leads to the increased spontaneous and evoked neurotransmitter release. The effects of metals on this pathway are shown in this figure where a green color indicates an activation/upregulation and a red color indicates an inhibition/downregulation.

could be a consequence of the inhibition of the Ca^{2+}-ATPase [20]. 1 μM *arsenic* trioxide (As_2O_3) caused an irreversible increase in $[Ca^{2+}]_i$ in human neuroblastoma cells (SY-5Y) and in human embryonic kidney 293 cells. This rise of $[Ca^{2+}]_i$ was independent of extracellular calcium, but dependent on intracellular calcium stores. Blocking of IP_3 receptor and ryanodine receptors with their specific blockers reduced the increase in $[Ca^{2+}]_i$ indicating their involvement in this process [88]. *Cisplatin* also increased $[Ca^{2+}]_i$ in a concentration-dependent manner in human cervix adenocarcinoma cells but not in human osteosarcoma cells. It is unlikely that the increase in $[Ca^{2+}]_i$ is induced by entry of extracellular calcium, but more likely through activation of IP_3 receptor as described above [88].

In addition, $[Ca^{2+}]_i$ could indirectly be affected by several mechanisms. For example, $[cis\text{-}(NH_3)_2Pt(H_2O)_2]^{2+}$, a form of platinum, caused an uncoupling of oxidative phosphorylation one minute after exposure in a concentration-dependent manner, which resulted in a release of Ca^{2+} from the mitochondria. *Cisplatin* did not produce the same effect even at a concentration of 500 μM [90]. However, another study by Gemba et al. showed that mitochondrial uptake of Ca^{2+} in rat kidney cortical mitochondria was decreased 24 hours after exposure to 500 μM *cisplatin* [91].

Methyl-mercury (*MeHg*) 0.5–1 μM caused an increase in $[Ca^{2+}]_i$ *in vitro* in rat cerebellar granule neurons, which decreased cell viability (Table 4). This increase in cell death was prevented 3.5 hours after exposure by using two calcium channel blockers (ω-conotoxin and nifedipine) and a calcium chelator (1,2-bis(2-aminophenoxy) ethane-N,N,N9,N9-tetracetic acid tetrakis (acetoxymethyl) ester (BAPTA)). The effect of the calcium channel blockers could indicate that they inhibit the *MeHg* interaction with the target site or block of the entry of *MeHg* in addition to the effects on $[Ca^{2+}]_i$. BAPTA may have reduced calcium-induced cell death at 3.5 hours after exposure but was unable to prevent *methylmercury*-induced cell death at 24.5 hours. That may indicate that calcium-independent pathways of cell death are involved [92]. The increase of $[Ca^{2+}]_i$ by *methylmercury* is caused by release from intracellular stores and through an influx of Ca^{2+} into the cell [89].

In HeLa cells, *trimethyl-tin* caused spikes in $[Ca^{2+}]_i$ as well as sustained increases. The spikes were of variable size and duration and required 0.25 μM *trimethyl tin*. The sustained increase in intracellular calcium was partially reversible and dependent on the concentration of *trimethyl tin* used, where a 5 μM concentration caused an 8% increase in $[Ca^{2+}]_i$. These effects were independent of external calcium concentrations; however, the increase in $[Ca^{2+}]_i$ was reduced when the internal calcium stores were compromised [7].

Overall, any of the metals affecting any channel or active transport mechanism that involves calcium, at the cellular membrane or the internal stores (as described above) could potentially change $[Ca^{2+}]_i$.

2.2.4. Calmodulin (CaM). Calmodulin is a calcium binding protein. Ca^{2+}/calmodulin activates CaMK, which phosphorylates synapsin I and opens voltage-activated calcium channels by phosphorylation. Thereby Ca^{2+}-influx is increasing which is crucial for releasing the neurotransmitter from vesicles [52, 93].

Aluminum (Al^{3+}) decreased the biological activity of CaM both *in vitro* and *in vivo* where inhibition *in vivo* is largest in the hippocampus (36.56%), followed by the cerebral cortex (31.76%) and the corpus striatum (22.49%) [20]. *Lead*, however, had an opposite effect as *lead acetate* enhanced CaM activity both *in vitro* and *in vivo* resulting in an increase in CaM-dependent synaptic vesicle protein phosphorylation including the phosphorylation of proteins such as synapsin I. This was proposed as a mechanism for increased spontaneous release of neurotransmitter and depletion of neurotransmitters norepinephrine and acetylcholine following exposure to *lead* [53].

2.3. Transporters

2.3.1. Ca^{2+}-ATPase. Ca^{2+}-ATPase activity in male albino rat synaptic plasma membranes was reversibly inhibited by Al^{3+} (up to100 μM). This inhibition was concentration dependent with an IC_{50} of 10 μM and resulted in an increase of $[Ca^{2+}]_i$ [20] (Table 3).

2.3.2. Na^+/K^+-ATPase. Cisplatin caused a concentration and time-dependent decrease in Na^+-K^+ATPase activity in liver and kidney cells [90]. *Lead* also affected Na^+-K^+ATPase activity, and one study showed lowered RBC membrane Na^+/K^+-ATPase activity below 60% in 77% of patients with Pb-Rbc $\geq$ 40 μg/100 mL while only 40% had the same decrease in activity who had a Pb-Rbc $\leq$ 40 μg/100 mL [94]. *Mercury* compounds, *cadmium, triethyltin, and trimethyltin* also inhibit Na^+-K^+ATPase activity [95–97]. *Triethyl lead* altered the microviscosity of the plasma membrane of ascites tumor cell and also completely inhibited Na^+-K^+-ATPase at concentrations 5–20 μM possibly through direct interaction with its catalytic subunit [98].

2.3.3. Mitochondrial ATPase. Mitochondrial ATPase was inhibited in adult rat brain homogenates with an IC_{50} of 260 μM by *triethyltin* [99]. *Trimethyl tin* has also been shown to affect mitochondrial ATPases *in vitro* [97].

2.4. Synaptic Vesicle Associated Proteins

2.4.1. Synaptotagmin I. Synaptotagmin I is a membrane protein, which is hypothesized to be a Ca^{2+}-sensor in Ca^{2+}-dependent neurotransmitter exocytosis. It has a short intravesicular N-terminus and the cytoplasmic part is composed mostly of two C2 domains, C2A and C2B. The C2A domain is known to bind two Ca^{2+}-ions and the binding affinity shows a correlation with the Ca^{2+} dependence of exocytosis [100]. Synaptotagmin I binds phospholipids and syntaxin in a Ca^{2+}-dependent manner. The binding to syntaxin is associated with exocytosis. The C2B domain of synaptotagmin I also has Ca^{2+} binding sites and is involved in the Ca^{2+} dependent self-association of synaptotagmin I into multimers [100].

Synaptotagmin I was recently described as a target site for *lead*. Bouton and coworkers suggested a competitive interaction between Pb^{2+} and Ca^{2+} for the Ca^{2+} binding sites in the C2A domain of synaptotagmin I. At nanomolar concentrations Pb^{2+} induced the binding of synaptotagmin I to phospholipids with an EC_{50} of 8 nM. This made it a thousand times more potent than Ca^{2+} at increasing phospholipid binding to synaptotagmin I. Binding of Pb^{2+} also increased the stability of the secondary structure of synaptotagmin I. A concentration of 2 μM free Pb^{2+} protected a 32 kDa fragment of synaptotagmin I from proteolytic degradation. It required 11 μM free Ca^{2+} to protect the same size of synaptotagmin I. The same authors showed that Pb^{2+}, unlike Ca^{2+}, did not induce the interaction of synaptotagmin I and syntaxin. Overall, the interaction of Pb^{2+} was competitive with Ca^{2+} and nanomolar concentrations of Pb^{2+} could inhibit the ability of micromolar concentrations of Ca^{2+} to induce the interaction of synaptotagmin I and syntaxin [101].

Four binding sites of Cu^{2+} in the cytoplasmic C2A domains of synaptotagmin I are discussed, three of which are common to Ca^{2+}, and one of which is unique to Cu^{2+}. It was suggested that Cu^{2+} has a competitive interaction with Ca^{2+}, but Cu^{2+} has a greater affinity for the binding sites common to these metals. Also it was determined that Cu^{2+} caused a conformational change in the protein, which may make it less susceptible to trypsin cleavage [102]. Kathir and colleagues looked at the interactions between Cu^{2+} and the C2B domain of p40 synaptotagmin I, which is formed by an alternative translation of the synaptotagmin I gene at the Met103 of the p65 synaptotagmin I. They determined that these interactions stabilized synaptotagmin I bound to phosphatidyl serine vesicles [103].

2.4.2. Synapsin I and p38. Synaptic vesicle associated proteins, synapsin I and p38, in rat CNS decreased on acute exposure of rat to *trimethyl tin*. This decrease was both concentration and time dependent; however, 12 weeks after the exposure, the levels returned to normal. The decline was not a result of loss of tissue which also occurs with *trimethyl tin* exposure but was significantly greater that the reduction in tissue [104].

2.5. Neurotransmitters. Release of neurotransmitters is modulated by multiple mechanisms. How metals interfere with some of these pathways has been described above. The following paragraph focuses how metals modulate neurotransmitter levels, their release, and uptake in the presynaptic button.

2.5.1. Effect on Neurotransmitter Metabolism. Treatment of PC-12 dopaminergic neuronal cells with 10 μg/mL *copper* nanoparticles (Ø 90 nm) caused a decrease in dopamine and its metabolites 3.4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). This indicates that the decrease in dopamine may be attributed to a decrease in production and an increase in the breakdown of dopamine [105].

In the same cell line, 10 μg/mL *manganese* nanoparticles (Ø 40 nm) caused a suppression of the tyrosine hydroxylase gene expression, which is involved in the synthesis of dopamine [105]. *Aluminum* has been shown to decrease

Table 3: Effects of metals on transporters, neurotransmitters, and neuropeptides (↑—activation/upregulation, ↓—inhibition/downregulation).

Target		Transporters			Neurotransmitters						Neuropeptides
		Ca^{2+} into mitochondria	Ca^{2+} ATPase	Dopamine transporter	Glutamate	Aspartate	GABA	Glycine	Dopamine	Acetylcholine	Substance P, neuropeptide K, and neurokinin
Al	Effect		↓								
	Conc		0–100 μM								
	Ref		[2]								
Cd	Effect				↓[v]	↓[v]	↑[v]	↑[v]			↓
	Conc				10–30 μM	10–30 μM	10–30 μM	10–30 μM			5[iii] μM
	Ref				[31]	[31]	[31]	[31]			[32]
Cu	Effect								↓		
	Conc								10[i] μg m^{-1}		
	Ref								[33]		
Hg	Effect			NC					h		
	Conc			400 μM					6 mg/kg, 400 μM		
	Ref			[34]					[35], [34]		
Mn	Effect				↓	↓	↓		↓		
	Conc				20–200 nm[iv]	20–200 nm	20–200 nm		10[ii] μg mL^{-1}		
	Ref				[36]	[36]	[36]		[33]		
Ni	Effect								↑		
	Conc										
	Ref								[37]		
Pb	Effect				↑		↑			↑	
	Conc						50 μmol L^{-1}			100 μmol L^{-1}	
	Ref				[38]		[39], [38]			[39]	
Pt	Effect	↓	NC								
	Conc	0.5 mM	500 μM								
	Ref	[40]	[41]								
Sn	Effect									↑	
	Conc									10–100 μM	
	Ref									[42]	

[i] Upregulates expression of monoamine oxidase, decreases production, and increases depletion
[ii] Downregulates expression of tyrosine hydroxylase gene
[iii] Reduces expression of precursor gene
[iv] Another study shows that uptake of glutamate into astrocytes is reduced through the decreased expression of glutamate aspartate transporter; this may result in increase in glutamate levels in the synapse [43]
[v] Shows effect on neurotransmitter release.

Table 4: Effects of organic metals on presynaptic targets (↑—activation/upregulation, ↓—inhibition/downregulation).

Target			MeHg			Me_3Pb			Et_3Pb			Me_3Sn			Et_3Sn		
			Effect	Conc	Ref	Effect	Conc	Ref	Effect	Conc	Ref	Effect	Conc	Ref	Effect	Conc	Ref
Voltage-gated channels	Calcium channels	L															
		N	↓	1.3 μM	[8]												
		T															
		R	↓	1.1 μM	[8]												
	All[(i)]		↓	0.25–1 μM	[44]	↓	0.5–50 μM	[45]									
	Potassium channels		↓	2.2 μM	[46]												
ATPases															↓	260 μM	[47]
	Na^+K^+ATPase								↓	5–20 μM	[48]	↓	5–20 μM	[48]	↓		
Transporters	Na^+-dependent GABA transporter								↓	10 μM	[49]	↓	10 μM	[49]	↓		
Pathways	IP_3		↑	3 μM	[50]												
	Intracellular Calcium		↑	(0.5–1) μM	[51]												
	Synapsin I											↓		[52]			
	p38											↓		[52]			
Neurotransmitters	GABA								↑[(iii)]	10 μM	[49]	↑[(ii)]	75 μM	[53]	↓		
	Dopamine											↓		[54]			
	Norepinephrine											↑[(ii)]	43 μM	[53]	↓		
	Serotonin											↑[(ii)]	24 μM	[53]	↓		

[(i)] Paper does not describe which subtype is affected
[(ii)] Decreases uptake of neurotransmitter into synaptosomes, thereby probably increasing the amount in cleft
[(iii)] Release of neurotransmitter from vesicles is being measured.

striatal dopamine content and inhibit the enzyme dopamine-β-hydroxylase, which converts dopamine to norepinephrine [106].

Among organic metals, *trimethyl tin* hydroxide treatment of rats on alternate days from days 2–29 of life was shown to decrease the amount of dopamine in the striatum without affecting dopamine metabolites homovanillic acid and dihydroxyphenylacetic acid [107].

2.5.2. Effect on Neurotransmitter Release. Stannous chloride increased the amplitude of end-plate potentials in frog neuromuscular junction. A concentration of 10–100 μM $SnCl_2$ increased the quantum of end plate potentials (EPP). However, the miniature end plate potential (MEPP) was not affected. Hattori and Maehashi (1988) suggested that this was due to an increase in the evoked neurotransmitter release while there was no effect on spontaneous release of neurotransmitter. Also, $SnCl_2$ did not increase MEPP amplitude or acetylcholine (Ach) potential, indicating that the sensitivity to ACh was not altered [108].

Lead (Pb^{2+}) in concentrations of at least 100 nM was found to increase the spontaneous release of glutamate and GABA from the presynaptic terminal of rat hippocampal neurons. This effect was found to be concentration dependent and partially reversible and the suggested mechanism of action was through an intracellular signaling pathway [109]. Similarly, it is likely that other metals also affect neurotransmitter release through their interaction with the voltage-gated ion channels, intracellular signaling pathways, and synaptic vesicle associated proteins.

One study looked at the effects of *cadmium* on synaptic transmission by perfusing the amygdala of rats with 10–30 μM $CdCl_2$. There was an inhibitory effect on the release of excitatory neurotransmitters glutamate and aspartate while the release of inhibitory neurotransmitters glycine and GABA was stimulated [12]. *Aluminum*, as described in previous chapters, affects $[Ca^{2+}]_i$ and, therefore, as expected, inhibits the release and uptake of GABA from synaptosomes by inhibiting Ca^{2+}/calmodulin-dependent calcineurin. It also inhibited pyruvate-supported calcium-evoked acetylcholine release in synaptosomes while in differentiated SN56 cells it decreased acetylcholine release on short-term exposure and increased release on long-term exposure [106].

2.5.3. Effect on Neurotransmitter Reuptake. Trimethyl tin, in vitro, inhibited the uptake of neurotransmitters GABA, norepinephrine and serotonin, with an IC_{50} of 75, 43, and 24 μM in a concentration-dependent manner in mouse forebrain synaptosomes. *In vivo*, at 2 and 14 hours after *trimethyl tin* exposure, uptake of GABA, and serotonin was decreased whereas there was no significant decline in norepinephrine. These changes in uptake of neurotransmitters could explain their altered levels in the synaptic cleft [110]. However, unlike *trimethyl tin*, *triethyl tin sulfate* had no effect on the levels of dopamine, GABA or acetylcholine in rat brain on exposure for 6 days a week from days 2 to 29 of life in mice [107].

Triethyl lead caused a concentration-dependent inhibition of Na^+-dependent high-affinity GABA uptake with an IC_{50} of 10 μM in rat brain synaptosomes. These results were not dependent on Na^+ and GABA concentration-indicating that competition with Na^+ and GABA were not the mechanism of action. Triethyl lead also caused a time- and chloride-dependent decrease in ATP [111]. Skilleter showed that *trialkyl lead* at 1 μM causes a decrease in pyruvate uptake by mitochondria in a KCl medium which could possible explain the decline in ATP [112]. However, since the inhibition of GABA uptake occurs before a significant decline in ATP, Seidman and Verity suggested that the inhibition could be due to a defect in GABA binding to uptake site [111].

2.6. Neurofilaments and Microtubules. In vivo exposure of Wistar rats to *arsenic* caused a dose-dependent decrease in neurofilament M and L proteins in the sciatic nerve [113]. These components are required for the formation of a heteropolymer in the cytoskeleton. Since the mRNA expression of these proteins was unaffected, it is possible that the decrease was a consequence of proteolysis. Caplain, which is a calcium-activated cytoplasmic protease, has been implicated in this phenomenon due to the increase in cytoplasmic calcium caused by trivalent *arsenic* [8].

Triethyl lead also affects microtubules [114–116] and neurofilaments [117]. 50 μM *triethyl lead* caused an inhibition of assembly and a disassembly of microtubules *in vitro* in porcine brain [114]. *Triethyl lead* also caused a reversible perinuclear coil formation of neurofilaments *in vivo* in mouse neuroblastoma cells, which was not associated with a significant change in the microtubules. *In vitro*, *triethyl lead* caused bulging and constriction of isolated neurofilaments from porcine spinal cord, and an unraveling of fibers in preformed filaments [117].

2.7. Summary of Presynaptic Effects of Metals. To summarize, presynaptically, voltage-gated sodium, potassium and calcium ion channels are affected by metals such as Al^{3+}, $Cd^{2+}Pb^{2+}$, Hg^{2+}, *cisplatin*, Sn^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Sr^{2+}, Ba^{2+}, and La^{3+}. Mechanisms of effect included binding to a specific target, charge screening, shift of current-voltage curves, and competitive inhibition with the physiological ion or a combination of mechanisms [6, 11, 17, 19, 20, 23–32, 34, 41, 42, 44, 46–50]. Metals also interact with intercellular signaling pathways to modulate synaptic transmission. *Lead* modulated Gs, adenylate cyclase, PKC, and CaM [10, 53, 54, 85]. Adenylate cyclase activity was also modulated by *cadmium, mercury, and zinc* [54, 82, 83] while PKC was inhibited by *lead, aluminum, and nickel,* [20, 69, 85, 86], and IP_3 was inhibited by *cisplatin and arsenic* [88]. Intracellular calcium was affected through interference with several targets including voltage-gated calcium channels, Ca^{2+} ATPases, and intracellular pathways. Al^{3+}, As_2O_3, and *cisplatin* modulated intracellular calcium [20, 88, 90, 91], and *aluminum,* and *lead* affected Calmodulin activity [20, 53]. Ca^{2+}-ATPase activity was inhibited by *aluminum* and Na^+/K^+-ATPase activity was modulated by *lead, cisplatin, mercury,* and *cadmium* [20, 90, 94–96]. Synaptotagmin I, a synaptic vesicle associated protein, was a target site for interaction with Pb^{2+} and Cu^{2+}

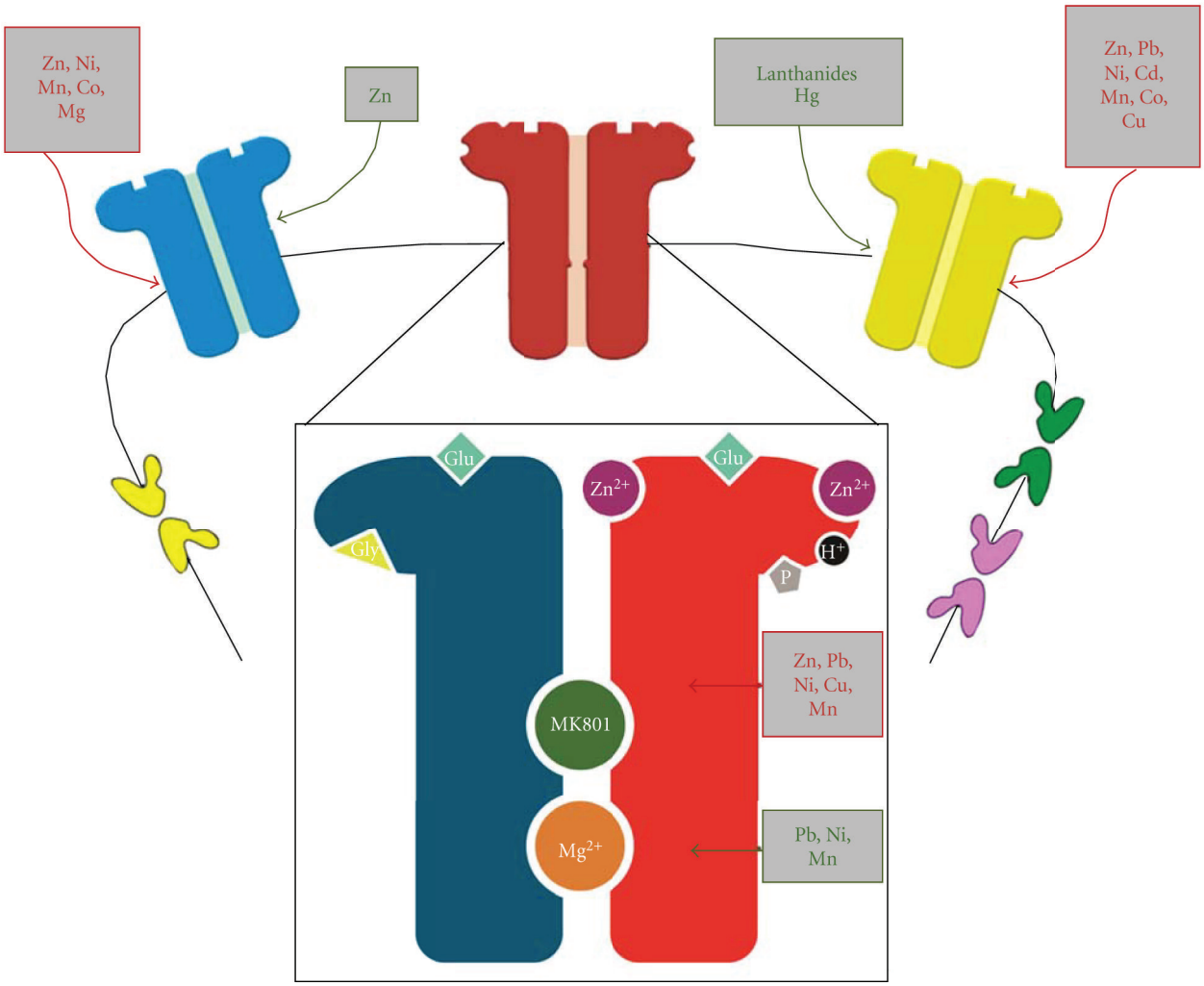


Figure 4: Postsynaptic ligand-gated ion channels as targets of neurotoxic metals. The main postsynaptic channels are the AMPA/kainate and NMDA receptors whereas the main inhibitory receptors are the $GABA_A$Rs. Each receptor represents a target for multiple metals. The NMDAR has many modulatory sites identified as it is more extensively studied. NMDAR is composed of a heteromer made of NR1 and NR2, each having multiple subtypes. In the diagram blue arm represents NR1 while the red arm represents NR2, the main modulatory subunit. Most metals have been shown to have effects on NR2 subunit (for values regarding the specific subtypes refer to Section 3.1 and Table 5).

[101–103]. Neurotransmitter release was possibly affected through interaction with many of the targets above as well as through interaction with synthesis and degradation of neurotransmitters and enzymes in the metabolic pathway, which resulted in modulation of neurotransmitter release by metals such as *copper, manganese,* and *tin* [101–103]. The mRNA expression of neurofilaments was affected by *arsenic* [113]. Often contradictory results were obtained regarding the effects of metals, which may indicate that metals had different effects on targets depending on the state of the metal, its concentration, the medium, the area of the brain, and whether the experiment was *in vivo* or *in vitro*.

3. Postsynaptic Targets

The activation of ligand-gated receptor channels is vital for controlling nerve cell inhibition or excitation and, therefore, fashioning the response of individual neurons, neuronal networks, and, ultimately, the entire brain. Consequently the resulting currents through the associated channels will either depolarize or hyperpolarize the postsynaptic terminal under different physiological conditions. The major *excitatory* neurotransmitter in the brain is L-glutamate. There are three classes of ionotropic glutamate receptors named according to their potent excitatory amino acids: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainate, and *N*-methyl-D-aspartate (NMDA). The AMPA and kainite-activated channels are designated non-NMDA receptor-channels and will be further discussed in Section 3.2 while metal actions at the NMDA/receptor channel complex are analyzed in the upcoming Section 3.1.

The most abundant *inhibitory* neurotransmitter in the brain is γ-aminobutyric acid (GABA), which acts on its own class of ligand-gated channels. However, these receptors

are subject to modulation by other compounds and ions, including metals [118] (Figure 4). As mentioned in the introduction, biological systems utilize metals because of their catalytic versatility, but the high affinity of these metals to specific binding sites could possibly severely impair synaptic transmission and, therefore, cause a malfunction of neuronal networks which might result in changes in perception, learning and memory, and finally change behavior, even at very low and environmentally relevant concentrations.

3.1. The NMDA-Receptor/Channel-Complex. The *N*-methyl-D-aspartate receptor (NMDAR) is a subtype of glutamate ionotropic receptors. The most widely distributed and studied NMDARs are tetrameric assemblies composed of two NR1 subunits and two of the four different NR2 types (named A, B, C, and D), of which NR2A and NR2B are most common [119, 120]. The physiological and pharmacological properties of these receptors are dependent on the NR2 subunit, although different NR1 splice variants may also influence channel performance [121, 122]. NMDAR subunits have a characteristic modular architecture consisting of two extracellular domains, the regulatory amino terminal domain (ATD) and the agonist-binding domain (ABD), and three membrane-spanning segments (M1, M3, and M4) and a reentrant hairpin-like pore loop, M2 [123].

The associated NMDAR channel requires simultaneous binding of two agonists, glutamate (Glu) and glycine (Gly), for opening (for review, [124]). Gly has its binding site in the ABD region of NR1 whereas NR2 ABD binds Glu [120]. The receptor-channel complex has unique properties such as a high Ca^{2+} permeability. Also, the functional activation of NMDAR channels is linked to a voltage-dependent *magnesium-* (*Mg-*) mediated block [125, 126]. Extracellular Mg^{2+} inhibits NMDA responses at membrane potentials close to the resting membrane potential [127]. Studies of the site of action of Mg^{2+} reveal that the N and N+1 site on NR2 subunit are important for the Mg^{2+} block [128]. When the membrane potential is sufficiently depolarized, Mg^{2+} leaves its binding site and even potentiates NMDA responses in low glycine concentrations. This potentiation is shown to be due to increased NMDAR affinity to glycine, in all neurons [129]. However, there was also glycine-independent potentiating effect of Mg^{2+}, which appeared to be largely voltage-independent and subunit specific, being seen only with NR2B-containing receptors. This potentiation has an EC_{50} of ~2 mM [130].

All of these effects reveal the complicated modulation by Mg^{2+} on NMDAR currents. Some data suggest that Mg^{2+} and spermine may completely or partially share a common binding site; similar observations are obtained using spermine [130, 131]. Three different steps in the action of these two substances could be distinguished: (1) increase in glycine affinity, seen in all neurons; (2) voltage-dependent block, also seen in all neurons; and (3) glycine-independent potentiation that was subunit specific [132, 133].

NMDARs contain a number of distinct recognition sites for other endogenous and exogenous ligands, which modulate their functions, such as divalent metal cations, as explored in the later sections (Figure 4).

Zinc is the second most prevalent trace element in the body. Most of the *zinc ions* (Zn^{2+}) are trapped within proteins, but some of it is loosely bound (chelatable zinc) [134]. In the mammalian brain, chelatable *zinc* is distributed mainly in the forebrain and localized almost exclusively within synaptic vesicles of a subset of glutamatergic axon terminals [135]. Since it is accumulated in synaptic vesicles, it has been assumed that *zinc* is released, with glutamate, during neuronal activity. Many studies have showed evidence of quantal corelease of *zinc* and glutamate (for review, [134]).

NMDARs are the best characterized synaptic zinc targets. At low micromolar concentrations, Zn^{2+} selectively inhibited NMDAR-mediated responses. The major effect was through voltage-independent, noncompetitive inhibition seen as a decrease in the opening probability of the channel [136–138]. However, at concentrations higher than 20 μM, Zn^{2+} could also produce voltage-dependent inhibition, probably by binding inside the pore at the Mg^{2+} blocking site [139]. It had been proposed that *zinc* is an endogenous ligand controlling NMDARs functions [140].

An important consideration in NMDAR function and pharmacology is that the Zn^{2+} binding to NR2A and NR2B subunits is associated with discrete subunit selectivity [141]. NMDARs containing the NR2A subunit had a very high sensitivity to extracellular Zn^{2+} (IC_{50} ~15 nM) [133, 142]; however, this inhibition never exceeded 60–80% [142]. The mechanisms of this inhibition occurred in different steps [143]: in the first step Zn^{2+} bound in the interlobe cleft of the NR2A-NTD promoting its closure, which would exert tension on the linkers connecting NTDs to ABDs. This effect would secondarily cause a disruption of the ABD dimer interface. In turn, this disruption relieved the strain on the transmembrane segments, and with proton binding, it allows the closure of the channel gate [133]. This mechanism of enhancement of proton inhibition was supported by subsequent work [144].

Zinc has a much lower affinity to the NR2B subunit, compared to NR2A, with voltage-independent inhibition (IC_{50} ~ 1 μM) [122, 145]. It was suggested that the mechanism of inhibition might be similar to the mechanism described for NR2A receptors. However, *zinc* inhibition of NR2B receptors appeared to not be dependent on pH [122], suggesting that this inhibition might occur through a different mechanism [133]. Studies showed that Zn^{2+} bound with high affinity to a site in NR2A ATD region [146] and with a lower affinity to a site in the same region of the NR2B [145]. The affinities of NR2C and NR2D to Zn^{2+} described to be even higher (IC_{50} > 10 μM) [134].

Lead (Pb^{2+}) is an exogenous heavy metal, which has been a public health concern due to its widespread contamination and its multiple toxic effects. Effects of acute exposure to Pb^{2+} in the micromolar range were originally described in cultured and acutely dissociated neurons as a reversible inhibition of the NMDAR current [19, 147–150]. These studies outlined several features of the effects of Pb^{2+} (for details, see review [151]). First, the inhibition was specific for NMDA channels, which were significantly more sensitive

Table 5: Effects of metals on postsynaptic ligand-gated ion channels (↑—activation/upregulation, ↓—inhibition/downregulation).

Target		NMDA	AMPA/kainate	GABA-A
Lead	Effect	(1) ↓ open channel probability		(1) ↓ (35%)
		(2) 60% ↓ in current (reversible)		
		(3) >80% block		
		(4) ↓		
		(5) ↓		
		(6) ↓ receptor binding		
		(7) ↓		
	Conc	(1) 1–10		(1) 1m M
		(2) 50		
		(3) 100		
		(4) IC_{50} = 1.52–8.19		
		(5) IC_{50} = 8.78 (in 0 Zn); IC_{50} = 1.26 (10 Zn) at high site, 94 at low site		
		(6) IC_{50} = 300 (adult); 60 (neonatal)		
		(7) IC_{50} (free) = 0.55		
	Ref	(1) [16]		(1) [10]
		(2-3) [14]		
		(4) [20]		
		(5) [9]		
		(6) [12]		
		(7) [5]		
Zinc	Effect	(1) ↓ open channel probability	(1) ↑	(1) ↓
		(2) Channel block	(2) ↓	↓ current in voltage independent, noncompetitive manner
		(3) NR2A block	(3) ↑	
		(4) NR2B block	(4) ↓	
		(5) ↓	(5) ↑ (16% to kainate, 15% to glu peak and steady state)	
		(6) ↓ receptor binding (76%)	(6) ↓	
		(7) ↓	(7) ↑ AMPA response	
		(8) ↓	(8) ↑ desensitized Kainate responses	

Table 5: Continued.

Target		NMDA	AMPA/kainate	GABA-A
			(9) ↓ AMPA and kainate responses	
	Conc	(1) 1–10 μM	(1) 50	(1) 100 (dose dependent)
		(2) >20	(2) 1 mM	(2) IC_{50} = 19
		(3) nM	(3) <300	
		(4) μM	(4) >500	
		(5) High affinity: IC_{50} = 0.77; low affinity: IC_{50} = 153	(5) 10	
		(6) 1 mM	(6) IC_{50} = 700	
		(7) IC_{50} (free) = 1.3	(7) EC_{50} = 30	
		(8) IC_{50} = 42.9	(8) EC_{50} = 13	
			(9) IC_{50} = 1.2-1.3 mM	
	Ref	(1-2) [11]	(1-2) [17]	(1) [13]
		(3) [17, 21]	(3-4) [7, 25]	(2) [10]
		(4) [17]	(5-6) [15]	
		(5) [9]	(7-9) [22]	
		(6) [12]		
		(7) [5]		
		(8) [22]		
Magnesium	Effect	(1) ↑ NMDA-R affinity to glycine in all receptors	(1) ↓ (27%)	
		(2) ↓ elementary current at +ve potentials(+20 to +80)		
		(3) ↑ glycine and voltage-independent and subunit specific		
		(4) external channel block, voltage dependent		
	Conc	(1) 10 mM	(1) 20 mM	
		(2) 10 mM		
		(3) 2 mM		
		(4) IC_{50} (−100 mV) = 2–15		
	Ref	(1–3) [6]	(1) [15]	
		(4) [2]		

Table 5: Continued.

Target		NMDA	AMPA/kainate	GABA-A
Manganese	Effect	(1) ↓ (activity dependent, channel blocker)	(1) ↓ (46%)	(1) Little or no effect
	Conc	(1) Ki = 35.9 (presence of glu and gly); Ki = 157 (no glu nor gly)	(1) 25 mM	(1) 1 mM
	Ref	(1) [8]	(1) [15]	(1) [10]
Copper	Effect	(1) ↓ (2) ↓ receptor binding (54%) (3) ↓ (channel block) (4) ↓ (5) ↓ voltage independent, noncompetitive	(1) ↓ (2) ↓ kainite-induced current (3) ↓ efficacy of kainate	(1) ↓ (voltage independent)
	Conc	(1) ND (2) 1 mM (3) Ki = 195 (no coagonists); two sites (9.4, 248) with glu and gly (4) IC_{50} = 15 (5) IC_{50} (free) = 0.27	(1) (2) IC50 = 4.3 (3) 30	(1) IC_{50} = 5
	Ref	(1) [17, 23] (2) [12] (3) [8] (4) [30] (5) [28]	(1) [17] (2-3) [30]	(1) [10]
Cobalt	Effect	(1) ↓	(1) ↓ (2) ↓	(1) ↓ (2) ↓ (29%)
	Conc	(1) 2 mM	(1) 2 mM (2) IC_{50} = 6.1 mM	(1) 2 mM (2) 1 mM
	Ref	(1) [3]	(1) [3] (2) [15]	(1) [3] (2) [10]

Table 5: Continued.

Target		NMDA	AMPA/kainate	GABA-A
Nickel	Effect	(1) NR2A: ↓, NR2B: ↑ (2) NR2A ↓ (100% at +ve potentials) (3) NR2B ↓ (4) NR2B ↑ (voltage independent)	(1) ↓ (kainite-induced current) (2) ↓ (glu-induced current)	(1) ↓(20%)
	Conc	(1) 30 (2) IC_{50} = 36 at −60 mV and 81 at +40 mV (3) IC_{50} 138 at −60 mV and 442 at +40 mV (4) 3		
			(1) IC_{50} = 420 (2) IC_{50} = 2.6 mM	(1) 1 mM
	Ref	(1) [16] (2–4) [21]	(1-2) [15]	(1) [10]
Mercuric chloride	Effect			(1) ↑ 130% (2) ↑ (270%)
	Conc			(1) 1 (2) 100
	Ref			(1) [1] (2) [19]
Methyl mercury	Effect	(1) ↓ receptor binding		(1) ↓ amplitude to 82.4%
	Conc	(1) IC_{50} = 0.95 (neonatal); 70 (adult)		(1) 100
	Ref	(1) [12]		(1) [55]
Cadmium	Effect	(1) ↓ receptor binding (58%) (2) ↓ (39% of control) (3) ↓ (4% of control)	(1) ↑ (kainate to 108% and QA to 115%) (2) ↓ (kainate to 79% and QA to 60%)	(1) ↓ (18%)
	Conc	(1) 1 mM (2) 50	(1) 50 (2) 1 mM	(1) 1 mM

Table 5: Continued.

Target		NMDA	AMPA/kainate	GABA-A
		(3) 1 mM		
	Ref	(1) [12] (2-3) [18]	(1-2) [18]	(1) [10]
Lanthanide	Effect	(1) ↓ NMDA response in a voltage-independent manner	(1) ↑ (2) ↓	(1) ↑ (300% max) and ↑ as the potential more −ve
	Conc	(1)) IC_{50} = 2	(1) 1–100 (2) >100	(1) EC_{50} = 231
	Ref	(1) [27]	(1-2) [27]	(1) [10]
Trimethyl-tin (TMT)	Effect	(1) ↓ (35%) reversible	(1) ↓ (20%) irreversible	(1) ↓ (30%) irreversible
	Conc	(1) 100	(1) 100	(1) 100
	Ref	(1) [4]	(1) [4]	(1) [4]

to Pb^{2+} inhibition than other glutamate channels. Secondly, the channel block was independent of voltage [148–150], and therefore the interaction site was likely to be located away from the electric field, or outside the conducting pore. Thirdly, the effect was noncompetitive since increasing the glutamate or glycine concentration could not overcome the block of the current [148, 152]. Biochemical studies suggested that the inhibitory effects of Pb^{2+} on NMDA receptors were age- and brain-region specific [152–154]. One important observation in Pb^{2+} neurotoxicity was that the hippocampus appears to be more sensitive than other brain regions [153, 155, 156].

The effect of Pb^{2+} on glutamate and NMDA-evoked currents depended on the subunit composition of the receptor-channel complex. Concentration-dependent Pb^{2+} inhibited the currents activated by either Glu or NMDA in oocytes expressing NR1-2A or NR1-2B (Table 1, [157]). Yamada and colleagues [158], however, showed that higher concentrations were needed than mentioned before, although, there were methodological differences between the two studies, which could account for the different results (for details, [157]).

Also, Pb^{2+} at low concentrations (<1 μM) acted as a positive modulator of agonist action on NR1-2AB and NR1-2AC receptors whereas at higher concentration *lead* inhibited NR1-2AB and NR1-2AC receptors, but with less potency compared to NR1-2A or NR1-2B [157, 159].

There is incongruity whether *lead* acts via the *zinc* binding site or through a different site. A set of experiments demonstrated that in the presence of increasing amounts of Pb^{2+}, there was a concentration-dependent downward shift of the Zn^{2+} inhibition curve; also, the values of IC_{50} for Zn^{2+} inhibition decreased as a function of increasing Pb^{2+} concentrations. The effects of Zn^{2+} on Pb^{2+} curve and IC_{50} were analogous [160]. These findings suggested that the two metals act via independent binding sites, which is in line with the observation that increasing concentrations of Pb^{2+} did not affect the Zn^{2+} IC_{50} [154]. However, these results were in contrast with other findings which report that the two cations compete for the same binding site [161, 162].

Nickel (Ni^{2+}) is a trace element, which is essential for many biological organisms, but could also induce toxicity. The effects of Ni^{2+} on NMDA channel activity were described as a voltage-dependent and "Mg^{2+}-like" inhibition [127]. Later work showed a potentiation of homomeric NR1a channels [121] and an inhibition of NR1-2A channels [13, 146]. In more recent studies, it is suggested that, besides the voltage-dependent Mg^{2+}-like inhibition, Ni^{2+} causes a potentiation of NR2B-containing channels and a voltage-independent inhibition (Zn^{2+}-like inhibition) in those neurons containing NR2A [163].

Nickel also caused a reduction of single channel current amplitude at negative voltages while the dependence on membrane voltage was slightly steeper for NR2A than NR2B [13]. Several analogies with Mg^{2+}-like inhibition indicated that it might interact with either the N or N+1 site in the pore-forming region of the NR2 subunit [13].

Support for the above theory came from an experiment showing that a single mutation in the NR2B site at the N+1 site would completely abolish the voltage-dependent block Ni^{2+} [123]. The N+1 residue had been shown to be a critical binding site for Mg^{2+} block in NR2A subunit [128]. Moreover, at positive potentials the effects of Ni^{2+} were highly subunit dependent. NR2A-containing channels were blocked in a voltage-independent manner whereas NR2B containing channels were facilitated [13] (see Abbreviation section). However, at higher concentrations (IC_{50}= 442 μM), a voltage-independent inhibition was also present in NR1-2B channels [123]. The voltage-independent inhibition site of Zn^{2+} was investigated as a potential site for Ni^{2+} inhibition, but this did not seem to be the case. Besides the difference in blocking affinity, Zn^{2+} inhibition was pH dependent [122, 164] while Ni^{2+} inhibition was not [163]. Also, Zn^{2+} inhibition was never more than 60–80% (as described in section 3.2.1), while Ni^{2+} inhibition approached 100% at positive potentials [123]. Other results also showed that mutations that affect the inhibition of Zn^{2+} did not modify Ni^{2+} sensitivity [146].

The NR2B-selective potentiation was suggested to share the site of action with spermine, as Ni^{2+} partially obscured the effect of spermine when they were applied concurrently [123].

Although the actions of *zinc, lead, and nickel* on NMDARs were intensively investigated, there are also some reports that other metals have an effect on these receptors and channel activity.

Copper (Cu^{2+}) is an endogenous metal in the human brain [165], and it is an established fact that *copper* represents an integral part of neurotransmission [166]. It is released from synaptic vesicles following neuronal depolarization [167]. The concentration of *copper* in the synaptic cleft could reach up to 100 μM [168]; later studies estimated the concentration of *copper* released into the synaptic cleft to be in the range of ~15 μM [169, 170]. However, the topographic distribution showed marked variations between different brain areas [171]; the highest concentration of *copper* has been found to be in the hypothalamus [172].

Cu^{2+} acts on NMDA receptors and reduces the current—induced by 50 μM NMDA—in a concentration-dependent manner with an IC_{50} of 15.9 μM. This block was completely and quickly reversible, even in the absence of antioxidant dithiotreitol, suggesting that the inhibition was not an oxidizing effect [173]. Further studies showed that Cu^{2+} inhibition was characterized by voltage-independent, but use-dependent mechanism of action, as the degree of inhibition was dramatically decreased in the absence of agonists [174].

Another trace metal required for normal brain function is *manganese (Mn^{2+})*. In the human brain, Mn^{2+} is most concentrated in the globus pallidus, caudate, and putamen, but also found in other areas [175]. Mn^{2+} produces a strong voltage-dependent block in response to NMDA [176]. It was, also, a competitive antagonist of MK-801 binding to the NMDAR-channel. Its inhibitory effects were activity-dependent since Mn^{2+} was a more potent inhibitor in the presence of NMDA coagonists (Glu and Gly) than in their absence [177]. Taking these studies together, they indicate that Mn^{2+} is an NMDAR channel blocker. Interestingly, the inhibitory constant for Mn^{2+}, in the absence of Glu and Gly, was significantly different in neuronal membranes from the cerebellum relative to other brain regions; however, in the presence of the agonists, Mn^{2+} was equally potent in inhibiting NMDARs in different brain regions [177].

3.1.1. The Glycine-Binding Site of the NMDA-Receptor/Channel Complex. The NMDAR glycine-binding site was susceptible to modulation by divalent cations, especially when the glycine site was not saturated. Low, extracellular concentrations of Mg^{2+} potentiated NMDAR currents. The potentiation was the result of an increase in the affinity of NMDAR for glycine [129, 130, 178]. The mean glycine EC_{50} value was 100–133 nM in control conditions and was reduced to 60–62 nM in the presence of 10 μM Mg^{2+} [178]. This increase in affinity was also demonstrated by decreasing the inhibitory potency of NMDAR glycine-site antagonists upon the addition of potentiating concentrations of Mg^{2+}[129, 178, 179]. Ca^{2+} had the same effect as Mg^{2+} [129, 178, 179].

Concentrations of Pb^{2+} and Zn^{2+} higher than 10 μM inhibited NMDAR potentiation by Ca^{2+} and Mg^{2+} [178]. These findings were supported by other studies, which showed that increasing concentrations of Ca^{2+} diminished the inhibition of NMDAR currents by Zn^{2+} [136], or Pb^{2+} [180]. It is suggested that these divalent cations act on the same site, and Ca^{2+} and Mg^{2+} have opposite effects on glycine binding compared to Pb^{2+} and Zn^{2+} [178].

Even the presence or absence of glycine modulated the effects of some of the cations: for example, Cu^{2+} and Mn^{2+} are both potent NMDAR channel inhibitors (as shown above), but in the presence of glycine and glutamate, Cu^{2+} was more potent than Mn^{2+}, and in the absence of glycine, Mn^{2+} was slightly more potent [177].

3.2. AMPA and Kainate Receptors. The two classes of ionotropic glutamate receptor-channels, which are designated non-NMDA channels, are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and kainate receptors, named after their most potent excitatory amino acids. The AMPAR channel is also activated by kainate (for review, [181]). Also, AMPARs mediate the fast excitatory synaptic transmission in the CNS [182].

AMPA/kainate receptor-gated channels are permeable to Na^{+} and K^{+} and more or less impermeable to Ca^{2+}; however, there is a subpopulation of central neurons, which contain AMPA/kainate receptors with enhanced Ca^{2+}-permeability [183, 184]. This Ca^{2+} conductance triggered by the AMPA/kainate receptors seemed to be dependent on the absence of the GluR2 subunit [119, 182, 185].

Extracellular calcium ions produced rapid and reversible voltage-independent inhibition of AMPARs, with both Ca^{2+} permeable and Ca^{2+} impermeable AMPAR being equally sensitive [186]. The Ca^{2+} effects were agonist dependent, more prominent in the case of AMPA compared to Glu or kainate. These data suggested that Ca^{2+} enhances desensitization, as two well-known antidesensitization agents prevented Ca^{2+} inhibition through Ca^{2+} binding to a modulatory site in the AMPAR [186].

Effects of *zinc* on AMPA/kainate receptors have also been explored. Zn^{2+} appeared to have a dual effect on AMPAR: at micromolar concentrations, it enhances AMPA receptor responses whereas at millimolar concentrations, it inhibits them [136, 187]. These effects of Zn^{2+} appear to be subunit specific as well. Experiments using cloned AMPAR expressed in oocytes demonstrate that, in normal calcium-containing solution, *zinc* could potentiate current from homomeric GluR3 receptors over a narrow range of 4–7.5 μM Zn^{2+} while homomeric GluR1 receptors could not be potentiated, but are inhibited by 10 μM Zn^{2+} [188]. Additionally, in calcium-free solution, the inhibition caused by Zn^{2+} on GluR1 shifted to ≥1 mM and potentiation was attainable reaching a maximum of ∼200% at 50 μM Zn^{2+}. Also, GluR3 showed maximum potentiation not significantly different from GluR1 potentiation. The presence of GluR2 subunit in heteromeric expression of GluR2/GluR3 prevented the potentiation by Zn^{2+}, but also allowed inhibition (with 500 μM Zn^{2+}, current was 39% of control). The presence of GluR2 rendered the effects of Zn^{2+} independent of Ca^{2+} levels (for details, [189]).

The effects of other divalent metals effects were explored on these channels but less extensively compared to NMDARs. Various metals caused inhibition of Ca^{2+} impermeable AMPAR with the following rank order of inhibition: $Ni^{2+} > Zn^{2+} > Co^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$ (for values, refer to Table 5) [190]. The proposed mechanism of action is that complexes of divalent cations and AMPAR agonists compete with the free agonists rather than the cations themselves. This mechanism fits the data in which a competitive type of inhibition is observed; in addition, an increase in agonist concentration reduce the inhibitory effects of divalent metals less than that of DNQX (the classical competitive AMPAR antagonist) [190].

3.3. GABA Receptor. γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system. The synaptic transmission mediated by $GABA_A$ receptor-channel complex leads to a hyperpolarization of the cell membrane due to the fast activation of postsynaptic chloride channels upon the exposure to GABA [191]. The $GABA_AR$ is comprised of pentameric combination of α1–6, β1–4, γ1–3, δ 1, and/or ε1 subunit subtypes that form an intrinsic chloride ion channel, and each subunit comprises four domains. $GABA_ARs$ have some recognized allosteric binding sites such as barbiturates, benzodiazepines, and picrotoxin [192, 193]. The properties of the allosteric binding sites were influenced by the subunit subtype composition of $GABA_AR$ (for review [194]).

3.3.1. Inhibitory Effects of Metal Ions. The $GABA_AR$ response to GABA-evoked currents was modulated by a number of divalent metal cations. *Zinc, cadmium, nickel, manganese, cobalt, lead,* and *copper* inhibited the response to GABA. The inhibition by divalent metals has consistently been shown to be reversible with no or little voltage dependence [14].

Zn^{2+} had the potential to directly interact with the $GABA_AR$ to influence inhibitory postsynaptic currents (IPSC) amplitudes and kinetics [118]. Zn^{2+} suppressed the GABA-induced chloride current with a K_d of 19 μM in a noncompetitive, voltage-independent manner, and without interference with any of the allosteric sites on the GABA-R [195]. Using cultured hippocampal neurons, studies showed that Zn^{2+} reduced the amplitude, slowed the rise time, and accelerated the decay of mIPSCs. Evidence indicated that inhibition of mIPSCs by Zn^{2+} was attributed to an allosteric modulatory site located on the extracellular domain of $GABA_A$ receptors [196, 197]. In accordance with the previous hypothesis, single-channel studies have also shown that Zn^{2+} reduced the opening frequency with no evidence of flickering [198–200].

From another perspective, the effects of Zn^{2+} were subtype-specific. The *zinc*-sensitivity of the channels seemed to be dependent on the absence of γ subunits, as its presence in any combination with other subunits led to the formation of $GABA_A$ receptors almost insensitive to Zn^{2+} [201, 202]. Furthermore, the presence of a δ subunit enhanced *zinc* sensitivity [202, 203]. The exchange of a particular subunit with other members of the same subunit family (α1 versus α3, β1 versus β2, and γ1 versus γ2) did not alter the large difference in Zn^{2+} sensitivity between $GABA_AR$ containing or lacking γ subunit [202]. Later studies showed that recombinant $GABA_AR$, which contain α4, α5, and α6 subunits, were more sensitive to *zinc* than those that contain α1 subunits [204–207]. Given that the majority of synaptic $GABA_AR$ are of the α-, β-, γ-isoform [208], together with the above studies, indicate that a likely target of Zn^{2+} modulation is an extrasynaptic α-, β- or δ-receptor [118].

In rats dorsal root ganglion (DRG) neurons, Cu^{2+} at concentration of 15 μM, suppressed the peak amplitude of the GABA-induced current to approximately 50%; the blocking was exerted and reversed quickly, and it was independent of membrane potential [195].

The similar blocking profiles of Cu^{2+} and Zn^{2+} led to the question whether they shared a common binding site. Competition experiments showed that Zn^{2+} suppression of GABA-induced current was decreased with increasing concentrations of Cu^{2+}, suggesting that Zn^{2+} and Cu^{2+} act on the same allosteric site to inhibit $GABA_AR$ [55].

In a later study, the *copper*-induced block of $GABA_AR$ in Purkinje cells developed slowly, was poorly reversible, and decreased with increasing GABA concentrations. The block occurred at low concentrations indicating a high affinity with an IC_{50} ∼35 nM [209]. The copper block of $GABA_AR$ in Purkinje cells seemed to have a higher affinity compared to the block in DRG [195] and olfactory bulb neurons [210]. Another difference between these tissues was that Cu^{2+} in DRG cells interacts in a noncompetitive manner while in Purkinje, Cu^{2+} decreased the potency of GABA without affecting the maximal response. A possible explanation for this discrepancy might be different subunit composition of the $GABA_AR$ [210].

The effects of multiple divalent metals (*cadmium, nickel, manganese, zinc,* and *barium*) were shown in a study of GABA responses of embryonic chick spinal cord neurons. The results were suggestive of an allosteric mechanism of inhibition of $GABA_AR$ currents. Through combination

experiments they showed that the ions acted at a common site but possessed different intrinsic efficacies with the following rank: *Zn* > *Cd*> *Ni*> *Mn*. *Ba* was thought to bind to the site but lacked efficacy as an inhibitor of the GABA response [14]. The rank of efficacy was supported by other experiments [195].

3.3.2. Excitatory Effects of Metal Ions. *Lanthanides* comprise a series of 15 metals starting with *lanthanum (La)* and ending with *lutetium*. In sub-millimolar concentrations, lanthanum ions modulate GABA-induced currents [55, 191, 211].

La^{3+} increased the affinity of GABA for the receptor in a concentration-dependent manner with an $EC_{50} = 231\,\mu M$ and maximum enhancement to about 300% of control with 1 mM. This potentiation was completely and quickly reversed, but was more enhanced as the potential became more negative (1.6% per 10 mV) [195]. Also, this effect was independent of the presence or absence of barbiturates, benzodiazepines, picrotoxin, or Zn^{2+}/Cu^{2+}, indicating that it was bound to a site different from all of the binding sites of the above substances [55]. La^{3+} did not activate transmembrane currents, it only potentiated GABA-induced currents; also La^{3+} did not affect the amplitude of the maximum response induced by GABA. These data together suggested that La^{3+} increased the affinity of $GABA_AR$ to its agonist [212]. *Other lanthanides* exhibited enhancing actions, and the efficacy increased with increasing the atomic number, such that Lu^{3+} (1 mM) increased the current to 1230% of control [55]. However, previously it was reported that *lanthanides* generate inward currents on their own in the absence of GABA [55]. This controversy might be due to use of different tissues; as La^{3+} did not activate transmembrane currents in CA1 hippocampal pyramidal neurons whereas La^{3+} generated inward currents in DRG neurons.

Additionally, recombinant $GABA_AR$ studies suggested that changing the α-subunit subtype from $\alpha 1$ to $\alpha 6$ alters the effects of *lanthanum* from potentiation to inhibition at comparable concentrations. These studies also suggested that the maximal inhibition of $GABA_AR$ current by La^{3+} in $\alpha 6$-containing receptors is greater in the presence of δ subunit (83%) than in the presence of γ subunit (32%) [213].

Another cation that might affect $GABA_AR$ is *mercury (Hg)*. In its inorganic form, $GABA_AR$ channel complex was strongly stimulated by low concentrations of *Hg* [214]. *Mercuric chloride* (100 μM) increased the GABA-induced current to 270% of control, and increased it to 115% of control with 0.1 μM [55] indicating its high potency.

3.4. Summary of Postsynaptic Effects. The main **postsynaptic targets** are the ligand-gated receptors including, but not limited to, NMDA, AMPA/kainite, and GABA receptors. Of those, NMDAR channels are the most widely studied receptors due to their association with disease status. Relatively fewer studies have been done on other targets, which could lead to underestimation of their roles in metal toxicity.

Two main mechanisms established for metals effects on NMDAR: Mg^{2+}-like inhibition, which is voltage dependent, or Zn^{2+}-like inhibition, which is voltage independent. *Lead* and *copper* were found to inhibit NMDAR in Zn^{2+}-like pattern. However, *copper*-mediated inhibition of NMDAR was use dependent which was also true for *manganese*-mediated inhibition [160–162]. *Nickel* on the other hand, showed an Mg^{2+}-like inhibition at negative potentials. However, it had different effects on NR2B and NR2A containing channels at positive potentials. It caused a potentiation of NR2B-containing channels and a Zn^{2+}-like inhibition in those containing NR2A. However, at high concentrations, NR2B-containing receptors also showed Zn^{2+}-like inhibition at positive potentials [163]. Another major target on the NMDAR was glycine-binding site with multiple metals affecting it. Ca^{2+} and Mg^{2+} potentiated NMDAR currents whereas Pb^{2+} and Zn^{2+} inhibited NMDAR currents. All of these four divalent metals might act on the same binding site with different effects [178]. Also, the presence or absence of glycine affected the potency of Cu^{2+} and Mn^{2+}, such that in the presence of Gly, Cu^{2+} was more potent, whereas in the absence of Gly, Mn^{2+} was slightly more potent [177].

The main inhibitory receptors in the brain, $GABA_A$, are also modulated by a variety of metals. Certain metals suppressed the GABA-induced chloride current while others augmented it. Multiple divalent metals had inhibitory effects on GABA-induced current. Cu^{2+} and Zn^{2+} suppressed $GABA_AR$ in an equipotent manner, and there was some evidence that they act on the same site. Other divalent metals also showed inhibitory effects through an allosteric mechanism of inhibition, and they demonstrated a common site of action but with different intrinsic efficacies with the rank $Zn^{2+} > Cd^{2+} > Ni^{2+} > Mn^{2+}$ [14].

Interestingly, *Lanthanides* exhibited enhancing effects of GABA-induced currents. The effect was completely and quickly reversible. The efficacy increased with increasing atomic number [55]. However, recombinant $GABA_AR$ studies suggested that changing the α-subunit subtype could alter the effects of lanthanum from potentiation to inhibition in a comparable concentration range.

Mercury was another metal, which had inhibitory and excitatory effects. In its inorganic form, $GABA_AR$ channel complex was strongly stimulated by low concentrations of *Hg*. However, *methyl mercury* was a potent inhibitor of GABA-induced current, and this effect was irreversible [55]. This showed how organic metals might behave differently compared to inorganic cations; however, there is less information in the literature about the effects of organic metals. One of the most toxic organic metal is *trimethyl-tin* (*TMT*). At a concentration of 100 μM, around 20–30% of AMPAR and $GABA_AR$ currents were inhibited whereas 35% of NMDAR ion currents were blocked [215].

4. Disruption of Synaptic Plasticity

Multiple reports have demonstrated that human exposure to environmentally concentrations of certain metals can result in cognitive deficits.

Arsenic consumption, mainly through contaminated water, has been found to be associated with impairment

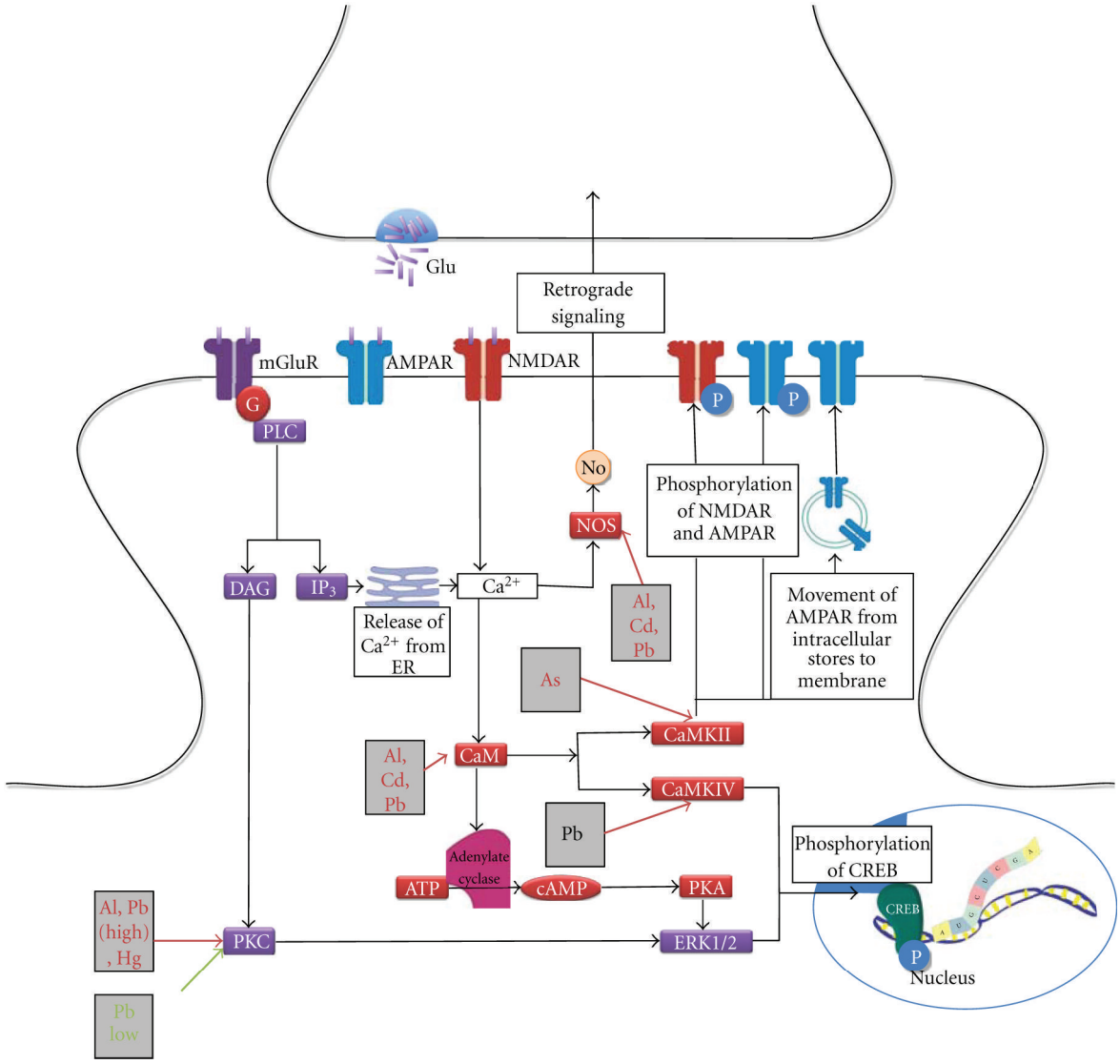


Figure 5: Proteins involved in the formation of long-term potentiation (LTP) and toxic effects of metals. LTP consists of different forms: early-phase LTP (E-LTP), which lasts only a few hours, and late-phase LTP (L-LTP), which lasts for several days. E-LTP includes short-term potentiation (STP), which is dependent on NMDA receptor activation and Ca^{2+}/calmodulin and LTP-1 that involves protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase- (CaMK-) dependent phosphorylation. While STP can be formed by activation of NMDA and calmodulin dependent enzymes, LTP-1 requires activation of PKC via DAG that is produced after the activation of mGluRs. PKC and CaMKII then phosphorylate AMPA and NMDA receptors. L-LTP consists of the later phases of LTP, which are LTP-2 and LTP-3. LTP-2 requires synthesis of new proteins and receptors whereas LTP3 requires gene transcription. Activation of adenylate cyclase and cAMP-dependent activation of PKA are required for the formation of the later phases of LTP. LTP-3 depends on the activation of extracellular signal- related kinase 1/2 (ERK1/2) and CaM kinase IV, which in turn phosphorylate CREB and lead to new protein synthesis. Other factors such as p38 mitogen-activated protein kinase (p38 MAPK) leads to the formation of long-term depression (LTD). Several of the molecules required to produce these different forms of LTP have been identified and are targets for metal toxicity, which have been shown (red arrows indicate inhibition whereas green arrows indicate activation by metals. Black arrows indicate activation that occurs during normal formation of LTP).

of long-term memory and a reduction in the verbal IQ of children [216, 217]. *Lead* has been studied extensively for its role in disruption of synaptic plasticity in an attempt to explain the cognitive deficits observed in children with elevated blood lead levels. The CDC currently considers blood Pb^{2+} level of 10 μg/dL to be the threshold for impairment of cognitive function in children [218], although recent studies have observed that cognitive impairment can occur even at blood lead levels <10 μg/dL [219]. There have been reports that *aluminum* also affects synaptic plasticity, which has been implicated in the pathogenesis of Alzheimer's disease, although this topic is highly debated. It has been argued that these detrimental effects on learning, memory, and cognition, which are associated with exposure to metals, may be linked to the disruption of processes that are involved in synaptic plasticity. The formation of long-term potentiation (LTP) is impaired in mice that have inborn low learning capacity indicating the crucial role for synaptic plasticity as the basis of learning and memory. Impairment of LTP has been observed with exposure to *lead* [220]. Moreover, studies have shown that *aluminum* also impaired hippocampal long-term potentiation (LTP) and long-term depression (LTD) in rats both *in vivo* and *in vitro* [221, 222]. Also, multiple metals have been shown to have different concentrations in patients with Parkinson's disease compared to healthy individuals, and the levels of aluminum have been identified as a potential diagnostic marker [223].

To understand how metals and their compounds affect learning and memory, their effects on different stages of LTP and LTD were compared to identify specific sites of interaction for particular metals as well as targets common to more than one metal.

Recently, it has been shown that LTP consists of different succeeding forms: early-phase LTP (E-LTP), which lasts only a few hours, and late-phase LTP (L-LTP), which lasts for several days [224–226]. Several of the molecules required to produce these different forms of LTP have been identified and are targets for metal toxicity [224, 225, 227] (refer to Figure 5 and Table 6).

Early-LTP. it includes short-term potentiation (STP), which is dependent on NMDA receptor activation Ca^{2+}/calmodulin; and LTP-1 that involves protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase-(CaMK-) dependent phosphorylation. While STP can be formed by activation of NMDA and calmodulin-dependent enzymes, LTP-1 requires activation of PKC via DAG that is produced after the activation of mGluRs. The activity of mGluR-PKC is important for both increasing activity as well as increasing number of AMPA receptors. PKC and CaMKII then phosphorylate AMPA and NMDA receptors.

Late-LTP. there are two later phases of LTP named LTP-2 and LTP-3. LTP-2 requires synthesis of new proteins and receptors whereas LTP-3 requires gene transcription. Activation of adenylate cyclase and cAMP-dependent activation of PKA are required for the formation of the later phases of LTP. LTP-3 depends on the activation of extracellular signal-related kinase 1/2 (ERK1/2) and CaM kinase IV, which in turn phosphorylate CREB, and this leads to new protein synthesis. p38 mitogen-activated protein kinase (p38 MAPK) is involved in the formation of long-term depression (LTD), and c-JUN-N-terminal kinase (JNK) is thought to participate in LTD [228, 229].

4.1. Disruption of Long-Term Potentiation by Exposure to Metals in Adults

4.1.1. Calmodulin. Calmodulin (CaM) is a regulatory protein that is activated by $[Ca^{2+}]_i$. This protein is found in high concentrations in CNS neurons and is involved in the activation of several other proteins. Some of the CaM-regulated proteins that modulate synaptic plasticity include adenylyl cyclases (AC1 and AC8), protein kinases, calcineurin, calmodulin kinases (CAMK I, II, and IV), nitric oxide synthase, and Ca^{2+} conducting channels. CaM has four Ca^{2+} binding sites. Ca^{2+} binding to CaM leads to a conformational change that exposes a hydrophobic domain which enhances the binding of CaM to other target proteins [226]. It has been hypothesized that in absence of Ca^{2+}, the concentration of free CaM is regulated by neurogranin that binds CaM and releases free CaM in response to PKC and Ca^{2+} [226, 230, 231]. This important molecule has been identified as a target of several neurotoxic metals such as *aluminum, cadmium,* and *lead*.

CaM, when incubated with increasing concentrations of *aluminum* (Al^{3+}) (from 0–1000 μM), showed decreased activity. This decrease in activity, measured by the ability of CaM stimulate activator-deficient cAMP phosphodiesterase was concentration dependent, [20]. Yet, another study showed that an $[Al^{3+}]$: [CaM] ratio of 3 : 1 resulted in 50% decrease in phosphodiesterase activity, and maximal inhibition was observed at a ratio of 4 : 1 [60].

Recently, using highly specific monoclonal antibodies that detect the different conformational states of CaM and monoclonal antibodies against *Al*-CaM complex, researchers found that on dissolving CaM with $AlCl_3 \cdot 6H_2O$ (in increasing concentrations from 0–480 μM), the antibody specific to Ca^{2+} calmodulin conformation (the active form) mAb CAM-1, did not recognize the *Al*-CaM complex (at *Al* concentrations of 240–300 μM) indicating that the CaM was in the inactive conformation. Moreover, the antibodies against the *Al*-calmodulin complex were found to bind to their antigen in the presence of Ca^{2+}. This shows *Al* binds *CaM,* even in the presence of Ca^{2+}, and CaM undergoes a conformational change into an inactive form. Equilibrium dialysis and atomic adsorption studies indicated that Ca^{2+} remained bound to CaM simultaneously with *Al*. When the *Al*-chelator citrate was added to the solution only partial restoration of CaM activity occurred, suggesting that some of the *Al* ions became inaccessible for chelation [62].

The effects of *Cadmium* (Cd^{2+}) were observed *in vivo,* where adult male rats, received 6 mg Cd^{2+}/kg body weight daily for four weeks. Brain CaM activity was determined by measuring the stimulation of phosphodiesterase activity. A

Table 6

Target		Al Effect	Al Concentration	Al Ref	As Effect	As Concentration	As Ref	Cd Effect	Cd Concentration	Cd Ref	Hg Effect	Hg Concentration	Hg Ref	Pb Effect	Pb Concentration	Pb Ref
Pumps	Ca^{2+} ATPase	↓ *in vitro*	10 mg/kg/day Al^{3+}	[1]												
Protein Synthesis	NMDAR NR1													↕ dev.	750 ppm PbAc	[13]
														↑ dev.	750 ppm PbAc	[14]
	NMDAR NR2A													↓ dev.	750 ppm PbAc	[13]
														↓ dev.	750 ppm PbAc	[14]
	NMDAR NR2B													↕ dev.	750ppm PbAc	[13]
														↕ dev.	750 ppm PbAc	[14]
Enzymes	CaM	↓ *in vitro*	10 mg/kg/day Al^{3+}	[1]				↓ *in vivo*	6 mg/kg/day $CdCl_2$	[15]				↑ *in vitro*	30 μM Pb^{2+}	[21]
		↓ *in vitro*	IC_{50} —15 μM $AlCl_3$	[3]				↓ *in vitro*	IC_{50} —0.47 mM $CdCl_2$	[8]						
		↓ *in vitro*	$AlCl_3$—240 μM	[9]				↓ *in vitro*	10 nM $CdCl_2$	[4]						
	PKC	↓ *in vitro*	10 mg/kg/day Al^{3+}	[1]							↓ *in vitro*	IC_{50}—1.5 μM Hg; IC_{50} —0.22 μM CH_3Hg	[2]	↓ dev.	0.1% PbAc	[5]
		↓ *in vivo*	0.3% $AlSO_4$ for 4 months	[18]							↓ *in vitro*	IC_{50}—0.08 μM $HgCl_2$; IC_{50}—1.32 μM CH_3Hg; IC_{50}—0.90 μM $C_6H_5Hg^+$	[7]	↓ *in vitro*	IC_{50} —2.12 μM PbAc	[10]
											↓ *in vivo*	10 mg MeHg/kg	[16]	↓ *in vivo*	1500 ppm PbAc	[17]
														↑ *in vitro*	10^{-13} to 4×10^{-4} M PbAc	[19]
														↓ *in vitro*	$> 4 \times 10^{-4}$ M PbAc	
	NOS	↓ *in vivo* ↓dev.	2.5% $AlSO_4$ for 3–5 weeks 3% $AlSO_4$	[20] [27]	↓ *in vivo*	37 ppm $NaAsO_2$	[6]	↓ *in vitro*	100 μM $CdCl_2$	[11]				↓ *in vivo*	125 ppm PbAc	[12]
														↓ dev.	0.2 % PbAc	[22]
														↓ *in vitro*	IC_{50}—0.36 Pb^{2+}	[11]
	CaMK II													↓dev.	1500 ppm PbAc	[25]
	CaMK IV				↓ *in vivo*	4 ppm As_2O_3 for 60 days	[23]									
Transcription factors	P38 MAPK							↑ *in vitro*	5–100 μM $CdCl_2$	[28]				↑ *in vitro*	5 μM Pb^{2+}	[30]
	ERK1/2 phosphorylation							↑ *in vitro*	100–200 μM $CdCl_2$	[28]				↑dev.	2 mg/kg/day PbAc	[30]
														↑ *in vitro*	5 μM Pb^{2+}	[30]
	CREB phosphorylation													↓ dev.	1500 ppm PbAc	[29]

Julka and Gill [20], Nihei and Guilarte [56], Guilarte and McGlothan [57], Vig et al. [58], Sandhir and Gill. [59], Siegel and Haug [60], Cox and Harrison [61], Levi et al. [62], Ohtani-Kaneko et al. [63], Rajanna et al. [64], Xu et al. [65], Inoue et al. [66], Rajanna et al. [64], Saijoh et al. [67], Nihei et al. [68], Johnson et al. [69], Coppi et al. [70], Hermenegildo et al. [71], Zarazua et al. [72], Mittal et al. [73], García-Arenas et al. [74], Chetty et al. [75], Llansola et al. [76], Wang et al. [77], Toscano et al. [78], Rigon et al. [79], Cordova et al. [80], Toscano et al. [81].

significant decrease in the CaM activity was observed after Cd^{2+} treatment. CaM bound to Cd^{2+} was also detected in the brains of rats exposed to $CdCl_2$. It was proposed that, since Cd^{2+} has an ionic radius similar to Ca^{2+}, it might interact with the Ca^{2+}-binding sites on the CaM [58, 61].

Lead was also found to interfere with CaM activity *in vitro* and *in vivo*. *In vitro* incubation of CaM with *lead* (Pb^{2+}) increased the activity of calmodulin in terms of its ability to stimulate cAMP phosphodiesterase and a maximum increase was observed at 30 μM lead concentration whereas at higher concentrations the calmodulin activity was inhibited. CaM-dependent cAMP phosphodiesterase activity increased up to a concentration of 100 μM, following which there was a sharp decline in activity with higher concentrations of *lead*. The involvement of phenomenon of mimicry of calcium by *lead* as a mechanism of toxicity has been proposed. The affinity of *lead* to CaM is stronger than that of calcium and *lead* can displace calcium from calmodulin [59]. An *in vitro* study done on CaM purified from bovine brain showed that Pb^{2+} mimics a natural ligand and raises the maximal activation slightly above the activation by Ca^{2+} [232].

4.1.2. Protein Kinase C (PKC). Protein Kinase C is a Ca^{2+} and phospholipid-dependent serine/threonine kinase that is a receptor for DAG and phorbol esters. There are two classes of PKC. The classical group of PKC consisting of four isozymes: PKC-α, PKC-βI, PKC-βII, and PKC-γ, are Ca^{2+}-dependent and require Ca^{2+} as well as DAG or phorbol ester for their activation. The second group of PKC isoforms consists of five isozymes: PKC-δ, PKC-ε, PKC-η, PKC-θ, and PKC-μ. These do not require Ca^{2+} for their activation by DAG or phorbol ester. Various isozymes of PKC are involved in the formation of LTP. For instance, a null mutation in PKC-γ prevented the induction of LTP [233]. PKC is activated postsynaptically when metabotropic glutamate receptors (mGluR) are activated leading to the formation of DAG and release of intracellular Ca^{2+}, which activates PKC. The mGluR- PKC pathway then increases the number and activity of AMPA receptors [224]. The PKC activity is affected by metal ions such as Al^{3+}, Pb^{2+}, *Hg,* and organic metals such as *methylmercury (MeHg;* refer to Figure 5).

Aluminum (Al^{3+}; 0–100 μM) decreased *in vitro* PKC activity (determined by transfer of ^{32}P from γ-^{32}P-ATP to lysine rich histone in the presence of Ca^{2+} and phosphatidyl serine), and this effect was concentration dependent [20]. *In vivo*, rats fed *aluminum* ($AlSO_4$) orally were found to have more PKC in the particulate fraction of the brain homogenate compared to the soluble fraction. Normally PKC is translocated from the cytosol to the membrane when it is activated. Application of Al^{3+} caused a 70% increase in the total activity of PKC resulting in a greater fraction of it being translocated to the membrane, and hence the presence of greater fraction of PKC in particulate fraction compared to the soluble fraction [69].

Lead acetate upon *in vitro* incubation with PKC from adult rat brains significantly inhibited PKC activity with an IC_{50} of 2.12 μM [64]. However, it was found that while very low concentrations of Pb^{2+} (10^{-13} to 4×10^{-4} M) increased PKC activity, higher Pb^{2+} concentrations ($>4\times10^{-4}$ M) caused an inhibition of PKC activity. When recombinant human PKC iso-enzymes were examined, low concentrations of Pb^{2+} had very little activating effect on PKC-γ but inhibited it at higher concentrations ($>4 \times 10^{-4}$ M) [70]. *In vivo*, on exposure of adult rats to 1500 ppm *lead acetate*, there was a decrease in protein expression of PKCγ by 32% in the cytosol of hippocampal cells and 25% in the membrane fraction [68]. Another study comparing the effects of Pb^{2+} on the PKC in the brain *in vivo* and *in vitro* found a considerable increase in PKC activity *in vitro, but* failed to find a considerable change in PKC activity *in vivo* [234].

In vivo methylmercury chloride administration in rats in five doses of 10 mg/kg body weight leads to a decrease in the enzymatic activity of the cytosolic PKC extracted from the brain, although it did not induce any change in second messenger binding as measured by binding of [^{3}H]PDBu [67].

4.1.3. Ca^{2+}/Calmodulin Kinases. There are two types of Ca^{2+}/calmodulin kinases (CaMK) involved in LTP: CaMKII and CaMKIV. The Ca^{2+}-CaM complex generally activates these kinases. CaMKII is a serine/threonine protein kinase consisting of 12 subunits that are activated when activated calmodulin is associated with them [235]. Studies have shown that CaMKII blockers impede the ability to generate LTP. CaMKII can also be autophosphorylated at Thr^{286} and its activity becomes independent of Ca^{2+}-CaM. This autophosphorylation occurs after the formation of LTP. It is suggested that after activation, CaMKII phosphorylates the AMPA receptor subunit as well as GluR1 and NMDA receptors and therefore enhances their conductance [236, 237].

CaMKIV is also activated similarly but the downstream targets are different for CaMKIV. Experiments have shown that upon activation, CaMKIV can phosphorylate CREB, which in turn mediates the transcriptional control of protein synthesis required for the long-term maintenance of LTP [238].

In vivo, rats exposed to 4 ppm *arsenic trioxide* (As_2O_3) for 60 days showed about a 4-fold decrease in expression of CaMKIV compared to a control group of rats as elucidated by microarray analyses. Western blot analyses reflected similar findings. Moreover, the decrease in expression of the β-subunit of CaMKIV was greater than the decrease in α-subunit expression [77].

4.1.4. Nitric Oxide Synthase. Nitric oxide synthase (NOS) is an enzyme that produces nitric oxide (NO) by oxidizing L-arginine using molecular oxygen and NADPH [239, 240]. There are different kinds of NOS expressed in several cell types. Endothelial cells express constitutive endothelial NOS (eNOS) that is activated by Ca^{2+}. Macrophages express inducible NOS (iNOS), and its expression is inducible by cytokines. Neurons express constitutive Ca^{2+}-activated neuronal NOS (nNOS). Following the activation of NMDA receptors and the influx of Ca^{2+}, it is believed that nNOS

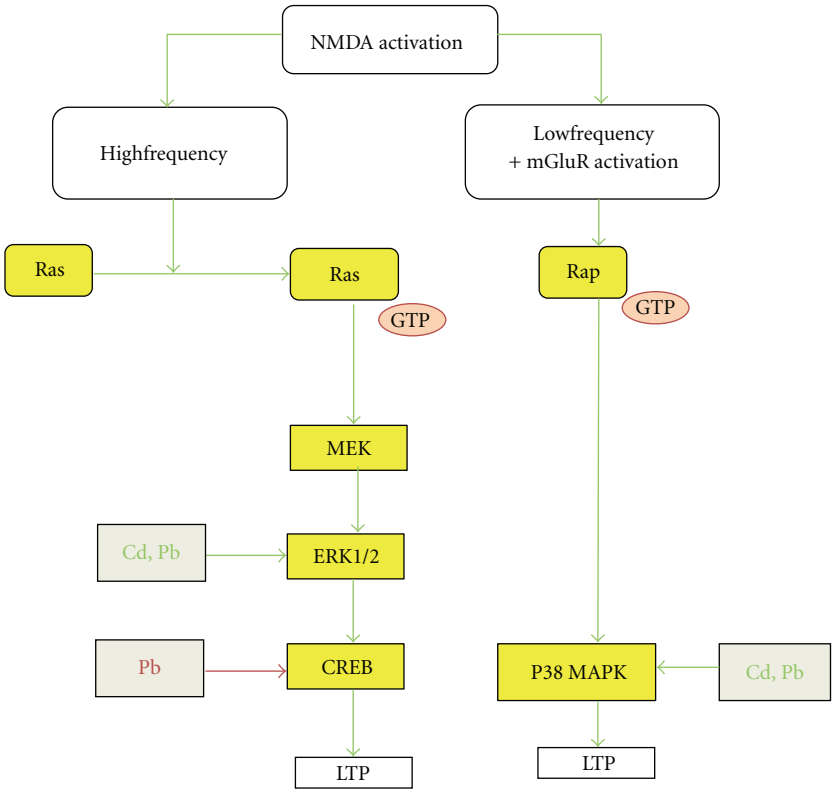


Figure 6: Molecules involved in the transcriptional control of LTP and LTD and effects of metals. LTP-3 depends on the activation of ERK1/2 and CaM kinase IV, which in turn phosphorylate CREB, and this leads to new protein synthesis. Other factors such as p38 mitogen-activated protein kinase (p38 MAPK) are involved in the formation of long-term depression (LTD) and c-JUN-N-terminal kinase (JNK) is thought to participate in LTD. A green color indicates an activation or an increase, and a red color indicates an inhibition or a decrease.

produces NO, a retrograde signal, that diffuses into the presynaptic membrane to enhance presynaptic neurotransmitter release by the production of cGMP during the formation of LTP [241, 242].

In vivo, chronic exposure to *aluminum* (Al^{3+}) resulted in the reduced formation of NO after activation of NMDA in the rat cerebellum, as a consequence of decreased calmodulin and NOS [71].

In vivo arsenic exposure to 37 ppm sodium *arsenite* for 10 days, reduced NMDA-induced NOS activity (as measured by sampling of extracellular fluid by means of microdialysis). The maximal NMDA-induced increase of NOS activity (estimated by measuring the changes in extracellular citrulline in the exposed groups) was only 170 ± 24% while under control conditions it reached 278 ± 27% ($P < 0.001$) [72].

In vitro incubation of NOS with 100 μM of *cadmium* ($CdCl_2$) resulted in a significant reduction in brain NOS activity as measured by the conversion of radioactive arginine to citrulline. When incubated, the activity of NOS was decreased with an IC_{50} value of 0.22 mM [73].

In vitro incubation of NOS with *lead.* inhibited NOS activity with an IC_{50} of 0.36 mM [73]. *In vivo*, the cNOS activity in the hippocampus and cerebellum (measured by citrulline radioactivity following incubation with radioactive arginine) was decreased in rats that were exposed to 125, 250 and 500 ppm *lead acetate* for 14 days. This decrease was completely reversible by increasing the free Ca^{2+}-concentration. The decrease in NOS activity correlated with blood lead levels [74].

4.1.5. Extracellular Signal-Regulated Kinases (ERK1/2). The extracellular signal-regulated kinases (ERK1/2) are serine threonine kinases that are activated when extracellular signals lead to an increase in intracellular Ras-GTP (GTP-bound form of Ras). Ras-GTP produced by an increase in guanyl nucleotide exchange factors (GEF), a decrease in activity of GTPase-activating proteins (GAPs) or a combination of both then leads to the activation of the enzyme MAPK/ERK kinase (MEK). MEK than activates ERK1 and ERK2 by phosphorylating them. ERK1 and ERK2 (also known as p44 and p42 MAPK) target transcription factors, cytoskeletal proteins, regulatory enzymes, as well as other kinases. In the postsynaptic membrane, calcium influx through the NMDA receptors leads to production of Ras-GTP that can then trigger the cascade leading to phosphorylation of ERK1 and ERK2. CREB maybe one of the targets of the ERK1/2 pathway involved in LTP [229, 243]. CREB, a member of the basic leucine zipper (bZip) family, is a transcription factor that is responsible for initiating new protein synthesis for the maintenance of L-LTP. PKA, CAMK and MAPK can activate CREB by phosphorylation at

serine-133. On phosphorylation other proteins such as CREB binding protein are recruited to form a complex, which initiates transcription of CRE containing genes [244–250] (refer to Figure 6).

Exposure of hippocampal slices of rats to Cd^{2+} activates ERK1 and ERK 2 but only at very high concentrations (100–200 μM $CdCl_2$) [79, 229].

On incubation of ERK1/2 with 5 μM Pb^{2+} *in vitro* for 3 hours, there was significant increase in ERK1 and ERK2 phosphorylation in hippocampal homogenates [80].

4.1.6. P38 Mitogen-Activated Protein Kinase (p38 MAPK). Parallel to the ERK1/2 pathway, which is involved in long-term potentiation, another MAPK cascade, which involves p38 MAPK is involved in long-term depression (LTD). Inhibition of p38 MAPK was shown to inhibit a form of hippocampal LTD that involved the activation of mGluR. Inhibition of ERK1/2 by blocking MEK had no effect on this form of synaptic plasticity. The pathways upstream and downstream of p38 MAPK are yet to be elucidated [229, 251] (refer to Figure 6).

Hippocampal slices of postnatal day 14 rats were exposed *to* Cd^{2+} in concentrations between 5–100 μM for 3 hours. A western blot analysis showed that this increased the activity of p38 MAPK, which is involved in the inhibition of LTP [79, 229].

Incubation of ERK1/2 with 5 μM Pb^{2+} for 3 hours resulted in a significant increase in p38 MAPK phosphorylation in hippocampal homogenates [80].

4.2. Disruption of Long-Term Potentiation by Exposure to Metals during Development. Developmental exposure to "neurotoxic" metals differs from exposure in an adult in various ways. The developing brain is more vulnerable than the adult one. The basic circuitry of the brain is laid down during development and any disruption of receptors, neurotransmission, and neurogenesis can prevent the brain from maturing normally. Inappropriate activation of the unspecific receptors in the developing brain can interfere with the normal "tuning." Moreover, the blood-brain barrier is not laid down till approximately six months of age in humans. This absence of blood brain barrier allows toxic agents to enter the brain freely and interfere with its development. Developmental exposure to metals also raises the issue of what Costa et al. labeled as "silent" neurotoxicity. This is when the deleterious effects of various neurotoxic insults do not manifest until several months or years post-partum. For instance, in Guam's disease, unknown neurotoxic agents cause damage to the CNS, which do not become apparent until decades later. Here we discuss developmental exposure of metals and their effects on the molecules involved in the formation of LTP, which are important for the development of memory and learning [252].

N-methyl-D-aspartate receptors (NMDAR) are Ca^{2+} channels, which play an essential role in several forms of synaptic plasticity (see Section 3.1). They have glutamate receptors present which are involved in excitatory synaptic transmission in various parts of the brain. Its unique properties, such as Mg^{2+} block and high permeability to Ca^{2+}, give NMDAR the ability to contribute to the formation of long-term potentiation and long-term depression. Several subunits of NMDA receptors have been identified: NR1 that is ubiquitously expressed; NR2 subunit family that has four distinct types (A, B, C, and D) and two NR3 subunits. The expression of the various subunits is different in different stages of development. For instance, NR2B and NR2D expression is present during the neonatal period and NR2A and NR2C are present in the later stages of development [253]. Due to its many binding sites (especially those for divalent cations), which change in their affinity to their agonists during development, a variety of (toxic non-physiologic) metals might bind to these NMDAR with a high affinity and thereby impair their function.

Lead causes impairment of long-term potentiation in different regions of the hippocampus following chronic *lead* exposure [254, 255]. This has been associated with a disruption in the normal functioning of the NMDA receptors (NMDAR). NMDAR currents decrease after *in vitro* exposure to 5 μM *lead* in hippocampal cells [152]. This can be attributed to the observation that Pb^{2+} alters expression of the different subunits of NMDAR, which has been observed in the hippocampus and cerebral cortex. Additionally, a decrease in expression of NR2A subunit mRNA and proteins in the hippocampus have been seen [56, 57]. Also, the expression of NR1 subunit mRNA in the hippocampus and the cerebral cortex of rats increases [57], but this finding was not supported by another study by Nihei and Guilarte [56], which found no change in the expression of the NR1 subunit protein.

The effects of cadmium (Cd^{2+}) on calmodulin expression were determined in an *in vitro* study done on embryonic rat (ED 15) cerebral cortex, where the cortical slices were incubated with 10 nM *cadmium chloride* for 24 hours. This experiment showed a reduced the amount of calmodulin expression following *cadmium* exposure [63].

Nitric oxide synthase (NOS) was affected by the developmental exposure of rats to *aluminum (Al^{3+})* and *lead (Pb^{2+})*. Prenatal exposure of developing rats to *aluminum* sulfate (3%) decreased the content of neuronal NOS by 62 ± 12% in the cerebellum [76].

Perinatal exposure to Pb^{2+} decreased NOS activity, as well as NOS expression. Chetty et al., using western blot analysis of nNOS in developing rat brain after perinatal exposure to 0.2% *lead* acetate, found a significant decrease in nNOS protein levels at postnatal day (PND) 21 and 35 in cerebellum, and at PND 21 in hippocampus [75].

Developmental *lead* exposure also affected PKC-γ and CaMKII function. PKC-γ is activated by binding of Ca^{2+} or DAG and on activation, it translocates to the membrane. To determine the effects of Pb^{2+} on PKC-γ and other PKC-subtypes, pregnant rats were exposed to 0.1% *lead acetate*, dissolved in distilled deionized water (DDW) from gestation day 6 to postnatal day 21 (PND). With western blot analysis the expression on PKC-γ was determined. Pb^{2+} reduced PKC-γ mRNA expression significantly in hippocampus and frontal cortex at PND 1, 5, and 10, with greater effect on the membrane PKC-γ than on the cytosolic PKC-γ. Additionally

there was a decrease in the activity of PKC-γ following exposure to *lead*. The PKC-γ activity was determined by measuring the amount γ-^{32}P transferred to histone per min per mg protein. In the hippocampus and the frontal cortex, both total and calcium-dependent PKC activities were significantly inhibited [65].

Moreover, rats exposed to 1500 ppm Pb^{2+} during development demonstrated a reduction in the $V_{\max}$ of CaMKII (examined by measuring the phosphorylation of a biotinylated substrate for CaMKII) and reduced expression of CaMKII β subunit in the hippocampus, but showed no changes in the sensitivity of calmodulin to CaMKII. In other words, the decrease in CaMKII activity was not due to impairment in its ability to bind CaM [78].

Various metals also inhibited the enzymes related to the transcription of new proteins involved in the formation of LTP. Two such targets are ERK1/2 and CREB.

Prenatal exposure to *aluminum* sulfate (3%) slightly increased the content of ERK [76]. Also, *in vivo* developmental exposure to 2 mg/kg of Pb^{2+} increased both ERK1 and ERK2 phosphorylation in rat hippocampal neurons [80].

On developmental exposure to 1500 ppm of Pb^{2+}, a decrease in the amount of phosphorylated CREB was observed in both the hippocampus (25% decrease) and the cerebral cortex (25% decrease) but there were no significant changes in unphosphorylated CREB levels [81]. Also, significant changes in the binding kinetics of CREB to CRE were observed in the hippocampus. The K_{d} and $B_{\max}$ both were decreased by 38% and 30%, respectively, in the hippocampus but no significant changes in binding kinetics were observed in the cortex [244].

4.3. Summary of Long-Term Effects. Metals affect various mediators of synaptic plasticity. Calmodulin (CaM) activity is affected by *aluminum, cadmium,* and inorganic *lead*. Both *aluminum* and *cadmium* inhibited CaM activity [20, 58, 60–63] whereas inorganic *lead* first increased CaM activity at lower concentrations (possibly by mimicking calcium), but then at higher concentrations it decreased CaM activity [59, 232]. CaM is the central modulator of NMDAR-mediated synaptic plasticity and a majority of the regulators of synaptic plasticity depend on CaM for their activation. Thus, interference with CaM function will indirectly affect the function of numerous LTP- and LTD-related proteins such as adenylyl cyclase, Ca^{2+}/Calmodulin kinases, nitric oxide synthase, and Ca^{2+} channels [226]. Al^{3+}, Pb^{2+}, and *MeHg* affect PKC activity, which is involved in the formation of LTP-1. *In vitro* studies demonstrated that in rat brain, Pb^{2+} inhibited PKC activity at low concentrations but increased PKC activity at higher concentrations. However, when recombinant human PKC-γ was used, an opposite trend was observed [70]. Exposure to Pb^{2+} *in vivo* resulted in a decrease in protein expression whereas *MeHg* decreased the activity of PKC. Al^{3+} inhibited PKC activity *in vitro* but ironically, oral administration lead to increase in PKC activity [20]. *Arsenic trioxide* (As_2O_3) decreases the expression of Ca^{2+}/calmodulin kinase IV (CaMKIV) with a greater decrease in the β-subunit than the α-subunit *in vivo* [77]. Nitric oxide synthase (NOS) activity is decreased by various metals such as Al^{3+}, As^{2+}, Cd^{2+}, and Pb^{2+} [71–73]. The components of the transcription pathway, p38 MAPK and ERK1/2 were phosphorylated more when incubated with Pb^{2+} and Cd^{2+}. As discussed earlier, P38 MAPK is involved in the induction of LTD whereas ERK1/2 is involved in induction of LTP-3 [79, 80, 229].

5. Discussion and Conclusion

Most metals act on multiple modulators of synaptic transmission. Heavy metals such as *mercury, lead,* and *arsenic* interfere with normal functioning of molecules both presynaptically and postsynaptically. They also target molecules involved in synaptic plasticity. As discussed above *in vivo* and *in vitro* studies have shown that metals inappropriately inhibit or activate various molecules involved in synaptic transmission and synaptic plasticity. Even though the current pool of the literature gives us valuable insights into the mechanisms of metal toxicity at the synapse, there are many limitations of the current studies.

Firstly, there is hardly any sufficient information with regard to the effect of metals at ***different stages of development***. Postsynaptically, there is a strong suggestion that the different effects on development are due to different subunit expression. As discussed in Section 3.2, Pb^{2+} was a more potent inhibitor of Glu-activated currents in NMDAR expressing NR2A or NR2B compared to receptors expressing both these subunits [157]. At the same time, Pb^{2+} showed high- and low-affinity components for its inhibition in PN14 and PN21 hippocampal membranes. These data suggested that the high-affinity Pb^{2+}-sensitive site was associated with receptors expressing NR2A or NR2B subunits, while the low-affinity site was associated with receptors expressing both subunits (to see the effects on other brain areas, review [153]). The support to this hypothesis came from studies showing that the developmental pattern of NR2A and NR2B mRNA in the hippocampus was similar to that in the data presented [256].

This developmental aspect is not well studied for most of the metals and also for AMPA/kainate and GABA receptors. The effects of divalent metals on AMPA/kainate receptors seem to be dependent upon the subunit composition as well, particularly the presence of GluR2 subunit which rendered the channel impermeable to Ca^{2+}. Zn^{2+} and Co^{2+} both had dual effects on AMPA-Rs: at micromolar concentrations they enhanced AMPA receptor responses whereas at millimolar concentrations, they had inhibitory actions. Various metals caused inhibition of Ca^{2+} impermeable AMPA-R, the inhibition was fast, reversible, and voltage independent. The rank order of activities was $Ni^{2+} > Zn^{2+} > Co^{2+} > Ca^{2+} > Mn^{2+} > Mg^{2+}$ [190]. The proposed mechanism of action was that complexes of AMPAR agonists and divalent cations compete with the free agonists for the binding sites.

Prenatal exposure to heavy metals also leads to various changes in the LTP machinery in the developing brain as discussed before. *Lead*, for instance, changed the expression pattern for NMDAR subunits, and decreased the expression

and activity of PKC-γ, CaMKII β, and nNOS in various areas of the brain. These results could explain why the development exposure to some metals causes cognitive deficits in children.

Moreover, a majority of the studies were done *in vitro*, and the *in vivo* studies were done in rats. In most *in vitro* studies, either brain homogenates or purified target molecules were incubated with a given metal. These studies therefore may not accurately depict the physiologic effects of the metals since they do not undergo the physiological process of absorption from the gut as in the human body, and the alterations that may occur in the blood before the metals reach the target tissue. Also, the solutions used for the preservation of the cells may interfere with the experimental results rendering them inaccurate. Moreover, the contents of the media and the forms of metals used were inconsistent between studies. Consequently properties such as solubility of metals in the media and presence of anions and pH were variable and beyond the scope of this study to discuss. It is definitely a limitation of *in vitro* studies, which makes it difficult to compare the different experiments, even when an identical concentration of the same metals was used. However, the ease of carrying out the experiment and lack of requirement for storage space for animals make them a likely choice for most researchers. *In vivo* studies may be closer to the physiologic processes; however it becomes harder than to vary the concentrations of metals and monitor them at the selected site of interest.

In addition, most of the findings presented in this review both *in vivo* and *in vitro* were based on studies done using rats. Even though it is easy to measure concentrations of metals, levels of proteins and enzymes in rats, elucidating the clinical manifestations in animal models can be challenging. Also, it is hard to find whether the effects in rats are similar to those in humans and if the effects in rats are representative of the effects in humans. Higher cognitive functions in humans might alter the presentation of the toxicity in ways that cannot be adjusted for because much of the mechanisms of the functioning of the human nervous system are not fully understood today.

Another important limitation of the currently available literature is that the majority of studies discussed were on effects of inorganic metals on the brain cells or brain molecules, and very few centered around organic metals which are perhaps even more significant than inorganic metal toxicity since in some cases the organic forms are more toxic than the inorganic forms, as for *mercury* [257]. There is a rapidly growing body of evidence that the majority of metals may actually be methylated to their organic form as the body attempts to detoxify metals. For *arsenic*, in the past it was believed that conversion of arsenic to *monomethyl arsenic* and *dimethyl-arsenic* was a method of detoxification; however, the view has changed since then with the recognition that methylated metabolites of trivalent arsenic are carcinogenic [258]. *Antimony, mercury, lead, tin, and selenium* are known to cause public health problems in their methylated forms. *Cadmium, cobalt, mercury, and nickel* reportedly undergo biomethylation; however, the effects of biomethylation have been studied more in unicellular organisms rather than plants and animals, therefore it has been suggested that although biomethylation does occur in plants and animals, the rates are likely to vary on the basis of the animal and the metal, and its concentration [259].

There are some situations that have not been considered in most experimental designs. One such issue that arises with the study of metals is the problem that most studies are not reflective with regard to the actual exposure in nature where humans are simultaneously exposed to more than one metal. Very few studies have targeted this issue, most likely due to the complicated nature of conducting an experiment with many variables and determining the contribution of each. One study by Platt and Büsselberg who examined the effects of combinations of Pb^{2+}, Zn^{2+}, and Al^{3+}, on voltage-activated calcium channels by simultaneous application of various combinations of two metals determined that regardless of the order in which the metals were added, the actions were in fact additive [260]. Whether this is the case with other metals is not certain and there is not enough data in the literature describing effects of combinations of metals. Another limitation of the study is that some targets may not be as relevant as others in causing the clinical symptoms of metal toxicity; however, it is not possible to know at this stage the exact contribution of each target.

Finally, the most important objective is to put these effects of metals into practical use. This can be done by using the data of the toxic concentrations of metals to make a meaningful decision in regard to their acceptable blood levels. There is evidence to suggest that currently accepted levels for some metals are still not "safe" levels. Even at the currently accepted blood *lead* levels of 10 μg/dL, it is causative of preterm labor and adverse pregnancy outcome [261]. Therefore, there is a need to reevaluate the accepted blood concentrations of metals in light of the newer evidence as it appears.

Metal neurotoxicity is a field, which is abounding with the literature and excellent research; however, in the current literature some metals are highlighted while for other metals (or metal compounds) hardly any data are available. There is an emphasis on certain metals such as *lead*, whose harmful effects are well known while there is very little known about certain metal groups such as *lanthanides* and *actinides*.

There are certain targets where metal actions have been excessively examined such as voltage-activated calcium channels while there is little known about the effects of metals on parts of signaling pathways such as phosphodiesterases and IP_3. This raises the need to evaluate new targets for metals, which have not been studied before, which may prove to have a groundbreaking effect in the field of neurotoxicity.

To summarize, exposure to different metals occurs due to industrial activities, environmental, and food chain contamination. This paper elucidated the various targets of metals in synaptic transmission and synaptic plasticity. Exposure to metals had varied effects on different synaptic targets, which were dependent on the form of metal, the concentration of metal, route of exposure (*in vitro* or *in vivo*), the medium used, and even the duration of exposure in some cases.

Abbreviations

$[Ca^{2+}]_i$: Intracellular calcium concentration
ABD: Agonist-binding domain
AC: Adenylate cyclase
Ach: Acetylcholine
Al: Aluminum
$AlCl_3$: Aluminum trichloride
AMPA: α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
As: Arsenic
As_2O_3: Arsenic trioxide
ATD: Amino terminal domain
ATP: Adenosine triphosphate
ATPase: Adenosine triphosphatase
Ba: Barium
BAPTA: 1,2-bis(2-aminophenoxy)ethane-N,N,N9,N9-tetracetic acid tetrakis(acetoxymethyl) ester
B_{max}: Maximal binding
Ca: Calcium
CaM: Calmodulin
CaMK: Ca^{2+}/calmodulin-dependent protein kinase
cAMP: Cyclic adenosine monophosphate
Cd: Cadmium
$CdCl_2$: Cadmium chloride
CDDP: *cis-Diammine-dichloroplatin*
CH_3Hg: Methylmercury
CNS: Central nervous system
Co: Cobalt
Cr: Chromium
CRE: cAMP response elements
CREB: Ca^{+2}/cAMP response element-binding protein
Cu: Copper
DAG: Diacylglycerol
DDW: Distilled deionized water
DOC2: Double C2 domain
DOPAC: 3.4-dihydroxyphenylacetic acid
DOPAC: 3.4-dihydroxyphenylacetic acid
DRG: Dorsal root ganglion
E-LTP: Early-phase LTP
ED: Embryonic day
eNOS: Eendothelial NOS
EPP: End-plate potential
ERK1/2: Extracellular signal-related kinase 1/2
Et_3Pb: Triethyl lead
Et_3Sn: Triethyl-tin
Fe: Iron
GABA: γ-Aminobutyric acid
GAP: GTPase-activating protein
GEF: Guanyl nucleotide exchange factors
$G_{i/o}$: Inhibitory Sguanine nucleotide binding protein/other guanine nucleotide-binding protein
Glu: Glutamate
GluR: Glutamate Receptor
Gly: Glycine
GPCR: G-protein-coupled Receptors
Gpp(NH)p: 5′ Guanylylimidodiphosphate
G_s: Stimulatory guanine nucleotide binding protein
GTP: Guanosine-5′-triphosphate
Hg: Mercury
HVA: Homovanillic acid
IC_{50}: Concentration for 50% inhibition
iNOS: Inducible NOS
IP_3: Inositol triphosphate
Inositol triphosphate: Inhibitory postsynaptic currents
IQ: Intelligence quotient
JNK: c-JUN-N-terminal kinase
K: Potassium
KCl: Potassium chloride
K_d: Dissociation constant
L-LTP: Late-phase LTP
La: Lanthanum
Li: Lithium
LTD: Long-term depression
LTP: Long-term potentiation
mAb: Monoclonal antibody
MAPK: Mitogen-activated protein kinase
Me_3Pb: Trimethyl lead
Me_3Sn: Trimethyl-tin
MeHg: Methylmercury
MEK: MAPK/ERK kinase
MEPP: Miniature end-plate potential
Met: Methionine
Mg: Magnesium
mGluR: Metabotropic glutamate receptors
mIPSC: Miniature inhibitory postsynaptic currents
Mn: Manganese
mRNA: Messenger ribo nucleic acid
Na: Sodium
NADPH: Reduced nicotinamide adenine dinucleotide phosphate
Ni: Nickel
NMDA: N-methyl-D-aspartate
NMDAR: N-methyl-D-aspartate receptors
nNOS: Neuronal NOS
NOS: Nitric oxide synthase
NT: Neurotransmitter
Pb: Lead
PKA: Protein kinase A
PLC: Phospholipase C
PND: Postnatal day
Ras: Rat sarcoma family
RBC: Red blood cells
Sn: Tin

SNAP-25: Synaptosome-associated protein 25 kDa
SNARE: SNAP and NSF attachment receptor
$SnCl_2$: Stannous chloride
Sr: Strontium
STP: Short-term potentiation
Thr: Threonine
VGCC: Voltage-gated calcium Channel
V_m: Membrane voltage
Zn: Zinc.

Authors' Contribution

S. Sadiq, Z. Ghazala and A. Chowdhury contributed equally.

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Research Article

Early Effects of Long-Term Neurotoxic Lead Exposure in Copper Works Employees

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The situation of exposure in a copper works facility in Germany enabled early lead-induced neurotoxic effects to be investigated in the workers. The aim of the investigation was to study the long-term effects of small doses of lead on psychometric/psychophysiological performance of workers. The study involved 70 male lead exposed workers and 27 male controls with no neurotoxic exposure. All test persons were subjected to the method of investigation involving performance data, physiological strain data, and the subjective state. It was found that of the psychometric performance parameters, only the mainly motor performance parameters had a potential for being neurotoxic early indicators. Preferably centrally influenced performance parameters were found to be less suitable early indicators. The lead-exposed subjects exhibited a slowed poststrain resetting behaviour of the vegetative nervous system, which correlated with the individual blood lead level. This was attributed to vagus depression, which had already started in the prevailing situation of exposure and was reflected by diminished cardiac phase duration variability. Our results indicate that it is necessary to more critically choose the lead level standards in the air on the working area. Heart rate variability may be affected even at small lead concentration.

1. Introduction

Although the neurotoxic action of lead is known for centuries, it has not yet been adequately elucidated which effects would be useful early indicators of a clinically latent lead intoxication. Lead, as a trace element, is not necessary for the organism, and is known to be toxic in almost all organ systems. Lead, because of its high affinity for the nervous system, produces neurological effects and impairments which have been described frequently. These effects might be subdividing into those occurring in the central, the peripheral, and the autonomic nervous systems [1].

1.1. Central Nervous System (CNS). According to reports in the literature, lead-exposed subjects have increasingly experienced CNS-induced complaints [2–15].

A great number of studies have focussed on deterioration of psychic or psychomental performance, describing mainly disturbed mood and affectivity [8, 16–19] as well as impaired performance such as enhanced fatigue symptoms, poor concentration, impaired memory, dysmnesia, and blocking of thought processes [9, 20]. The symptom verified most frequently has been a diminished reaction speed [9, 13, 20–22].

Neuropsychological data reported in the literature have demonstrated that continuous low-degree lead exposure impairs sensorimotor and primary central information processing. Stollery et al. [23] observed in higher-exposed workers (41–80 μg/dL) a longer sensorimotor reaction time, in particular for simple tasks, and impairment of the short-term memory. Araki et al. [24] found in lead-exposed workers statistically significant changes in evoked potentials which disappeared after one year of nonexposure. Murata et al. [25], Hirata and Kosaka [26], and Abbate et al. [27] described similar effects for the early visually evoked potentials after lead exposure. For early auditory evoked potentials, latency alterations have been demonstrated after chronic lead exposure [25, 28, 29].

1.2. Peripheral Nervous System. In the literature, there has been evidence suggesting that the response of the peripheral

nervous system to chronic lead exposure is more pronounced when compared to the CNS [26, 30–32]. Ulnaris extensor muscle paralysis in hands and feet is a typical symptom of lead intoxication.

In the search for effects of lead intoxication in the peripheral nervous system, measuring the motor nerve conduction velocity has turned out to be a useful approach even though this issue has been controversially discussed in the literature [4, 25, 26, 33–36].

As proposed by Ogawa et al. [37], determining the latency of Achilles jerk is a method that lends itself to describing lead-induced subclinical impairment of the peripheral nervous system. Investigations conducted by Stollery et al. [38] revealed delays of motor reactions in lead-exposed subjects. A marked slowing of the simple reaction time by lead was noted by Winneke [19]. Bjetak [39], in a study of lead-exposed workers, found that sensorimotor performance was affected even though memory and reaction tests did not reveal any difference compared to controls.

The effects of low-dose lead occupational exposure on neurobehavioral functions are still not well defined by occupational literature [40].

1.3. Autonomic Nervous System. Lead exposures may affect cardiovascular health through the autonomic nervous system [41, 42]. A method suitable to describe the function of the autonomic nervous system is the cardiac-rhythm analysis or analysis of heart rate variability (HRV) [43]. Reduced heart rate variability has been associated with sudden cardiac death and heart failure [44]. Abnormal cardiac autonomic function may be an important contributor to the pathophysiology of vascular disease, heart failure, and myocardial ischemia and their consequences, including sudden cardiac death [42].

Despite the wealth of literature published on this issue, work investigating the influence of harmful substances on this division of the nervous system has been scarce [25, 42, 45–50]. These publications gave accounts of a significantly reduced parasympathetic activity in lead-exposed workers when compared to nonexposed controls. The study from Park et al. [41] provides evidence that people with higher past exposures to lead are at increased risk of adverse health outcomes from air pollution. However, Gennart et al. [51] reported that in 98 lead-exposed workers studied, blood levels of lead (40–75 μg/dL) did not exert any influence on the autonomic nervous system as judged from sinus arrhythmia. The effects of lead on the heart rate variability have not yet been established [52]. Reference [53] found that the validity and precision of the studies on the association between lead exposure and decreased heart rate variability are often limited by small sample sizes, limitations in the assessment of lead exposure, and lack of control for established cardiovascular risk factors and other confounders.

From these sources dealing with the various divisions of the nervous system and a potential effect which lead may have, in the search for early effects, it is reasonable to conclude the following.

Early forms of a neurotoxic action of lead, with no other pathological clinical findings, show numerous individual features of manifestation making the scientific description of lead-induced neuronal disorders difficult. Hence, in the search for early effects of neurotoxic lead exposure, it is only a multifactorial approach that can be pursued. The multilevel concept proposed by Fahrenberg [54], which comprehensively includes performance, strain, and subjective feeling, may serve this purpose.

Whilst in the past 30 years useful schemes of reducing the levels of harmful substances in companies and in the general environment in industrial nations have substantially reduced the lead exposure, there was a copper works facility in East Germany where workers in various jobs had been definitely continuously exposed to levels of up to 25% above the German threshold limit values (DE-MAK) of lead in air (0.1 mg/m^3) over a period of more than ten years. The MAK value (maximale arbeitsplatz-konzentration) is defined as a maximum permissible concentration of a chemical compound present in the air within a workplace, which, according to current knowledge, does not impair the health of the employee or cause undue annoyance. Under these conditions, exposure can be repeated and of long duration over a daily period of 8 hours, constituting an average working week of 40 hours. MAK values are those from the Deutsche Forschungsgemeinschaft (DFG). For the USA and for Sweden, permissible exposure limits are 0.15 mg/m^3 and 0.05 mg/m^3, respectively. This rare situation of exposure, being substantially improved through rehabilitation measures after the unification of Germany, brought about the present study, as it offered a chance for objectifying neurotoxic effects induced by occupational lead exposure.

The aim of the investigation was to define proper and sensitive indicators as screening methods of early neurologic effects after occupational exposition by lead using psychometric and psychopathologic procedures.

2. Subjects and Methods

The investigation schedule involved all the available male workers of a copper works facility who had had a history of several years occupational chronic lead exposure. These 70 males satisfied the criteria for being included in the study: voluntary participation, no pathological clinical findings, definite average lead exposure (0.13 ± 0.09 mg/m^3 air) within the threshold limit value (MAK) range (see above), aged over 35 years, and not less than five years of uninterrupted work in the area of exposure. They formed the group of exposed subjects (E) with mean blood levels of lead (BPb) of 30.6 ± 10.2 μg/dL over the past 12 years; the internal dose time-weighted average (TWA) calculated as proposed by Hänninen et al. [16] was 29.7 ± 10.2 μg/dL. Out of the 70 exposed subjects (E), 21 had a higher exposure (hE, BPb continuously >35 μg/dL) and 49 had a lower exposure (lE, mean BPb over the period under investigation <35 μg/dL). The mean BPb over the 12-year period under investigation was 43.0 ± 6.1 μg/dL for the hE group and 25.3 ± 6.3 μg/dL for the lE group. On the day of examination, the current BPb level was 30.4 ± 15.5 μg/dL in Group E, 42.9 ± 12.7 μg/dL in Group hE, and 24.0 ± 12.7 μg/dL in Group IE males. The TWA

values for the hE and IE exposure groups were determined as 41.9 ± 6.2 μg/dL and 24.5 ± 6.4 μg/dL, respectively.

On the analogy of the internal dose TWA, one can follow the procedure described by Bleecker et al. [55] to calculate the external lead-in-air lifetime-weighted average exposure (LWAE) of exposed subjects while allowing for their accurate duration of stay in the areas of exposure, along with the lead-in-air concentrations measured. The LWAE value determined for the 21 hE subjects was 0.17 ± 0.11 mg/m^3 and that for the 49 lE subjects 0.10 ± 0.08 mg/m^3.

Compared to these lead-exposed workers were 27 male controls (C) working in the iron and steel industry, with no history of occupational exposure to heavy metals or solvents and with criteria for being included in the study identical to those of exposed subjects. The control group was not more similar in sample size, because we subdivided afterwards our exposed subjects into both groups, hE (n = 21) and lE (n = 49), and compared the controls with this both exposed group.

Criteria for exclusion from the present study for both groups were evidence of nervous lesions or unusual psychic signs, known diabetes mellitus, manifest arterial hypertension or cardiac insufficiency, and abuse of alcohol and/or drugs.

The following age information applies to the subjects studied: Group E mean age 43.4 ± 5.4 years (35–52 years), Subgroup hE mean age 41.3 ± 4.8 years (36–50 years), Subgroup lE mean age 44.3 ± 5.4 years (35–52 years), and Group C mean age 45.2 ± 4.9 years (35–52 years). Analysis of variance did not reveal any age difference between the groups.

All test persons involved were subjected to an identical test programme which included psychometry of various performance areas (1st level), determination of physiological strain reactions during the tests (2nd level), and inquiry on subjective state of health (3rd level). The test battery used the following PC systems: Swedish performance evaluation system (SPES) [56–58] and COMBITEST [59, 60].

(1) Performance Level:

(i) capacity of short-term memory (memory span for numbers and labyrinth test),

(ii) central information processing speed (initiation time (INT) in case of single-choice reaction (SCR) to a visual signal),

(iii) movement time (MOV) in case of single-choice reaction (SCR) to a visual signal,

(iv) concentration power and load capacity (reaction time under selection requirements in case of a multiple-choice reaction task (MCR) with adaptive mode),

(v) psychomotor coordination, sensorimotor performance (outtime and speed reached in pursuit test),

(vi) psychomotor response (maximum frequency of the preferred hand in the tapping test).

(2) Physiological Strain Level: The heart action potentials were recorded by means of modified thoracic Nehb anterior leads. The R-R intervals were recorded within 1 ms accuracy. Immediately after the experiment, the process of heart period duration covering the time of the test phases identifiable by markers was visualized on monitor. Further processing of the R-R intervals by means of fast Fourier transformations (FFT) as well as calculation of the cardiac rhythm parameters and power density spectra were conducted after the test on the basis of the registered data. When plateau (steady state) was seen in cardiac performance, the following values were calculated from the R-R intervals or the cardiac phase duration (CPD) values, in addition to the heart rate (HR):

(i) HR as a mean heart rate value on entire recording,

(ii) absolute sinus arrhythmia (SA_a) as proposed by Eckoldt [61]

$$SA_a = \frac{1}{n}\sum_{i=1}^{n}|CPD_{i+1} - CPD_i| \text{ (ms)}, \quad (1)$$

where CPD = cardiac phase duration (ms) or duration of phases of cardiac cycle, n = number of successive CPDs considered (not less than 200), and i = no. of CPD,

(iii) the total power density spectrum (TP) of the CPDs as a short-term estimate of the total power of spectral density in the range of frequencies between 0 and 0.5 Hz (ms^2) representing the overall activity of the autonomic nervous system,

(iv) the power density bands

(a) VLF (0.0–0.5 Hz) = thermoregulatory band or very low-frequency band, reflecting overall activity of some slow mechanisms of sympathetic function (ms^2),

(b) LF (0.05–0.15 Hz) = circulatory band or low-frequency band, reflecting mixed sympathetic and parasympathetic activity (ms^2),

(c) HF (0.15–0.5 Hz) = RSA (respiratory sinus arrhythmia) band, or high-frequency band, reflecting parasympathetic activity and corresponds to N-N variations (time between two heartbeats) caused by respiration (ms^2),

(v) the relative proportions of these bands in the total power density spectrum

(a) relative UF band share of TP-VLF (%),

(b) relative LF band share of TP-LF (%),

(c) relative HF band share of TP-HF (%).

A major vagal influence on the SA_a and the HF band share in the total power density spectrum with regard to cardiac regulation has long been postulated.

Ambulatory Holter monitoring was done on a total of 70 exposed workers and 27 controls. Arrhythmia diagnosis was based on standard electrocardiographic criteria. The HRV analyses follow the guidelines published in 1996 by the HRV Task Force [62]. Ambulatory electrocardiography was

obtained using a Tracker tape recorder (Reynolds Medical, Hertford, UK) at a sampling rate of 128 Hz in the occupational department.

(3) Psychological-State Level:

(i) Subjective state (EZ) as proposed by Nitsch [63, 64] and

(ii) state during the past six months as determined by means of the SPES.

While no premorbid-intelligence determination was conducted in the subjects, there is good reason to assume that as studied by Seeber [65], it covaries with the standard of education and qualification, and the subjects studied (exposed subjects and controls) were of the same standard.In a supporting approach, a search analysis for drugs and/or metabolites including caffeine and nicotine was conducted at the University's Institute of Clinical Pharmacology, as the results of performance tests may be modified by such substances.

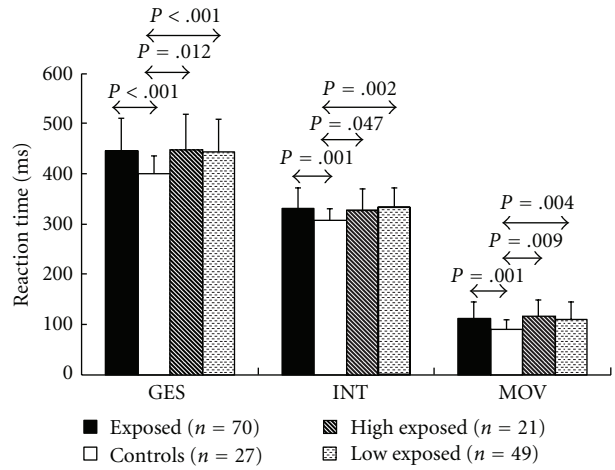

Figure 1: Mean values of total reaction time (GES), initiation time (INT), and movement time (MOV) of lead-exposed subjects (E) and controls (C) in the single-choice reaction task after visual stimulus.

3. Statistical Analysis

All statistical analyses were performed with the statistical software package SPSS for Windows (Version 15.0). The normality of the variable distributions was evaluated. The Student's t-test (normal distribution) or Mann-Whitney test (not normal distribution) were applied to test statistically significant differences between workers and controls. Results were presented using tables displaying the mean with the standard deviation (SD). The analysis was performed using a critical error probability of 0.05 (5%). Moreover, statistical evaluation was performed by means of multivariate methods and single-factor analysis of variance, Mantel-Haenzel's test.

4. Results

Effect variables known from the literature, such as sex and circadian influence on measurements, were insignificant in this study, as it only involved males, and measurements were consistently taken at the same time of the day.

Multivariate biostatistical analysis (ONEWAY procedure using Scheffe's test; SPSS for Windows) was performed to identify the lead exposure-related variability of the parameters studied versus other variance-generating sources (age, standard of education/qualification, prior case histories, and motivation). No statistically significant difference was found with respect to the effect factors mentioned, and hence, a monocausal consideration can be presented below excluding lead as an influencing variable.

Only essential results of the investigation are presented here.

(1) Performance Data. Out of the performance data which are mainly influenced centrally, viz INT, the total duration and the number of errors in the labyrinth test, the time needed, and the interstimulus interval achieved in the adaptive MCR test (see above), it is only for the initiation time INT of the single-choice reaction task (SCR) that statistically significant differences were seen between exposed subjects and controls. The mean values of this parameter were 330.3 ± 42.8 ms for the 70 lead-exposed subjects and 306.0 ± 26.5 ms for the controls, with $P = .001$ (cf. Figure 1), suggesting a slowing of the central information processing speed in exposed subjects.

Mainly motor performance parameters are the movement time (MOV) in the SCR, the sensorimotor performance, outtime (OUT1 and OUT2) during the first and the second halves of the pursuit test (PUR), and the maximum tapping frequency (FRQ1 and FRQ2) during the first and the second halves of the tapping test. Table 1 shows the results of these tests. The movement time was 112.4 ± 35.4 ms in lead-exposed subjects, thus being markedly slower when compared to the controls (91.4 ± 21.6 ms) with $P = .001$. Similarly, in the tapping test, the tapping frequency of the preferred hand of exposed subjects was lower than that seen in the normal controls, in particular during the first half of the test, the respective values being 5.4 ± 0.7 Hz and 5.8 ± 0.6 Hz ($P = .03$).

The total reaction time (GES) in the single-choice reaction task comprises the preferably centrally induced INT and the motor-induced MOV. Again, exposed subjects (444.2 ± 68.1 ms) were seen to be markedly slower than the controls (398.4 ± 39.1 ms) with $P < .001$.

Selecting from the group of 70 lead-exposed subjects (E) the higher exposed (hE) and the lower exposed (lE) workers, comparison to the controls revealed significantly impaired performance for those exposed to the harmful substance as can be seen in Table 1.

(2) Physiological Strain Data. The subjects performing the multistage psychometric test battery did not exhibit any qualitatively different deflection of the physiological reaction

Table 1: Statistically significant performance difference between lead-exposed subjects (E) involving the subgroups of high exposed (hE) and low exposed (lE), and controls (C) in the tapping test and in the single-choice reaction task.

Test performance	Exposed (E)	Controls (C)	High exposed (hE)	Low exposed (lE)	*P* (E-C)	*P* (hE-C)	*P* (lE-C)
Tapping							
FRQ1 (frequency 1. Half of test) (Hz)	5.42 ± 0.69	5.78 ± 0.64	5.42 ± 0.56	5.42 ± 0.74	.030	.056	.049
Single form visual reaction test							
GES (total time) (ms)	444.2 ± 68.1	398.4 ± 39.1	446.0 ± 73.8	443.5 ± 66.4	<.001	.012	<.001
INT (initiation time) (ms)	330.3 ± 42.8	306.0 ± 26.5	327.1 ± 44.9	331.6 ± 42.3	.001	.047	.002
MOV (movement time) (ms)	112.4 ± 35.4	91.4 ± 21.6	115.7 ± 35.4	111.0 ± 35.6	.001	.009	.004

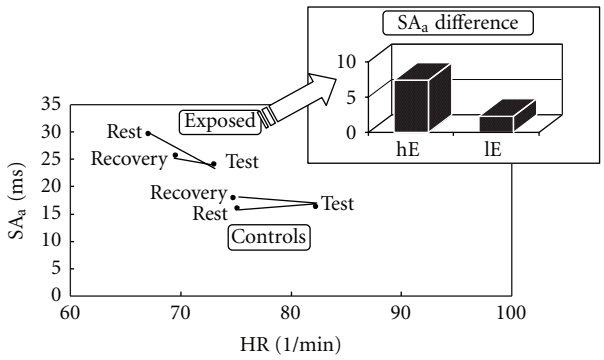

Figure 2: Absolute sinus arrhythmia (SA_a) versus heart rate (HR) in lead-exposed subjects (E) and controls (C) at the three stages of rest, "memory span for numbers" test, and recovery (vegetative pattern) as well as SA_a differentials between rest and recovery in the two subgroups of high-exposed (hE) and low-exposed (lE) subjects.

parameters HR, SA_a, and the spectral power density of the CPD as well as the arterial blood pressure. However, the controls exhibited higher average HR and lower average SA_a (see Table 2).

Still, care should be taken in interpreting the latter finding, as the tonicity prevailing prior to lead exposure was not known. After repeated measurements of HR and SA_a at rest, normal subjects can be classed into a predominantly vagotonic group I (low HR and high SA_a) and a predominantly sympathicotonic group II (comparatively high HR and low SA_a) [66, 67]. According to Ward's cluster analysis (SPSS), among the 70 lead-exposed copper workers, there were 32 vagotonic subjects (Group I) and 28 sympathicotonic subjects (Group II), whereas 10 subjects of Group E could not be statistically assigned. Of the 27 controls, two were classed as Group I and 22 Group II, while three could not be classed at all. Thus, a greater proportion of exposed subjects could be classed into the group of vagotonic subjects (I) which, in terms of the regulation theory, is more favourable.

Figure 2 shows the tonicity pattern in terms of HR and SA_a at rest, under the strain of the "memory span for numbers" test, and during recovery.

The mean initial condition (rest) for the two groups appears to be different: controls exhibited a high average HR of 75.2 min^{-1} and a low SA_a of 16.0 ms. The respective values for the lead-exposed subjects were 67.1 min^{-1} and 29.6 ms. When under strain, both groups responded with an increase in activity that was characterised by rising HR and diminishing SA_a. Return to the initial tonicity after recovery showed differences between lead-exposed subjects and nonexposed controls. The former had a marked deficit when compared to the controls; after a 5-min recovery, they were still far away from the initial tonicity, their heart rate being 69.6 min^{-1} and their sinus arrhythmia by Eckoldt SA_a 25.7 ms. After an identical recovery period, the controls exhibited a much more pronounced relaxation as can be seen from the vegetatively induced cardiovascular parameters of HR = 74.8 min^{-1} and SA_a = 17.8 ms versus the values at rest given above. From the higher sinus arrhythmia SA_a during recovery versus rest, it appears that Group C was more relaxed at the end of the test series when compared to the beginning, which was not true for Group E. When Group E was subdivided into high-exposed (hE) and low-exposed (lE) subjects, it was found that the recovery tonicity, expressed as the difference between SA_a at rest and SA_a during recovery, was further away from the tonicity at rest the higher the workers' exposure to lead had been. This effect can also be seen in Figure 2.

The result of an FFT of successive interbeat intervals represents a power density spectrum which is usually subdivided into three frequency bands A (thermoregulatory effects; 0–0.05 Hz), B (blood pressure regulation; 0.05–0.15 Hz) and C (respiratory sinus arrhythmia; 0.15–0.5 Hz). The absolute power density of the cardiac phase duration spectrum and its three bands A, B, and C, as a whole, was higher for the exposed subjects compared to the controls, a finding which is consistent with the varied group structure by the individual tonicity as outlined above. To permit a lead-modified vagal tone to be identified, one should have a closer look at the regulative dynamics of the frequency band pattern at different strain conditions. Comparison of the relative frequency bands for the two groups of subjects at rest, under test strain, and during subsequent recovery after 5 min reveals different band distributions for lead-exposed subjects and controls as demonstrated by Figure 3.

It can be seen that in the lead-exposed subjects, the relative pattern of the C band (respiratory sinus arrhythmia) did not change between the three experimental stages, whereas in the controls, a statistically significant difference (P = .01) was observed, typically between the test strain (23.9%) and subsequent recovery (19.9%). This was also true for the B, or cardiovascular, band. The exposed subjects did not exhibit any difference between the three test stages, but

Table 2: Comparison of a number of cardiovascular parameters of lead-exposed subjects (E) involving the subgroups of high-exposed (hE) and low-exposed (lE), and controls (C) at rest and during recovery.

Physiological strain data	Exposed (E)	Controls (C)	High exposed (hE)	Low exposed (lE)	*P* (E-C)	*P* (hE-C)	*P* (lE-C)
HR rest (1/min)	67.1 ± 10.9	75.2 ± 11.7	64.4 ± 10.9	68.3 ± 10.7	.002	.003	.015
SA_a rest (ms)	29.6 ± 17.6	16.0 ± 16.6	34.1 ± 21.2	27.5 ± 15.5	.001	.002	.005
LF (%) rest	49.9 ± 15.8	62.7 ± 16.1	48.7 ± 14.6	50.5 ± 16.4	.001	.005	.004
HF (%) rest	36.2 ± 17.4	18.3 ± 15.3	35.8 ± 16.4	36.4 ± 18.0	<.001	.001	<.001
HR recovery (1/min)	69.6 ± 9.1	74.8 ± 13.1	67.6 ± 10.4	70.5 ± 8.5	.068	.045	.137
SA_a recovery (ms)	25.7 ± 12.9	17.8 ± 13.9	26.7 ± 14.6	25.3 ± 12.2	.010	.037	.019
HF Recovery (ms^2)	4067.5 ± 3545.7	1627.5 ± 2789.7	3885.5 ± 3673.1	4152.4 ± 3523.7	.001	.019	.001
HF (%) Recovery	34.5 ± 23.9	19.9 ± 17.7	30.5 ± 23.2	36.3 ± 24.3	.002	.079	.002

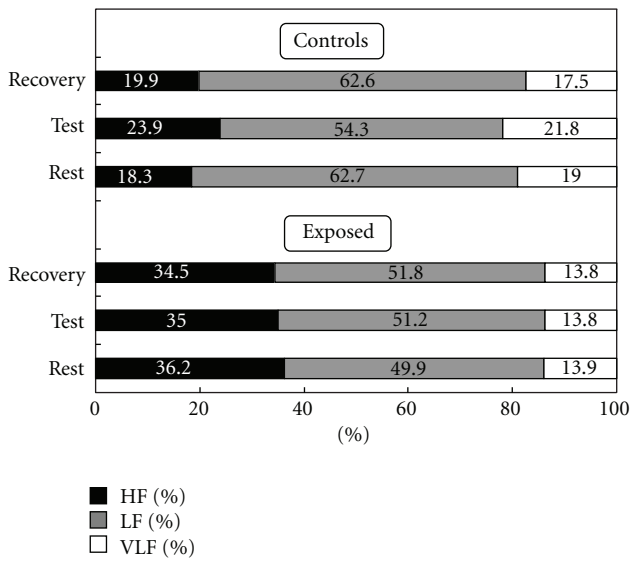

Figure 3: Relative frequency band percentages of cardiac phase duration variability after Fast Fourier Transform for lead-exposed subjects and controls at the stages of rest, "memory span for numbers" test, and recovery.

a difference did exist between rest and test ($P = .05$) as well as between test and recovery ($P = .03$).

(3) Psychological State Data. From the SPES questions relating to the psychological state, 16 were selected which are logically connected with a neurotoxic exposure in question: (1) = "physically tired in the morning," (2) = "mentally tired in the morning," (3) = "general sensation of lack of energy," (4) = "feelings of vertigo or fainting," (5) = "lack of initiative," (6) = "difficulties falling asleep," (7) = "disturbed sleep," (8) = "waking up too early," (9) = "finding it hard to concentrate," (10) = "anxious, restless, out of balance," (11) = "being forgetful," (12) = "feeling down without reason," (13) = "being easily upset," (14) = "headaches," (15) = "feeling clumsy or shaky," and (16) = "prickling sensation, numbness in limbs." The answers of "never", "occasionally", "often", and "very often" scored points from 0 to 3. A statistically significant difference between exposed subjects and controls was noted for questions 1, 2, 3, 4, 9, 11, 12, and 15 ($P < .05$; see Table 3). Each of these significant differences concerned greater complaints experienced by the lead-exposed subjects versus controls.

At the end of the psychometric test series, each subject was shown Nitsch's EZ self-rating scale. In evaluating the subjective opinions, a positive exertion attitude too was considered as this feature is known to have a major influence on psychometric test results. Yet, similar to the "motivation" factor, no statistical differences were observed between the groups and, hence, the varied findings obtained in the performance tests are not thought to be attributable to differences in exertion attitude or motivation.

5. Discussion

In the light of the great expectations the occupational medicine is to meet when it comes to detecting vocationally induced disturbances of health in good time such that primarily successful preventive action can be taken, early indicators hitherto not considered from the clinical viewpoint are also needed for neurotoxic substances. This approach is in line with workers' increasing demand for comprehensive occupational medical care.

In East Germany, an old nonferrous metal works facility was available for the investigation in which, "ideally suited" for the study, 70 male workers had been continuously exposed to occupational lead over not less than 5 years, with exposure levels being roughly identical to the current German threshold limit value for lead of 0.1 mg/m^3. Of these 70 males, 21 had had a verified permanent lead concentration of more than 35 μg/dL over the past 5–10 years. The average level among the normal population of Germany has been reported to range from 5 to 8 μg/dL [68], and there has been a trend towards lower concentrations.

Considering the literature on psychometric performance impairment, the lead-exposed subjects examined in the present study were expected to exhibit effects which relate to the short-term memory, the discrimination capacity, or the speed of information processing as well as the motor reaction time. In fact, only few of the present performance findings came up to the hypothetical expectations of being early indicators of a lead-induced neurotoxic harm. Those were predominantly the motor performance features of movement time in the single-choice reaction task and the tapping

Table 3: Results of the survey (using the SPES method) of complaints experienced by lead-exposed subjects (E) involving the subgroups of highexposed (hE) and lowexposed (lE), and controls (C) during the past six months. In the 16 answers: 1 denoted "occasional", 2 "often", and 3 "very often " (The answers of 1, 2, and 3 to the particular question were percentages within a group; the balance of 100% did not complain of such symptoms).

Parameter		Exposed (E)	Controls (C)	High exposed (hE)	Low exposed (lE)	*P* (E-C)	*P* (hE-C)	*P* (lE-C)
SPES1 (physically tired in the morning)	1	51.4	22.2	42.9	55.1	<.001	.025	<.001
	2	10.0	3.7	9.5	10.2			
	3	5.7	0	4.8	6.1			
SPES2 (mentally tired in the morning)	1	2.29	0	19.0	24.5	.005	.019	.004
	2	14.3	0	0	2.0			
	3	0	0	0	0			
SPES3 (general sensation of lack of energy)	1	44.3	18.5	42.9	44.9	.006	.029	.008
	2	2.9	0	4.8	2.0			
	3	14.3	0	0	2.0			
SPES4 (feelings of vertigo or fainting)	1	41.4	14.8	47.7	38.8	.017	.057	.023
	2	5.7	3.7	4.8	6.1			
	3	4.3	3.7	0	6.1			
SPES5 (lack of initiative)	1	42.9	25.9	42.9	42.9	n.s.	n.s.	n.s.
	2	2.9	3.7	4.8	2.0			
	3	0	0	0	0			
SPES6 (difficulties falling asleep)	1	24.3	14.8	9.5	30.6	n.s.	n.s.	n.s.
	2	8.6	18.5	14.3	6.1			
	3	10.0	0	14.3	8.2			
SPES7 (disturbed sleep)	1	32.9	14.8	14.3	40.8	n.s.	n.s.	n.s.
	2	11.4	14.8	28.6	4.1			
	3	5.7	0	9.5	4.1			
SPES8 (waking up too early)	1	22.9	22.2	23.8	22.4	n.s.	n.s.	n.s.
	2	15.7	3.7	9.5	18.4			
	3	8.6	7.4	9.5	8.2			
SPES9 (finding it hard to concentrate)	1	57.1	10.2	57.1	57.1	.003	.011	.006
	2	5.7	3.7	9.5	4.1			
	3	14.3	3.7	0	2.0			
SPES10 (anxious, restless, and out of balance)	1	24.3	11.1	28.6	22.4	n.s.	n.s.	n.s.
	2	4.3	3.7	0	6.1			
	3	0	0	0	0			
SPES11 (being forgetful)	1	60	16.3	61.9	59.2	<.001	.003	.002
	2	10	3.7	14.3	8.2			
	3	2.9	0	0	4.1			
SPES12 (feeling down without reason)	1	25.7	0	38.1	20.4	.011	.004	.028
	2	2.9	3.7	0	4.1			
	3	0	0	0	0			
SPES13 (being easily upset)	1	38.6	25.9	38.1	38.8	n.s.	.059	n.s.
	2	7.1	3.7	9.5	6.1			
	3	4.3	0	9.5	2.0			
SPES14 (headaches)	1	30.0	14.8	33.3	28.6	.094	.078	n.s.
	2	5.7	3.7	9.5	4.1			
	3	5.7	3.7	4.8	6.1			
SPES15 (feeling clumsy or shaky)	1	22.9	3.7	23.8	22.4	.034	n.s.	.027
	2	4.3	0	0	6.1			
	3	14.3	3.7	0	2.0			
SPES16 (prickling sensat., numbness in limbs)	1	41.4	40.7	47.7	38.8	n.s.	n.s.	n.s.
	2	2.9	7.4	0	4.1			
	3	7.1	3.7	9.5	6.1			

frequency in the tapping test, the total reaction time in the single-choice reaction task, and the initiation time, which may be considered a parameter of central information processing. The present findings failed to meet the great expectations for the adaptive multiple-choice reaction task. This contrasts sharply with Lilienthal et al. [69] who described multiple-choice reaction tests as providing more meaningful information in case of a lead-induced damage in question compared to single reactions. However, the present psychometric findings revealing an impaired performance for some of the lead-exposed subjects compared to the controls need to be qualified when the doses involved are considered. Indeed, an increasing individual lead dose did not bring about a statistically significant impairment of performance data. It is because of the pronounced difference between groups that early diagnosis in occupational medicine cannot do without single-reaction and tapping tests.

To objectify a lead-induced neurotoxic damage not yet identified by the clinician or occupational physician, use should be made of physiological strain parameters as well as psychological state and subjective experience data, in addition to the performance data referred to above.

According to the hypothesis proposed, workers after many years of lead exposure were assumed to exhibit greater strain reactions than the nonexposed controls, provided the performance was comparable. While the specificity of the strain parameters of heart rate was partly to characterise a varied strain pattern, results were obtained for the cardiac rhythm response (heart rate variability) which were not expected when the study was planned on the grounds of the literature. This relates to the slowed restoration of the initial vegetative condition in the lead-exposed subjects once they have performed their test tasks. In fact, a simple vegetative tonicity comparison between exposed subjects and controls at rest does not consistently lend itself to indicating the expected effect of a restricted cardiac phase duration variability and, thus, vagus depression by lead. In this respect, we go against a number of other workers [25, 45–47] who described this effect for lead workers at rest. In the latter publications, it has been tacitly assumed that the restricted HRV found was solely caused by exposure to heavy metals, while disregarding the fact that even normal subjects not occupationally exposed to harmful substances are generally known to differ greatly from each other because of their HRV at rest, whether inherited and/or acquired as a result of their conduct of life (in particular sports activities). Therefore, unless sufficient previous knowledge is available, sectional comparison of HRV variables for various groups of subjects is not suited to provide meaningful information on the effect of an individual factor. The effect of a slowed restoration of the initial vegetative condition as noted in this study does not take into account the interindividually varied vegetative tonicity at rest, and hence, it is believed to be a measure more appropriate to describe lead-induced vagus depression. A slower adjustment of cardiovascular parameters due to diminished vagus efferences was described as early as 1977 by Schwarz [70] who performed vagus blocking experiments.

Andrzejak et al. [71] described that in copper smelters, occupational exposure to lead HRV is lower than in healthy subjects, which results rather from the decreased parasympathetic than from the increased sympathetic activity.

A longitudinal study performed at intervals of several years in case of persisting exposure would offer another approach to demonstrating the effect of vocationally absorbed lead. In fact, a repeated study conducted among workers of a company after about 4 years revealed a progressive restriction of the HRV [50].

As already mentioned, a study also included search for early indicators of a clinically concealed lead-induced neurotoxic damage at the level of subjective state and experience too. The hypothesis of an impaired subjective sensation of the state of health and the mental state was confirmed for a number of categories in lead-exposed workers versus controls, and the findings were statistically significant. In this context, effects with regard to tiredness (or lassitude) and lack of energy deserve particular mention. This is essential to valuation of limits as the copper workers studied had been exposed to the German threshold limit value (DEMAK). However, verification of impaired subjective state and experience through years of exposure to lead within statutory limits does not apply to all of the categories included in the questionnaire or factor groups of the SPES method as well as Nitsch's self-rating scale. Still, applied occupational medicine should include these methods in its activities of monitoring lead-exposed subjects, especially since they can be implemented without any need for major equipment. It is admitted, though, that studies of subjective state and mental experience alone are not sufficient to reliably ascertain the neurotoxic effect of lead within the bounds of exposure hitherto considered harmless.

Some studies described the bone lead levels as a good indicator of exposure and lead toxic effects [41] but unfortunately not possibly practicable in the occupational studies in our institute. Park et al. [44] have reported that associations between patella bone lead levels on heart rate variability are stronger among study participants with metabolic syndrome and with individual component of metabolic syndrome.

As the occupational and environmental medicine is increasingly required to advance into spheres where marked effects by harmful substances fail to occur (being undisputable that in companies and in the general environment, weak pollutant-induced effects occur, particularly in sensitive subjects), an interdisciplinary approach involving several clinical and theoretical disciplines is becoming essential. In the present study, an exemplary attempt was made to get psychophysiology, clinical psychology, and cardiology involved in an issue of occupational medicine. Modern occupational medicine should proceed along these lines so as to meet the self-set high standard of optimal prevention for all working people.

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Research Article

Cytotoxicity of Environmentally Relevant Concentrations of Aluminum in Murine Thymocytes and Lymphocytes

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The effects of low concentrations of aluminum chloride on thymocytes and lymphocytes acutely dissociated from young mice were studied using flow cytometry with a DNA-binding dye. We demonstrate a rapid and dose-dependent injury in murine thymocytes and lymphocytes resulting from exposure to aluminum, as indicated by an increase in the entry into the cell of the DNA-binding dye, propidium iodine. A 60-minute exposure to 10 μM $AlCl_3$ caused damage of about 5% of thymocytes, while 50% were injured after 10 minutes at 20 μM. Nearly all thymocytes showed evidence of damage at 30 μM $AlCl_3$ after only 5 minutes of incubation. In lymphocytes, injury was observed at 15 μM $AlCl_3$ and less than 50% of cells were injured after a 60-minute exposure to 20 μM. Injury only rarely proceeded to rapid cell death and was associated with cell swelling. These results suggest that aluminum has cytotoxic effects on cells of the immune system.

1. Introduction

Aluminum is one of the most abundant elements on earth but has no known biological function in living organisms [1]. Exposure to aluminum and its associated toxicity are well documented in plants and animals [2, 3]. In humans, aluminum toxicity was first described as osteomalacic dialysis osteodystrophy [4]. Although aluminum has been primarily recognized as a neurotoxin and etiologic agent of dialysis dementia [5, 6], other detrimental health effects have been documented [7]. Some authors report aluminum-induced genotoxicity [8]. Others associate exposure to aluminum with osteodystrophy [9], anemia [10], and altered calcium homeostasis [11]. In addition, underlying conditions such as renal failure, leukemia, and diabetes increase aluminum retention in human and animal subjects due to impaired absorption and excretion, which in turn exacerbates its toxic effect [12, 13].

Evidence regarding the effect of aluminum on the immune system is limited and conflicting. Some researchers report immunosuppression, while others portray aluminum as an efficient adjuvant in vaccines [14, 15]. Exposure to low concentrations of aluminum was reported to cause immunopotentiating effects, whereas exposure to high levels caused immunosuppression [16, 17]. Some authors report that long-term exposure to low concentrations of aluminum resulted in elevated intracellular levels in lymphocytes, which might be a contributing factor in the reported immunosuppression [18]. However, none of this evidence is very convincing in the absence of clear understanding of the mechanism(s) of immunotoxicity [19].

Human exposure to aluminum other than during dialysis occurs primarily through ingestion of food and water, utilization of personal care products and cookware, and consumption of medications and administered vaccines [20–22]. Elevated levels of aluminum in soils have been implicated in the higher frequency of neurodegenerative disorders in the Kii Peninsula and natives in Guam [23].

Due to rapid urbanization, anthropogenic contaminants accumulate in various environmental media, including source water and sediment, food and pharmaceuticals, air, and dust [24, 25]. While numerous studies have been conducted on aluminum toxicity, nearly all of them have investigated effects of exposure to high concentrations. These concentrations are not representative of typical environmental exposure levels and cannot be associated with ordinary circumstances for people with normal renal function.

Due to its abundance in nature and in man-made products, cumulative daily uptake of aluminum by humans is difficult to estimate. Study of aluminum is further complicated by the fact that a variety of complexes are formed in solution [26], and these various forms may have different toxicities and biological effects. Based on available information, we have attempted to estimate the range of daily exposure in humans and study the effects of $AlCl_3$ solutions on isolated immune system cells at concentrations that are environmentally relevant. In the present study, we have investigated the effects of low concentrations of aluminum on thymocytes and lymphocytes that were acutely dissociated from young mice. Our data indicate that exposure to aluminum results in a dose- and time-dependent damage of the plasma membrane of thymocytes and lymphocytes but does not cause acute cell death to any significant degree.

2. Materials and Methods

2.1. Reagents. Aluminum chloride (III) of 99.95% purity grade was purchased from Sigma-Aldrich (St. Louis, Mo, USA) and was dissolved in distilled water. Propidium iodide (PI) and Annexin V-FITS apoptosis detection kit were purchased from Sigma-Aldrich (St. Louis, Mo, USA).

2.2. Preparation of Thymocytes and Lymphocytes. These investigations were reviewed and approved by the University at Albany Animal Care and Use Committee. Thymocytes were acutely dissociated from the thymus gland of four-week-old ICR male mice (Taconic Biotechnology, Inc., Germantown, NY) as previously described [27], while lymphocytes were separated from the spleen. Mice were rapidly decapitated with a guillotine, and the thymus and spleen were removed. To obtain cell suspensions, the organs were gently ground between frosted glass microscope slides. Red blood cells in spleen tissue were lysed with RBC-lysing buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.2–7.4). The suspensions of remaining white cells were then filtered by gravity through a cell strainer (70 μm) to obtain a more uniform single-cell suspension. Unless otherwise specified, all experiments were conducted at 37°C with freshly prepared Tyrode's solution (148 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES, pH 7.4). The strained suspensions were washed three times with Tyrode's solution. Before being loaded with fluorescent dyes and exposed to $AlCl_3$, murine thymocytes or lymphocytes were incubated in Tyrode's for 30 minutes to recover from injury of dissociation.

2.3. Loading Thymocytes with Dyes. The viability of thymocytes was determined using propidium iodide (PI), a DNA-binding probe that enters the cell only if the plasma membrane is damaged. This dye was added to a sample tube containing approximately 2×10^6 cells 5 minutes prior to a measurement. The effect of immediate exposure of thymocytes and lymphocytes to aluminum was assessed at 0 time, when cells were first preloaded with PI and then analyzed immediately after addition of $AlCl_3$. To distinguish necrosis from apoptosis, we used PI and the Annexin V-FITS apoptosis detection kit. Annexin binds to phosphatidyl serine, which in healthy cells is found only on the inner membrane leaflet but moves to the outer leaflet early in the process of apoptosis. Necrosis, unlike apoptosis, is accompanied by cell swelling and is not associated with movement of phosphatidyl serine. Five thousand cells per sample were analyzed using a BD LSRII flow cytometer. The obtained data were processed via utilization of BD FACSDiva software.

2.4. Statistical Analysis. Experimental data values were obtained from at least six independent measurements and are presented as mean ± standard deviation of the mean. Statistical analysis was performed using the Student's paired t-test and two-way ANOVA, and a P value of < 0.05 was considered significant.

3. Results

Figure 1 shows how cell injury was detected in our experiments. Histogram A shows untreated cells where a gate (R1) was selected to differentiate between healthy and injured cells. In conventional practice the term "cell death" is used when the PI fluorescence intensity is more than two decades brighter than unstained cells [28]. This is shown in histogram 1(b), where 2% ethanol caused significant thymocyte cell death. Exposure to aluminum resulted in a gradual increase in PI intensity (Figures 1(c) and 1(d)) but did not result in the large increase associated with dead cells. This indicates that aluminum has resulted in some leakage of PI through the plasma membrane, but not to the degree that is seen when membrane integrity is totally lost and cells are dead. Thus we used the term "damage" or "injury" to describe changes associated with aluminum toxicity. All cells in area R1 were considered to be damaged throughout the experiments reported here.

Figure 2 shows the dose and time dependence of damage induced by $AlCl_3$ in thymocytes. Cell injury was rapid and took place within minutes. We observed significant injury as quickly as the measurements would be taken after exposure to 10 μM $AlCl_3$. Cellular damage increased with concentration and exposure time, and after a 10-minute exposure to 20 μM $AlCl_3$ close to 50% of the cells showed injury. Nearly all cells were damaged at concentrations of 30 and 40 μM $AlCl_3$ after only 5 minutes of incubation (Figure 2). The curve showing cell damage as a function of concentration reached a plateau after 10 minutes.

A similar pattern of injury was observed with lymphocytes (Figure 3), although they were somewhat less sensitive to aluminum toxicity. Significant lymphocyte injury was observed only at a concentration 15 μM, as compared with 10 μM for thymocytes. Less than 50% of lymphocytes incubated with 20 μM $AlCl_3$ for 60 minutes were damaged. Moreover, the damaging effect of 30 μM $AlCl_3$ on lymphocytes was less pronounced and did not reach the plateau until after 25 minutes of exposure. Our data suggest that

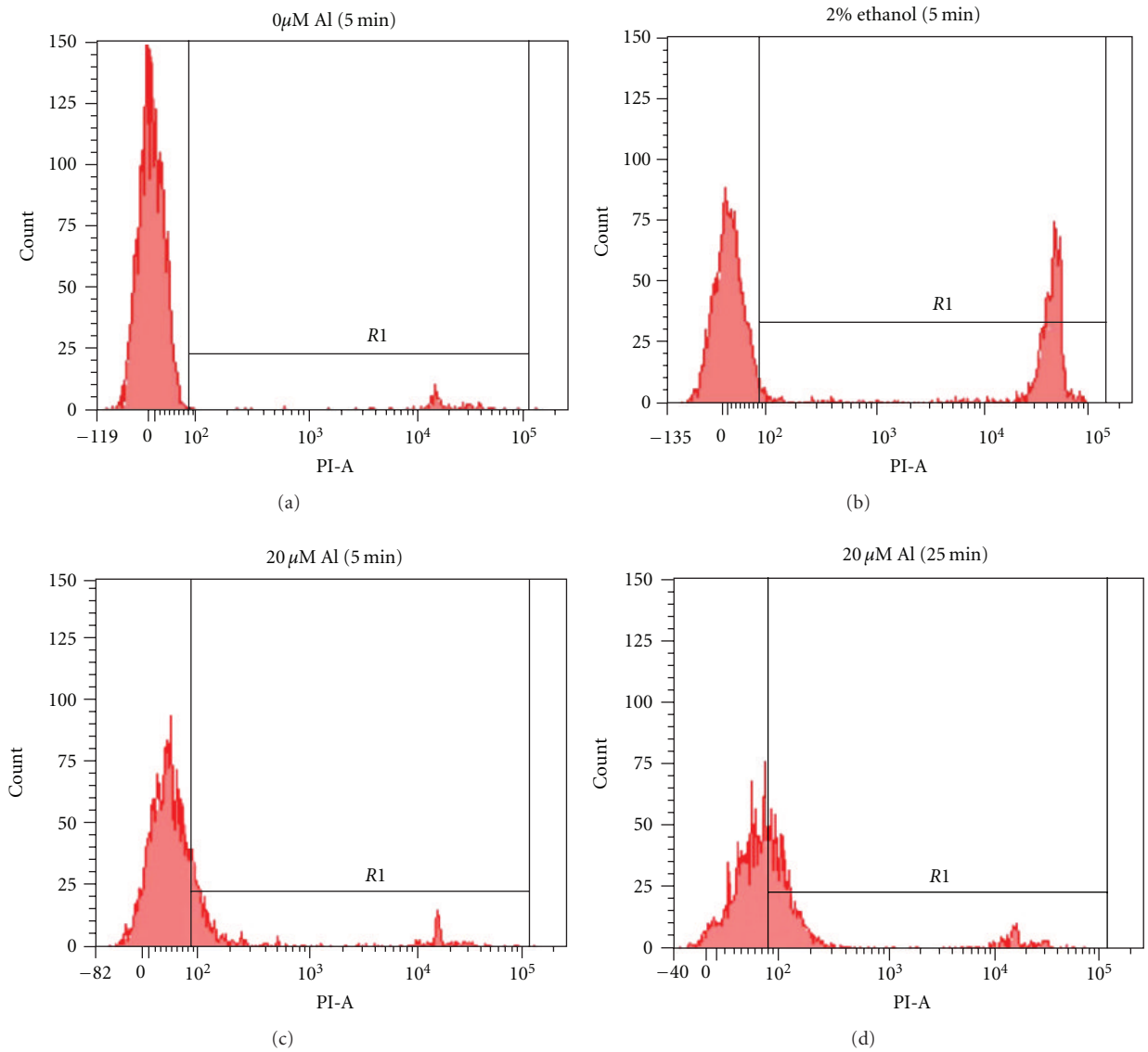

Figure 1: Histograms showing the effects of aluminum exposure to thymocytes. PI fluorescence intensity (x-axis) is plotted against cell count (y-axis). Histogram (a) shows untreated thymocytes where most of the cells have low PI intensity, which is characteristic of healthy cells whose membranes exclude PI. In (b) thymocytes were exposed to 2% ethanol, and a large number of cells show very high PI intensity. These are dead cells, whose plasma membrane has lost integrity. (c) and (d) show the gradual increase in PI intensity in thymocytes exposed to 20 μM of $AlCl_3$ at five- and 25-minute exposure. The number of dead cells did not increase with $AlCl_3$ exposure. There is, however, a shift in the distribution of healthy cells to the right, indicating an increased uptake of PI, reflecting cell damage. For purposes of quantitation, all cells falling under the bar labeled R1 are considered injured.

lymphocytes exposed to aluminum are less sensitive than thymocytes.

To determine the nature of the observed cell injuries, we performed experiments which employed the apoptotic detection kit and investigated changes in cell size. Figures 4(a)–4(d) show scattergrams of PI versus Annexin V fluorescence in control and exposed thymocytes. The rationale for this study is that while aluminum does not actually kill thymocytes, it might trigger early events associated with apoptosis. Since Annexin-V detects the movement of phosphatidyl serine to the outer leaflet of the plasma membrane, an increase in Annexin-V fluorescence is indicative of early apoptosis. Region Q3 includes live cells (PI-negative and Annexin V-negative), whereas region Q4 contains apoptotic cells (PI-negative and Annexin V-positive). Dead cells are represented in Q2 region (both PI- and Annexin V-positive), while quadrant Q1 shows damaged cells (PI-positive and Annexin V-negative). Toxicity of aluminum was evident after a very brief exposure resulting in a visible increase in the number of damaged cells (Figure 4(b)). With a 20-minute exposure to 20 μM $AlCl_3$, the cell population from the Q3 region moved to the Q2 region, without any shift to the Q4 area, leaving less than half of thymocytes undamaged. This observation indicates that thymocytes are not undergoing an

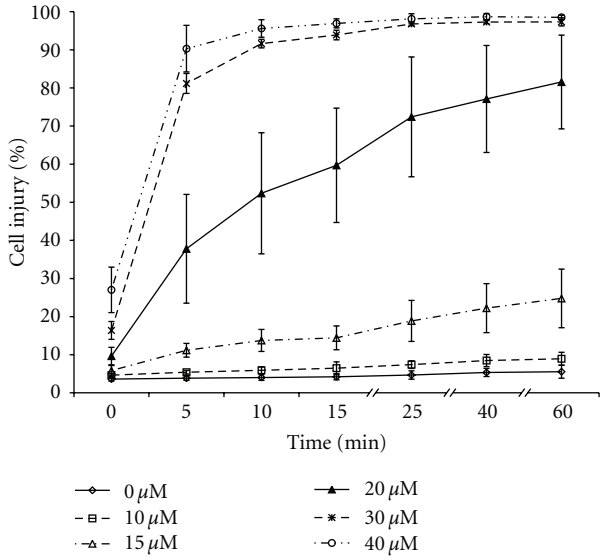

Figure 2: Dose and time dependence of $AlCl_3$-induced injury in thymocytes. The cells were treated with a range of concentrations of $AlCl_3$ (0–40 μM) at various time points. Thymocytes were considered to be damaged when the level of fluorescence intensity of PI in the cells was higher than the level in untreated cells. Values are mean ± SD obtained from six independent measurements (based on Student's paired t-test). The concentration curves are all statistically significantly different at the $P < .05$ level by ANOVA analysis. There are no significant changes with time between 25, 40, and 60 minutes, nor between 10 and 15 minutes, but all other time differences are significant.

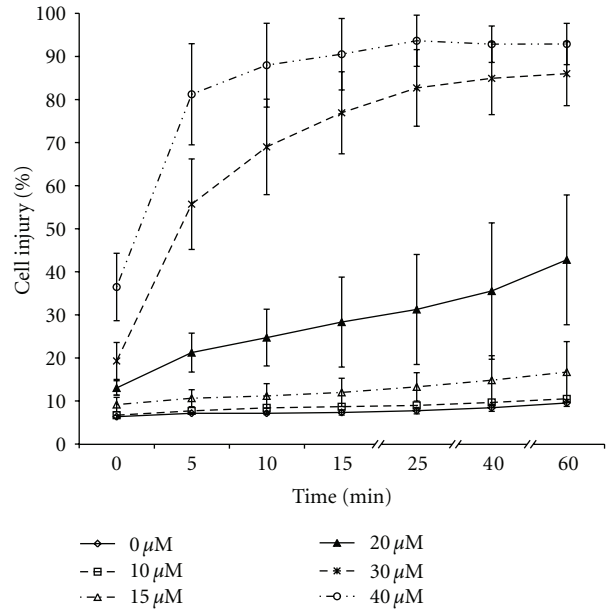

Figure 3: Dose and time dependence of lymphocyte injury with exposure to various concentrations of $AlCl_3$ (0–40 μM) at various time points. Other conditions were as described in the legend to Figure 2. All concentration curves are significantly different from each other at the $P < .05$ level by ANOVA with the exception of 0 and 10 minutes. There were no significant changes with time between 25, 40, and 60 minutes, nor between 10 and 15 minutes, but all other time periods were significantly different at the $P < .05$ level by ANOVA.

apoptotic process. Rather the shift of cells from the Q3 to the Q1 region suggests damage to these plasma membranes and, if any, a necrotic pathway. Consistent with this conclusion is the result shown in Figures 4(e) and 4(f), which plots side scatter (SSC), a measure of cell granularity, against forward scatter (FSC), which is related to cell size. In the presence of $AlCl_3$ (20 μM) for 20 minutes, there is a clear increase in the forward scatter, which indicates an increase in cell size. Necrosis is accompanied by an increase in cell size, whereas apoptosis is associated with cell shrinkage.

4. Discussion

Due to its ubiquity, environmental exposure to aluminum may play an important role in the etiology of several diseases [29]. Human ingestion of aluminum from food and beverages represents the major source of intake [30]. It is estimated that the average dietary intake of aluminum in adults ranges from 2 to 3 mg per day. These levels are not considered harmful to people with normal renal function [13]. Dietary exposure is higher in young children and teenagers [31]. However, these exposures do not include intakes associated with the use of personal care products, over-the-counter medication, inhalation of dust, and vaccines. In addition, aluminum becomes more soluble and, thus, even more bioavailable in acidic conditions [32]. Thus, many people with underlying medical conditions are even more vulnerable to aluminum-induced toxicity due to their exposure to higher concentrations of this metal. In other words, total daily aluminum intake by the human body varies broadly and is presumably higher than the levels referenced above [33].

In this study we attempted to estimate the range of aluminum concentrations that would be representative of typical daily exposure levels for humans, designating such concentrations as "environmentally relevant". This is important because even though these are cellular studies, one would hope that the results obtained would be relevant to what would be observed in an intact animal or human. Our results suggest that concentrations of aluminum that would be expected in humans can result in subtle changes in the physiology of immune system cells. While the injury we have observed was seen in acute studies, there may be long-term alterations in immune system function as a consequence.

Thymocytes were somewhat more sensitive to aluminum toxicity than lymphocytes, exhibiting statistically significant cell injury almost immediately after exposure to 10 μM of $AlCl_3$, while lymphocytes showed cell injury only at 15 μM. These results show that while both cell types responsible for immunodefense are quite sensitive to aluminum, thymocytes are somewhat more vulnerable. The reason for this difference is unclear but may reflect their less mature status.

The injury observed in both thymocytes and lymphocytes was very rapid, occurring in some cases as quickly

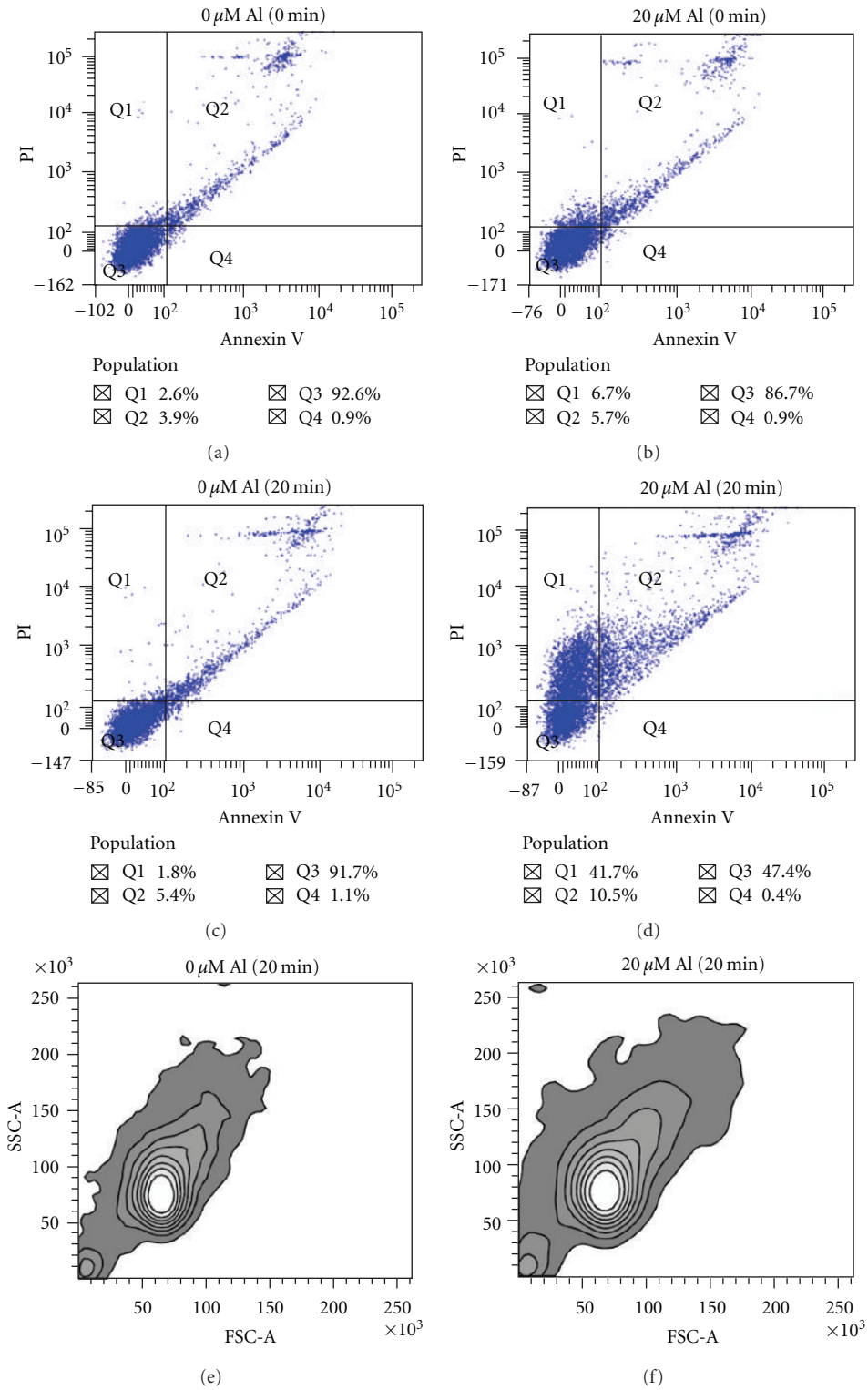

Figure 4: Thymocytes were exposed to 0 μM (a, c) and 20 μM (b, d) $AlCl_3$ at 0 and 20 minutes. Various staining patterns signify different cell populations. Region Q3 includes live cells (PI-negative and Annexin V-negative), whereas region Q4 contains apoptotic cells (PI-negative and Annexin V-positive). Dead cells are represented in Q2 region (both PI- and Annexin V-positive), while quadrant Q1 shows damaged cells (PI-positive and Annexin V-negative). Upon a 20-minute exposure to 20 μM $AlCl_3$ the cell population from the Q3 region moved to Q1 region, without a clear shift to the Q4 area first. This fact indicates that thymocytes are not undergoing the apoptotic process. Contour plots (e, f) show fluorescence intensity with regard to forward scatter and side scatter in the control (e) and in the presence of 20 μM $AlCl_3$ for 20 minutes (f). The increase in forward scatter on exposure to $AlCl_3$ is indicative of an increase in size (i.e., swelling).

as measurements could be made. While the mechanism responsible is not clear, the speed of the injury suggests a direct effect on the plasma membrane. This action increases the permeability of the membrane to PI but does not result in total loss of membrane integrity over the period of time we have studied. We conclude that aluminum causes acute damage to the plasma membrane to a degree that allows some entry to PI, but not to such a degree that membrane integrity is completely lost. In acutely isolated cerebellar granule cells, aluminum has been found to cause a rapid neurotic cell death [34] while toxicity of cultured neurons has been reported to induce either apoptosis [35] or a combination of neurosis and apoptosis [36].

Two points are especially important. The concentrations of aluminum studied are environmentally relevant, being ones to which humans are commonly exposed. Secondly, the time course of cell damage was very quick, suggesting a direct damage to the thymocyte/lymphocyte plasma membrane. These results may be relevant to the study and understanding of the mechanism(s) of chronic exposure to low concentrations of aluminum, which may result in long latency and slow progression of disease [23]. In addition, alteration of plasma membrane integrity associated with exposure to aluminum could make cells more permeable to other unwanted substances. Given the prominence of aluminum in the environment and the susceptibility of thymocytes, further investigation of the effects of aluminum on immune system function is warranted.

5. Conclusions

We have investigated the immunotoxicological effects of exposure to environmentally relevant concentrations of aluminum. We have documented a dose- and time-dependent injury in murine thymocytes and lymphocytes, which results from exposure to low levels of $AlCl_3$ (10 to 40 μM). Less than 5% of thymocytes were damaged after a 60-minute exposure to 10 μM $AlCl_3$, while 50% were injured after 10 minutes at 20 μM $AlCl_3$. Nearly all thymocytes sustained damage at 30 μM $AlCl_3$ after only 5 minutes of incubation. Notable lymphocyte injury was observed at 15 μM $AlCl_3$, and less than 50% of cells were injured after a 60-minute exposure to 20 μM. Our data suggest that lymphocytes are less sensitive to aluminum than thymocytes, perhaps due to their more advanced cell maturation. The damage is accompanied by cell swelling, which is consistent with damage to the plasma membrane.

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Review Article

Recognizing and Preventing Overexposure to Methylmercury from Fish and Seafood Consumption: Information for Physicians

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Fish is a valuable source of nutrition, and many people would benefit from eating fish regularly. But some people eat a lot of fish, every day or several meals per week, and thus can run a significant risk of overexposure to methylmercury. Current advice regarding methylmercury from fish consumption is targeted to protect the developing brain and nervous system but adverse health effects are increasingly associated with adult chronic low-level methylmercury exposure. Manifestations of methylmercury poisoning are variable and may be difficult to detect unless one considers this specific diagnosis and does an appropriate test (blood or hair analysis). We provide information to physicians to recognize and prevent overexposure to methylmercury from fish and seafood consumption. Physicians are urged to ask patients if they eat fish: how often, how much, and what kinds. People who eat fish frequently (once a week or more often) and pregnant women are advised to choose low mercury fish.

1. Introduction

All forms of mercury are toxic: elemental, inorganic, and organic forms. Methylmercury (MeHg) is the major organic form we are exposed to when we eat fish. All fish and shellfish contain some MeHg, but larger, longer-lived predatory fish generally have the highest levels. MeHg is particularly hazardous because it can cross the blood-brain barrier. Manifestations of MeHg poisoning are very variable and may be difficult to detect unless a test for blood or hair mercury is performed.

Exposure to elemental mercury in vapor form, for example, from broken thermometers or fluorescent light bulbs, can cause acute adverse effects. Inorganic mercury compounds can cause kidney toxicity, but exposure is uncommon except in certain occupational settings. This document focuses on MeHg exposure from fish and shellfish consumption. For information on other forms of mercury relevant to human health (e.g., from vaccines, silver-colored amalgams, and skin lightening creams) please see information from the Agency for Toxic Substances and Disease Registry [1].

In 2004, the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA) issued a joint advisory on fish consumption and MeHg [2]. The EPA/FDA advice is targeted to the higher risk populations of women of childbearing age, pregnant and nursing women, and young children (<6 years old). But fish consumers of all ages and genders who eat several meals of fish per week, or who regularly eat fish with higher levels of MeHg, are at risk of exceeding the EPA reference dose (RfD), a level of exposure that is prudent for all people to use as a guide to safe fish consumption.

Table 1: Fish with highest MeHg Contamination.

King mackerel
Tilefish (Gulf of Mexico)
Tuna (Bluefin, Bigeye)
Shark
Swordfish

Data derived from FDA, WA DOH, CT DPH, and EDF websites (links in resources section).

State health departments in most states have issued advisories about MeHg contamination of locally caught sport fish. Some state health departments include advice on commercially caught fish, but most do not. As a result, many consumers are unaware that MeHg in commercially caught fish can have health effects. Table 1 identifies commercial fish with the highest MeHg levels.

Data from the National Health and Nutrition Examination Survey (NHANES) conducted by the Centers for Disease Control and Prevention (CDC) show that blood mercury levels are strongly correlated with fish consumption. Levels are higher among people with higher incomes, among ethnic groups that eat more fish, such as Native American, Asian and Pacific Island populations [3], and among residents of US coastal regions [4]. A recent New York City Department of Health & Mental Hygiene study estimated that almost 25% of adults in New York City and almost 50% of Asian New Yorkers have blood mercury levels above 5 μg/L [5]. Nationally, about 7% of the NHANES sample exceeded that level [4]. The data illustrate there are identifiable, specific groups of people that are most vulnerable to overexposure to MeHg from fish consumption. With all of the public debate around the benefits and risks of fish consumption, we see informing physicians about MeHg in fish as an important way to reach the persons at greatest risk of overexposure to MeHg from fish and seafood consumption.

2. How Does Mercury Get into Fish?

Mercury occurs in the environment both naturally and as a result of human activities. The largest source of mercury emissions is coal combustion for energy production (cement kilns and chlor-alkali plants are also sources). When coal is burned, elemental mercury and inorganic mercury compounds are released and can be carried long distances in the air before they fall out onto land or water bodies. In water and wetlands, inorganic mercury is transformed by microorganisms to a more toxic form, MeHg. MeHg biomagnifies in the aquatic food chain and larger predatory fish such as shark, swordfish, king mackerel, and certain species of tuna accumulate some of the highest levels.

3. What Happens in the Body When MeHg Is Consumed?

More than 95% of the MeHg consumed in fish is absorbed in the gastrointestinal tract and transported to the blood stream, whence it is distributed to all organs. It takes only about 30–40 hours for an ingested dose of MeHg to be completely distributed throughout the body. Some organs have a higher affinity for MeHg than others. It crosses the blood-brain barrier and accumulates in the brain, where it can damage the central nervous system. About 10% of the MeHg in the body is in the brain; there, it is slowly demethylated to inorganic mercury, which crosses the blood-brain barrier very poorly.

Mercury—MeHg and demethylated—is gradually removed from the body, primarily via liver bile and feces, but some is also excreted in urine, sweat, and breast milk and some is stored in hair and nails. The MeHg level in blood is assumed to reflect the total amount in the body. The half-life of MeHg in blood is about 50–70 days in adults but can vary significantly; the half-life is longer in neonates and research suggests that genetic variation may account for additional differences.

MeHg crosses the placenta, and levels in umbilical cord blood are about 1.7 times as high as the mother's blood levels. To keep fetal blood mercury below the EPA reference level of 5.8 μg/L, the mother's blood level should thus not exceed 3.5 μg/L [6]. The developing nervous system is known to be particularly vulnerable to MeHg; effects depend on both the dose and the timing of exposures. Prenatal exposure to MeHg can result in cognitive deficits [7], motor skill effects [8], attention deficits, language skill deficiencies [9, 10], and decreased learning capacity and memory [11, 12].

Fish consumption also has well-documented nutritional benefits that improve cognitive test performance in children of women who ate low-mercury fish during pregnancy. Mothers-to-be should therefore be encouraged to eat fish, but advised to choose only low-mercury varieties [9, 10].

Research has also shown positive cardiovascular effects of fish consumption, probably due to omega-3 fatty acids [13]. But recent studies have also linked negative cardiovascular effects with the MeHg exposure associated with fish consumption, including increased heart rates and blood pressure [14] and a greater risk of myocardial infarction [15–17]. MeHg may also affect immune system function [18]. The important message is that the benefits of eating seafood clearly exceed the risks from MeHg, as long as the fish consumed are mostly low in mercury [19–21].

4. How Can You Identify Patients with Health Effects from MeHg?

Clinical manifestations (see Table 2) vary with the degree and length of exposure, and symptoms may not appear for some length of time after high exposure begins. Symptoms can vary significantly from individual to individual. Some studies suggest that symptoms may emerge when the body's ability to compensate for the damage is depleted [22, 23]. Other research suggests that genetic variation, specific food interactions that effect mercury metabolism, and other characteristics of individuals influence the manifestation of symptoms [18, 24, 25].

Patients with chronic lower level exposure to MeHg can experience nonspecific health effects such as fatigue, difficulty concentrating, hair thinning, muscle and joint

Table 2: Signs and symptoms of MeHg poisoning.

Lower level exposures
Sleep disturbance
Headache
Fatigue
Difficulty concentrating
Depression
Memory loss
Diminished fine motor coordination
Muscle and joint pain
Gastrointestinal upset
Hair thinning
Heart rate disturbance
Hypertension
Tremor
Numbness or tingling around the mouth
Highest level exposures
Numbness or tingling in hands and feet
Clumsy gait, difficulty walking (ataxia)
Slurred speech
Tunnel vision
Diminished visual acuity

Table 3: Cases of MeHg poisoning.

A 40-year-old lawyer who ate fish three or four times a week, primarily sea bass, could not sleep and lost his ability to concentrate. His hair contained 13 ppm mercury and his blood level was 58 μg/L.
A middle-aged sales manager ate fish eight or nine times a week, usually choosing tuna, swordfish, halibut, or sea bass. She experienced chronic fatigue, muscle aches, memory and concentration loss, and thinning of hair. When diagnosed, her blood mercury level was 76 μg/L.
A 66-year-old guitarist experienced a loss of fine motor coordination that affected her ability to play her instrument. She also had muscle weakness, thinning hair, and hand tremors. She had been eating swordfish and tuna steaks four to five times a week. Her blood mercury was 38 μg/L.
A 64-year-old anthropologist who ate fish nine times a week, often choosing tuna, swordfish, sea bass, and halibut, suffered from chronic fatigue, headaches, memory loss and, hair loss. Her blood mercury level at diagnosis was 21 μg/L.
A 10-year-old boy who had always been an "A" student began having problems concentrating and completing assignments in school. He lost his ability to catch a ball and developed hand tremors. He had eaten a can of tuna every day for a year. His blood mercury level was above 60 μg/L.

Cases excerpted from Groth [27].

pain, sleep disturbance, and gastrointestinal upset. Classical signs and symptoms of higher level MeHg exposure include (in order of typical appearance of symptoms) numbness and tingling around the mouth, numbness and tingling in hands and feet, clumsy gait or difficulty walking (ataxia), slurred speech, visual field constriction, coma, convulsions, and death. Patients with very high MeHg exposure may also exhibit symptoms described for lower exposures.

Multiple research studies and personal observations by the authors of this document indicate that individual patients vary widely in sensitivity to MeHg toxicity. The milder symptoms have been seen in some patients at relatively low blood mercury levels [15, 26–28]. A review of 24 cases of symptomatic MeHg poisoning found a range from 7 to 125 μg/L blood mercury, and the majority of cases had levels below 40 μg/L [27]. Table 3 briefly describes some cases of MeHg exposure. People vary in their susceptibility to mercury and not everyone will experience negative health effects. Patients at greatest risk of developing MeHg poisoning are those who eat fish often and who prefer higher-mercury seafood varieties such as swordfish or tuna [29]. Thus it is important that health care professionals ask patients about their diet in order to make the connection between MeHg exposure and seafood consumption.

5. Laboratory Tests and Their Interpretation

A blood analysis should be done for patients with suspected elevated MeHg exposure from fish and shellfish consumption. A hair sample may also be analyzed; the level in hair reflects longer-term exposure and helps distinguish organic (methyl- or ethyl-) mercury exposure from inorganic or elemental mercury exposure. Urine tests primarily reflect inorganic and elemental mercury exposures. In general a low urine mercury test (<10 μg/L) in combination with elevated blood (>5 μg/L) or hair (>1 μg/g) mercury points to MeHg exposure from seafood consumption. While most clinical analyses of blood, nail clippings, or hair are for total mercury, almost all mercury present is in the form of MeHg.

Blood Test. While the EPA has defined criteria for excessive blood mercury in women of childbearing age, there are no generally recognized guidelines for acceptable blood mercury in the rest of the population. Geometric mean blood levels in the USA based on NHANES data are <1 μg/L for those age 29 and under and about 1 μg/L for those 30 and older. Blood mercury levels tend to increase with age and peak in the 5th or 6th decade, depending on race and ethnicity [30]. The Centers for Disease Control and Prevention define the laboratory criteria for diagnosis of excessive mercury exposure as a blood level above 10 μg/L [31]. Some state departments of health, including New York [32], require laboratories that measure blood mercury to report levels above 5 μg/L to the state heavy metals registry. The authors of this document believe that a blood mercury level above 5 μg/L calls for counseling of patients with regard to fish consumption emphasizing low mercury species.

Blood mercury tests reflect recent exposures as well as chronic accumulation. Blood labs instruct patients not to eat seafood for three days before a mercury test, but patients should be advised to follow their normal diet prior to testing.

Hair Test. Most people have hair mercury levels well below 1 μg/g (ppm), the EPA reference level. Neuropsychological

functional deficits have been reported in adults with an average hair level of 4.2 ppm [33], while prenatal neurodevelopmental effects have been associated with maternal hair levels of 1.2 ppm or higher [9]. A 2009 literature review looking at neurodevelopmental effects on the fetus estimated that a lowest observable adverse effect level in maternal hair might be as low as 0.3 μg/g [34], thus supporting a precautionary approach that includes counseling those planning to be pregnant, or who already are pregnant, to choose low mercury fish.

6. Recommended Action for Those with High Blood or Hair Mercury

The primary advice for patients with MeHg poisoning is to stop eating fish temporarily or shift to very low-mercury fish. Once their blood mercury has declined to a lower level (<5 μg/L) and symptoms have resolved, low-mercury fish and shellfish can be reintroduced to the diet.

Chelation can be a valuable medical intervention for *inorganic* mercury poisoning, but it poses its own risks and, except in rare cases, is not generally warranted for patients with elevated MeHg from fish consumption [11, 35]. Some practitioners mistakenly use a DMSA or DMPS provocation challenge when they test a patient's urine for mercury. This gives highly misleading results that overestimate, sometimes seriously, a patient's mercury exposure. There is also individual variability in response to chelation challenge or treatment.

7. Prevention and Risk Communication

To make healthy fish consumption choices, consumers need to know which fish are lowest in contaminants and higher in omega-3 fatty acids (see Table 4 for fish guidance considering both MeHg and PCB contaminant levels). Fish is a good source of protein and is low in saturated fat. Advise fish eaters to choose low-contaminant, high omega-3 fatty acid varieties, and to limit consumption of higher mercury fish. Pregnant women, women who are breastfeeding, women who plan to become pregnant within a year, and children less than 12 years old should eat *only* low-mercury fish.

Mercury accumulates in fish muscle and levels are not reduced by cooking. Persistent organic pollutants like PCBs accumulate in fat, and exposures can be decreased by removing skin and fatty tissue and letting fat drip off during cooking [36].

Table 4: Choose wisely.

Lowest contaminant levels
Anchovies☺
Arctic char
Atlantic mackerel (not king mackerel)☺
Catfish (U.S. farmed)
Cod
Haddock
Herring☺
Perch
Pollock (fish sticks)
Salmon (wild)☺
Sardines☺
Shellfish (oysters (Pacific☺), shrimp, clams, mussels, scallops)
Tilapia
Tuna (Skipjack/"chunk light", not yellowfin)
Trout (Rainbow, farmed)☺
Medium to high contaminant levels
Black sea bass☺
Grouper
Halibut
Lobster
Mahi mahi
Orange roughy
Rockfish/red snapper
Sablefish/black cod☺
Salmon (farmed)☺,∞
Spanish mackerel☺
Tuna (albacore☺/"white", yellowfin/ahi)
Highest contaminant levels
Bluefish∞
Croaker (White/Pacific)∞
Eel∞ (American, European; not Conger eel)
King Mackerel
Tuna (Bluefin∞, Bigeye)
Shark
Swordfish
Tilefish (Gulf of Mexico, not Atlantic)
Weakfish/Seatrout∞

☺ A good source of omega-3 fatty acids.
∞ May contain harmful PCB levels.
Data derived from FDA, WA DOH, CT DPH, and EDF websites (links in resources section).

8. How Much Fish Can You Eat?

A level of mercury consumption with no adverse effects has not been established. The EPA RfD is a guideline for acceptable daily exposure based on the best evidence available in 1999, but more recent studies, as noted earlier, suggest that more caution is justified, especially for pregnant and nursing women and children. The RfD assumes that prenatal cognitive effects are the most critical hazard and aims to prevent those effects. Given its narrow basis, the RfD is at best a guideline for acceptable exposure in other populations. Recent studies suggest adult cardiovascular health is sensitive to low levels of MeHg [37]. Neurobehavioral functions in adults can be impaired at doses similar to those that cause prenatal effects [28, 33]. Two recent epidemiological studies have found both beneficial effects of fish nutrition and adverse effects of MeHg exposure on prenatal cognitive development at MeHg doses near or below the RfD, that is, doses an order of magnitude lower than those recognized as harmful in 1999 [10, 12, 38]. To be on the safe side, physicians are advised to encourage frequent fish eaters to try

to keep their MeHg exposure *below* the RfD. This approach is especially important for pregnant women, but given the uncertainties, it may prudently be applied to other patients as well.

How many fish or shellfish meals are advised per week depends on *how often* one eats fish, a person's *body weight*, the *portion size*, the *mercury content* of the fish choice, and *individual health considerations* such as pregnancy status. Individual patients can also vary widely in their inherent susceptibility to toxic effects. Portion size is an important factor in exposure for many Americans in particular because we tend to eat large portions. According to the US Department of Agriculture, a serving is 2-3 ounces per meal for a recommended total daily total protein amount of 6-7 ounces of lean meat or fish, depending on your energy requirements [39]. Serving sizes at home and in restaurants are often larger [40] and thus should be factored into exposure considerations. Portion sizes for children should be approximately one ounce for every twenty pounds of body weight. For example, a serving for a 45 pound child would be about 2 ounces.

Adults who eat a very high MeHg fish such as swordfish or shark even as infrequently as once a month will generally exceed the EPA reference dose.

Many people consume both commercial and sport fish and thus total fish consumption should be considered. Check state public health departments for local fish advisory information (links are included in the resources section).

9. Other Contaminants of Concern

Fish also contain persistent organic pollutants (POPs) such as PCBs. Research suggests that POPs have their own negative health effects that may offset some of the benefits of fish consumption [41–44]. Fat from pork, beef, and chicken also contains POPs, usually at lower levels than in fish. But most Americans eat much more beef, pork, and chicken than fish, so fish is not the largest dietary source of POPs for most consumers.

Many people take fish oil supplements to obtain the benefits from the omega-3 fatty acids present in fish. Some brands of fish oil supplements specify that they have been molecularly distilled or purified to remove contaminants and contain no detectable mercury. As MeHg binds to proteins, it is not present in the fat (oil). However, PCBs and other persistent organic pollutants and halogenated natural products do accumulate in fat and may contaminate supplements. The issue is complicated by the presence of multiple brands of fish oils with varying sources of fish that contain varying contamination levels, as well as varying levels of purification. Lack of any government standards as to acceptable levels of contamination further complicates the issue. Until more is understood, the most prudent approach is to consume a variety of low mercury fish—generally smaller, nonpredatory fish—in order to obtain the health benefits. If supplements are desired, those derived from small, cold water fatty fish such as anchovies, sardines, and mackerel are reported to have lower levels of organic contaminants [45].

For those who wish to consider both MeHg and POPs in their fish choices, some advice does include both types of contaminants for commonly eaten commercial and sport fish (see Washington State Department of Health and Connecticut State Departments of Public Health as well as the Environmental Defense Fund and Sea Web Kid Safe Seafood web sites; web site addresses are in the Resources section).

10. Resources

10.1. Online Tools for Guiding Low MeHg Fish Choices. Several organizations offer easy to use online calculators that estimate safe fish intakes based on the consumer's body weight and the fish selected. These calculators are designed to keep exposure below the EPA RfD. MeHg levels in seafood vary by geographical location with certain species such as tuna showing more variability [46], therefore we suggest that you use online tools more for general guidance to help understand fish MeHg levels, and not as an absolute index of MeHg content. The most prudent advice is to eat low-mercury fish.

GotMercury.org (http://www.gotmercury.org/) and the Natural Resources Defense Council (http://www.nrdc.org/health/) both offer easy to use calculators that estimate safe intakes based on the EPA RfD and can help consumers make healthy seafood choices. Beware of some other online calculators that understate mercury risks. (e.g., the Howmuchfish.com website offers an on-line calculator that tells users they can consume ten times more methylmercury than the EPA RfD would indicate.)

Another option is an iPhone application called Fish4Health developed by researchers at Purdue University. Fish4Health lists seafood choices organized by mercury contamination levels and allows you to select the fish and the quantity you eat. It calculates and reports daily mercury intake and associated omega-3 fatty acid intake and keeps a log for you, letting you know if you are eating sufficient omega-3 fatty acids and if you exceed the EPA RfD for mercury. The application can be used by all seafood eaters who want to keep their mercury exposures below the EPA reference dose even though its target audience is pregnant and nursing women. (http://fn.cfs.purdue.edu/fish4health/iPhoneApp.html).

10.2. Online Seafood Advice. The *Environmental Defense Fund* Seafood Selector includes recommendations on how many meals one can eat of specific fish each month and stay below the EPA RfD for mercury. It also considers persistent organic pollutants and thus offers more broadly protective guidance than the joint FDA/EPA mercury advice. The EDF site also includes a sushi guide: http://www.edf.org/page.cfm?tagID=1540.

Sea Web Kid Safe Seafood considers MeHg and persistent organic pollutants and the advice is geared specifically for children: http://www.kidsafeseafood.org/.

The *Mercury Policy Project (MPP)* sorts top selling commercial seafood varieties into six categories by mercury content, offering finer distinctions on mercury content for those who seek the lowest-mercury fish: http://mercuryfactsandfish.org/.

10.3. Government Information Sources. The oversight of commercial and sport fish and resulting consumption advice is handled by separate government agencies. State, tribal and local governments are responsible for local sport fish advisories and the Food and Drug Administration governs the sale of commercial fish. Although most fish consumed in the USA is commercial fish, only some State Departments of Health offer advice that consider both sport and commercially caught fish (e.g., see Washington (http://www.doh.wa.gov/ehp/oehas/fish/default.htm) and Connecticut (http://www.ct.gov/dph/cwp/view.asp?a=3140&q=387460). Fish advisories for various states can be accessed through this EPA site: http://www.epa.gov/waterscience/fish/states.htm.

The *Environmental Protection Agency* (EPA) monitors mercury in the environment and regulates industrial releases. The EPA site also contains information on fish consumption. EPA issued a joint advisory with the FDA in 2004: http://www.epa.gov/waterscience/fish/advice/index.html.

Background information on the EPA FDA joint advisory on mercury in fish: http://www.epa.gov/fishadvisories/advice/factsheet.html.

EPA funded educational video modules for health professionals on the risks and benefits of fish consumption: http://www.fish-facts.org.

The *Food and Drug Administration* (FDA) is the agency responsible for the safety of commercial seafood. The FDA website offers information about MeHg, including the FDA database on mercury levels in different categories of fish and shellfish: http://www.fda.gov/Food/FoodSafety/Product-SpecificInformation/Seafood/FoodbornePathogensContaminants/Methylmercury/ucm115662.htm.

The *Agency for Toxic Substances and Disease Registry* provides data and educational resources on all forms of mercury in the environment (http://www.atsdr.cdc.gov/tfacts46.html).

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Research Article

Metals and Breast Cancer: Risk Factors or Healing Agents?

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Metals and metal compounds are part of our environment. Several metals are essential for physiological functions (e.g., zinc or magnesium); while the beneficial effects of others are uncertain (e.g., manganese), some metals are proven to be toxic (e.g., mercury, lead). Additionally there are organic metal compounds; some of them are extremely toxic (e.g., trimethyltin, methylmercury), but there is very little knowledge available how they are handled by organisms. Scientific evidence indicates that long-term exposure to (some) metallic compounds induces different forms of cancer, including breast cancer. On the other side, several metal compounds have clinical use in treating life-threatening diseases such as cancer. In this paper we discuss the recent literature that shows a correlation between metal exposure and breast cancer.

1. Introduction

According to the World Health Organization, breast cancer accounts for 16% of all types of cancer deaths globally (total deaths of cancer 7,600,000, total breast cancer deaths 460,000 [1]). It is the most common solid tumor diagnosed in women [2]. Although the incidence of breast cancer increases with age [3], certain lifestyle and environmental factors play an important role on breast cancer risk [4]. Such risk factors include the genetic background and environmental factors. For example, women who have inherited mutations in the BRCA1 or BRCA2 genes have substantially elevated risks of breast cancer [5].

Also an elevated lifetime estrogen exposure might be major risk factor for breast cancer [6]. However, the activation of estrogen receptors alone is not sufficient for the development of breast cancer [7] indicating that other factors play an important role in carcinogenesis. The underlining mechanism could rely on the ability of estrogen and estrogen metabolites to generate reactive oxygen species which induce DNA synthesis, increased phosphorylation of kinases, and activation of transcription factors, such as AP-1, NRF1, E2F, NF-κB, and CREB responsive to either oxidants (e.g., toxins, including metal compounds) or estrogen. Therefore, the genomic instability increases while the activation of transcription factors plays an important role in cell transformation, cell cycle, migration, and invasion [7].

Environmental factors also play a decisive role in breast carcinogenesis together with life-long dietary habits [4]. More and more evidence underlines that external factors are involved in the development of breast cancer: nutrition (obesity and alcohol consumption), smoking, and exposure to carcinogens (e.g., metal compounds) [4]. Multiple reports show that metallic compounds could function as estrogen disruptors [8], while other studies underline the connection between the exposure to metals or metal compounds and breast cancer risk [2, 9, 10].

The present paper discusses emerging data in support of the role of metal compounds in the development of breast cancer. It is envisioned that estrogen-induced metal-mediated signaling is a key complementary mechanism that drives the carcinogenesis process. On the other hand it also highlighted the beneficial effects of metal-derived compounds, which are used for the treatment of cancer (e.g., platinum compounds have been a breakthrough for the treatment of breast cancer) [11].

In the following sections the association of specific metals (and their compounds) in regard to their effects in inducing breast cancer are discussed as well as beneficial effects of metals in treating the same cancer.

2. Epidemiologic Studies Illustrating the Effects of Multiple Metals

A large number of epidemiologic studies associate potential risk factors for cancer with metals such as selenium (Se), zinc (Zn), arsenic (As), cadmium (Cd), and nickel (Ni), which are found naturally in the environment. Human exposure to these metals results from air, drinking water, and food [12, 13]. Other studies demonstrated that age-corrected breast cancer mortalities in different countries are inversely correlated with the dietary intake of Se and directly with the estimated intake of Cd, Zn, and chromium (Cr), suggesting that the anticarcinogenic properties of Se are counteracted by these elements [14]. As mentioned before, these metals can mimic the action of estrogen; the estrogenicity of various heavy metals was described in [15], for example, bis(tri-n-butyltin) > cadmium chloride > antimony chloride > barium chloride = chromium chloride > lithium hydroxide > sodium selenite = lead acetate > stannous chloride.

While an association between exposure to metals and the risk of the lung, breast, colorectum, prostate, urinary bladder, and stomach cancers is discussed, it was demonstrated that breast cancer patients have abnormal levels of copper (Cu), Zn, Se, and Cd [16], Interestingly, other evidence shows an inverse association between Se exposure and prostate cancer and lung cancer risk. There is also some evidence for an inverse association between Zn and breast cancer, while there is no association between exposure to Se and the risk of breast, colorectal, and stomach cancer and between Zn and the risk to develop prostate cancer [12]. Nevertheless, positive associations of breast cancer with Zn, iron, and calcium, but little association with Se, have been reported in [17].

In particular, a study of [16] showed that the genetic instability found in stage I breast cancer patients (frequency of micronucleated lymphocytes) was related with the blood levels of Cu, Zn, Se, and Cd. The authors found that the level of Cu, Zn, and Se was significantly lower in breast cancer patients, as compared to controls while the level of Cd was significantly higher in these patients. In breast cancer patients, the frequency of micronucleated lymphocytes showed complex associations with different concentrations of these elements. High Cd, low Zn, low Se, and both high and low Cu levels increased the micronucleus formation in lymphocytes. A similar correlation was found in the control group only in relation to high Se and Cd levels [16].

3. Arsenic

Arsenic (As) exposure constitutes one of the most widespread environmental carcinogens and is associated with increased risk of different types of cancers [18–20]. Arsenites are found in drinking water and are used in wood preservatives, insecticides, and herbicides. Epidemiological evidence has associated exposure to As in drinking water with an increased incidence of human cancers in the skin, bladder, liver, kidney, and lung [21, 22], and low concentrations of As_2O_3 induce carcinogenesis after long-term exposure [23].

Nonetheless, arsenic trioxide (As_2O_3) is also a component of traditional Chinese medicine [24]. In clinics it is successfully used to treat hematologic malignancies. However, the antitumor effects could not be replicated for solid tumors [11, 23, 25], although *in vitro* As_2O_3 induces apoptosis in other solid cancer cell lines including breast cancer cells [19, 20, 23, 26]. In either application, the precise molecular mechanisms through which As_2O_3 induces cell cycle arrest and apoptosis in solid tumors have not been fully understood [24]. Hopefully, new insights into how As_2O_3 binds to specific receptors and how they trigger signaling pathways might facilitate As_2O_3-based anticancer strategies and/or combination therapies in order to treat solid tumors [18, 24].

A large body of evidence indicates that arsenic compounds induce cell death in breast cancer cells and the induction of this effect is a possible endorsement for the treatment of breast cancer. For example, sodium arsenite mimics the effects of estradiol and induces cell proliferation in the estrogen-responsive breast cancer cell line MCF-7 while the S-phase recruitment was increased [22]. Interestingly, regarding the cell proliferation, a paradox effect was observed: lower concentrations ($<5\,\mu$M) of sodium arsenite induced cell proliferation while higher concentrations ($>5\,\mu$M) or longer treatment periods induced apoptosis [21]. In addition, As also influences the enzymes participating in the folate cycle [21].

Other studies indicate that arsenite, in environmental relevant concentrations ($5\,\mu$M/0.65 mg/L), is able to induce both replication-dependent DNA double-strand breaks and homologous recombination. Double-strand break formation was replication dependent and probably the result of conversion of a DNA single-strand break into double-strand breaks [18]. In addition, low arsenite concentrations (0.5–$5\,\mu$M) induce ROS production and ROS-related depolarization of the mitochondrial membrane, suggesting that mitochondria play an important role in the oxidative effects of As. In addition, when ROS-mediated DNA damage is measured by the presence of 8-OHdG DNA adducts in their nuclei, IκB phosphorylation, NF-κB activation, and increases in c-Myc and HO-1 protein levels were also observed. Therefore, these factors might play a relevant role in the arsenite-induced MCF-7 cell recruitment into the S-phase of the cell cycle and cell proliferation observed [22]. Nonetheless, the authors conclude that arsenite activates several pathways involved in MCF-7 cell proliferation, and, therefore, arsenite exposure may pose a risk for breast cancer in exposed populations.

Additionally it was found that, in MCF-7 breast cancer cells, As_2O_3 treatment changes the expression level of several genes that are involved in cell cycle regulation, signal transduction, and apoptosis. Important targets are represented by proteins which inhibit the cell cycle like p21 and p27. Liu et al. [23] and Wang et al. [24] showed that after 24 h exposure to As_2O_3 (0.01–$1\,\mu$M) cell proliferation significantly increased and a progression from the G1 to S/G2 phases occurred in the nontumorigenic MCF10A breast epithelial cell line. Several cell-cycle-associated genes were increased significantly, for example, cell division cycle 6 (CDC6) and cyclin D1 (CCND1) which are closely related

to cell cycle progression from G1 to S phase. In addition, the production of ROS was elevated, while activation of p38 MAPK, Akt, and ERK1/2 pathways was observed [23]. Arsenite also increases in MT1/2 and c-Myc protein levels concentration dependently [21].

For treatment of solid tumors a novel nanoparticulate formulation of As_2O_3 encapsulated in liposomal vesicles named "nanobins" [NB(Ni, As)] was synthesized in order to improve the therapeutic efficiency against breast carcinomas. The NB (Ni, As) agent was less cytotoxic *in vitro* than As_2O_3, and *in vivo* NB (Ni, As) dramatically improved the therapeutic efficacy of As_2O_3. These effects are possibly due to a reduced plasma clearance, an enhanced tumor uptake, and an induction of tumor cell apoptosis [25].

4. Cadmium

Cadmium (Cd) is a nonessential metal that is dispersed throughout the environment [27, 28]. It has been categorized as a human carcinogen by the US Environmental Protection Agency. Primary exposure sources include food and tobacco smoke [8, 9, 27, 29]. Cadmium is a ubiquitous carcinogenic pollutant and has multiple biological effects, and exposure is correlated with the occurrence of breast cancer in some US regional case-control studies [2, 9, 10, 29].

Gallagher et al. [2] as well as McElroy et al. [9] obser-ved a significant trend of an increased risk of breast cancer by elevated urinary cadmium concentrations, but the mechanisms of action of cadmium remain unclear [8, 30]. Cd affects multiple cellular processes, including cell proliferation, differentiation, and apoptosis [8]. Cd functions also as an endocrine disruptor, which stimulates estrogen-receptor-α (ER-α) activity and promotes uterine and mammary gland growth in and abolishes the cancer-protecting effects of Se in female inbred C3H mice carrying murine mammary tumor virus [14].

Cd modulates gene expression, affects the pattern of transcriptional activity, and, therefore, changes intracellular signals [31]. The modification of gene expression in MCF7 cells is blocked by antiestrogens. Therefore, these effects could be mediated by ER-α [29, 30]. In estrogen-responsive breast cancer cell lines, Cd stimulates proliferation and also activates the estrogen receptor independent of estradiol [28–30]. Cd activates extracellular regulated kinases, erk-1 and -2 in both ER-positive and ER-negative human breast cancer cells. High Cd concentrations from 50 to 500 nM induced a proliferative response SKBR3 cells, increased intracellular cAMP levels. Cd treatment activates raf-1, mitogen-activated protein kinase kinase, mek-1, extracellular signal-regulated kinases, erk-1/2, ribosomal S6 kinase, rsk, and E-26 like protein kinase, elk [28].

ER-α is required for both Cd-induced cell growth and modulation of gene expression. ER-α translocates to the nucleus in response to Cd exposure and potentiates the interaction between ER-α and c-Jun and enhances recruitment of this transcription factor complex to the proximal promoters of cyclin D1 and c-myc, increasing the mRNA expression [8]. Additionally, Casano and colleagues in 2010 [31] confirmed that treatment of breast cancer cells with 5 μM $CdCl_2$ induces a diversified modulation of the transcription patterns of p38, while [30] Sun and coworkers (2007) showed that treatment of MCF-7 cells with Cd resulted in induction of Hsp22. Cd increases breast cancer cell proliferation *in vitro* by stimulating Akt, ERK1/2, and PDGFRα kinases activity likely by activating c-fos, c-jun, and PDGFA by an ER-α-dependent mechanism [32].

In chronic Cd exposure (over 40 weeks) of the human breast epithelial cell line MCF-10A, secretion of matrix metalloproteinase-9 increased, followed by a loss of contact inhibition, increased colony formation, and increasing invasion. Furthermore, inoculation of Cd-treated cells into mice produced invasive, metastatic anaplastic carcinoma. Additionally, breast stem cell markers CK5 and p63 were found overexpressed indicating persistent proliferation, global DNA hypomethylation, and c-myc and k-ras overexpression [10]. Exposure of breast cancer cells to "subtoxic" levels of Cd significantly inhibited the angiogenic potential of the breast cancer cell line, suggesting the possibility that Cd might negatively regulate the production of proangiogenic factors in breast cancer cells.

Interestingly, melatonin prevents the Cd-induced growth of synchronized MCF7 breast cancer cells. Melatonin is a specific inhibitor of Cd-induced ER-α-mediated transcription, inhibits MCF7 cell growth induced by Cd, and regulates Cd-induced transcription in both ERE and AP1 pathways. Overall, the antiestrogenic properties of melatonin might be a valuable tool in breast cancer therapies [29].

In summary, Cd might exert a paradoxical effect in breast cancer: on the one hand, it could promote carcinogenesis, and, on the other hand, it could delay the onset of tumors by inhibiting breast cancer cell-induced angiogenesis [27].

5. Gold

Gold nanoparticles (GNPs) are regarded as a possible delivery vehicle for anticancer drugs and seem to have a great potential to be used in clinics. In breast cancer MDA-MB-231 cells the group of Jain [33] assessed the cellular uptake, intracellular localization, and cytotoxicity of GNPs. When GNPs were taken up, nanoparticles accumulated in cytoplasmic lysosomes. However, the GNP exposure did not increase radiation-induced double-strand breaks formation and did not inhibit DNA repair; but GNP chemosensitization was observed in MDA-MB-231 cells treated with bleomycin [33].

For some time it was assumed that the GNPs are but recent studies showed that this is not the case since it was demonstrated that they cause oxidative stress and even cell death, suggesting a possible biological mechanism for sensitization [33]. Using syngeneic mouse and human xenograft models of triple-negative breast cancer, Atkinson and his group demonstrated that local hyperthermia generated by gold nanoshells plus radiation eliminates radioresistant breast cancer stem cells [6]. Another study by Day et al. [34] describes the possibility of using near-infrared resonant gold-gold sulfide nanoparticles as dual contrast and therapeutic agents for cancer management via multiphoton microscopy followed by higher intensity photoablation which can be utilized to visualize cancerous cells

in vitro. When conjugated with anti-HER2 antibodies, these nanoparticles specifically bind SK-BR-3 breast carcinoma cells that overexpress the HER2 receptor, enabling the cells to be imaged via multiphoton microscopy [34].

6. Platinum

Cisplatin is a first choice chemotherapeutic drug for different types of cancer. Although there is increasing evidence that breast cancers are sensitive to cisplatin, its clinical success is often compromised due to dose-limiting nephrotoxicity and the development of drug resistance. To overcome these limitations, other platinum derivatives have been developed for the treatment of breast cancer, and several of them are still tested in clinical trials. In addition multiple drug combination therapies (with include cisplatin) have been employed [11, 35].

Multiple cellular effects have been described for cisplatin (for review see [11]). Recently it was demonstrated that cisplatin increases the intracellular calcium concentration dependently, and this increase of the intracellular calcium signal is directly related to cytotoxicity [36]. This is in agreement with similar results which were found earlier with other cancer cell lines [37].

Regarding the molecular effects triggered by cisplatin in breast cancer cells, Wong et al. [35], showed that inhibition of the mTOR, TGFbetaRI, NFκB, PI3K/AKT, and MAPK pathways sensitized basal-like MDA-MB-468 cells to cisplatin treatment. Nevertheless, the combination of the mTOR inhibitor rapamycin and cisplatin generated significant drug synergism in basal-like MDA-MB-468, MDA-MB-231, and HCC1937 cells but not in luminal-like T47D or MCF-7 cells. The synergistic effect of rapamycin plus cisplatin was mediated by the induction of p73. The authors conclude that a combination therapy with mTOR inhibitors and cisplatin could be a useful therapeutic strategy for the treatment of basal-like breast cancers.

A combination of gemcitabine and cisplatin was tested in metastatic breast cancer and was successful in phase II trials. This suggests that the combination of gemcitabine and cisplatin is a safe and tolerable regimen and useful as second-line combination for patients with anthracycline- and taxane-pretreated MBC. It is mostly used as a salvage regimen for progressive disease refractory to anthracyclines and taxanes and when liver dysfunction secondary to liver metastasis precludes these drugs [26, 38, 39].

It is also discussed whether a combination of cisplatin and TRAIL has the potential to improve the therapeutic outcome in triple-negative breast cancer (TNBC) patients. This approach was tested *in vitro* on normal and triple-negative breast cancer (TNBC cells) by Xu and coworkers [40]. Indeed, this combination significantly enhanced cell death in TNBC cell lines and inhibited the expression of EGFR, p63, survivin, Bcl-2, and Bcl-xL. Specific inhibition of EGFR and/or p63 protein in TNBC cells was observed while survivin played an important role in cisplatin plus TRAIL-induced apoptosis in TNBC cells. *In vivo* experiments resulted in a significant inhibition of CRL2335 xenograft tumors compared to untreated control tumors [40].

It was speculated that whole body thermal therapy would boost the efficacy of oxaliplatin chemotherapy with reduced toxicity. Indeed, elevating the temperature reduced the IC_{50} of oxaliplatin in MTLn3 cells, while the cellular uptake of platinum and platinum adducts increased. *In vivo*, 50% of all oxaliplatin treated rats 24 h before thermal therapy were immunologically cured; in 11% their primary tumor regressed but ultimately succumbed to metastases, and 17% experienced a limited response with increased survival. In uncured animals, the thermo-chemo-therapy had a delayed incidence and slowed growth of metastases [41].

The inhibitory activity of different anticancer metal complexes based on platinum, ruthenium, and gold metal ions was evaluated on the zinc-finger protein PARP-1, either purified or directly on protein extracts from human breast cancer MCF7 cells. The results by Mendes and coworkers [42] support a model whereby displacement of zinc from the PARP-1 zinc finger by other metal ions leads to decreased PARP-1 activity. *In vitro* combination on different cancer cell lines, including MCF7, showed synergistic effects [42].

New platinum compounds are yet to be studied for their potential to be used in anticancer treatment. A series of seven platinum (II) cyclobutane-1,1-dicarboxylato (cbdc) complexes {[Pt(cbdc)(L(n))(2)], 1–7}, derived from carboplatin were studied for their *in vitro* cytotoxicity activity against breast adenocarcinoma cells (MCF-7) and were found to be cytotoxic [43]. Recently, Paraskar and colleagues [44] reported a novel polymer, glucosamine-functionalized polyisobutylene-maleic acid, where platinum (Pt) can be complexed to the monomeric units. This complex self-assembles to a nanoparticle, which releases cisplatin in a pH-dependent manner. Those nanoparticles are rapidly internalized into the endolysosomal compartment of cancer cells and exhibited a significantly improved antitumor efficacy in breast cancers. Furthermore, the nanoparticle treatment resulted in a reduced systemic and nephrotoxicity, which was due to a decreased distribution of platinum to the kidney [44]. The *in vitro* antitumor activity of the [Pt(ox)(L(n))(2)] (1–7) and [Pd(ox)(L(n))(2)] (8–14) oxalato complexes involving N6-benzyl-9-isopropyladenine-based N-donor carrier ligands (L(n)) against breast adenocarcinoma (MCF7) were studied by Paraskar and coworkers [44]. This group found the tested complexes to be more cytotoxic compared to cisplatin, but they were non-hepatotoxic [44].

In MCF-7 cells [Pt(O,O′-acac)(γ-acac)(DMS)] had toxic effects at high concentrations, while subcytotoxic concentrations induced anoikis and decreased cell migration. This compound altered $[Ca^{2+}]_i$ homeostasis and triggered apoptosis. When cells were stimulated with ATP, the changes in Ca^{2+} levels caused by purinergic stimulation were altered due to decreased PMCA activity and due to the closure of Ca^{2+} channels opened by purinergic receptors. Conversely, [Pt(O,O′-acac)(γ-acac)(DMS)] did not affect the store-operated Ca^{2+} channels opened by thapsigargin or by ATP, but it provoked the activation of PKC-α and the production of ROS that were responsible for the Ca^{2+} permeability and PMCA activity decrease [45].

7. Lead

Breast cancer incidence in women has been related to industrialization suggesting that the associated widespread contamination of the soil, air, and the water by lead (Pb) and other industrial metals is a major risk factor. Due to its wide use, Pb is of particular concern. In levels as low as 0.5 ppm Pb (in drinking water), it promotes the development of mammary murine tumors in virus-infected female C3H mice [46]. It also accelerates tumor growth rates. Higher levels of Pb were found in blood and head hair samples of newly diagnosed patients with breast cancer, all with an infiltrating ductal carcinoma [46]. The Pb levels in the hair samples were directly correlated with the volumes of the tumors [46]. The same researchers also found evidence that Pb and other metals also interact with iodine, a vitally important essential trace element that most likely protects against breast cancer development [46].

On the other side, some new metal-organic lead structures have been developed over the last years, which actually exhibit cytostatic properties. However, the efficiency of such chemotherapeutics in the treatment of tumors might be limited by their low therapeutic index due to their short half-life, lack of tumor selectivity, and associated side effects [46].

8. Cymantrene-Peptide Conjugates

Cymantrene ($CpMn(CO_3)$) is a robust organometallic group, which is stable in air and water. In experiments done by Splith and coworkers [47], cymantrene derivatives were attached to the cell-penetrating peptide sC18 which acted as a transporter for the metal moiety. This group characterized the conjugates for their cytotoxic activity on human breast adenocarcinoma cells (MCF-7) and human colon carcinoma cells (HT-29). These researchers found that bioconjugates bearing two cymantrene groups were more active than the monofunctionalized ones and that, by the introduction of a cathepsin B cleavage site next to the organometallic group, the biologic activity was increased [47].

9. Selenium

The role of Se as a potential cancer chemopreventive and chemotherapeutic agent has been supported by epidemiological, preclinical, and clinical studies [48–50]. Se levels in hair and blood were inversely correlated with tumor volumes, which are consistent with the antiproliferative effects of Se [46].

While cell apoptosis is a critical mechanism mediating the anticancer activity of Se, the underlying molecular mechanisms still remain elusive [48]. The anticancer properties of Se might be due to the fact that it partially protects against oxidative stress [51]. The same group also assessed whether supplementation of BRCA1 mutation carriers with Se has a beneficial effect to oxidative stress/DNA damage since Se supplementation in patients may result in reduction of oxidative DNA damage. They found that BRCA1 deficiency contributes to 8-oxodG accumulation in cellular DNA, which in turn is a factor responsible for cancer development in women [51].

Se compounds modify gene expression. When breast epithelial cells (MCF-10A) were exposed to 100 nM sodium Se or high-Se serum, the expression of 560 genes including 60 associated with the cell cycle were affected. The group of Hawkes et al. [52] describes that selenoprotein W (SEPW1) was the only selenoprotein increased by both sodium selenite (specific) and high-Se serum (physiologic). SEPW1 small interfering RNA inhibited G1-phase progression and increased G1-phase gene transcripts while decreasing S-phase and G2/M phase gene transcripts, indicating that the cell cycle was interrupted at the G1/S transition. SEPW1 mRNA levels were maximal during G1 phase, dropped after the G1/S transition, and increased again after G2/M phase. SEPW1-underexpressing prostate cells had increased mRNA for BCL2, which can induce a G1 arrest and decreased mRNA for RBBP8 and KPNA2, which modulate the Rb/p53 checkpoint pathway. Altogether, these results suggest that SEPW1 and the G1/S transition are physiological targets of Se in breast and prostate epithelial cells [52].

Selenocysteine (SeC), a naturally occurring selenoamino acid, induces a caspase-independent apoptosis in MCF-7 breast carcinoma cells, accompanied by poly (ADP-ribose) polymerase (PARP) cleavage, caspase activation, DNA fragmentation, phosphatidylserine exposure, and nuclear condensation. Moreover, SeC induced a loss of the mitochondrial membrane potential (Delta Psi (m)) involving the expression and phosphorylation of Bcl-2 family members. Loss of Delta Psi (m) induced the mitochondrial release of cytochrome C and apoptosis-inducing factor followed by chromatin condensation and DNA fragmentation. MCF-7 cells exposed to SeC showed an increase in total p53 and phosphorylated p53 prior to mitochondrial dysfunction. Silencing and attenuating of p53 activation partially suppressed SeC-induced cell apoptosis. Furthermore, generation of reactive oxygen species and the induction of DNA strand breaks were found. Therefore, SeC could be a promising anticancer compound, which induces MCF-7 cell apoptosis by activating the ROS-mediated mitochondrial pathway and p53 phosphorylation [48].

Se might be beneficial in combination with other drugs in adjuvant therapy. Therefore, the combination of anticancer drugs with Se combinations was investigated. Li et al. [53, 54] investigated the therapeutic effect of methylselenocysteine (MSC) combined with tamoxifen in MCF-7 breast cancer xenograft. Indeed, treatment with tamoxifen together with MSC synergistically inhibited tumor growth compared to MSC alone and tamoxifen alone. MSC alone or MSC + tamoxifen significantly reduced ERα, PR and cyclin D1, Ki67 index, and microvessel density while increasing apoptosis in tumor tissues. These findings demonstrate a synergistic growth inhibition of ERα-positive breast cancer xenografts for a combination of tamoxifen with organic selenium compounds [53, 54].

The group of Li showed that combining doxorubicin with selenium resulted in an enhancement of apoptosis in MCF-7 human breast cancer cells [55, 56]. They found that mitochondrial activation of caspase-9 is in part responsible

for the synergy; while the death receptor pathway was involved in the activation of caspase-8. On the other hand, Se increased the expression of FADD, which is responsible for recruitment of caspase-8 to the Fas oligomer. Therefore, doxorubicin and selenium cooperatively activate Fas signaling by targeting key regulatory steps [56]. Se was capable of depressing doxorubicin-induced Akt phosphorylation, important in mediating the synergy between Se and doxorubicin. Se reduced the abundance of phospho-GSK3β induced by doxorubicin, whereas chemical inhibition of GSK3β activity muted the apoptotic response to the Se/doxorubicin combination. Se increased the transactivation activity of FOXO3A [55].

10. Conclusion

Metals and metal compounds interfere with breast cancer in multiple ways. On the one side, they are an important risk factor for the development of breast cancer, while on the other side their cytotoxicity might have also beneficial effects in inducing apoptosis and cytotoxicity in breast cancer cells. To highlight this delicate balance and to understand under which circumstances specifically cancer cells could be targeted by metals and their compounds, further research is needed.

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Review Article

Heavy Metal Poisoning and Cardiovascular Disease

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Cardiovascular disease (CVD) is an increasing world health problem. Traditional risk factors fail to account for all deaths from CVD. It is mainly the environmental, dietary and lifestyle behavioral factors that are the control keys in the progress of this disease. The potential association between chronic heavy metal exposure, like arsenic, lead, cadmium, mercury, and CVD has been less well defined. The mechanism through which heavy metals act to increase cardiovascular risk factors may act still remains unknown, although impaired antioxidants metabolism and oxidative stress may play a role. However, the exact mechanism of CVD induced by heavy metals deserves further investigation either through animal experiments or through molecular and cellular studies. Furthermore, large-scale prospective studies with follow up on general populations using appropriate biomarkers and cardiovascular endpoints might be recommended to identify the factors that predispose to heavy metals toxicity in CVD. In this review, we will give a brief summary of heavy metals homeostasis, followed by a description of the available evidence for their link with CVD and the proposed mechanisms of action by which their toxic effects might be explained. Finally, suspected interactions between genetic, nutritional and environmental factors are discussed.

1. Introduction

The potential association between chronic heavy metal exposure and cardiovascular disease (CVD) has a number of implications. Although the cardiovascular system is not typically viewed as a primary target of heavy metal toxicity, review articles covering their role as cardiovascular toxicant are scant, and the prime concern of most reviews has focused on the imbalance in the antioxidant protective mechanisms leading to oxidative stress in the cells as a major effect of their environmental exposure. Altered gene expression by environmental influence, particularly dietary components over gene regulation is expected to be responsible for heavy metal toxicity.

In this paper, we will give a brief summary of heavy metals homeostasis, followed by a description of the available evidence for their link with CVD and the proposed mechanisms of action by which their toxic effects might be explained. Finally, suspected interactions between genetic, nutritional, and environmental factors are discussed.

2. The Prevalence of CVD and Its Risk Factors

Despite recent significant advances in the treatment of CVD, it remains the number one cause of death in the developed world and accounts for almost one million fatalities each year in United States alone [1]. CVD also accounts for 82% of deaths in the developing countries [2]. The annual mortality rate of CVD is expected to reach 23.6 million deaths by 2030 [3]. The traditional risk factors for CVD do not account for all deaths [4]. Environmental, dietary, and lifestyle factors appear to be important, accounting for the dramatic recent changes in prevalence and would be of wide public health significance.

Confounding variables effects are being now evaluated as potential mediators (i.e., in the biological causal pathway), moderators (i.e., risk modifiers), direct causes, or otherwise parts of complex causal pathways [5]. These pathways can include connections between individual-level indicators (e.g., age, sex, race/ethnicity, socioeconomic status); behavioral risk factors (e.g., dietary habits); biological factors (e.g.,

genetics); social factors; heavy metals dose (i.e., both recent and cumulative); health conditions (e.g., diabetes, heart disease, and hypertension); other biological markers predictive of disease (e.g., homocysteine levels) that may be thought of as either outcomes by themselves or as intermediate pathological states that result in other conditions (e.g., renal dysfunction, cognitive declines).

The spectrum of risk factors for CVD ranges from purely genetic to behavioural and environmental factors in the broadest sense (Table 1). CVD is initiated by a coincidence of different risk factors. The latter two already show that behaviour and the environment (including the composition of nutrition) play an essential role in the majority of CVD. Patients differ in the time of onset, dynamics, and outcomes of CVD, indicating the complex pathophysiology of CVD. Different, genetically determined susceptibilities to environmental risk factors, interactions of the cardiovascular system with other organs like the immune system, and possible interactions between these risk factors within an individual are the likely causes of those differences. Despite an increasing understanding of genes, proteins, signalling pathways, cell-cell interactions, and systemic processes involved in CVD (initiation, progression, and outcome), the relevance of environmental factors is hardly investigated.

3. The Mechanisms of Atherogenesis

Atherogenesis is a multifactorial pathophysiological process of the arterial vasculature, which is characterized by progression from inflammation and smooth muscle cell proliferation to late stages that are marked by thrombotic and fibrotic obliterations of the vessels. Dysfunction of the endothelial cells leads to a series of events including inflammatory cell infiltration, platelet-thrombus formation, impaired nitric oxide (NO) homeostasis in the vessel and concomitant alteration of the cellular redox state [32]. Oxidized LDL particles are readily taken by macrophage scavenger receptors, leading to "foam cell" formation, that precedes atheroma development. Lipid aldehydes derived from LDL oxidation can also modulate expression of genes coding inflammatory mediators and adhesion molecules [33]. Reactive oxygen species can also function as signaling molecules that help to induce the activity of nuclear transcription factors such as nuclear factor Kappa B (NF-κB). The increased activity of these transcription factors is associated with upregulation of vascular adhesion molecules-1 (VCAM-1), cytokines such as interleukin-1beta (IL-1β) and tumor necrosis factor alpha (TNF-α) and chemokines including monocyte chemoattractant protein (MCP)-1 and interleukin (IL)-8 in the endothelium [34]. Many risk factors, including cigarette smoking, hypertension, diabetes mellitus, and hypercholesterolemia, can induce atherogenesis by modulation of inflammatory potential, oxidative stress, or NO perturbations in the endothelium.

There are several hypotheses to explain the initiation of CVD. Cumulative evidence from a large number of studies indicates that inflammation plays a pivotal role in atherosclerotic plaque formation [35]. Based on current knowledge, the hypothesis that best explains atherosclerosis pathophysiology is the "response to injury hypothesis" [34]. Lipid peroxidation is initiated by free radicals (e.g., superoxide anion, hydrogen peroxide, and lipid peroxide), which are produced in the body primarily as a result of aerobic metabolism [36, 37]. Transition metal ions, particularly divalent ions such as iron and copper, can further catalyze highly reactive free radicals formation in Fenton-type reactions [38]. LDL modification by oxidative damage is considered to be a key event in the development of atherosclerosis, and oxidized LDL particles are found in atherosclerotic lesions [33]. Although existing literature is limited, there are several mechanisms pointing to the atherogenic effects of heavy metals exposure by which they can promote lipid peroxidation and subsequent atherosclerosis.

4. The Toxic Effects of Heavy Metals

Heavy metals are commonly defined as those having a specific density of more than 5 g/cm^3 such as lead, mercury, aluminum, arsenic, cadmium, nickel. They are widely distributed in the earth's crust, but present at very low concentrations in the body. Their presence in the atmosphere, soil, and water, even in traces, can cause serious problems to all organisms. Their main impact on human health is principally through occupational exposure, environmental contamination, and accumulation in food, mainly in vegetables grown on contaminated soil. Arsenic and cadmium, in addition to mercury and lead, have been identified as the most probable causes of heavy metal-related disease observed in primary care medicine [39]. Exposure to one heavy metal contaminant is often accompanied by exposure to others. It is, therefore, expected that joint interactions may occur in populations exposed to mixtures of metals.

Heavy metals are toxic because they may have cumulative deleterious effects that can cause chronic degenerative changes [40], especially to the nervous system, liver, and kidneys, and, in some cases, they also have teratogenic and carcinogenic effects [41]. The mechanism of toxicity of some heavy metals still remains unknown, although enzymatic inhibition, impaired antioxidants metabolism, and oxidative stress may play a role. Heavy metals generate many of their adverse health effects through the formation of free radicals, resulting in DNA damage, lipid peroxidation, and depletion of protein sulfhydryls (e.g., glutathione) [42].

The importance of these metals as environmental health hazards is readily evident from the fact that they ranked in the top 10 on the current Agency for Toxic Substances and Disease Registry Priority List of Hazardous Substances [43]. This listing is based on the toxicity of the substance and the potential for exposure from air, water, or soil contamination. As a result of the extensive use of these metals and their compounds in industry and consumer products, these agents have been widely disseminated in the environment. Because metals are not biodegradable, they can persist in the environment and produce a variety of adverse effects. Maximum levels for heavy metals in food have been set in consideration for possible chemical contaminants.

Table 1: Classification of CVD risk factors.

Category	Examples	References
Nonmodifiable risk factors	(i) Advancing age (ii) Male gender (iii) Family history/genotype	(i) [6] (ii) [7] (iii) [8]
Metabolic risk factors	(i) Hypertension (ii) Diabetes mellitus/glucose intolerance (iii) Metabolic syndrome (iv) Hyperlipidemias (v) Obesity/overweight	(i) [9] (ii) [10] (iii) [11] (iv) [12] (v) [13]
Lifestyle risk factors	(i) Smoking (ii) Physical activity (iii) Diet	(i) [14] (ii) [15] (iii) [16, 17]
Novel risk factors	(i) Lipoprotein (a) (ii) Homocysteine (iii) Inflammatory markers (e.g., C-reactive protein) (iv) Prothrombotic factors (e.g., fibrinogen) (v) Trace elements (e.g., selenium, zinc, copper, chromium) (vi) Heavy metals (e.g., arsenic, lead, cadmium, mercury)	(i) [18] (ii) [19] (iii) [20–23] (iv) [24–27]

Although contaminated food may contain environmental toxins, they are also a very important source of nutrients, for example omega 3 fatty acids, which may prevent chronic diseases like CVD. Thus, an attempt has been made to allow people to obtain the beneficial health effects of natural food without excessive exposure to possible contaminants. Evaluations of heavy metals toxicity have been made by several international bodies, like the Center of Disease Control (CDC), World Health Organization (WHO), Occupational Safety and Health Administration (WHO-OSHA), International Programme on Chemical Safety (WHO-IPCS), Joint FAO/WHO Expert Committee on Food Additives (JECFA), and International Agency for Research on Cancer (IARC) (Table 2). Some of them have been classified as carcinogens of category 1 (cadmium). Currently, there are insufficient data to set a threshold value above which heavy metals would exert their negative effects. Defining this threshold might be fraught with difficulties since it might well be population-dependent due to differences in the population intake of dietary antioxidants or differences in their genetic-based defenses.

5. Potential Sources of Heavy Metals Contamination

The toxicity of heavy metals at high levels of exposure is well known, but a current concern is the possibility that continual exposure to relatively low levels of heavy metals may lead to chronic adverse health effects. Despite an overall decrease in human exposure to heavy metals in recent years, the potential for high intake of these contaminants still exists at many homes and in many occupational settings. Cosmetic products like lipsticks, eye makeup, Talcum powder, and skin lightening creams are potential sources of heavy metals exposure [44]. The presence of lead has been reported in traditional eye cosmetics such as Kohl and Surma [45]. Henna, a traditional plant product applied as temporary paint-on tattoos and hair dying, is reported to be very rich in heavy metals such as mercury and lead [46]. Other hidden sources may include ethnic folk remedies, toys, and certain imported candies and spices, [47–49]. Bottled Zamzam holy water, which is made available to pilgrims on sale, has been taken off the market recently for containing high levels of arsenic [50]. Tobacco plants have a special ability to absorb cadmium from soil and to accumulate it in the leaf [51]. Smoking of cigarettes and Shisha (hookah, narghile), a widely used smoking device in Saudi Arabia, is an important exposure route to cadmium [52].

6. The Metabolic Effects of Heavy Metals

The knowledge gained about the homeostasis of heavy metals has been substantial over more than a decade. Although they have no known metabolic function, when present in the body they disrupt normal cellular processes, leading to toxicity in a number of organs. They are relatively poorly absorbed into the body, but once absorbed are slowly excreted and accumulate in the body causing organ damage. Thus, their toxicity is in large part due to their accumulation in biological tissues, including food animals such as fish and cattle as well as humans. Distribution of heavy metals in the body relies on its binding to carrier molecules in the circulation. Metallothioneins are small proteins rich in cysteine residues, which accounts for the unique metal-binding properties of metallothioneins and play a major role in the dispersal and storage of heavy metals in the body. They also accumulate in hair and toenails (e.g., arsenic and mercury), which both can be used as indicators of long-term exposure in population studies. These heavy metals have a slow excretion rate from the body, as indicated by their long half-life time (e.g., half-life of lead is 27 year in cortical bone and 16 year in cancellous bone, half-life of cadmium is 10–30 years), compared with their uptake rate.

Table 2: Noncardiovascular harmful effects of heavy metals.

Heavy metal	Most affected organs	Chronic health effects	References
Arsenic	(i) Central nervous system (ii) Lungs (iii) Digestive tract (iv) Circulatory system (v) Kidneys	(i) Cancers (ii) Peripheral vascular disease, which in its extreme form leads to gangrenous changes (black foot disease, only reported in Taiwan) (iii) Skin lesions (melanosis, keratosis) (iv) Hearing loss (v) Reproductive toxicity (vi) Hematologic disorders (vii) Neurological diseases (viii) Developmental abnormalities and neurobehavioral disorders	[28]
Lead	(i) Central nervous system (ii) Erythropoiesis (iii) Kidneys (iv) Liver	(i) Cancers (ii) Kidney damage (iii) Neurological diseases (iv) Impaired intellectual ability and behavioral problems in children	[29]
Cadmium	(i) Kidneys (ii) Bone (iii) Liver (iv) Lungs	(i) Cancers (ii) Kidney damage (iii) Bronchiolitis, COPD, emphysema, fibrosis (iv) Skeletal damage, first reported from Japan, the itai-itai (ouch-ouch) disease (a combination of osteomalacia and osteoporosis)	[30]
Mercury	(i) Central nervous system (ii) Kidneys (iii) Liver (iv) Lungs	(i) Lung damage (ii) Kidney damage (iii) Neurological diseases (iv) Impaired intellectual ability and behavioral problems in children (v) Metallic mercury is an allergen, which may cause contact eczema (vi) Mercury from amalgam fillings may give rise to oral lichen	[31]

6.1. Arsenic. After ingesting inorganic arsenic compounds, the absorbed arsenic is metabolized primarily by the liver and excreted by the kidneys into the urine within a few days after exposure. Organic arsenic species in fish are also rapidly absorbed. In comparison to inorganic forms, organic compounds are much less extensively metabolized in the human body and more rapidly eliminated in urine with less than 5% was found to be eliminated in feces. In addition to gastrointestinal, dermal, or pulmonary uptake, exposure to organic arsenic species originates from methylation of inorganic arsenic inside the human body, which is regarded as a detoxification mechanism, since the methylated metabolites exert less acute toxicity and reactivity with tissue constituents than inorganic arsenic. The central site for arsenic methylation in the human body is the liver. These methylated metabolites can be eliminated in the bile. Factors such as dose, age, gender, and smoking contribute only minimally to the large interindividual variation in arsenic methylation observed in humans (reviewed by [53]).

6.2. Lead. The gastrointestinal absorption of lead is higher for children (30–50%) than for adults (5–10%). The absorbed lead is distributed to blood, soft tissue, and bone. In blood, red blood cells virtually bind all of the lead (98-99%), thus only 1-2% of blood lead are present in plasma. Gastrointestinal absorption and retention, the major pathway of lead intake, have been shown to vary widely depending on the chemical environment of the gastrointestinal lumen, age, and iron stores (nutritional status of the subject). Certain dietary components may act by increasing lead solubility, such as ascorbic acid, amino acids, vitamin D, protein, fat, and lactose, thus enhancing its absorption. Total body content of lead does not have a feedback mechanism which limits its absorption. Absorbed lead is mainly excreted in urine, whereas the feces contain predominantly unabsorbed lead. Being one of the calcium-like elements, lead follows the movement of calcium in the body to a large extent, and physiologic regulators of calcium metabolism usually affect the behavior of lead in a similar manner. Although bone has been considered a storage site for more than 90% of the total body burden, increased bone turnover in times of physiological (e.g., pregnancy or lactation) and pathological (e.g., osteoporosis) conditions release lead from bone. Lead can be remobilized from bone by competing with calcium for transport and for binding sites and is released, along with calcium, when bone is resorbed (reviewed by [54]). The mechanisms by which both elements enter and leave the bone are similar and through these mechanisms, bone lead equilibrates with blood lead [55].

6.3. Cadmium. The possible range of intestinal absorption rate for cadmium was established to be between 3 and 7% in humans and was used to assign an average 5% absorption rate in deriving a safe exposure level [56]. However, higher cadmium absorption rates (20–40%) were observed among young subjects and considered biliary excretion and reuptake via enterohepatic circulation to be the most likely possible reason. The duodenal iron transporter is upregulated by iron deficiency, which leads to an increased intestinal absorption of dietary cadmium. This is probably the main reason why the body burden of cadmium is generally higher among women [57] whose prevalence of iron depletion is higher than that of men. Once absorbed, cadmium binds avidly to metallothionein. Cadmium irreversibly accumulates in the human body, particularly in kidneys and liver. Because there is no efficient excretory mechanism for cadmium from the body and it is bound with high affinity to metallothionein within cells. Accumulation of cadmium mainly in liver and kidney and also in testes is due to the ability of these tissues to synthesize metallothionein, a cadmium-inducible protein that protects the cell by tightly binding the toxic cadmium ion. The kidney is regarded as critical organ for its accumulation and toxicity. Greater than one-third of body cadmium deposits are found in the kidney, especially in subjects with low environmental exposure. By far, the most toxicological property of cadmium is its exceptionally long half-life in the human body and thus its low excretion rate (reviewed by [30]).

6.4. Mercury. Dietary methylmercury is well absorbed from the gastrointestinal tract, readily enters the bloodstream, and is distributed to all tissues. About 5% of the body load is found in the blood compartment, and about 10% is found in the brain. 95% of the methylmercury in blood is bound to erythrocytes leaving 5% present in plasma. Less than 1% of the body burden of methylmercury is excreted per day, mainly via the feces. In the body, methylmercury is mainly, if not exclusively, bound to the sulfur atom of thiol ligands. Methylmercury is metabolized to inorganic mercury prior to elimination via feces, but the rate of conversion is slow (the half-life is about 70–80 days). In the liver and kidney, it is rapidly converted to inorganic mercury and stored as divalent mercury cation. This, together with the fact that the human body has no way of excreting mercury actively, means that mercury continues to accumulate in the body throughout life (reviewed by [31]).

7. Health Harmful Effects of Heavy Metals

The severity of adverse health effects is related to the chemical form of heavy metals and is also time and dose dependent. As mentioned earlier, heavy metals as environmental pollutants and promoters of oxidative stress are associated with a multitude of disadvantageous impacts on human health. There is a growing concern about the physiological and behavioral effects of environmental heavy metals in human population. Human intoxication has both acute and chronic effects on health and environment (Table 2). Albeit the toxicity of heavy metals at high levels of exposure is well known, a major concern of today is the possibility that continual exposure to relatively low levels of heavy metals may entail adverse health effects. Nevertheless, their contribution to CVD is still incompletely understood. Recent studies have shown that vascular effects of heavy metals may contribute to a variety of pathologic conditions including diabetes mellitus and hypertension [58, 59]. Mechanisms of action after heavy metal intoxication are less well studied and are still unclear.

7.1. Arsenic. Elemental arsenic is a metalloids found ubiquitously in nature. Humans are exposed to arsenic through medicinal, environmental, and occupational sources. Both organic and inorganic arsenic are present in various amounts in food-like marine fish. Organic forms are arsenobetaine, which account for 90% or more of the total arsenic in marine fish, and arsenocholine, in smaller amounts. However, inorganic forms of arsenic are much more toxic than the organic forms. Arsenic can exist in four valency states, trivalent (As^{III}) and pentavalent (As^{V}) arsenic are the major inorganic forms in natural water, whereas minor amounts of monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) can also be present. In the general population, the main exposure to inorganic arsenic is through ingestion of high-arsenic drinking water [60]. The safety level of arsenic in drinking water has been lowered from 50 to 10 ppb by the US Environmental Protection Agency [61].

Chronic arsenic intoxication seems to be an important public health problem in India, Bangladesh, Chile, Argentina, Hungary, Japan, and China [62]. Both environmental and occupational exposures to inorganic arsenic have been related to an increased cardiovascular mortality [63, 64]. Arsenic has been documented as the major risk factor of black foot disease, a unique peripheral vascular disease identified in endemic areas of arseniasis in Taiwan. However, other forms of peripheral vascular diseases have been shown to be caused by arsenic in other studies from several other countries.

Clinical studies have also reported other arsenic-induced cardiovascular effects including hypertension, diabetes mellitus, atherosclerosis, coronary heart disease, and stroke in a dose-dependent manner [65, 66]. Previous reviews of the role of arsenic in CVD were supportive of the possibility of an association, but the evidence was inadequate to establish a causal-effect relationship. A causal inference may be established if the data had a stronger effect in a susceptible subgroup of the population.

7.1.1. Epidemiological Evidence. The studies on arsenic-induced CVD were either occupational cohort studies or ecological correlation studies. Although epidemiological studies conducted in general populations strongly support long-term arsenic exposure as an independent risk factor for CVD, the studies of occupational populations are inconclusive [67]. Methodological problems might limit the causal interpretation of this relationship. Occupational studies may be subject to biases resulting from the healthy worker effect, which may underestimate the arsenic-related risk due to

the fact that the severely ill are ordinarily excluded from employment, and multiple exposure to various chemicals, and the correlation studies may have the problem of ecological fallacy. The limitation of nonoccupational studies includes the number of potential participants, accurate diagnosis of the cardiovascular endpoints, heterogeneity of exposure resources, limited exposure range which might be a challenge for epidemiological studies to have a valid long-term exposure measures, and interindividual variability to the cardiovascular effect of arsenic exposure.

Nonetheless the dose-response relationship and the biological plausibility for the association indicate that chronic arsenic poisoning is an independent risk factor for atherosclerosis [27]. Few epidemiologic studies in the US have reported the association of arsenic exposure with cardiovascular endpoints at low to moderate chronic levels in drinking water [68, 69]. Higher prevalence of ischemic heart diseases was found in subjects with cumulative arsenic exposure from drinking water, which was used as a marker of long-term exposure dosage, when compared to control subjects after multivariate adjustment [66]. Similarly, higher prevalence of hypertension was found among residents in endemic areas in Bangladesh of chronic arsenicism compared with those from nonendemic areas [70]. Inorganic arsenic exposure from drinking water, but not for the cumulative arsenic exposure, is also associated with an increased risk of developing type 2 diabetes mellitus [71]. Although hypertension and diabetes mellitus may partly explain the higher risk of CVD associated with arsenic exposure, the atherosclerotic effect of arsenic is independent because such an association persists even after controlling for the confounding effect of both factors. However, correcting for confounders in epidemiological studies is extremely challenging and is unlikely to completely account for their potential effects. An ecological study, conducted in the arseniasis-endemic areas of southwestern Taiwan, reported increased age-adjusted mortality from ischemic heart diseases compared with residents in nonendemic areas [64]. In Chile, acute myocardial infarction mortality increased following a period of high exposure to arsenic in drinking water and decreased after arsenic remediation had been implemented [72]. Likewise in southwestern Taiwan, mortality rates from ischemic heart disease were declining after the cessation of consumption of high arsenic well water [73]. However, results from studies conducted in endemic areas with chronic long-term arsenic exposure may limit the applicability to other populations, especially those with lower levels of arsenic exposure.

Except for a few studies using a prospective follow-up design, most are observational and cross-sectional. However, most of the existing epidemiological studies were conducted in populations with high levels of arsenic exposure, and little is known about the associations between chronic low level arsenic exposure via drinking water and CVD [74]. Furthermore, the heterogeneity of drinking water resources and the limited exposure range together pose a challenge for epidemiological studies to be conducted in other areas with low to moderate arsenic exposure levels that are relevant for most parts of the world and to have valid long-term arsenic exposure measures at the individual level.

7.1.2. Mechanism of Action. One of the suggested mechanisms by which arsenic exerts its toxic effect is through an impairment of cellular respiration by inhibition of several carbohydrates enzymes (i.e., gluconeogenesis and glycolysis pathways) and the uncoupling of oxidative phosphorylation [75]. This might explain the link between acute arsenic exposure and diabetic risk through its influence on the expression of gene transcription factors that are related to insulin pathways, such as, insulin upstream factor 1 (IUF-1) in pancreatic cells or peroxisome proliferative-activated receptor γ (PPARγ) in preadipocytes. Arsenic could also influence diabetes development by other mechanisms, including oxidative stress, inflammation, or apoptosis, nonspecific mechanisms that have been implicated in the pathogenesis of type 2 diabetes [76]. Future research should evaluate whether these mechanisms mediate the role of arsenic in diabetes development.

Other studies have suggested the involvement of oxidative stress in the pathogenic effects of arsenic exposure [77]. Available data suggest an important arsenic role based on a range of effects related to oxidative stress and vascular inflammation. Findings from mechanistic studies in animal/experimental studies suggest that arsenic causes inflammation in vascular tissues and activates oxidative signaling. The expression of chemokines and proinflammatory cytokines like monocyte MCP-1 and IL-6 has been induced in vitro by sodium arsenite in vascular lesions [78]. These observations are consistent with studies illustrating increased expression of circulating lymphocyte MCP-1 mRNA and plasma MCP-1 concentration in humans exposed to arsenic [79]. A more recent study also shows that occurrence of carotid atherosclerosis among subjects with genotypes of ApoE and MCP1 when exposed to high arsenic in drinking water [80].

Experimental studies have suggested that arsenic increases the production of reactive oxygen species [81]. Increased accumulation of arsenic in the vessel wall and increased atherosclerotic lesion formation were observed in the aorta of female ApoE-knockout mice given drinking water containing high concentrations of sodium arsenite without increasing plasma cholesterol. Characterization of these lesions illustrated increased macrophage accumulation and fibrosis in arsenic-exposed mice as compared to water-fed controls [82]. However, very little is known about the biochemical mechanisms by which low levels of arsenic exerts its proatherogenic effects.

Oxidative stress has been implicated in the pathophysiology of atherosclerosis [34]. The inflammatory process may be involved in the arsenic-induced atherosclerosis as shown by positive association between blood arsenic with plasma level of reactive oxidants (superoxide (O_2^-) and hydrogen peroxide (H_2O_2)) and by a negative association with antioxidant capacity [83]. Arsenic-induced oxidants, superoxide, and hydrogen peroxide have been implicated in in vitro studies [77]. Several cytokines and growth factors

involving inflammation were upregulated in persons with an increased arsenic exposure [79]. In individuals with arsenic-related skin lesions in Bangladesh, plasma levels of systemic inflammation and endothelial dysfunction markers (such as sICAM-1 and sVCAM-1) were positively associated with serum arsenic concentrations [84]. Markers of systemic inflammation and endothelial dysfunction were found to be predictive for CVD [85]. Thus, suggesting a possible mechanism through which long-term arsenic exposure may affect CVD development. Thus the biomarkers of early biological effects of ingested inorganic arsenic may include blood levels of reactive oxidants and antioxidant capacity, inflammatory molecules, as well as cytogenetic changes.

Oxidized lipids are present in all stages of atherogenesis, and they generate several bioactive molecules (e.g., peroxides and isoprostanes), of which aldehydes (Malondialdehyde and 4-hydroxy-trans-2-nonenal) are the major end products [86]. Increased plasma levels of free lipid aldehydes and increased accumulation of their protein adducts in atherosclerotic lesions were detected in experimental animals [87]. Since lipid aldehydes are highly reactive and can increase monocyte adhesion, cytokine production, and lipid uptake by scavenger receptors, it is conceivable that excessive generation of these aldehydes or decreased detoxification upon arsenic exposure exacerbates atherosclerotic lesion formation [88].

7.1.3. Combination of Gene-Environmental-Nutrient Interactions. Arteriosclerosis can occur following chronic arsenic poisoning irrespective of traditional coronary risk factors [89]. However, one would not expect every arsenic-exposed individual to develop CVD. This implies that other factors might affect the development and progression of arsenic-induced CVD. The epidemiological literature to date suggests that the cardiovascular effects of arsenic exposure are modified by nutritional factors, genetics, and arsenic metabolism capacity. These studies have clinical implications on the management and prevention of arsenic-induced CVD. It is known that both genetic and acquired susceptibility may modify the risk of arsenic-induced CVD [90].

Plausible mechanisms for the effect of arsenic on CVD include oxidative stress as previously explained, antioxidant enzymatic inhibition such as glutathione reductase, glutathione S-transferase, and glutathione peroxidase, and altered gene regulation, which might be implicated in the endogenous defense against arsenic's effect. Glutathione S transferases (GSTs) are a superfamily of enzymes that is important for the detoxification reactions in xenobiotic metabolism and plays a major role in cellular antioxidant defense mechanisms [91]. Glutathione has also been suggested to be a necessary component for arsenic metabolism probably in the initial reduction of arsenate to arsenite and in subsequent oxidative methylation. In a large study conducted in northeastern Taiwan with low-to-moderate exposure, the prevalence of carotid atherosclerosis was significantly associated with the genetic polymorphism of GST; P1 and p53 [92]. The induction of oxidative stress by arsenic may influence gene expression, inflammatory responses, and endothelial NO homeostasis [93], which play an important role in maintaining vascular tone [94]. A causal relationship was suspected in a study on human gene expression related to arsenic-associated atherosclerosis among residents of endemic areas in Taiwan. Significant differences in gene expression, encoding for several cytokines and growth factors involving inflammation such as IL-1β, IL-6, and matrix metalloproteinase 1, were found among groups with varying prolonged exposure levels to arsenic [79]. In a small study in residents of a high-exposed area in Taiwan, genes encoding for antioxidant enzymes, like NOS3, the gene for endothelial nitric oxide synthase; SOD2, the gene for manganese superoxide dismutase, and CYBA, the gene for p22 phox [a critical enzyme for superoxide production and an essential component of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX)], were found to be significantly associated with hypertension risk [95].

Several nutritional factors were investigated in relation to arsenic related cardiovascular effects. Selenium is a nutritionally trace element that has been known as an antagonist of arsenic toxicity [96]. Being also protective against oxidative stress, selenium supplementation was found to be effective in treating arsenism, an endemic chronic arsenic poisoning condition in China [97]. Consistent with this, some dietary deficiencies were found to interact with arsenic. For example, poor dietary selenium and zinc have been suggested as an underlying factor for arsenic toxicity in Taiwan and Bangladesh, well-known regions for their reduced selenium and zinc status worldwide [98].

Low serum carotene level has been suspected to increase the susceptibility to cardiovascular effects of arsenic exposure among residents of southwestern Taiwan villages with chronic arsenic exposure [99]. However, the potential mechanisms involved in the protective action remain to be studied. In the Health Effects of Arsenic Longitudinal Study (HEALS) in Bangladesh, the effect of low-level arsenic exposure on blood pressure was found to be highly correlated and was more pronounced in persons with lower intake of other micronutrients with known antioxidant action [100]. Arsenic exposure in the presence of inadequate intake levels of B vitamins and folic acid may affect blood pressure through its effect on the formation of S-adenosylhomocysteine and homocysteine. Historically, methylation of arsenic has been regarded as a detoxification pathway that takes place in the liver [101] and requires the conversion of S-adenosylmethionine to S-adenosylhomocysteine, which subsequently forms homocysteine, which requires sufficient levels of vitamin B2, B12, B6, and folic acid in body to be metabolized. Hyperhomocysteinemia, a novel cardiovascular risk factor [102], has been associated with high blood pressure [103]. Hence arsenic may contribute to the increase of homocysteine levels by consuming the S-adenosylmethionine pool and therefore enhance the subsequent cardiovascular risk.

Nevertheless, it is conceivable to presume that the findings from Taiwan and Bangladesh may not be generalizable to other populations due to several potential reasons like variations in the distribution of polymorphisms in genes involved in arsenic metabolism or response [104], differences

in arsenic species to which populations were exposed or other coexposures [105].

7.2. Lead. Lead exposure assessments have been based on its intake from food, water, or air. Possible routes for lead exposure are inhalation and swallowing. The four main sources of contamination of food are soil, industrial pollution, agricultural technology, and food processing. Worldwide, there are six sources that account for most cases of lead exposure: gasoline additives, food-can soldering, lead-based paints, ceramic glazes, drinking water pipe systems, and folk remedies [106]. Depending on the source, the concentration, and the bioavailability of lead determined by the physical and chemical form of lead, the relative contribution of each source may vary considerably. Although important measures have been implemented in a number of countries to decrease environmental lead exposure such as the use of unleaded gasoline, removal of lead from paint, solder of canned foods, and glazed ceramics used for storage and preparation of food, it is still a major environmental health problem in specific communities and targeted high-risk populations.

Even though safety standards of the WHO-OSHA for blood lead in workers have been established at 40 μg/dL, no safe level of lead exposure has yet been defined, as health risks associated with lead are found at ever lower doses. The CDC statement concerning lead poisoning in young children redefined elevated blood lead levels as that $\geq 10\,\mu$g/dL and recommended a new set of guidelines for treatment of lead levels $\geq 15\,\mu$g/dL [107]. However, it has been suggested that the criterion for elevated blood levels in children is too high in adults based on substantial evidence [108].

There is a great public health concern in the effects of environmental lead exposure on cardiovascular outcomes [109], especially the role of chronic low-level lead exposures in the pathogenesis of CVD [110]. Population-based studies on the cardiovascular effects of lead have focused largely on the association with hypertension, a leading risk factor for CVD morbidity and mortality [111]. The interrelationship between blood lead and blood pressure has been reviewed and reported to be statistically significant [112]. However, a major drawback of this meta-analysis was the inclusion of lead exposed subjects with occupational and non-occupational sources. In as much other cardiovascular events including, coronary heart disease, stroke, and peripheral arterial disease, were found to be associated with lead exposure, the exact role of lead in CVD is still incompletely understood [25].

7.2.1. Epidemiological Evidence. Lead intoxication has been shown to promote atherosclerosis in experimental animals [113]. Experimental findings in several species suggest that lead acts at multiple sites within the cardiovascular system [114]. Depending on the magnitude and the duration of lead exposure, cardiac and vascular complications are potentially life threatening. There are also indications that chronic lead exposure may affect systemic lipid metabolism [113]. Current evidence on lead-induced oxidative stress has been based mostly on in vitro experiments [115] or studies conducted in animals [116]. Chronic exposure has been also linked to atherosclerosis and increased cardiovascular mortality in man [117]. Several epidemiological studies among workers with high occupational exposure to lead have reported associations between lead exposure and oxidative stress markers [118]. Recent epidemiological studies have reported that low level lead exposure has a graded association with several disease outcomes such as hypertension and peripheral artery disease [119–121]. Although such diseases include components of oxidative stress, the relevance of oxidative stress to lead-related disease with low-level exposure has been criticized because mechanistic studies have been conducted at levels not typically observed in general population. The association between blood lead level and elevated blood pressure is still subject to controversy. However, lead has been postulated as causing hypertension by inducing an alpha adrenoceptor-mediated vasoconstriction [122]. Increased renin and angiotensin production, due to the nephrotoxicity of lead, could also be a factor in causing elevated blood pressure [123]. Lead-mediated impaired vasomotor tone, as a result of reduced NO bioavailability, may contribute to hypertension and hence atherosclerosis. Disturbances in calcium metabolism, particularly its role in modulating blood pressure through control of vascular tone, have been suspected as the likely mechanism of action. Moreover, cumulative evidence from clinical studies on the association between blood lead levels and CVD has yielded conflicting results [120, 124, 125]. Lack of consistency in findings could be due to differences among study cohorts in exposure/ toxicokinetic factors (e.g., dose, timing), in pattern of environmental characteristics (e.g., coexposures, comorbidity, developmental supports, assessment setting), in distribution of genetic characteristics that affect lead metabolism and racial background or the health worker effect [126, 127]. Methodological limitations are a great threat to validity of epidemiological studies, for example, misclassification of exposure and/or outcome may have occurred and resulted in further underestimation of the association of lead and cardiovascular end points.

Therefore, the results of these studies should be considered within the context of its possible limitations such as, the reliance on a single lead measurement, the use of different exposure measures, or residual confounding by sociodemographic determinants of lead exposure. The healthy worker effect may also lead to underestimate or invalidate the risk assessment of CVD.

7.2.2. Mechanism of Action. Acute lead exposure has been reported to affect cardiac function, and chronic lead exposure has been shown to affect the electrical and mechanical activity of the heart and to alter vascular smooth muscle function in experimental animals [128]. Many studies have focused on metal-induced toxicity and carcinogenicity, emphasizing their role in the generation of reactive oxygen and nitrogen species in biological systems. Metal-mediated formation of free radicals may enhance lipid peroxidation and changes in calcium and sulfhydryl homeostasis. By promoting reactive

oxygen species production, lead may trigger a cycle of oxidative stress and inflammation in the target tissues [129]. Depletion of cells' major sulfhydryl reserves seems to be an important indirect mechanism for oxidative stress that is induced by redox-inactive metals [130].

The precise mechanism explaining the hypertensive effect of lead exposure is unknown. However, an inverse association between estimated glomerular filtration rate and blood lead levels below 5 μg/dL has been observed in general population studies [108], indicating that lead-induced reductions in renal function could play a major role in hypertension. Other potential mechanisms include enhanced oxidative stress in the pathogenesis of lead-induced hypertension [131]. Although elevated blood pressure and impaired renal function are proposed mechanisms that mediate the effects of lead on clinical cardiovascular outcomes, other mechanisms are likely to be involved.

As has been mentioned earlier, CVD progression and outcomes rely to some degree on the presence of inflammation [34]. Increased expression and production of inflammatory markers in association with lead exposure have also been found in humans [132]. 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.

Various in vitro and in vivo studies have explored the underlying mechanisms by which chronic low level lead exposure can raise arterial pressure, thereby CVD development. These studies have identified the involvement of oxidative stress and inflammation [133], by promoting endothelial dysfunction [134], promoting vascular smooth muscle cells proliferation and transformation [135], and impairing NO homeostasis [136]. NO plays multiple physiological roles in vascular wall including endothelium-mediated vasodilatation, inhibition of platelet activation and smooth muscle cell migration and proliferation, and suppression of the proinflammatory mediators through NF-κB inactivation [32]. Diminished NO bioavailability may be caused by inhibition of the endothelial NO synthase (eNOS) expression, a lack of substrate or cofactors for eNOS, alterations of cellular signaling such that eNOS is not appropriately activated, and, finally, NO inactivation through interaction with reactive oxygen species (O_2^-). Furthermore, antioxidant therapy with vitamin E and ascorbic acid supplementation raised NO availability in rats with lead-induced hypertension compared with no effect upon blood pressure or tissue nitrotyrosine (a marker of NO oxidation) in control rats [137]. These findings support the notion that exposure to lead causes functional NO deficiency, in part by reactive oxygen species mediated NO inactivation. Given the critical role of NFκB in many aspects of atherogenesis, their activation by lead exposure may play a part in the development of hypertension [138].

Experimental studies have suggested plausible mechanisms whereby lead contributes to alterations of vascular resistance and hypertension by causing disturbances in calcium metabolism, particularly its role in modulating blood pressure through control of vascular tone [139]. It has been shown that lead can compete with calcium for the transport by channels and pumps involved in movements of ions across the cell membrane and between cytoplasm, endoplasmic reticulum, and mitochondria, thereby contributing to the changes in cytosolic calcium ions known to be involved in the regulation of vascular tone and vascular smooth muscle contraction [140]. In addition, lead may affect the calcium-mediated control of vascular smooth muscle contraction via serving as a substitute for calcium in calcium-dependent signaling pathways by interacting with calmodulin and calcium-dependent potassium channels [141]. Uncontrolled release of calcium ions from the mitochondria has been reported to occur during oxidative stress, a condition resulting from the imbalance between the production of free radicals and the counteraction by the cellular antioxidant defenses [142]. Lead may also increase pressor responsiveness to catecholamines, which may be a consequence of the lead effect on the intercellular messenger protein kinase C and its role in smooth muscle contraction [143].

7.2.3. Combination of Gene-Environmental-Nutrient Interactions. The exact mechanism by which lead induces oxidative stress is not fully understood. However, at least certain circumstances (i.e.) including genetic predisposition, nutritional influence, and environmental coexposure are expected to interact and therefore are linked in an attempt to explain such mechanism(s) of lead-induced toxicity.

Nutrition is an important susceptibility factor suggesting that people with poor nutrition are particularly susceptible [144]. Nutritional factors are often considered as important modifier of the metabolism and toxicity of lead [145]. This can be explained by lead-induced oxidative stress, an effect that is augmented by lead-induced inhibition of several of the antioxidant systems. This supposition was confirmed by studies which showed extensive accumulation of reactive oxygen species (as markers of NO oxidation) in kidney, brain, and cardiovascular tissues of untreated rats with lead-induced hypertension and its reversal by antioxidant therapy using high doses of vitamin E and vitamin C [137]. Essential elements, such as calcium, zinc, iron, selenium, and antioxidant vitamins have shown to counteract the toxic effects of lead [146]. The joint effect of high lead and low antioxidant micronutrients levels should be considered as a modifying factor in atherosclerosis, and their role in determining risk should be investigated. These nutritional facts suggest a novel approach to strategies for treating environmental lead toxicity with micronutrients supplementations.

Certain genetic polymorphisms can lead to differences in the level of susceptibility to adverse effects of lead environmental exposure. A better understanding of the genetic factors that influence susceptibility to lead-induced intoxication could have significant importance for public health and intervention initiatives [147]. It is therefore reasonable to conjecture that genetic disposition could lead to differences in susceptibility to lead poisoning among the human population. Researchers have identified a small number of genes that induce susceptibility to environmental toxicants, and much interest has developed in that area. Three polymorphic genes have been identified that can influence the bioaccumulation and toxicokinetics of lead in humans, the

6-aminolevulinic acid dehydratase (ALAD) gene, the vitamin D receptor (VDR) gene, and the hemochromatosis (HFE) gene.

One of the most important mechanisms of lead toxicity is its inhibitory effect on the heme biosynthetic pathway enzymes; ALAD and ferrochelatase. Polymorphisms of the ALAD gene have been associated with the accumulation and distribution of lead in the blood, bone, and internal organs. Lead binds the enzyme's sulfhydryl group, which normally binds zinc, preventing the binding of aminolevulinic acid (ALA), the normal substrate. ALAD activity has been used as a sensitive marker for the detection of lead intoxication [148]. Concomitantly, ALA accumulates in blood and urine and may contribute to lead-induced toxicity to the brain. Its urinary excretory level has been used as a biomarker for early lead exposure [149].

$1\alpha 25(0H)_2D_3$ (calcitriol), the circulatory form of vitamin D in blood, is involved in calcium absorption. It binds to VDRs in gut, kidneys, and bone. The VDR gene has been implicated in the control of calcitriol levels in serum, which normally regulates calcium absorption and can in turn affect lead levels. The high-affinity VDR appears to activate genes that encode calcium-binding proteins such as calbindin-D, which is involved in intestinal calcium transport. Because of their similar biochemical nature as divalent cations, calcium and lead often affects the normal function of calcium-dependent systems [150]. These data suggest that calcium and lead are cotransported through the gut into the blood, and from there the two metals may be codistributed to calcium-rich tissues such as the bone [151]. In addition, lead toxicity may impair calcitriol hormonal synthesis in the kidney, therefore interfering with calcium absorption [152]. Together, these data show that the interactions between lead, calcium, and calcitriol are complex and induce modifications of mineral and vitamin levels.

Hemochromatosis is the genetic form of iron load in which patients lack a functional HFE protein involved in iron homeostasis due to mutations in the HFE gene that may also influence lead absorption [153]. At least two mechanisms for the increased absorption of lead in hemochromatosis gene carriers have been postulated. HFE protein binds to the transferrin receptor, reducing its ability to bind to transferrin and thus decreasing the absorption of iron in the gut [154]. Consistent with us, an important association has been made between iron deficiency and increased lead absorption and hence toxicity [144]. HFE protein may also influence the expression of other metal transporters such as divalent metal transporter in the gut that modify the absorption of other metals in addition to iron [155]. This is compelling data that iron status influences lead toxicity.

Therefore, differences in the expression rate of the polymorphic genes in response to nutritional influence over their activities are highly suggestive but not conclusive in considering environmental link with both genetic and dietary elements.

7.3. Cadmium. Cadmium is a widespread toxic metal contaminating many areas, either naturally or because of industrial use as in regions of Belgium, Sweden, UK, Japan, and China. Modes of exposure are either through intake of contaminated food (e.g., leafy vegetables, grains, organ meats, and crustaceans), drinking water, or by inhalation of polluted air or occupational in industries. Cadmium presence in tobacco smoke further contributes to human exposure as the tobacco leaves accumulate cadmium in a manner similar to certain food from plants. Smokers have approximately twice the cadmium body burden of nonsmokers [156].

Regardless of the route of exposure, cadmium is efficiently retained in the organism and remains accumulated throughout life. In addition to its cumulative properties, cadmium is also a highly toxic metal that can disrupt a number of biological systems, usually at doses that are much lower than most toxic metals. A European risk assessment report proposed that cadmium deleterious effects may occur at levels as low as 0.5 μg/g creatinine based on data from the most recent European studies [157].

A threshold value for safe dietary cadmium exposure level has been set to be below 2.5 μg/kg body weight per week [158]. Furthermore, it was noticed that subgroups of the population, such as vegetarians, women in reproductive phase of life, smokers, and people living in highly contaminated areas may exceed the tolerably weekly intake by about 2-fold.

Chronic cadmium exposure is associated with hypertension and diabetes [24, 159]. However, the exact influence of cadmium on the cardiovascular system remains controversial. More importantly, these data show that cadmium may exert effects on the cardiovascular system at extremely low exposure levels. In vitro studies data revealed that low-dose cadmium levels (well below toxic concentrations) may contribute to the initiation of pathophysiological changes in the vessel wall [160].

Several important reviews have outlined the cardiovascular effects of cadmium in man. Evidence from prospective studies reveals potential causal relationships of blood cadmium and blood pressure but not the relationship between urinary cadmium and hypertension [161]. An inverse relationship between urinary cadmium levels and blood pressure was reported in another meta-analysis [24]. These paradoxical relationships were evident in both high- and low-exposure populations and thus contradict earlier assumptions that this inverse association only reflected higher cadmium exposures. A limitation common to all these studies and thus to this meta-analysis is that the outcome was not consistently defined across studies; therefore, lack of association might reflect outcome misclassification.

7.3.1. Epidemiological Evidence. The cardiovascular effects of cadmium have been observed in in vitro studies and in experimental animal models [162, 163]. Increased cardiovascular mortality was documented for men living in areas with increased potential for cadmium exposure, thus suggesting that cadmium is at least a comorbidity factor if not a causative factor [57].

Epidemiologic studies of the association of environmental cadmium exposure with blood pressure end points are

inconsistent. Discrepancies across epidemiological studies might be due to that some studies have strengths including prospective designs [164], blood pressure values entry as a continuous variable to avoid outcome misclassification bias [165], while other studies, however, have been limited by small sample sizes, lack of adjustment for potential confounders, and lack of standardization of blood pressure measurements [161]. Sample selection considerations and exposure measurement error are additional limitations in these studies [166].

Cadmium exposure also potentiates some diabetic complications related to renal tubular and glomerular function. Epidemiological evidence shows higher susceptibility for persons with diabetes to develop cadmium induced renal dysfunction [167]. A study examining the data from National Health and Nutrition Examination Surveys (NHANES) reported a significant association between high urinary cadmium levels and high fasting blood glucose levels in a dose dependent manner, as well as more susceptibility among the diabetic subjects for the cadmium-induced renal effects [168]. Thus, suggesting that cadmium may be a cause of prediabetes and diabetes mellitus in humans. On the contrary, less agreement exists about the clinical significance and predictivity of the urinary cadmium level as a surrogate marker of body content and the tubular biomarkers of renal dysfunction.

NHANES data reported that peripheral arterial disease might be associated with blood and urinary cadmium, thus suggesting that cadmium is involved in arterial dysfunction [58]. Different cadmium biomarkers may provide different information regarding the timing and source of exposure. However, the use of these biomarkers has been inconsistent across epidemiological studies. In general, urinary cadmium level reflects the body burden over long-term exposure among people with lower, nonoccupational exposures, and blood cadmium, with a half-life of 3-4 months, is considered an indicator of recent exposure [56]. Alternatively, urine and blood cadmium are sometimes considered biomarkers of ongoing and long-term cadmium exposure, respectively [156].

Cadmium may exert its adverse cardiovascular effects by promoting atherosclerosis and by inducing disadvantageous cardiac functional and metabolic changes [169]. Recently blood cadmium level was independently associated with myocardial infarction [170] and early atherosclerotic vessel wall thickening as estimated by intimamedia thickness ratio [171]. In contrast no correlation was observed between blood cadmium and measures of arterial function [172]. Epidemiological studies did not firmly establish a link between cadmium and CVD due to confounding effects, for example, coexposure to other heavy metals, unadjusting for smoking habits. Moreover, disagreement between exposure studies might be attributed to the use of different exposure measures with different pathophysiological significance of blood and urinary cadmium.

7.3.2. Mechanism of Action. It has been long hypothesized that cadmium may contribute to the pathogenesis of CVD via a number of proposed mechanisms, such as partial agonism for calcium channels, direct vasoconstrictor action, and inhibition of vasodilator substances such as NO [173]. The exact mechanism whereby cadmium affects the cardiovascular system is not known, although experimental studies have suggested several plausible possibilities [174]. Because cadmium levels used in experimental models are much higher than exposure in the general population, the relevance of these mechanisms to the pathogenesis of CVD is uncertain. A primary mechanism for cadmium toxicity is its effect on cells which has been ascribed to the oxidative stress promoting cadmium action, as observed in vivo [162], and most importantly, the depletion of glutathione and alteration of sulfhydryl homeostasis [42], thus indirectly increasing oxidative stress and lipid peroxidation [175]. However, results from other studies were inconclusive in supporting a direct effect of cadmium [171]. It has been argued that reasons are the concentration of cadmium applied, as well as the upregulation of antioxidant defense in endothelial cells in response to cadmium may define the presence of reactive oxygen species in endothelial cells [176].

Cadmium is absorbed mainly through the respiratory and digestive tracts and under conditions of chronic exposure; cadmium is transported in blood bounded mainly to metallothionein. Metallothioneins are heavy metal-binding proteins that can protect against heavy metal toxicity and oxidative stress. The vascular wall has been shown to be a target organ of cadmium deposition [177]. However, other important issues are not yet fully understood, like the form of cadmium which is taken up by cells (i.e., free ion or protein bound), their expected amounts in circulation, and the precise uptake route of cadmium by the cells. Albeit, several ion channels and transporters have been described to transport cadmium across the plasma membrane, for example, calcium-channels [178], plasma membrane-associated DMT-1 [179]; it is unclear whether these mechanisms are also active in endothelial cells.

Apart from direct uptake of cadmium by endocytosis into cells of the vessel wall, cadmium may also be taken up by cells of the immune system and may enter the vessel wall via infiltration of the vessel wall, for example, by cadmium-laden monocytes [180]. Given the fact that the critical role of monocytes/macrophages transdifferentiate into foam cells and necrotic foam cell death in many aspects of endothelial dysfunction, their excessive production by cadmium plays a major part in the initiation and promotion of atherosclerosis. Cadmium uptake could also occur via disruption of endothelial integrity and subsequent cadmium-mediated endothelial cells death. Formation of gaps between endothelial cells usually follows, allowing for cadmium diffusion from the blood stream into the medial layer [59]. Vascular wall cells seem to allow for a sufficient transport of cadmium across the endothelium and are capable of retaining high amounts of cadmium mainly in the smooth muscle cells [177]. Effects on smooth muscle cells include an interaction with ion homeostasis and Ca^{2+} flux, cytotoxic effects, but also the stimulation of smooth muscle cell proliferation at low cadmium concentrations [181], thus, allowing for subsequent lipid accumulation in the vessel wall and a modification

of lipid profiles towards a more atherogenic state [34]. Ample of evidence pinpoints the induction of endothelial cell death by cadmium which is thought to be fundamental in the atherosclerosis-promoting properties of cadmium [59]. However, data on the mode of cell death are contradictory. Cadmium-induced endothelial necrosis would, in addition to damaging the integrity of the vascular endothelium, also contribute to vascular inflammation.

7.3.3. Combination of Gene-Environmental-Nutrient Interactions. There is evidence for nutritional influence over the rate of intestinal absorption of cadmium (i.e.) increased cadmium if the nutritional intake of calcium, iron, or zinc is low [182]. Moreover, cadmium exposure interferes with the homeostasis of other metals, and, reciprocally, cadmium effects depend on the body status for some essential metals [183]. Cadmium is acquired by transport mechanisms developed for essential metals, most likely to be one of the following divalent cations: zinc (Zn^{2+}), iron (Fe^{2+}), manganese (Mn^{2+}), and calcium (Ca^{2+}). It follows that the mechanisms of cadmium toxicity must be considered with respect to the systems regulating different aspects of these metals turnover in the body. Considering that cadmium substitutions at the metal sites of metalloproteins were performed in vitro only and the scarcity of data demonstrating such occurrences in vivo, care should be taken before interpreting cadmium toxicity data with a simple molecular explanation. Yet, cadmium replacement of other metals in cellular proteins does occur as in metallothionein [184].

Furthermore, metallothionein may, apart from binding and thereby inactivating the major portion of cadmium ions, also serve as a source for constant levels of intracellular free cadmium ions [59]. On the other hand, recent epidemiological studies are indicating the protective effect of the antioxidant property of zinc against cadmium toxicity probably by metallothionein stimulation [171]. To date, mechanisms of cadmium-zinc interaction and their impact on the oxidative status derived from in vitro studies and limited number of human studies [185]. The wide variety of different doses, dose ratios, element administration modes, and exposure lengths of cadmium and zinc often yielded contradictory results.

Cadmium intoxication results in an induction of metallothionein gene transcription and an increase in metallothionein production [186]. Cadmium also affects several genes involved in the stress response to pollutants or toxic agents, as in heat shock proteins that are highly implicated in cardiovascular pathophysiology [187]. Many genes involved in cell cycle regulation are overexpressed after exposure to cadmium, and many proteins are upregulated; for example cadmium stimulates the expression of ICAM-1 [188].

Cadmium affects cell cycle progression, proliferation, differentiation, DNA replication, and repair as well as apoptotic pathways [147, 158]. In addition to its role as a generator of reactive oxygen species, involved in the occurrence of DNA damage, cadmium may also reduce cellular antioxidants levels [189]. The reduction of activities of several antioxidant proteins (catalase, glutathione reductase, total glutathione), mediated by cadmium, may cause the accumulation of reactive oxygen species in cells [190]. Indirectly, this overproduction of oxidant molecules may be also responsible for the generation of abnormal or misfolded proteins and lipid peroxidation [191].

7.4. Mercury. Mercury is an environmental pollutant that presents at low levels in water systems (lakes, rivers, oceans, etc.) but bioconcentrate in the aquatic food chain, as in some fish species (particularly fatty fish) that can also contain other environmental contaminants such as polychlorinated biphenyls, dioxins. The global cycle of mercury begins with the evaporation of mercury vapor into the atmosphere. More concern about the release of volatile mercury that will become part of the local mercury cycle and repollute the environment again, into the ambient air. Mercury exists in three forms: elemental or metallic mercury, inorganic mercury compounds, and organic mercury. It is used in glass thermometers as elemental mercury and in dental amalgam fillings as inorganic mercury compounds. Organic mercury is found mainly in fish as methylmercury and in some vaccines as ethylmercury (thimerosal).

The US Environmental Protection Agency has reduced the recommended safe daily intakes of methylmercury from 0.5 to 0.1 μg/kg body weight [192]. In the absence of advisories for local waters which are available, US Environmental Protection Agency and US Food and Drug Administration have also issued recommendations on fish consumption among women of childbearing age and young children based on methylmercury content; commonly eaten fish and shellfish that have lower levels of mercury should be limited to two meals per week.

In recent years, more attention has been given to other health effects of methylmercury exposure, following the epidemiological findings from Finland, confirming that high mercury content in hair was associated with an increased progression of atherosclerosis and risk of CVD [193]. It is noteworthy that these adverse effects on CVD have been observed at methylmercury levels much lower than those associated with neurotoxicity.

7.4.1. Epidemiological Evidence. Mercury exposure has been shown to promote atherosclerosis both in vivo and in vitro [194, 195]. The potential harmfulness of mercury in CVD was first observed in the Kuopio Ischemic Heart Disease Risk Factor (KIHD) study cohort [26]. Several follow-up studies on KIHD study cohort confirmed their observations [196, 197]. In agreement with KIHD study results, it has been suggested in the European Multicenter Case-Control Study on Antioxidants, Myocardial Infarction, and Cancer of the Breast (EURAMIC) study, that high mercury content may diminish the beneficial effects of fish consumption on cardiovascular health [198]. Likewise, in the Health Professionals Follow-up Study (HPFS), increased cardiovascular risk following mercury exposure among dentists, who have an occupational exposure to mercury vapor via amalgam, was consistent with the results from the KIHD

and EURAMIC studies [199]. However, these findings were not supported by prospective studies conducted in Sweden [200]. Unlike the previous studies, this population included women and had too low mercury levels, and the range of mercury may have been too narrow to exert sufficient statistical power to detect an association. Apparent discrepancies might be attributed to methodological limitations which are very common in epidemiological studies. In many studies, there are uncertainties in exposure quantification, outcome ascertainment (e.g., misclassification bias), and a lack of information about exposure to other metals and traditional risk factors that are of confounding effects.

Current uncertainties in the role of mercury in the development of hypertension and diabetes mellitus could be attributed to limited available data [201, 202], and other shortcomings like differences in study design or exposure assessment, lack of sensitive biomarker, and lack of standard criteria for hypertension and diabetes assessment.

7.4.2. Mechanism of Action. Mercury-induced oxidative damage has been observed both in vivo and in vitro, including myocardial tissues. The mechanisms by which mercury exerts its cardiovascular effects are not fully understood. However, exposure to mercury can lead to oxidative stress induction [203], sulfhydryl groups depletion [204], altered mitochondrial function, and apoptosis [205].

Mercury-induced redox imbalance may be caused by either increased reactive oxygen species generation or by reduced antioxidants defense capacity. This is supported by observations that antioxidants, both enzymatic and nonenzymatic, can protect against methylmercury toxicity [194]. However, most information is currently derived from animal experimental models and thus implications for human populations consuming mixed diets can only be speculative at this time.

Mercury can bind to and thus forming complexes with thiol-containing compounds targeting proteins such as glutathione [206], which plays a critical role in regenerating vitamins C and E from their oxidized byproducts. In addition, glutathione-mercury complexes appear to be the primary form in which mercury is transported and eliminated from the body, further decreasing cellular defenses against oxidation. Furthermore, its high affinity for thiol groups and its ability to bind selenium to form an insoluble complex could reduce antioxidative defenses and promote free radical stress and lipid peroxidation in the human body [26]. This interaction between mercury and selenium may represent one mechanism through which mercury increases the risk of CVD, for instance by reducing the bioavailability of selenium or by impairing the activity of glutathione peroxidase. On the contrary, reciprocal interactions are expected, that is, high selenium levels could protect against excess mercury. However, at present, there is very little evidence from human studies to support the hypothesis.

It has been demonstrated that mercury alters the structural integrity of the mitochondrial inner membrane, resulting in loss of normal cation selectivity [194].

Other possible mechanism by which mercury can promote lipid peroxidation and subsequent atherosclerosis is by inhibiting the activation of NF-κB. Mercury may bind to the sulfhydryl groups present in NF-κB and thus impair the activation of NF-κB and attenuate its effects on gene expression [207]. Mercury has been also shown to suppress NO production in in vitro studies by inhibiting the NF-κB pathway and, in that way, inactivating the expression of inducible nitric oxide synthase (iNOS) gene [208]. iNOS catalyzes the production of NO, which has an important role in the maintenance of vascular regulation and immune system [94]. There is some evidence from in vitro studies that mercury can induce changes in platelet aggregation by binding to the thiol groups present in the platelet membrane [209]. However, the exact role of mercury in CVD-related endothelial, inflammatory, and immune functions warrants further investigation.

7.4.3. Combination of Gene-Environmental-Nutrient Interactions. The cardiovascular effect of mercury at lower exposure levels is still subject to controversy. As in the limited understanding of the mechanisms of mercury toxicity [210], nutritional consideration may often be concurrent with or may be additive to genetic predisposition to mercury exposure [211]. However, more focus was attributed to mercury retention by various organs in efforts to explain nutrient mercury interactions [212].

Even though there is ample evidence on food interaction with mercury metabolism at the physiologic level, less certain are the effects of nutrients that might influence bioavailability, toxicodynamics, and transport to target organs and influence the immunologic, biochemical, or cytologic functional responses to mercury.

Food-like fish has been implicated in the alteration of mercury metabolism. In terms of macronutrient intakes such as fat intake, a positive correlation between dietary mercury and low-density lipoprotein cholesterol levels was observed [213]. Unsaturated fatty acids were also correlated with mercury exposure in populations frequently consuming seafood and fish [198]. However, evidence for protective or antagonistic effects is often complex and highly dependent on metabolic conditions. Studies on the effects of macronutrients on mercury metabolism are expected to shed some light on possible interactions between different nutrients as they have been shown to modulate toxicokinetics and dynamics of mercury metabolism [214].

Micronutrients may modify mercury toxicity due to their antioxidant properties. Certain phytochemicals found in the diet reportedly protect against methylmercury toxicity [215]. Such a role is subject to controversy as some antioxidants were found to enhance mercury toxicity in vitro [216]. It is still unknown if these effects are related to antioxidant/prooxidant activity or other aspects of mercury metabolism. Of all trace elements, selenium, because of protective effects observed in animal studies, has received the most attention as a potential protector against methylmercury toxicity in populations consuming seafood [217]. Mercury has a high affinity for selenium, and it readily binds selenium to form

insoluble mercury selenide complexes [218]. Through this interaction, mercury could reduce the bioavailability of selenium and impair the activity of glutathione peroxidase, thus promoting lipid peroxidation and, subsequently, atherosclerosis. Nonetheless, the combination of high mercury and low selenium was not associated with higher CVD risk [199]. These observations could have been due to limited number of subjects in stratified analysis.

Environmentally induced changes in gene regulatory mechanisms along with dietary interactions may exacerbate mercury intoxication. Metallothionein protein, rich in sulfhydryl groups, helps in scavenging and reducing the toxic effects of mercury. Metallothionein induction is not only seen with mercury but various other metals like cadmium, zinc, and copper [219]. Toxic effects of mercury also induce a number of stress proteins which include heat shock proteins and glucose-regulated proteins that have also been implicated in cardiovascular pathophysiology [220].

8. Conclusions

Detrimental effects of heavy metals on the cardiovascular system have been less well defined. A potential proatherogenic effect even if modest compared to other traditional risk factors would have a significant impact in sensitive population groups. However, some issues need to be taken into consideration before one can draw any definitive conclusions. For example, adjustment for confounding variables has been performed in some studies; however, it does not ensure the independence of their association, as it is not possible to measure every conceivable variable.

Studies summarized in this review point to the harmful effects of heavy metals exposure on the development of CVD. Heavy metals are suspected of inducing pathophysiological changes relevant to atherogenic events including increased oxidative stress, inflammatory response, and coagulation activity. In addition, there are several suggested biological mechanisms that support this hypothesis. The combination of a susceptible genetic background and dietary elements along with environmental coexposure to heavy metals may also explain some aspects of their cardiovascular effects.

However, the exact mechanism of CVD induced by heavy metals deserves further investigation either through animal experiments or through molecular and cellular studies. Such study designs are optimal to define the cellular and subcellular mechanisms through which they affect the cardiovascular system. Basic insights from the science of cell and molecular biology coupled with improved analytical capabilities have led to the development of better biomarkers embracing the genetic field. There is also a pressing need for the use of sensitive biomarkers in early detection of low-level exposures from new technologies such as nanotechnology. The genetic mechanisms investigated in these studies may also offer new avenues for risk assessment research. Regarding experimental animal models, doses and exposure should be adjusted to long-term low exposure levels that are usually found in human population. Findings based on these studies can lead to the identification of a coherent and consistent biological research pathway for biomarker validation and acceptance into public health practice. Furthermore, large-scale prospective studies with followup on general populations using appropriate biomarkers and cardiovascular endpoints might be recommended to identify the factors that predispose to heavy metals toxicity in CVD.

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Review Article

Metals and Disease: A Global Primary Health Care Perspective

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Metals are an important and essential part of our daily lives. Their ubiquitous presence and use has not been without significant consequences. Both industrial and nonindustrial exposures to metals are characterized by a variety of acute and chronic ailments. Underreporting of illnesses related to occupational and environmental exposures to chemicals including metals is of concern and presents a serious challenge. Many primary care workers rarely consider occupational and environmental exposures to chemicals in their clinical evaluation. Their knowledge and training in the evaluation of health problems related to such exposures is inadequate. This paper presents documented research findings from various studies that have examined the relationship between metal exposures and their adverse health effects both in developing and developed countries. Further, it provides some guidance on essential elements of a basic occupational and environmental evaluation to health care workers in primary care situations.

1. Introduction

Metals are ubiquitous. They are an important and essential part of our daily lives. Their benefits are many. They have contributed immensely to rapid advances in health care, information technology, telecommunications, construction, and other sectors of industry. Additionally, metals such as iron, copper, zinc, and molybdenum are essential for innumerable biological processes and enzymatic reactions that occur in the human body. But their presence in our environment presents health risks and hazards. They have the potential to cause acute toxicity. Additionally, through their insidious mode of action metals are notorious for promoting many chronic conditions including carcinogenesis. They present serious challenges. The public health professionals, safety staff, and policy makers who develop policies and implement safety programs to protect the health of workers and other members of society must become conversant with the hazards that metals present and develop programs aimed at preventing and controlling human exposure. Additionally, it is crucial that primary and other basic and frontline health workers possess adequate knowledge and skills to evaluate and manage the exposures and counsel patients.

Here is some basic information about metals. All chemical matter consists of pure chemical substances called elements. There are three types of elements: metals, nonmetals, and metalloids. Metals, which account for about two-thirds of all elements, are good conductors of both electricity and heat. Examples of metals include iron, copper, mercury, and zinc. Unlike metals, nonmetals are poor conductors of electricity and heat. Examples of nonmetals are hydrogen, carbon, and halogens. Metalloids, also called semimetals, have intermediate properties. They are semiconductors and are vital in computers and industry, generally. Arsenic, antimony, and bismuth are classical examples of nonmetals. Alloy is a mixture of two or more elements of which metal is a primary element. Steel, of which iron is a primary constituent, is an alloy. Trace metals are those that make up less than one gram of the human body. Copper and zinc are examples of trace metals. All metals can exert toxic effects. Dose, route of exposure, and duration of exposure determine whether a metal can exert its toxic effect or not.

Metals are also classified according to their atomic weights: heavy and light metals. Heavy metals, such as cadmium and mercury, have higher atomic weights. Many of them are toxic. But there are other heavy metals such as molybdenum, which are essential for normal human physiology. So, it is not true that all heavy metals are toxic. Light metals can also be toxic. Beryllium is a case in point.

In general, exposures to metals occur in two ways: one, via their presence in the environment (air, food, water, and soil) and two, by undergoing transformation in their structure. In such transformations metals can exhibit a higher level of toxicity. Examples of transformations include mercury changing to more toxic methyl-mercury and the increasing concentration of metals moving up the food chain on account of their binding capacity to sulfhydral groups present in proteins [1]. The exposure to metals can occur at work, home, or in the community environment. Occupational exposure to metals is of serious concern and is discussed later in the paper.

Why should we be so concerned about metals and metalloids? There are several reasons for the concern: recent increase in production of chemicals (including metals), serious health risk associated with metal exposures, and underreporting of medical problems related to occupational and environmental exposures to chemicals, to name a few [2–4].

In this paper, we discuss the growing worldwide concern surrounding exposure to chemicals and metals. We report on exposure incidents and findings of some studies that have examined the relationship between metal exposures and their adverse health affects both in developing and developed countries. Special situations such as exposures among children and in home environments are also discussed. Relevant toxicological aspects of hazardous metals such as arsenic, lead, and mercury and their effects on human health are summarized. Finally, we provide some guidance on essential elements of a basic occupational and environmental evaluation in primary care situations. Our review is aimed at basic and primary health care workers who provide care to individuals exposed to chemicals but are unfamiliar with basic clinical aspects of occupational and environmental health evaluations and toxicology [5]. Due to the diverse nature of the subject, we are unable to discuss every clinical aspect of toxicology of metals and their impact on health. However, our discussion sensitizes health care workers to essential basic information and initial steps they can take to integrate chemical exposure assessments in their day-to-day clinical work.

2. Growing Concern over Exposure to Chemicals and Metals

The production of chemicals (including metals and their variants) around the world has increased dramatically in recent years. It has been reported that there has been a 10-fold increase in the global output of chemicals worldwide [2]. This trend is likely to escalate in years ahead. Many experts believe that with recent advances in metal technology and increased contamination of the environment due to energy production, the potential for exposure to metal toxicity has increased in recent years. What is even more worrisome is the production of chemicals in many developing countries where public health laws are either weak or insufficient to protect the health of their workers and residents.

Chemicals including metals account for significant mortality and morbidity. The World Health Organization (WHO) estimates that more than "25 percent of total burden of disease is linked to environmental factors including exposure to toxic chemicals." It is believed that lead, a heavy metal, for example, is thought to be responsible for 3 percent of cerebrovascular disease burden worldwide [2]. In a recent carefully conducted analysis, Pruss-Ustun and colleagues of WHO have estimated that 4.9 million deaths (8.3 percent of total mortality worldwide) are attributable to environmental exposure and inappropriate management of selected chemicals [3]. In communities of low-income nations, particularly those with marginal resources, the consequences of such exposures can be grave. A case in point is the serious risk that arsenic-contaminated groundwater presents to many people living in developing countries. In Bangladesh, for example, where half the population is exposed to arsenic-contaminated drinking water from tube wells, the risk of adverse effects is significantly high. It has been reported that in 2001 arsenic-contaminated water caused 9,100 deaths and 125,000 disability adjusted life years (DALYs) in this Asian nation [4]. Disability adjusted life years is a measurement that "combines the burden due to death and disability in one single index. One DALY can be thought of as 1 lost year of healthy life" [2].

One concern that merits attention is the failure by health care workers to recognize many occupational and environmental health-related problems [6]. Reasons for this, among other factors, include lack of training of health care providers and medical students in occupational and environmental medicine, low level of suspicion for work- and environment-related health problems, and inadequate management or failure of management of such problems [5, 7–9]. In general, health care workers who take care of their patients have limited knowledge and skills in evaluating those patients who suffer from occupational health-related problems [7].

3. Review of the Literature

In both the developing and developed countries the ubiquitous presence and use of heavy metals have not been without significant consequences. The most highly developed nations of the world have been both the primary beneficiaries of the advances spurred by industrialization and the sometimes-unwitting source of problems that have accompanied these advances. Many nations of the developing world, such as China and India, are rapidly becoming epicenters for all types of manufacturing and industrial activities and are expected to suffer the inevitable human and environmental consequences of unbridled industrial expansion.

In recent years, interest in heavy metal exposures has further intensified among populations previously thought

to be less vulnerable to such exposures by virtue of living in the more developed communities. Many of these nations are often in the forefront of research and the development of regulatory protections to benefit both individuals with potential occupational exposures and those who may be environmentally exposed. Additionally, because of increasing international trade, unexpected points of exposure intersection have been identified, resulting in enormous concern. The concerns over unacceptable levels of metal concentration in certain products sold to consumers are legitimate. Increased governmental regulations and disclosure of product contents on the part of manufacturers are required.

3.1. More Developed Countries. Metals are of particular concern as they play essential roles in the manufacture of thousands of products destined for use in the developed world and beyond. The negative aspects of occupational and environmental exposures to heavy metals continue to be researched and documented. Both industrial and nonindustrial exposures are characterized by a variety of acute and chronic ailments, the specifics of which depend on the metal in question as well as how it is being handled [3, 10].

Exposure to mercury (in various elemental, inorganic, and organic forms) has long been linked to neurological problems, to acute toxicity associated with inhalation of mercury vapor, to cardiovascular, renal, and reproductive problems [11] and has been implicated as a possible contributing factor to chronic immune disorders. Although its risks have been well characterized and, as a consequence, has been phased out of some once-common usages particularly in developed countries, it continues to be utilized in industries as diverse as cosmetics, lighting, electrochemistry, and pharmaceuticals. In addition to industrial concerns, an ongoing mercury-related issue centers on methyl mercury's presence in the food chain, specifically in fish and other seafood.

Stringent measures to control industrial contamination of waterways are only part of the solution to mercury contamination. According to the US Department of Energy, a certain amount of the mercury found in the atmosphere results from the combustion of coal and other fossil fuels [12]. Through precipitation, atmospheric mercury eventually finds its way into surface water where bacterial action transforms inorganic mercury into the toxic methylmercury, which accumulates in the food chain. Recent genomic research has further illuminated potential pathways for the bacterial mercury methylation mechanisms [13].

Over the recent past, considerable interest was stirred in the health problems attributed to mercury-containing dental amalgams. Researchers have been unable to confirm the presence of amalgam-related disease in persons exposed occupationally or otherwise to mercury-containing amalgams [14, 15]. Thus, the practice of using amalgams continues although the use of resin composites has grown as techniques to strengthen these ceramic-plastic composites have improved.

Though known for thousands of years thanks to its many useful applications, lead's toxic properties have become more widely recognized over the past 150 years (though it is believed that even the Romans were aware of some of its less salubrious qualities). Writing in his monumental work, *De Architectura*, Vitruvius, who lived more than 2000 years ago, strongly advised against the use of lead for water pipes and noted that those who worked with lead looked unhealthy [16]. Nevertheless, lead has been used through the ages for a myriad of purposes including piping, soldering, ceramic glazes, paints, glassware, construction, bullets, batteries, and more.

According to the Agency for Toxic Substances and Disease Registry (ATSDR), in the US, significant numbers of workers are regularly exposed to lead as a direct consequence of their employment [17] in industries such as smelting and lead battery production. In addition, ATSDR notes that nonoccupational settings also may provide sources of exposure from old leaded paint surfaces, to water from lead-contaminated pipes and to cigarettes. Despite the fact that leaded paints have been banned in various countries around the world beginning in 1909 (when Austria, Belgium, and France prohibited the use of white lead interior paint) and that leaded gasoline began to be phased out in the early 1970s [18], lead remains a potent force in the environment.

In the developed world, grave concerns continue over the effect of lead exposure on children's cognitive abilities. Since 1971, blood lead levels requiring intervention for children have been reduced from 40 μg/dL to the current <10 μg/dL. But ongoing research suggests that there is no "safe" blood level in children and that cognitive impairment may occur at much lower levels [19, 20].

Cadmium, a human carcinogen, became a more visible presence on the heavy metal stage as a component in nickel-cadmium batteries as well as its use in a number of industrial applications including automotive and aircraft industries as well as its presence in plastics and paints. Following the European Union's restriction of cadmium use in batteries in 2008, cadmium dealers began to seek out other markets for the material as demand was reduced [21].

Beryllium is used for aerospace components, precision instruments and also brings with it a variety of health risks. Dermatologic, respiratory, and malignant diseases may all result from unprotected exposure to beryllium [10]. Its use in electronic components has made it a key heavy metal problem in terms of waste management, as it joins other heavy metals in less environmentally sensitive landfills and incinerators.

Between 2007 and 2011 hundreds of news reports and scholarly papers were published detailing concerns over toxic materials contained in various products including children's toys and jewelry. In 2011, US researchers determined that inexpensive jewelry (often meant for children and originating in manufacturing facilities outside the United States) contained cadmium concentrations some one hundred times the exposure limit [22]. Testing commissioned by the Associated Press in 2010 found that objects such as illustrated drinking glasses featuring popular characters contained excessive lead concentrations [23]. These and other revelations regarding exposure in children to potentially damaging materials continue to be of major concern.

One other issue that merits some discussion is related to the use of metals in the developed world with profound implications for the developing world. The disposal of electronic waste is a case in point. Contained within the materials dubbed "e-waste" are metals including mercury, lead, beryllium, and cadmium. These metals are contained within components of many electronic devices currently in extraordinarily wide distribution throughout the world today. In the developed world, many of these obsolete devices find their way into landfills and incinerator facilities. A significant amount of e-waste, however, is transported to the developing world [24, 25] as some waste management companies try to circumvent increasingly stringent policies designed to protect the environment by requiring recycling and/or predisposal extraction of toxic materials. The United Nations Environment Programme (UNEP) cautioned in its 2010 report "Recycling—from E-Waste to Resources" that China, for example, improperly handles much of this waste, utilizing unregulated incinerating techniques to recover valuable metals. Moreover, though China has banned e-waste imports, these materials continue to arrive and are handled together with China's own dramatically high levels of e-waste—over 2 million tons annually (only the US produces more e-waste.) [26, 27]. A number of other countries in the developing world are also at risk for damaging effects as a consequence of e-waste, and the United Nations has made this problem an important and ongoing focus of UNEP and other relevant agencies.

3.2. Less Developed Nations. Developing countries are undergoing rapid industrial development. Occupational and environmental exposures in many communities of these nations are common and have been extensively reported in medical literature. Most experts agree that government regulations and laws are weak in many developing countries and do not always provide adequate protection to the public and workers. The use of obsolete and outdated technology further contributes to these exposures. A blind eye is turned to the regulations and requirements for protecting human health and the environment in order to gain rapid economic growth.

Despite frequent reports of their adverse health effects, the chemical exposures continue to present challenges in the developing world. Heavy metal exposures of lead, arsenic, and mercury remain the main threats to human health [26]. Dental amalgam, contaminated fish and food, and fertilizers are possible sources of mercury exposure in many communities [27]. In 1961 in Pakistan, Agrosan GN (a mixture of phenyl mercury acetate and ethyl mercury chloride) poisoning was reported due to eating of the seed wheat, which had been treated with the chemical. The incident resulted in several fatal cases [28]. In rural Iraq, in early 1972, an epidemic of methyl mercury poisoning occurred after ingestion of homemade bread prepared from wheat. The bread had been treated with a methyl mercury fungicide [29]. Mercury is also used in large amounts in the gold mining industry thereby presenting health risk to the industry workers. It has been reported that the dramatic rise in the price of gold has led to increased illegal mining, often in developing countries [30]. This process is unregulated and raises serious concerns.

Arsenic, another metal toxicant, is commonly used in the manufacture of wood preservatives and pesticides, widely used in the developing world. In the general population, exposure to arsenic is predominantly through food intake and drinking water. The exposure through food is more common; however, in some countries presence of inorganic arsenic in drinking water has been reported to be a major source of exposure. In several countries around the world like Chile, Bangladesh, and China inorganic arsenic is present in groundwater that is primarily used for drinking [31]. Also, with arsenic, there is a recognized interaction with smoking that increases the risk of cancer.

Developing regions bear the brunt of the highest burden of lead exposure. Lead is commonly found in paint, lead-tainted soil, and battery manufacturing industry. The general population is exposed to lead pollution roughly in equal proportion from food and air. A major source for cadmium exposure is cigarette smoking, which is widespread in the developing world. However in the nonsmoking general population, food remains the most important source of cadmium exposure in most countries.

Despite its rapid industrialization, Thailand continues to grapple with the problem of lead pollution. In one pilot study, garage workers were found to have significantly high levels of lead in their blood [32]. Lead exposure monitoring amongst the high risk workers in Thailand such as in mechanics and dye sprayers has been clearly overlooked, and specific control measures for these high risk occupations have not been set [32]. In another study from Taiwan investigators observed that the occupational lead exposure, herbal drug use, and drinking water from certain sources are important risk factors for high blood lead in the general population [33].

In an extensive review of human exposure to lead in Chile, the investigators observed that lead pollution in Chile persists [34]. They identified city and home soil, as well as soil near the highways as major sources of this pollution. Clusters of exposure among certain occupational groups were also noted.

In many countries, leaded gasoline continues to pose as a major health exposure problem with autos and trucks emanating leaded exhaust fumes and other contaminants into the environment [35, 36].

Even though overall awareness in regards to metal toxicity has increased worldwide, most experts agree that chemical exposure incidents in many communities either go unnoticed or when noticed the attention they receive is marginal. Developing countries need to develop stringent policies and enforce public health laws to protect the public from chemical exposures. Additionally, more public health research is warranted to assess the magnitude of the problem and identify all the sources of exposures.

4. Special Considerations

4.1. Children. Children are more susceptible to environmental hazards than adults due to several reasons. Generally,

children drink more water, breathe more air, and eat more food per unit weight as compared to adults. Children spend a significant amount of time on the ground and floor. All of these reasons increase a child's opportunity for exposure to metals. Additionally, since normal childhood development includes hand-to-mouth behavior, children are more likely to come into contact with metal toxicants in dust, carpets, or in the soil. Due to differences in children's metabolism and behavior, and poorly developed mechanisms for detoxification, children may not be able to get rid of the toxic substances efficiently. This may result in higher levels of exposure among children within the same environment when compared with adults [37]. Since children play more outdoors they become more vulnerable to short-term illness and other types of derangements from ambient air pollution [38].

Fresh water and ocean fish may contain large amounts of mercury. Excessive consumption of fish by pregnant women and children lead to significant mercury exposure. "The developing fetus and young children are thought to be disproportionately affected by mercury exposure, because many aspects of development, particularly brain maturation, can be disturbed by the presence of mercury" [39]. This was exemplified in Minamata Bay, Japan in the 1950s where large quantities of mercury was discharged into the bay, and subsequent ingestion of the contaminated fish by mothers during pregnancy led to 41 deaths and at least 30 cases of profound brain injury in infants [40]. Clearly minimizing mercury exposure is essential to optimal child health.

In many countries, children continue to be chronically exposed to a range of common pollutants like lead and organic pollutants at background levels [41]. Children absorb a larger percentage of inhaled and ingested lead than adults do. Furthermore, inorganic lead can penetrate the blood-brain barrier in children but not in adults. This is so because the barrier is not fully developed in children [42]. Due to these reasons, children are highly susceptible to lead exposure and subsequent nervous system damage [42]. It is estimated that the prevalence of elevated levels of blood lead in children worldwide is approximately 40%, with children in developing countries at greater risk of exposure than those in the developed countries [43].

Cadmium, another metal of concern, has a long half-life of 10–30 years in bones and kidneys. As a result children exposed to the metal end up suffering more from cadmium exposure than adults. Many plants, especially rice plant, can absorb cadmium from soil. Also cadmium is an ingredient of tobacco and tobacco smoke. In many Asian developing countries or communities where rice is a staple food and prevalence of smoking is widespread, cadmium exposure presents a serious threat to the health of children [44].

It should be mentioned that soil pica, which is ingestion of high amounts of soil, presents a serious risk of metal toxicity to children who engage in this activity. Soil in many communities can be contaminated with lead paint, chips, pesticides, and take—home contaminants such as mercury.

Even though all children are affected by environmental exposures, there is a disproportionate risk to children living in poverty or in certain ethnic and minority communities. Poverty compounds the risk of exposure and impending health effects since it is clearly associated with inadequate housing (with flaking lead-based paint leading to lead exposure), poor nutrition, and inadequate access to healthcare [44].

4.2. Reproductive Hazards. Exposure to chemicals and metals can impair reproductive processes in men and women. The data from the US [45] suggests that the prevalence of reproductive problems related to environmental toxicants is rather low. Data from low-income nations on the subject is inadequate to make any definitive comments.

While employed women are more likely to have better pregnancy outcomes than those who are not, there are certain occupations and exposures that cause adverse pregnancy outcomes. Exposure to toxins in the first trimester can be serious. Pregnant women at risk can develop lead exposure of the fetus which can also cause neurobehavioral and low birth weight problems. Adverse reproductive effects such as spontaneous abortions and birth defects due to lead and mercury exposures have been well documented [45]. Adverse reproductive effects in men have also been reported. Metals such as lead and mercury are known to cause spermatotoxicity.

4.3. Domestic Exposures. There are several sources of chemical exposures in domestic environment. Hobbies such as painting and welding, cleaning agents, second hand smoke, water supply, and job situations of household members (take home contamination) are examples of such sources. Take home contamination, which is transmission of chemicals from workplace to homes, is often overlooked. This type of exposure affects the immediate family members of the contaminated worker. The clothes, shoes, and other personal belongings of these workers should never be brought home for washing and/or cleaning. Small children are very susceptible to such exposures. There have been several reports of lead contamination among children of workers who are exposed to lead at their workplace [46].

One other issue that deserves special mention is the exposure to metals through the use of oral and topical herbal remedies commonly used worldwide. The two alternative systems of medicine, which rely on extensive use of herbs, are Ayurveda, a traditional healing system practiced in India, and the Traditional Chinese Medicine (TCM), commonly used in China. Many patients in the Western nations also use both systems.

It has been documented that some Ayurvedic herbal preparations, which contain metals, have been associated with adverse health effects. In one Center for Disease Control and Prevention report 12 cases of lead poisoning were found to be associated with the use of Ayurvedic remedies [47]. In two other studies Saper et al. have shown that some of these herbal preparations, which are available in the US, may contain potentially harmful levels of arsenic, mercury, and lead [48, 49]. TCM herbal remedies are also known to be associated with metal contamination and toxicity. Metals such as lead and arsenic have been implicated in these negative outcomes [50, 51].

5. Metals of Concern

It is evident that many metals present serious risk to human health. In this section we provide a brief summary of lead, arsenic, and mercury, which have been implicated in various occupational and environmental exposures around the world resulting in serious morbidity and mortality. They are the main threats to human health. Examples of other metals that can produce adverse health effects, but not mentioned in this discussion, include cadmium, cobalt, zinc, and aluminum.

Arsenic is a metalloid, which cannot be destroyed. It is present in the Earth's crust. Arsenic compounds are classified as either organic or inorganic. Arsenic compounds have no smell or taste, and therefore it is hard to detect their presence in food, water, or air. Inorganic arsenic which is found in soil and rocks is mainly used as a wood preservative to prevent its rotting. When attached to small particles it can stay in air for several days and carried to distance sites. It is also present in very small amounts in potable water, wines, and seafood [52–54]. Arsenic can be ingested or inhaled; its main route of excretion is via urine.

Lead is a grayish blue metal, which exists in the Earth's crust. It can leach into drinking water and enter food items. Its corrosion resistance and low melting point properties make lead an attractive substance for its extensive use in pipes and batteries. Its primary route of exposure is ingestion by way of drinking water, lead-containing paint or chips and contaminated dust. It is excreted in urine and feces. Children are particularly susceptible to its toxicity [54, 55].

Mercury exists in three forms: elemental (metallic), organic (methyl mercury), and inorganic. Methyl mercury is the most toxic form and exerts its effect by accumulating in the central nervous system. It is formed by microorganisms in soil and water. It is found in fish; swordfish and sharks have the highest level of mercury in their bodies. Elemental mercury, also called quicksilver, is found in household items such as thermometers, fluorescent light tubes/bulbs, and thermostats. It is slowly absorbed and less toxic than methylmercury [56, 57].

Adverse health effects due to metal exposures have been extensively documented [3, 10, 51–57]. Types of exposure to arsenic, lead and mercury, and their health effects appear in Table 1.

Table 1: Sources of exposure to arsenic, lead and mercury.

General population	Occupational populations
Arsenic	
(1) Air, drinking water, and food. Food is the predominant source. (2) Sawing and sanding, or burning of wood treated with arsenic-containing preservatives. Sawdust can be inhaled. (3) Arsenic also used in herbicides and as additives in animal feed.	(1) Workers involved in copper and lead smelting and wood treatment. (2) Workers who deal with arsenic-containing pesticides.
Lead	
(1) Lead-contaminated food and water, and also dust and lead paint. (2) Through foods from improperly glazed pottery. (3) Herbal remedies may contain lead. (4) Hobbies that use lead: soldering, making stained glass, and firing ranges.	Workers engaged in industries: lead smelting, battery manufacture, steel welding, construction, printing, radiator repair shops, rubber production, firing ranges, and printing.
Mercury	
(1) Dental amalgam fillings. (2) Practicing rituals that use mercury. (3) Damaged mercury-containing household items: thermometers, blood pressure devices, and fluorescent light bulbs. (4) Eating fish high in methyl-mercury. (5) Breathing contaminated air from hazardous waste sites that contain mercury. (6) Fungicides that contain mercury.	(1) Occupations in which there is a potential risk for mercury exposure—manufacture of electrical equipment, automotive parts that contain mercury, metal processing, and building parts and equipment that contain mercury (electrical switches, blood pressure devices). (2) Dentists and their assistants from breathing mercury vapor.

Adapted from References: [52–57].

6. Incorporating Occupational and Environmental Assessment in Primary Care: A Global Viewpoint

It is evident that metals present serious and significant health risks. The hazards that metals present are a function of the toxic properties of metals: duration, dose and route of exposure, and health history of the individuals exposed to them. Controlling and preventing metal exposures will require a multidisciplinary and integrated strategy warranting close collaboration between the government, employer, academic and research institutions, and nongovernmental organizations. Examples of initiatives in such a strategy include screening and surveillance of exposures, public education and awareness programs, environmental control of exposures, availability of adequate and accessible employee health services, worker safety programs, and medical programs aimed at protecting the health of all the citizens especially vulnerable populations namely children, elderly, and workers at risk.

It is beyond the scope of this paper to discuss all of the above programs. Our discussion, which is aimed at primary care workers, presents relevant information that will encourage and allow primary health care workers to integrate essential components of occupational and environmental assessments in their day-to-day clinical and public health practice. While much of the information presented in this section applies to chemical exposures, we provide examples

and illustrations related to metal exposures so as to stay focused on the theme of our paper.

Why should frontline health care pay attention to occupational and environmental chemical exposures in the evaluation of their patients? Several reasons come to mind. One, exposures to hazardous chemicals is common [58–60]. In one primary care setting study, about 17% patients perceived their health problem being work related; 75% gave a history of prior exposure to one or more toxic agents [58]. In a recent study Pruss-Ustun and team estimate that of all deaths that occurred in 2004, 4.9 million were attributable to chemicals [3]. Two, most individuals who suffer from any problem including chemical exposure, make their first health contact with a primary care worker, who provides an initial evaluation of their problem. Many of these problems manifest as commonly occurring medical problems or nonspecific symptoms frequently seen in primary care situations. What is of concern is that many work-related problems including exposure problems are missed because primary care workers rarely consider and address occupational and environmental factors in their clinical evaluation [5, 61]. In general, clinicians' level of suspicion concerning environmental and occupational illness is usually very low [5, 58]. They do poorly when it comes to taking occupational exposure history [62]. Therefore, it is imperative that health care workers possess adequate knowledge and skills so that they can recognize early symptoms and signs of chemical toxicity, and when necessary refer them to experts and or agencies responsible for evaluating and managing chemical exposures.

Any chemical exposure requires a comprehensive evaluation consisting of (a) obtaining a thorough medical and exposure history, (b) detailed physical examination, and (c) performing medical tests as might be indicated. Opinion of professional experts is invariably sought in documented exposures. It is not our intent to discuss the details of this type of evaluation. Instead, we focus on basic elements of exposure history that could be easily integrated in day-to-day routine primary care practice situations. Additionally, practical information on examination and tests that primary care workers could use in their day-to-day practice is provided. Any assessment tools and clinical protocols that are developed by practitioners should be based on the specific needs of those exposed, community environment, potential exposures, available resources and the level of training of health care workers. Information presented is general and can be incorporated in any clinical protocol used by primary care workers around the world.

6.1. Taking Exposure History: Basic Elements. Taking basic exposure history on every patient is important. Obtaining such history does not require detailed knowledge of toxicology. In seeking history the health worker should consider all possible exposures that may occur in the community where the patient lives and/or works. Exposure history can be done by asking a few questions or requiring patients to complete a simple form, the language of which should be simple and easily understood by the patient. Patient should be informed why exposure history is important.

There are many occupational and environmental exposure history-taking approaches [5, 58, 62], available to clinicians. Most focus on obtaining the following information:

(a) current job of the patient—job title, type or nature of work, and any protective equipment on the job,

(b) patients perception whether or not their presenting symptoms are related to their work or the environment they live in,

(c) information on whether others at home or work present with similar problems,

(d) employment history and chronology of jobs held; temporal relationship is explored,

(e) relationship between work and health problems,

(f) environmental (nonwork) exposures—hobbies, smoking, household, herbal products, and community,

(g) specific environmental and/or occupational exposures—fumes, dust, metals, and chemicals,

(h) history of any comorbid conditions.

While all of the above information is vital, obtaining detailed time-consuming occupational and environmental history could be counterproductive [63, 64]. This especially applies to primary care situations where practitioners focus more on providing care to the presenting problem of the patient.

Therefore, any environmental and occupational history taking approach should be designed such that it is easy to use and provides a snap shot of any exposures. One such approach that has been developed by the South Carolina Family Practice Residency programs uses the simple mnemonic WHACS [65]. It appears (verbatim) below:

W: what do you do?

H: how do you do?

A: are you concerned about any of your exposures on and off work?

C: coworkers or others exposed?

S: satisfied with your job?

Another initial and quick approach [58] focuses on the following four items:

(1) kind of work patient does?

(2) any relationship between work and health problems of the patient?

(3) symptoms or problem better or worse at work or at home ?

(4) any exposure to metals, chemicals, dusts, or fumes at home, work, or out in the community?

Additional questions can also be included to seek information on hobbies, use of herbal products, and exposure of coworkers or others at home.

Goldstein, in a recent Journal of Occupational and Environmental editorial, suggests even a simpler approach for occupational exposures [64]. The author recommends an initial question "What is your job?" followed by the "second question" which is "What is the riskiest part of your job?". He argues that physicians have limited time and, in order to engage primary care workers in the initial evaluation of occupational health problems of their patients, the "second question" informs the patient that occupational health is important. The patient may respond identifying a particular risk which may then prompt the physician to ask a third question, "What are you doing to avoid that risk?". The author suggests that this modest, empathetic, and interactive approach could be helpful in time-constrained primary practice situations.

While physical hazards such as radiation and noise are not the focus of this paper, questions on exposure to them could be incorporated in these approaches. Additional and detailed questioning may be warranted in some situations. The details of such questions are found in standard textbooks and references [5, 58] and other resources (Agency for Toxic Substances and Diseases Registry, Occupational and Safety Health Administration, and National Institute for Occupational Safety and Health websites).

Since most occupational and environmental health care is rendered by primary care workers it is imperative that health care workers use simple and quick approaches to obtain exposure history. Examples of three such approaches are described above. Keeping the above guidance and principles in mind it should be easy to develop custom-tailored and novel approaches to meet the needs of the community and/or primary practitioners.

6.2. Examination and Medical Tests. Most primary care practitioners do not provide complete occupational and environmental exposure assessments and therefore do not require special skills to diagnose occupational and environmental health problems. However, some practitioners may benefit from practical information on examination and tests that they could use in their day-to-day practice in certain situations.

Since most metals affect multiple organs and systems, it is recommended to conduct a complete systemic examination with a special focus on blood, cardiac, gastrointestinal, lung, liver, central nervous system, and kidney. Complete blood count, urine analysis, kidney function, and liver function tests are usually helpful. Chest X-ray and pulmonary function may be performed where relevant. Metallic content in blood, urine, and tissues may be used to confirm the diagnosis. Each metal produces a constellation of symptoms and a clinical picture unique to the metal. The tests required for exposures are metal specific. See Table 2 for routes of exposure, potential health effects, and specific tests required to diagnose and/or monitor the exposure. The information provided in Table 2 is not comprehensive, but it provides some general guidance on clinical aspects of exposure to arsenic, mercury, and lead—metals to which public and workers can be exposed. Medical and exposure history guides the nature of examination and tests to be performed.

6.3. Guidance and Referral. If there is a suspicion of metal related exposure or illness, it is vital to evaluate and manage the patient while taking necessary steps to prevent future exposures. It is beyond the scope of this paper to describe the details of relevant preventive and management strategies. However, it is worthwhile mentioning briefly that primary care workers could consider taking certain steps examples which include (a) counseling the patient aimed at prevention and health promotion, and treatment, (b) referring the patient to a specialist or designated health care facility, (c) partnering or collaborating with health care providers and/or government agencies, and (d) notifying their supervisors, appropriate government/environmental agency and/or employer as may be indicated. Various actions taken by primary care workers will depend upon their scope of practice, training and responsibility, availability of resources, nature of the problem/exposures, local laws, and patient needs and preferences.

7. Challenges

Exposures to metals cause significant mortality and illness. Failure to recognize occupational and environmental health problems in health care settings remains a challenge. Inadequate training of primary care practitioners and health care students in the occupational and environmental health disciplines is of concern. The priority given to these subjects in the medical and nursing schools curricula is very low. This must receive immediate attention.

Long- and short-term exposures resulting in delayed onset of occupational and environmental illnesses will continue to defy scientists in better understanding the causes of such illnesses. Reports of presence of metals in various herbal products and their impact on human health are worrisome. The interaction between metal exposures and disease risk factors such as smoking and obesity will require a closer examination. Lack of adequate regulatory controls in many nations is also of concern.

Establishing national registries for occupational and environmental health problems and investing in data collection and monitoring will require additional resources and intersectoral collaboration.

8. Conclusion

Exposure to chemicals is a serious public health problem that affects wildlife, soils, water, and air and can have very harmful human health effects. Exposures to chemicals including metals must be identified promptly, and individuals exposed to them must be evaluated and managed without delay. Vulnerable populations, namely, children, pregnant women, workers, and those at risk in community situations deserve our highest priority. Programs aimed at (a) providing basic occupational and environmental health education and training to health care workers, (b) creating public awareness

Table 2: Routes of exposure, health effects, and diagnosis/medical monitoring for arsenic, lead, and mercury.

	Route of exposure	Health effects	Diagnosis/medical monitoring
Arsenic (inorganic and organic)	Inhalation, oral, dermal	(1) Acute exposure: nausea, diarrhea, GI bleeding, cardiovascular effects, shock, and death. Liver, kidney damage and seizures have been reported. (2) Chronic exposure: hyperpigmentation of skin, warts, corns, heart disease, neuropathy, liver damage, anemia and peripheral vascular disease (gangrene of lower limbs), and increased risk of skin, liver, lung and bladder cancer. Arsenic in drinking water can also cause diabetes and hypertension.	Urinary arsenic level is the most reliable indicator of recent exposure to arsenic. Arsenic in hair and fingernails can indicate exposure to high levels in the past 6–12 months.
Lead	Inhalation oral dermal	(1) Hematologic: decreased heme synthesis enzymes, anemia. (2) Cardiovascular: elevated blood pressure. (3) Cognitive, neurobehavioral, and psychological effects. (4) Gastrointestinal: colic or abdominal cramps. (5) Peripheral neuropathy; encephalopathy (at high levels). (6) Reduced fertility. (7) Immune system: alterations in T cell, reduced IgG serum levels. (8) Children: lethargy, loss of appetite, anemia, colic, neurological impairment, and impaired metabolism of Vit D. Exposure in uterus and during childhood can result in impaired neurological development, IQ deficits, and growth retardation. Lead-based paint is a common source of lead exposure.	Lead in whole blood is a reliable test. Erythrocyte protoporphyrin (EP) test can also be used but it is not sensitive to detect high levels of lead in children.
Mercury (elemental or metallic, organic-methyl mercury and inorganic)	Inhalation oral food (fish), dental work.	(1) All forms of mercury are toxic to the CNS. (2) Exposure to high levels can damage brain, kidneys, and developing fetus. (methyl mercury is the most toxic form). (3) Toxicity to brain results in irritability, tremors, visual changes, and memory problems. (4) Mercury salts can cause abdominal cramps, diarrhea, and kidney damage.	Acute exposure is best measured by mercury in blood and chronic exposure by mercury in urine.

Adapted from References: [52–57].

about exposures to chemicals and their adverse health effects should be developed and implemented without delay. Ongoing epidemiological, public health, and clinical research on the subject will enhance our efforts in controlling metal exposures and contamination of the environment. Health care providers, scientists, academicians, environmental health departments, and employers must work together and make every effort to prevent human exposure to chemicals and metals. The problems associated with metals are not disappearing even as new control measures are implemented and more regulations are enforced. However, the lessons learned from the past may help limit the inevitable impact of heavy metal use as part of advancing industrialization in both less and more developed nations around the world.

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Review Article

Arsenic Exposure and the Induction of Human Cancers

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Arsenic is a metalloid, that is, considered to be a human carcinogen. Millions of individuals worldwide are chronically exposed through drinking water, with consequences ranging from acute toxicities to development of malignancies, such as skin and lung cancer. Despite well-known arsenic-related health effects, the molecular mechanisms involved are not fully understood; however, the arsenic biotransformation process, which includes methylation changes, is thought to play a key role. This paper explores the relationship of arsenic exposure with cancer development and summarizes current knowledge of the potential mechanisms that may contribute to the neoplastic processes observed in arsenic exposed human populations.

1. Introduction

Arsenic (As) is a chemical element classified as a metalloid. The most common oxidation states in the environment are +3 (As^{III}, also known as arsenite) and +5 (As^{V} or arsenate), which exhibit different grades of toxicity [1]. Arsenic compounds can be found in organic (when linked with carbon and hydrogen) and inorganic (when combined with oxygen, chlorine, and sulfur, among other elements) forms [2].

Long term ingestion of inorganic arsenic has been associated with several human diseases. There are various sources of ingested arsenic, such as food (mainly in fish and seafood, algae, and cereals), air (coal-fired power generation and smelting), and water [3]. Of the various sources of arsenic in the environment, long-term exposure of arsenic in drinking water likely poses the greatest threat to human health [4]. Given its daily and widespread consumption, occurrence of arsenic in drinking water has been increasingly recognized as a major public health concern in several regions of the world over the past decades [5–7]. In fact, groundwater used for drinking contaminated with naturally occurring inorganic arsenic in Bangladesh represents one of the largest mass poisoning of a population in history [8]. Worldwide, an estimated 160 million people live in regions with naturally elevated levels of arsenic in drinking water, due to the presence of arsenic-rich geological formations [7].

Arsenic is a natural component of rocks containing copper or lead, which can result in release of arsenic into water or air in zones of intensive mining activities [6]. Due to these geological conditions and/or anthropogenic activities, soil and water supplies in these areas contain high concentrations of arsenical compounds [9]. This situation is compounded in extremely arid zones (such as in Northern Chile), where water sources are scarce and contaminated water serves as a drinking and irrigation supply. This can lead to massive chronic poisoning—called arsenicosis—affecting local populations [10]. Arsenic-contaminated drinking water represents an important public health issue, especially for developing countries.

Due to its physical characteristics (no odor, no color, and no flavor), arsenic exposure is often unnoticed, especially when ingested through drinking water. In this context, long-term effects are a major health concern in affected areas. The World Health Organization (WHO) and the U.S. Environmental Protection Agency have recommended a threshold of 10 μg/L for inorganic arsenic concentration in drinking water [11, 12]. Unfortunately, millions of people are exposed to toxic levels and are at increased risk for

the adverse health effects of arsenic [6, 13]. Concentrations exceeding this threshold have been described in Bangladesh, India, China, Argentina, Mexico, Canada, USA, and Chile, among other countries [14].

There is a strong body of evidence linking arsenic with a variety of health problems, from acute toxicities to chronic diseases which can take years to develop. Arsenic-related diseases include skin lesions, hypertension, ischemia, some endemic peripheral vascular disorders (e.g., "black foot disease"), diabetes, severe arteriosclerosis, neuropathies, and, significantly, many types of cancer [15–18]. Cancer-death risk associated with daily consumption of 1.6 liters of water with inorganic arsenic (50 μg/L) has been estimated to be 21/1,000 [19].

Arsenic has been classified as a class I human carcinogen by the International Agency of Research on Cancer (IARC), meaning that there is sufficient evidence of carcinogenicity to humans. Despite evidence in humans, animal models fail to replicate these observed effects, hampering elucidation of the exact mode(s) of action underlying arsenic related carcinogenicity [20]. Skin and several types of internal cancers, including bladder, kidney, liver, prostate, and lung have been associated with arsenic ingestion [10, 21–25]. Skin cancer is the most common form of neoplasm associated with arsenic ingestion, while lung cancer corresponds to the most deadly [13, 26]. Interestingly, arsenic (specifically arsenic trioxide or As_2O_3) has been used as a chemotherapeutic agent for several types of cancer, with some studies showing high percentage of response in patients with acute promyelocytic leukemia (APL) [27, 28]. We will also discuss this issue in further sections.

2. Common Arsenic-Induced Malignancies

2.1. Skin Cancer. The relationship between arsenic and skin cancer has been well documented over the past several decades [29, 30]. The first inferences were made through observations of an increased frequency of skin cancer cases following treatment with Fowler's solution (1% potassium arsenite), formerly used for a variety of skin and hematological disorders [31]. Bowen's disease (intraepithelial carcinoma or carcinoma *in situ*), basal cell carcinoma (BCC)- and squamous cell carcinoma (SqCC) are the most common malignancies found in patients with long-term exposure to arsenic. Merkel cell carcinoma, an uncommon and highly aggressive cutaneous neoplasm, has been also documented at a lower frequency [32–35].

Arsenic-related skin SqCC can develop either *de novo* or progress from Bowen's disease, whereas arsenic-related BCC develops usually in multiple foci and areas of the body covered from sun exposure, in contrast to cases originating from other skin carcinogens, such as UV-light [36–38]. Arsenic-related Bowen's disease can appear 10 years after arsenic exposure, while other types of skin cancer can have a latency period of 20 or 30 years [39]. A dose-response relationship and cell-type specificity have been described for arsenic-related skin cancer [40, 41]. Additionally, normal human epidermal keratinocytes exposed to varying noncytotoxic/slightly cytotoxic concentrations of inorganic arsenic exhibit gene expression changes associated with molecular pathways relevant to arsenic-related skin carcinogenesis, such as oxidative stress, increased transcriptional levels of keratinocyte growth factors, and modulation of MAPK and NF-κB pathways [42].

Premalignant skin lesions are relatively early manifestations of arsenic toxicity and are often considered precursors to arsenic-induced skin BCC and SqCC tumors [43]. These lesions include dermal manifestations such hyperpigmentation (a finely freckled, "raindrop" pattern of pigmentation or depigmentation) and hyperkeratosis (skin thickening, mainly at palms and the feet). These lesions are commonly found in chronically exposed populations and are considered a diagnostic criterion of arsenicosis [44]. Moreover, some genetic susceptibilities to these arsenic-related skin lesions have been proposed, since they do not occur in every exposed individual [45]. Hyperkeratosis can appear with shorter periods of arsenic exposure, and it has been described that these lesions give rise to the majority of arsenic-induced skin cancer [46, 47]. Additionally, it has been demonstrated that a significant proportion of fatal cases of skin cancer occurred in patients with prior signs of arsenicosis, such as keratosis and hyperpigmentation [31, 48].

In addition to directly affecting the carcinogenic process, it has been demonstrated that arsenic toxicity can also be potentiated by other environmental carcinogens. For example, arsenic-exposed individuals with a history of smoking and chronic exposure to environments with high fertilizer use may be more susceptible to cancer-prone skin lesions than those without these risk factors, even at the same level of arsenic exposure [43]. Arsenic can act as a cocarcinogen with UV light in a synergistic mode of action, leading to development of hyperkeratosis [49, 50]. Additionally, the same mode of action was observed between high levels of arsenic (over 100 μg/L) and tobacco smoking with respect to risk of skin lesions in men [51].

2.2. Lung Cancer. There exists a significant dose-response relationship between arsenic concentration in water and incidence of lung cancer and other malignancies for both men and women [52]. The association between lung cancer and ingested arsenic was discovered following therapeutic application of this metalloid in psoriasis patients treated with Fowler's solution [53–55]. Thereafter, an increased lung cancer risk following exposure to arsenic in drinking water was demonstrated by several case-control and cohort-type studies [20]. Consistent, positive, and statistically significant associations among individuals exposed to high concentrations of arsenic in drinking water and increased risk of lung cancer have been detected [56, 57]. Based on large epidemiology studies in 1999, a report from the National Research Council (NRC, USA) concluded that there was sufficient evidence suggesting that the ingestion of arsenic in drinking water causes lung cancer, among other types of malignant neoplasias [58]. After this publication, other major arsenic and lung cancer epidemiological studies were published [25, 59]. Due to mounting evidence, the NRC

study was reevaluated in 2001, concluding that the carcinogenic effects of arsenic in humans are significant, and that lung (and bladder) cancer should continue to be the focus of arsenic risk assessment for regulatory decision making [60].

Interestingly, the increase risk of lung cancer associated with arsenic seems to be cancer subtype specific. For example, where SqCC incidence had decreased worldwide and overwhelmingly associated with cigarette smoking; in Northern Chile, a high proportion of SqCC frequently occurs in never smokers who have been chronically exposed to arsenic [25, 61].

As mentioned, Bangladesh represents the largest mass poisoning of a population in history, as groundwater used for drinking is contaminated with naturally occurring inorganic arsenic [8]. In rural areas in Bangladesh, arsenic contamination in drinking water from tube wells is associated with lung cancer in males, with lung SqCC being the predominant histological subtype in areas with arsenic concentrations above 100 μg/L [62]. In these areas, the lifetime mortality risks of lung cancer are 159.1/100 000 for males and 23.1 for females (per 100 000 population) [63].

Blackfoot disease (BFD) is an endemic, peripheral arterial disease characterized by severe systemic arteriosclerosis and spontaneous gangrene resulting in amputation, common to individuals exposed to arsenic in Southwestern Taiwan [18]. In zones affected by BFD, increased incidence and subsequent mortality rates for lung cancer have been demonstrated, especially among those who used arsenic-contaminated well water for $\geq$40 years [64]. Smokers in this area have a 4.1-fold higher relative risk for lung cancer, suggesting a possible synergistic relationship between arsenic and tobacco exposures in terms of lung tumorigenesis [65]. Also, short-term exposure (5 years) of arsenic-contaminated drinking water ($\geq$0.05 μg/L) can also result in elevated lung cancer risk [66].

The Andean zone in South America is another area where the relationship between chronic arsenic exposure and lung cancer has been demonstrated. A dose-response relationship between arsenic in drinking water and lung cancer was found in central regions of Argentina [67], where arsenic concentrations in water supplies were >100 μg/L, even reaching as high as 2000 μg/L [68]. A correlation between increased arsenic concentration in drinking water and lung cancer incidence was also discovered in Northern Chile [25]. In this area, lung cancer mortality increased ten years after the initiation of high-level exposure (arsenic concentration >90 μg/L in 1958) [10].

Chronic exposures to water contaminated with low concentrations of arsenic do not, however, show the same strong associations with increased cancer incidence and mortality. For example, a study carried out in Denmark [69] did not find any significant association between exposure to low concentrations of arsenic in drinking water (0.05–25.3 μg/L) and risk of melanoma or lung cancer, among other types of neoplasias. Similarly, another study conducted in Belgium did not find a significant correlation between exposure to drinking water containing relatively low arsenic concentrations (20–50 μg/L) and lung cancer mortality [70].

Genetic factors are thought to modulate susceptibility to arsenic-induced lung cancer. Carriers of *CYP1A1** 2*A*/*GSTM*1 homozygous deletion genotype show increased odds ratios for lung cancer, especially among smokers [71, 72]. In addition, our group has recently proposed that genomic aberrations in arsenic induced lung cancers exhibit distinct molecular characteristics. Using a whole genome tiling-path comparative genomic hybridization (CGH) array platform (described in [73]), we analyzed DNA copy-number alterations (CNAs) among lung SqCC cases from a Northern Chilean population chronically exposed to inorganic arsenic in drinking water (Figure 1) [74]. We identified unique patterns of chromosomal disruption and gene dosage related to SqCC from never smokers in Northern Chile, which did not correlate with normal DNA copy-number variations (Figure 1). This has led to the growing hypothesis that lung SqCC in arsenic-exposed individuals could represent a molecularly distinct form of this disease [74].

3. Carcinogenic Mechanisms of Arsenic Exposure

3.1. Arsenic Biotransformation as a Toxicity Activation Mechanism. Arsenic metabolism implicates a series of reduction and oxidation reactions. Pentavalent arsenical species are reduced to trivalent species, and oxidative methylation occurs to yield methylated tri- and pentavalent metabolites [75]. However, more than a detoxification mechanism, it has been proposed that methylation can activate the toxic and carcinogenic potential of arsenic, since it has been demonstrated that mono/dimethylated arsenical species (both tri/pentavalent) can affect gene transcription, and are more potent enzyme inhibitors and cytotoxins than non-methylated species [76, 77]. Moreover, since the arsenic biotransformation pathway uses S-adenosylmethionine (SAM) as a methyl group donor, arsenic can also interfere with a number of cellular processes that require methyl groups, leading to the idea that alteration of epigenetic mechanisms can also participate in arsenic-induced carcinogenesis [78]. A variety of these arsenic associated toxic events have been elucidated in cell line and animal models (Table 1).

After reduction of As^{V} to As^{III} by purine nucleoside phosphorylase, As^{III} is methylated via a As^{III}-methyltransferase, using SAM as a methyl group donor [79], producing mono and dimethylated trivalent species, such as monomethylarsonous acid (MMA^{III}), dimethylarsinous acid (DMA^{III}), and equivalent pentavalent species (monomethylarsonic acid or MMA^{V}, and dimethylarsinic acid or DMA^{V}. Interestingly, there is little evidence of methylated arsenic metabolites in skin, and *in vitro* studies have demonstrated that keratinocytes display very slow rates of arsenic methylation, and only mono-methylated species are produced [45, 80]. It has been proposed that As^{III} could be one of the responsible agents in arsenic related skin carcinogenicity, since it acts as a cocarcinogenic to mouse skin [81]. Additionally, individuals with arsenic-related skin lesions or skin cancer exhibit lower levels of dimethylated species in urine (in contrast to monomethylated species) [82–85], indicating that a lower

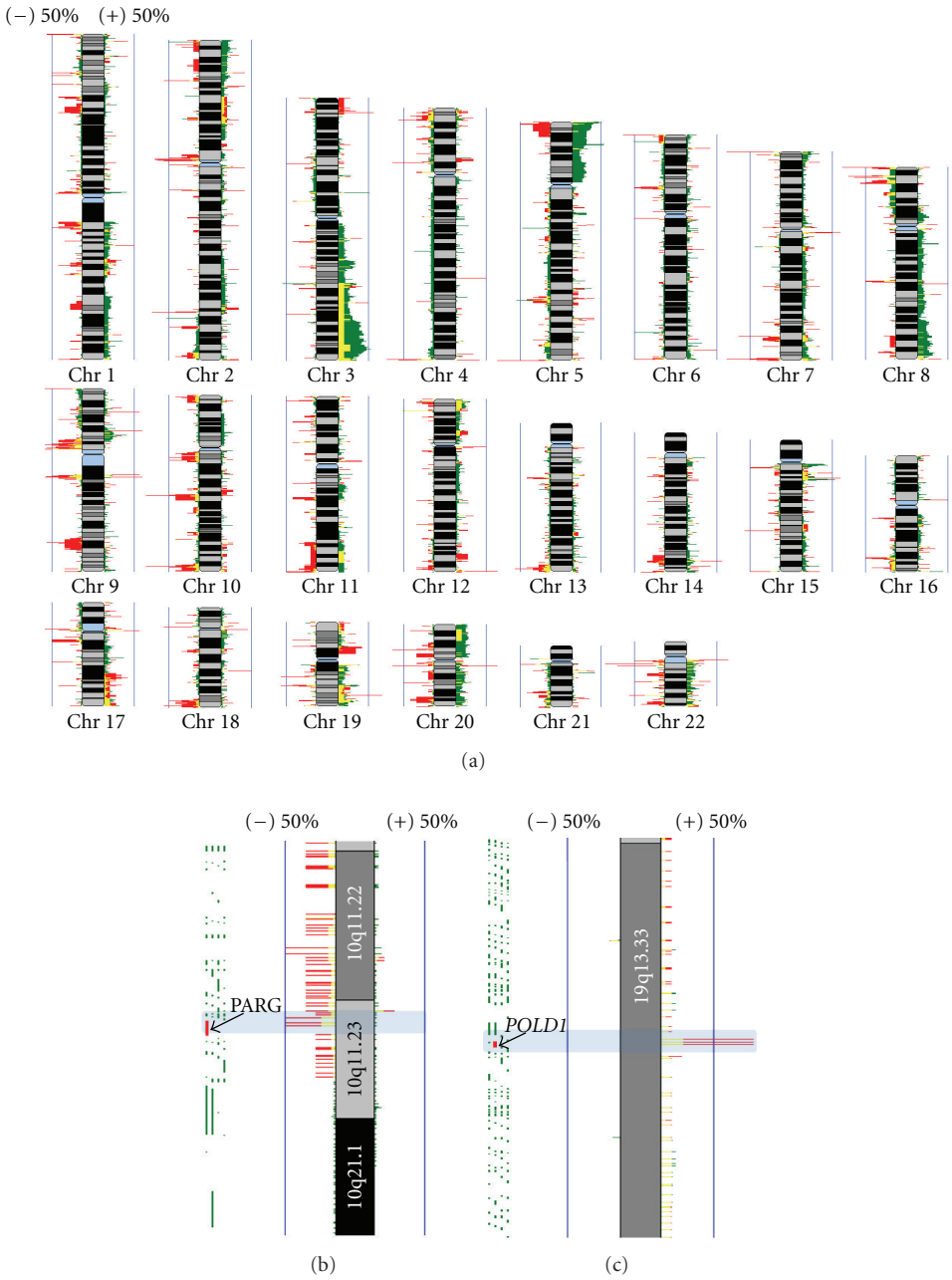

Figure 1: Genome-wide identification of arsenic-related and smoking independent DNA copy number alterations in lung squamous cell carcinoma (SqCC). Genomic copy number profiles for lung SqCC biopsies ($n = 52$) were obtained using whole genome aCGH. SqCC tumors from smokers ($n = 42$), comprised ($n = 30$) samples from North American with no known arsenic exposure, and ($n = 12$) samples from Northern Chile from individuals chronically exposed to arsenic. SqCC tumors from never smokers ($n = 10$) were from chronically arsenic-exposed individuals from Northern Chile. (a) Frequency plot of arsenic-related and smoking independent copy number differences in SqCC. The frequency of DNA gain/loss for each probe was calculated and plotted for each group, where smokers (dark green) and never smokers (red). Regions exhibiting similar alteration in both groups are denoted in yellow. The magnitude of green and red bars represents percent alteration for each probe per group (0–100%, with blue vertical lines representing 50% frequency). DNA gains and losses are represented to the right and left of each chromosome, respectively. Analysis was restricted to autosomes, with any differences based on sex subtracted from further analysis. A high frequency of copy number alteration, previously undescribed for SqCC were evident in arsenic exposed tumors from never smokers, particularly for chromosome 3q. (b) Detail of DNA losses at 10q11.23 specific to never smokers are highlighted in a light-blue rectangle. PARG, previously shown to mediate cell death in response to genotoxic stimuli (PMID: 19571039), is indicated in red. (c) Recurrent DNA gain found in never smokers at 19q13.33. This segment contains the *POLD1* gene, a DNA polymerase delta complex, involved in DNA replication and repair (red probe).

Table 1: Changes in functions associated to arsenic-related carcinogenicity.

Type of alteration	Cell model/type	As species	Reference
Associated to oxidative stress			
DNA strand break	Human fetal lung fibroblast (2BS cells)	As^{III}	[121]
DNA strand break	Human alveolar epithelial type II (L-132) cells	DMA^{V}	[122]
Single-strand DNA breaks, DNA-protein adducts, sister chromatid exchanges	Human fibroblast cell lines	As^{III}	[123]
Formation of apurinic/apyrimidinic sites	Human alveolar epithelial cell line (L-132)	DMA	[124]
Induction of 8-OHdG	Human breast cancer MCF-7 adenocarcinoma epithelial cells	As^{III}	[125]
Increases 8-oxo-G levels through $(CH3)_2AsOO$		DMA^{V}	[126, 127]
Presence markers for oxidative stress were detected, including 8-oxodG	Mouse bronchiolar Clara cells	DMA^{V}	[128]
Double-strand DNA breaks	Mammalian cells		[96]
Epigenetic changes in DNA methylation/histones modification/miRNA expression			
Alteration of methylation in p53 promoter	A549 cell line	As^{III} As^{V}	[106]
Inductor of hypermethylation of the p16INK4a and RASSF1A CpG islands in nontumor lung tissues (including hyperplasia and adenoma) and lung adenocarcinomas	Lungs of mice exposed during 18 months	As^{V}	[107]
Increase of dimethylated H3K9	Human BEAS-2B cell line	As^{III}	[112]
Increased H3K9 dimethylation and decreased H3K27 tri-methylation (gene silencing), increasing H3K4 tri-methylation (gene-activating mark), increases histone methyltransferase G9a protein levels	Human A549 cell line	As^{III}	[112]
Changes to histone H3 acetylation, DNA promoter methylation, and decreases expression of the *DBC1*, *FAM83A*, *ZSCAN12*, and *C1QTNF6* genes	Human nontumorogenic cell lines		[113]
Altered expression of hsa-miR-210, -22, -34a, -221, and -222	Human lymphoblastoid cells	As^{III}	[116]
Reduction in levels of miR-200	Immortalized p53-knocked down human bronchial epithelial cells (HBECs)	As^{III}	[117]
Decrease in expression of miRNA-9, -181b, -124, and -125b	Chick embryos	As^{III}	[118]
Other changes			
Amplification of the dihydrofolate reductase gene	Mouse 3T6 cells	As^{III}	[129]
MAPK activation; phosphorylation of ATF-2 and c-Jun, elevated IL-8 release	Human BEAS 2B line	As^{III}	[130]
Induction of p53-independent expression of GADD45 protein (a G2/M cell-cycle checkpoint protein)	Human BEAS 2B line	As^{III}	[131]
Stabilization of GADD45 alpha mRNA through nucleolin	Human BEAS 2B line	As^{III}	[132]
Mostly decreased expression for transcripts involved in angiogenesis, lipid metabolism, oxygen transport, apoptosis, cell cycle, and immune response	Lung of mice exposed	As^{III}	[133]

Table 1: Continued.

Type of alteration	Cell model/type	As species	Reference
Induction of the expression of genes involved with cancer, the cell cycle, cellular proliferation, DNA replication, recombination and repair, lipid metabolism, cell-cell signaling and interaction, molecular transport, and immunological disease pathways in Ogg1$^{-/-}$ mice	Lungs of Ogg1$^{-/-}$ mutant mice exposed	DMA^{V}	[134]
Enhanced centrosome amplification in p53-compromised cells. Resistance to arsenite-induced G2/M cell cycle arrest and arsenite-induced apoptosis in p53-compromised cells. Reductions in arsenite-induced enhancement of p53, p21, and Gadd45a expressions (at 5–10 μM), Higher (200%) cell colony formation in p53-inhibited BEAS-2B cells (5 μM)	H1355 cells (human lung adenocarcinoma cell line with mutation in p53) Human BEAS-2B line p53-inhibited BEAS-2B cells	As^{III}	[135]
Increased expression of ER-alpha and genes related to estrogen signaling in the fetal lung of female mice	Lung samples from gestation day 18 female fetal C3H mice	As^{III}	[136]
Downregulation of (validated genes): Tpi1, Ldha, and Pgk1. Upregulation of (validated genes): Cox6a2; ^{V}ariable: Id1, Gpnmb	Rat lung epithelial cell line (L2)	As^{III}	[137]
Increased cell viability ($\leq$0.5 μM). Downregulation of APE1 and Polβ mRNA (above 1 μM)	GM847-immortalized human lung fibroblast	As^{III}	[138]
Increased plating efficiency (cell growth advantage), micronuclei incidence (marker of chromosomal instability), gene amplification (PALA resistance), invasive capabilities; anchorage-independent growth (oncogenic transformation); lost of β4 integrin expression; upregulated phosphorylation of Rb and ERK; decreased expression of p53 protein	h-TERT-immortalized human small airway epithelial cells	As^{III}	[139]

methylation activity could predispose individuals to arsenic-related skin malignancies.

3.2. Arsenic-Induced Oxidative Stress. Cellular induced damage derived from arsenic biotransformation leading to carcinogenic processes have usually been described to occur through oxidative stress by generation of toxic species, such as reactive oxygen species (ROS) leading to genomic aberrations. Generation of ROS has been described as one of the earliest and most important mechanisms of arsenic-induced carcinogenicity [86–91]. Oxidative damage (measured as guanine oxidation) is significantly associated with skin tumors associated with arsenic exposure [92, 93]. It has also been shown that oxidative stress can modify gene transcription profiles of human hyperkeratosis, affecting several cancer-relevant pathways, such as the Wnt/β-catenin and calcium signaling pathways [94, 95]. Both single- and double DNA strand breaks are characteristic of most cancer types and have been shown to be induced by chronic arsenic exposure, even at low concentrations [96, 97].

3.3. Epigenetic Changes. Arsenic, arsenic metabolites, and metabolism, directly and indirectly, affect normal epigenetic transcriptional regulation at both the level of DNA methylation, histone maintenance, and miRNA expression (reviewed in [98, 99]). As previously mentioned, biotransformation and reduction of arsenic leads to the formation of highly toxic methylated arsenic species which act as potent cytotoxics and enzyme inhibitors. Since this process utilizes SAM, the cell's own methyl group donor, arsenic is thought to interfere with the cell's ability to maintain normal epigenetic regulation via the disruption of normal DNA methylation patterns, histone modification, and expression of microRNAs (miRNAs), possibly by the depletion of cellular pools of methyl groups (Figure 2). Epigenetic modifications do not alter the DNA sequence itself but instead result in chemical modifications to DNA or histone tail residues. Cells and tissues exposed to arsenic display epigenetic aberrations that mimic early hallmarks of cancer, providing evidence for an epigenetic role in arsenic-mediated tumorigenesis. Epigenetic regulation of gene expression is a highly dynamic process that can be modulated by existing therapeutics [100, 101] which may potentially apply to arsenic-related malignancies.

3.3.1. DNA Methylation. In the human genome, DNA methylation occurs at the 5-carbon position of cytosine in CpG dinucleotide sequences, resulting in 5′-methylcytosine (5mC), often within short evolutionarily conserved regions enriched for CpG dinucleotides, called CpG islands [102, 103]. When located in promoter regions of genes, CpG islands are typically unmethylated (~90%); however, promoters without CpG islands are frequently methylated [104]. Therefore, the bulk of methylated DNA in the human genome occurs in repetitive DNA sequences, where it is thought to have an important role in silencing transposable elements and maintaining genomic stability. The transfer of the donor methyl group from SAM to the cytosine in a CpG dinucleotides is catalyzed by DNA methyl transferase (DNMT) enzymes, responsible for the *de novo* methylation

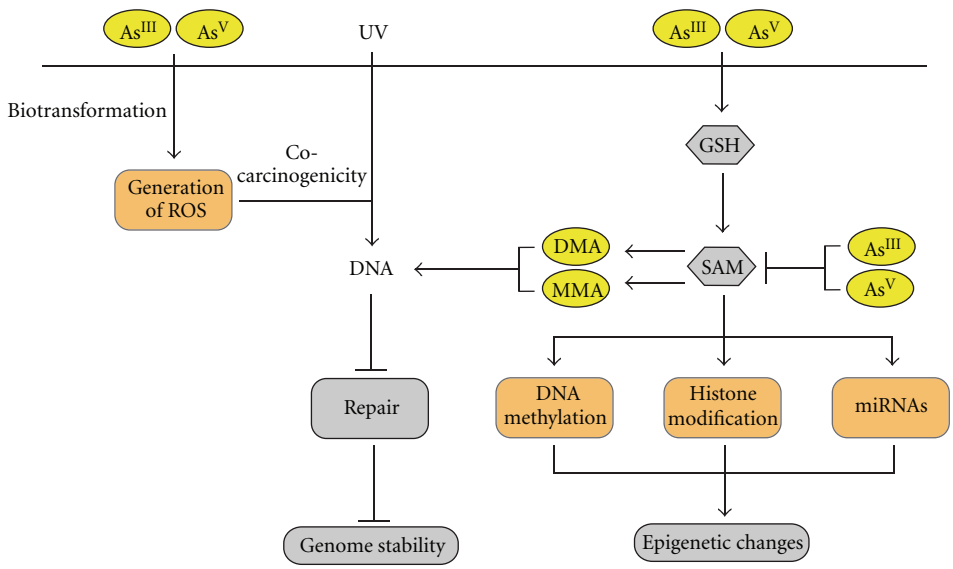

Figure 2: Schematic representation of proposed arsenic-induced carcinogenic mechanisms. Arsenic can enter cells in both tri- or pentavalent forms (As^{III} or As^{V}). Inside cells, As^{V} is converted to As^{III}, with subsequent methylation to monomethylated (MMA) and dimethylated (DMA) species. The methylation of inorganic arsenic consumes both S-adenosylmethionine (SAM) and glutathione (GSH). Cellular damage derived from arsenic biotransformation can occur through generation of reactive oxygen species (ROS), and through epigenetic mechanisms: changes in DNA methylation patterns (by depletion of cellular pools of methyl group), histone modification, and altered expression of microRNAs (miRNAs).

throughout development (DNMT3a, DNMT3b, and DNMT3L) and maintenance of methylation patterns in somatic tissue (DNMT1). Aberrant DNA methylation is implicated in a vast spectrum of diseases and disorders and is one of the earliest and most frequent aberrations in cancer. DNA hypomethylation is associated with genomic instability and the reexpression of parasitic DNA, in addition to activation of genes normally silenced by methylation in a tissue-specific manner. Conversely, aberrant DNA promoter hypermethylation is strongly linked to transcriptional gene silencing, particularly for tumor suppressor genes (TSGs) and cancer.

DNA methylation patterns observed in human cancers resemble those of arsenic-related premalignant and malignant cells and tissues. For example, the DNA methylation patterns of several well-known cancer genes have been studied in the context of, and found to correlate with, arsenic exposure in *in vitro* cell models and in populations exposed to arsenic. The activation of proliferative genes in rat liver epithelial cell lines which undergo malignant transformation following chronic, low-level arsenic exposure is associated with aberrant DNA hypomethylation [105]. Exposure to As^{III} and As^{V} in the lung cancer cell line (A549), resulted in promoter hypermethylation and subsequent transcriptional silencing of *p53* [106]. Mice, chronically exposed to As^{V} through drinking water, acquire frequent promoter hypermethylation of the tumor suppressors $p16^{INK4A}$ and *RASSF1A* in lung tumor tissues [107]. A study analyzing 351 cases of bladder cancer cases found that arsenic exposure was associated with promoter methylation of *RASSF1A* and *PRSS3* [108]. Intriguingly, in one study, individuals with no cancer, but who were exposed to high arsenic concentrations (>251 μg/L), had a significant degree of DNA hypermethylation in promoter regions of *p53* and $p16^{INK4A}$ compared to nonexposed controls [109], suggesting that arsenic related-hypermethylation events may be some of the earliest causal tumorigenic events. Collectively, these and other findings (see [98, 99]) support the notion that arsenic-induced changes to DNA methylation play a role in tumor formation.

3.3.2. Histone Modification. Histones proteins enable condensation of double-stranded supercoiled eukaryotic DNA into nucleosomes, which are made up of two copies each of H2A, H2B, H3, and H4 proteins. The N-terminal tails of histones are accessible to modifying enzymes, which function in catalyzing posttranslational modifications to the amino acid residues residing within the histone tail, including acetylation, methylation, ubiquitination, sumoylation, and phosphorylation amongst others [110, 111]. Specific patterns of these modifications, commonly referred to as the "histone code", correlate with transcriptional states of associated genes as well as to disease phenotypes. Working in conjunction with transcriptional coactivators or repressors, histone modifying enzymes catalyze the addition or removal of these modifications to generally induce or maintain an (1) open euchromatic state, through the addition of acetyl groups (via histone acetyltransferases) or (2) a closed or heterchromatic state, through the addition of methyl groups (via histone methyltransferases) or removal of acetyl groups (via histone deacetylases) on specific histone residues. Therefore, transcriptional activity of associated genes correlates with the formation of euchromatin or heterochromatin.

Arsenic metabolites have been shown to modulate normal histone patterns. As^{III} has also been shown to modify methylation patterns on H3K4, H3K9, and H3K27 [112]. A549 cells exposed to 2.5–5 μM of As^{III} exhibited an increase in H3K9 dimethylation and a decrease in H3K27 trimethylation, both of which are associated with heterochromatin (gene silencing), and a decrease in H3K4 trimethylation which is associated with euchromatin formation (an activation mark). When the normal bronchial epithelial cell line (BEAS-2B) was exposed to 1-2 μM of As^{III}, an increase in dimethylation of H3K9 was observed.

Arsenic compounds were also shown to induce malignant transformation of human nontumorigenic cell lines through changes to histone H3 acetylation, DNA promoter methylation, and decreases expression of the *DBC1*, *FAM83A*, *ZSCAN12*, and *C1QTNF6* genes [113]. For each of these underexpressed genes, DNA methylation inversely correlated with the histone acetylation levels for their respective promoter regions, leading authors to conclude that changes in histone H3 acetylation occur during arsenic-induced malignant transformation.

3.3.3. MicroRNAs. miRNAs are small, noncoding RNA species that orchestrate the expression of genes involved in many key aspects of cell biology by degradation and translational inhibition of their target mRNAs (reviewed in [114]). In humans, more than 1400 miRNAs have been identified to date (miRBase data base; Release 17, April 2011). miRNAs inhibit gene expression by binding to the 3′-untranslated region of mRNAs through imperfect base pairing; consequently, a single miRNA can negatively regulate the expression of multiple and sometimes upwards of hundreds target genes. As a result, miRNAs deregulations are implicated in diverse human pathologies, including cancer (reviewed in [115]).

An increasing number of studies show that arsenic exposure can alter miRNA expression levels *in vitro* and *in vivo*. Human lymphoblastoid cells exposed to sodium As^{III} over six days showed altered expression of five miRNAs (hsa-miR-210, -22, -34a, -221, and -222) [116]. The authors hypothesized that these alterations could be a consequence of changes in methylation patterns, since the same alterations were observed when cells were grown under folate-deficient conditions, which can lead to reduced levels of SAM. Furthermore, overexpression of hsa-miR-222 was confirmed in human peripheral blood-derived cells from individuals with insufficient dietary folate. The induced changes in miRNA expression could be reversed by the restoration of folate, suggesting that continuous exposure to agents like arsenic may be necessary to permanently alter the expression of miRNAs.

Chronic exposure to As^{III} has also been shown to induce malignant transformation and epithelial-to-mesenchymal transition (EMT), in concert with reduction in levels of miR-200 family members in immortalized p53-knocked down-human bronchial epithelial cells (HBECs) but not in p53-intact HBECs [117]. Interestingly, stable expression of miR-200b alone was capable of entirely reversing and preventing As^{III}-induced EMT and malignant transformation. Arsenic exposure depleted the miR-200 s through the induction of EMT-inducing transcription factors zinc-finger E-box-binding homeobox factor 1 (ZEB1) and ZEB2 and increased methylation of miR-200 promoters.

A recent study examining the global expression of miRNAs and mRNAs of chick embryos after arsenic exposure revealed a dramatic decrease in expression of miRNA-9, -181b, -124, and -125b [118]. NRP1—a transmembrane receptor involved in angiogenesis—which is upregulated at the mRNA level in arsenic-treated chick embryos was found to be a target gene of miR-9 and miR-181b. Overexpression of miR-9 or miR-181b suppressed As^{III}-induced NRP1 expression, cell migration and tube formation, supporting involvement of these miRNA species in As^{III}-induced angiogenesis via NRP1 gene activation.

Despite its carcinogenic potential, As^{III} has also been used as a treatment option for APL, which is frequently associated with a gene fusion involving the retinoic acid receptor alpha (RARA) and the promyelocytic leukemia protein (PML) gene [119]. Saumet et al. have shown that PML-RARA is able to transcriptionally repress several miRNAs associated with critical pathways linked to leukemogenesis, such as HOX proteins and cell adhesion molecules [120]. Expression of these miRNAs was restored by retinoic acid and As^{III}, suggesting that, in APL, these agents may function to inhibit cell growth, at least in part by impacting miRNA expression.

4. Conclusion

Arsenic contamination of drinking water remains a serious public health problem, affecting hundreds of millions individuals worldwide. The more severe effects, such as cancer, are evident up to several decades after exposure has ceased. Although mitigation measures have been taken, the natural origin of this contamination keeps this problem an active preoccupation that requires strategies to monitor arsenic concentrations in drinking water and to define markers associated with early health effects.

Overall, reviewed literature indicates that arsenic exposure exerts deleterious health effects primarily through the induction of oxidative stress, alterations to DNA methylation, histone modification, and miRNA expression. Understanding these events in the context of arsenic toxicity may provide powerful biomarkers for arsenic-induced carcinogenicity and elucidation of early steps in arsenic-induced malignancies that may be reversed by targeted therapies or preventative chemotherapeutics. Larger, carefully designed epidemiologic studies will be required to more comprehensively examine the presence and consequence of these alterations in populations affected by arsenic contamination. Since synergistic cocarcinogenicity, especially in skin and lung cancer, occurs in arsenic-exposed individuals, considerations of other environmental agents should be taken into account in these studies. Elucidation of the mechanisms underlying the initiation and promotion of carcinogenesis related to arsenic's biotransformation processes and metabolites is of foremost importance to the development of early detection and treatment regimes for affected individuals.

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