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Review Article

Mechanisms Of Heavy Metal Toxicity in The Male Reproductive System

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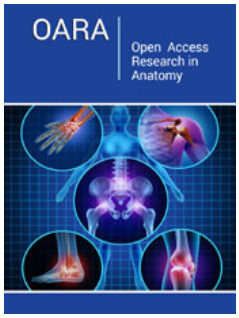

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Abstract

Heavy metals are major polluters of the environment. They disrupt the functions of the digestive system, nervous system, respiratory system, reproductive system, etc. These elements induce generation of intracellular Reactive Oxygen Species (ROS), which mediate multiple changes in the cellular behaviour by altering signalling pathways and epigenetic modifications or cause direct oxidative damages of biologically active macromolecules. Oxidative Stress (OS) is a strong molecular mechanism, which could cause different abnormalities in the DNA-molecule in the embryonic cellular lineages during the spermatogenesis, but also, production of dysfunctional fetal cells and this, to cause male infertility. Also, these metals/metalloids profoundly affect protein homeostasis and cellular viability by interference with protein forming, folding and functions processes in living cells. Misfolded proteins are cytotoxic, as they may aggregate and/or interact inappropriately with other cellular components. The purpose of the current review is to be discussed some of the possible biochemical and physiological mechanisms, by which metals/metalloids affect or contribute to the disruption of male reproductive processes. Investigations on these mechanisms are important about the correct diagnostic of different pathological processes, but also about development of appropriate therapeutic and preventive against the toxicity, cause by heavy metals.

Keywords: Heavy metals; Protein folding; Protein aggregation; Reactive oxygen species; Oxidative stress; Male infertility

Introduction

Many metals, like zinc (Zn), iron (Fe), manganese (Mn), magnesium (Mg) and copper (Cu), perform vital functions and are toxic only in cases of overdose, but other as lead (Pb), arsenic (As), cadmium (Cd) and mercury (Hg) show high toxicity to living organisms. All cells in the organism maintain metal homeostasis within sub-toxic or physiological levels, respectively, and utilize metal detoxification mechanisms. Toxic heavy metals are difficult to metabolize, respectively they can accumulate in the body, as well as combine and inhibit vital cellular functions [1]. At the cellular level, heavy metals/metalloids interfere with membrane function and nutrient assimilation, perturb protein function and activity, cause DNA damages and/or impair DNA repair mechanisms, but they also generate Reactive Oxygen Species (ROS) and elicit Oxidative Stress (OS) [2]. The toxicity of a metal depends on its physicochemical properties, but mainly on its preference for certain ligands (chemical elements/molecules donors of electrons in complex compounds). The so-called “soft” transition metals, such as cadmium and mercury, prefer sulfur as their ligand, while “hard” as chromium (Cr), Mn, but also

the metalloids As, antimony (Sb), and selenium (Se), prefer more oxygen in their higher oxidation states and sulfur in the lower oxidation states. Cobalt (Co), nickel (Ni), Fe, Pb, Cu and Zn may use oxygen, sulfur or nitrogen as ligands [2]. There is a general consensus that proteins are key targets of heavy metals. These metals have also been shown to inhibit refolding of chemically denatured proteins *in vitro*, interfere with protein folding *in vivo*, and cause aggregation of nascent proteins in living cells [3]. Their involvement in the folding of nascent or non-native proteins has a profound effect on protein homeostasis and cellular viability. Presently, there is ample evidence that metals may increase the tendency to aggregate disease-related proteins and promote the progression of some neurodegenerative diseases [4-6]. During evolution, mechanisms are formed to control the quality of proteins, which protect cells from the harmful accumulation of protein aggregates. The malfunction of these quality-control systems may result in disease or cell death [7,8]. In our previous reviews have been shown a lot of literature data about the negative influence of toxic heavy metals on human health and particularly, on the male reproductive system [9,10]. Despite this, the mechanisms of the harmful effects of these elements on the male reproductive tract and fertility are not yet sufficiently elucidated. The purpose of this review is to discuss some of the possible biochemical and physiological mechanisms by which metals/metalloids affect or contribute to the disruption of male reproductive processes.

ROS-Mediated Pathways in Heavy Metals Toxicity

ROS-production in the organism could be accelerated by different exogenous factors, as radiation, heavy metals, viruses, bacteria and their toxins, but also xenobiotics (including some drugs) [11]. Heavy metals induce the generation of intracellular ROS, which mediate multiple changes to cell behaviour by altering signalling pathways and epigenetic modifications or cause direct oxidative damage of molecules. ROS could also directly participate in the regulation of signal transduction by redox signalling, but the over-rate production of these compounds could cause the death of the cell. The most often circulating forms of ROS are these, which contain active oxygen atoms, as superoxide anions (O_2•), hydroxyl radicals (OH•), hydroperoxyl/peroxyl radicals (HOO•/ROO•), hydrogen peroxide (H_2O_2), etc. Other organic atoms/molecules may be included in the class of ROS, such as carbon-containing radicals (or lipid peroxide radicals), which are derived by removing hydrogen from the unsaturated fatty acids as the result of lipid peroxidation. Nitrogen (peroxynitrite/ONOO-, nitric oxide/NO• or nitrogen dioxide/NO_2•) and other types of free radicals have also been described [11]. Another type are the thiol radicals (•SH), which are also derived from endogenous chemical substances, containing thiol (SH-) Groups as Glutathione (GSH), formed by hydrolytic breaking of disulfide (S-S-) bridges in the protein molecules. The strong toxicity of the thiol radicals has been proposed to be due to their possibility to react with oxygen species, which often leads to the formation of additional novel free radicals. GSH acts as an important line of defense against OS. The reduced form of glutathione (GSH) is a tri-peptide (γ-glutamylcysteinyl-glycine), which performs important physiological and metabolic functions in all cells, particularly being detoxification of free radicals, metals, and other electrophilic compounds [12]. GSH is the most often circulationg source of non-protein SH-groups in the mammalian cells [13]. Normal GSH content of a cell that is imperative to maintain balance between depletion and synthesis ranges from 1 to 10mM [14]. One of the ways of toxic action of metals is based on the fact that they are all redox-elements and thus, can generate ROS and/or OS, causing cellular damages in a variety of biological systems [15,16]. OS is a common factor in about half of infertile men, illustrating the role of heavy metals in activating transduction signaling pathways to initiate protective responses or to lead to oxidative damage in cells and tissues. As it is known, ROS can inhibit the production of sulfhydryl antioxidants, damage nucleic acids and inhibit DNA repair, and initiate membrane lipid peroxidation in many organs, including in the testis [11]. Any damage in the DNA of the sperm results in the impairment of fertility and could induce men infertility, cancer, or other disadvantages in the long term [17]. It is well documented that metal-induced generation of ROS can attack polyunsaturated fatty acids (PUFA), such as phospholipids. Lipid peroxidation (LP, a chain reaction in which ROS generate more new free radicals) is a biomarker for OS since the free radicals collect electrons from lipid molecules present inside the cell membrane [18], which ultimately destroys the plasmalemma and other membrane structures. Malondialdehyde is a major aldehyde product of LP and it serves as a marker for this process. The mechanism of free radical generation is specific to the type of heavy metal.

Lead toxicity

The variety of adverse effects due to increased tissue ROS levels is mainly related to Pb exposure [16]. This metal causes toxicity in living cells by following the ionic mechanism and that of OS. Pb induces OS by promoting H_2O_2 generation [19,20]. Generally, proteins are not easily damaged by H_2O_2 and other simple oxidants unless transition metals are available. Thus, protein damaged is usually metal-catalyzed and involves oxidative scission, tyrosine cross-links, loss of histidine residues, the introduction of carbonyl groups, and the formation of protein-centered alkyl (R•), alkoxyl (RO•) and alkyl peroxyl (ROO•) radicals [21]. Epidemiological studies on the male reproductive system show a positive correlation between Pb levels in seminal plasma (ejaculate) and ROS in germ cells [22], leading to a premature course of capacitation and acrosome reaction, processes related to the fertilizing ability of spermatozoa [22-24]. For example, a study on rat sperm exposed to ROS *in vitro* has demonstrated premature acrosome reactions and reduced penetration rate in the zona pellucida of the oocyte [25]. Data from a study of rats exposed to Pb for a long time showed an increase in the concentration of lipid peroxides in the reproductive

organs, suggesting that LP is an important molecular mechanism that disrupts reproductive processes, either in hormonal stages or during spermatogenesis [26]. Pb-induced OS has been shown to have a dose-dependent effect (from low to high doses of Pb) and shows different responses in various target sites of testicular tissue, including sperm [23]. Other experiments reported an increase in lipid peroxide concentration in the reproductive organs in rats chronically exposed to Pb [26]. However, exposure to Pb leads to an increase in ROS in the cells, but at the same time to a decrease in antioxidant levels. For example, one of the antioxidants, glutathione exists in both reduced (GSH) and oxidized (GSSG) states, the reduced form of glutathione gives its reducing equivalents (H+ + e -) from the thiol groups in cysteine of ROS and thus stable. In the presence of the enzyme glutathione peroxidase, this reduced form (after donating the electron) readily binds with another molecule and forms glutathione disulfide, which is its oxidized form - GSSG. Under OS, the concentration of the oxidized form of glutathione exceeds the concentration of the reduced form, while under normal conditions, GSH represents 90% of the total glutathione content and GSSG represents 10% [18]. Nevertheless, in people with protracted exposure to Pb, increased activity of superoxide dismutase (SOD) has been observed, which suggests an adaptive mechanism against the increased amount of ROS production induced by lead [27]. In this way, the possible oxidative cellular damage in reproductive tissues is closely associated with ROS production. The mechanism of the lead-induced carcinogenic process is also postulated to induce DNA damage, disrupt the DNA repair system and cellular tumor regulatory genes through the generation of ROS. Research data show that the ROS generation by Pb is a key point in the change in chromosome structure and sequence as a result of impaired transcription when Pb replaces Zn in certain regulatory proteins [28]. Therefore, the findings of the studies indicate that Pb-induced OS is an important molecular mechanism associated with both morphological and hormonal disorders during spermatogenesis leading to male infertility.

Arsenic toxicity

The metabolism of the element as in the cells, similarly to the other heavy metal, leads to the generation of ROS in them [29]. Arsenic induces the formation of singlet oxygen [30], O^{2-}•, H_2O_2, •OH, and ROO• in different cell lines during the reduction of the molecular oxygen [30]. Under physiological conditions, the formation of ROS by arsenic lay on the oxidation of inorganic arsenite (iAs III) to arsenate (iAs V) [31]. In humans, inorganic as can be methylated to organic matter by S-adenosyl-L-methionine (SAM), including monomethyl-arsonic acid (MMA) and dimethyl-arsic acid (DMA) with trivalent (MMA III and DMA III) and pentavalent forms (MMA V and DMA V), respectively [32]. Intermediate As forms, as dimethyl arsenic peroxyl radicals, could be generated during the metabolic processing of DMA [33]. In addition, the release of redox-active Fe from ferritin is caused by methylated types of As. Other mechanisms of ROS generation, induced by as toxicity in cell activity, have also been described [34]. Arsenic induces significant ROS generation mainly through the mitochondrial (Mit) electron transport chain, inhibiting the activity of enzyme succinic dehydrogenase and thus uncoupling oxidative phosphorylation with production of O^{2-}• (which gives rise to other forms of ROS) [35], and/or by activation of enzyme nicotine adenine disphosphonucleotide (NADPH) oxidase (Nox), which also contributes to O^{2-}• generation[34]. The endoplasmic reticulum (ER) is also thought to be a source of ROS caused by DMA III [36]. Arsenic (like Pb) has been shown to bind to glutathione and several antioxidant enzymes, thus decreasing the protective capacity of cells and inducing OS [37]. According to several studies, the interference of as with cellular antioxidants as GSH, SOD, catalase and other GSH-related enzymes [38,39] indirectly result to increased ROS levels. Furthermore, As can alter signal transduction pathways (for example, the influence of extra- and/or intracellular signaling molecules to genes functions) via ROS alteration or reversible oxidation of SH-groups in proteins, which could lead to activation or inhibition of transcription factors, regulating in this way gene transcription [40]. Many studies have shown that the major ROS-affected pathways in response to as including signaling pathway, mitogen-activated protein kinases (MAPKs), microRNAs (miRNAs), tyrosine phosphorylation system, mitophagy pathway, Nrf2-antioxidant response element (ARE), nuclear factor κB (NF-κB), and activator protein-1 (AP-1) [41,42].

Mercury toxicity

Mercury (both organic and inorganic) generated ROS and affects the antioxidant defense system of the cells by connection with SH-groups (or SH-containing residues). The main mechanism of biochemical action of Hg^{2+} is connected with the strong affinity of this ion to thiol/-SH groups (with high stability constants), which are main components for the structure and functions of different biomolecules (as GSH, cysteine, metallothionein, N -acetylcysteine, S -adenosyl-methionine, albumin and other proteins, including enzymes), presenting in both extra- and intracellular membrane structures, as well as in the cellular organelles [43]. In this way, Hg alters the intracellular thiol status, leading to free radical generation and abnormal synthesis of many proteins, including such, which affect the membrane permeability, causing functional anomalies in the cells and/or cellular apoptosis [44]. Hg can cause disruption to the mitochondrial membrane potential and interrupt with intracellular calcium homeostasis. Besides that, the binding of Hg may also occur to other sites - e.g., ligands containing amino or carboxyl groups generally less favorable (with almost 10 times lower Hg binding constant) than to SH-groups. Mercury generates mainly H_2O_2 and O^{2-}•, which in the presence of redox-active transition metals are converted into highly reactive OH• radical (as, by the reactions of Fenton and Haber-Weiss) [38]. Hg has been shown to induce cellular malignant growth through the generation of free radicals, inducing OS, as well as through disruption of DNA mo-

lecular structure, or the repair and maintenance system [45]. The molecular interactions of Hg with the SH-groups of SH-containing molecules are involved in the mechanisms of transport, accumulation, and toxicity of mercury ions in the tissues, including seminiferous tubules. On the one hand, GSH neutralizes the harmful action of Hg, but it is also a major factor of the cellular protection against OS, on the other. GSH increases the antioxidant capacity of mitochondria, thus providing their protection against H_2O_2, singlet oxygen, hydroxyl radicals, and lipid peroxides generated by Hg. In this relation, differences between the exposition to the influence of Hg and the chemical nature of the Hg, accepted in the organism, have been proved. Methylmercury (Me Hg, organic form) is usually bounded to one -SH-group to form a complex with thiol-containing molecules, while Hg^{2+} (inorganic mercury) binds to two GSH molecules by sulfur atom on the cysteinyl residue of GSH molecule [46]. Additionally, GSH facilitates the formation of metal complexes via non-enzymatic reactions. Hg^{2+} mediated depletion of GSH (reduced GSH) creates an OS condition characterized by increased sensitivity of the mitochondrial membrane to iron-dependent lipid peroxidation. The depletion of mitochondrial GSH and the increase of H_2O_2 in the inner mitochondrial membrane contribute to the acceleration of the exchange of Ca^{2+} and Mg^{2+} [47], which hampers mitochondrial function. On the other hand, an increase in GSSH level leads to the progression of OS, promoting the oxidation of cellular protein cysteinyl thiols, which ultimately leads to impaired protein function. Thiol-disulfide balance in the cell regulates metabolic pathways by activating or inactivating key enzymes. Because thiol transfer reactions are bidirectional, the balance is determined by the redox state of the cell. A lot of enzymes in the antioxidative protective systems prevent the disbalance between prooxidants and antioxidants. Antioxidant enzymes such as GSH reductase (GR), GSH peroxidase (GPx), SOD, etc., containing -SH groups in their active centers, and also content main metal ions in the role of co-factors (for instance, Zn, Se, etc.), are more prone to be attacked by Hg, which ultimately leads to the cessation of their activity [48].

Cadmium toxicity

There is increasing evidence that the mechanisms, by which Cd mediates impaired male fertility, by the production of ROS (mostly OH•, HOO•, O_2• radicals, but also H_2O_2, NO•, and NO_2•) and/or by suppression of the components of the antioxidant system in the testes, leading to OS. For instance, Cd exposure of adult rats (6.5mg/kg for 5 days) increases OS, including by activated processes of peroxidation and NO• formation, and decreased GSH level, catalase, SOD, glutathione peroxidase, and glutathione reductase, thus up-regulating the expression of pro-apoptotic protein BCL-2-associated-X-protein (Bax) and tumor necrosis factor-α (TNF-α) and down-regulating the expression of the anti-apoptotic gene (Bcl_2) in the testis, leading to a decrease of cell proliferation [49]. In experiments with adult mice exposed to Cd exposure (1mg/kg, i.p., for 5 and 8 weeks), an increase in lipid peroxidation and a decrease in SOD, catalase, peroxidase, and glutathione reductase activity in the testes were observed, leading to a decrease in sperm count and an increase in of abnormal germ cells [50]. The exposure of rodents (rats and mice) on the influence of Cd increases ROS levels and decreases the activity of enzymes SOD, glutathione peroxidase and glucose-6-phosphate dehydrogenase, glutathione-S-transferase in the mitochondrion, and may cause a remarkable reduction of the expression of LC steroidogenic enzymes (down-regulation of Star and Hsd3b1 and Hsd17b3) and TE synthesis [51]. According to the results of Koizumi & Li, (1992), the exposure of rats on Cd influence remarkably increases lipid peroxidation and formation of H_2O_2 in Leydig cells, decreasing in this way glutathione reductase and catalase activities after 12 hours of treatment and inducing LC tumors later [52]. The *in vitro* system also shows that Cd induces ROS production in various testicular cells. *In vitro* SC-germ cell co-culture shows that Cd induces ROS and decreases GSH, thus causing cytochrome c release, caspase-3 activation, and Sertoli cells apoptosis. Primary rat immature Leydig cells (ILCs) exposed to Cd have shown a reduced mitochondrial membrane potential (similarly the exposure on Hg and as) and increased ROS MAPK-extracellular-regulated kinase activity, increased cell death, and a decreased transcription of Hsd3b1 [53]. Cd exposure of rat R2C tumor Leydig cells (LCs) at 10 -160μM for 24h also causes mitochondrial damage and lowers Star expression levels, which leads to inhibited steroid secretion, possibly by increasing ROS levels and decreasing SOD2 activity [54]. Decreased testicular SOD and catalase activity due to Cd-generated ROS can lead to BTB disruption, in which case the application of vitamin C can inhibit TGF-β3 activation and p38 MAPK phosphorylation and antagonize Cd-induced BTB damage [55]. In addition, experiments with murine TM3 tumor LCs, exposed to Cd influence, have shown decreased LCs viability, increased cellular apoptosis due to increased ROS production, as well as activated JNK phosphorylation and c-Jun expression, leading to activated apoptosis-associated proteins cleaved caspase 3 and cleaved PARP, but decreased BCL_2. These effects can be reversed by the antioxidant N-acetyl-L-cysteine and/or JNK inhibitors [56].

Heavy Metals Mechanism of Action with Impaired Protein and Enzyme Activity

The review of literature data showed that proteins/enzymes are preferred targets for most heavy metals. Metal ions are well known to form relatively labile monodentate and highly stable pluridentate complexes with sulfur, nitrogen and oxygen atoms of proteins. In this aspect, metal toxicity can occur in two main directions of action: one is related to the inhibition and/or blocking of the physiological activity of specific, naturally folded proteins/enzymes (associated with increased ROS/OS generation), and the other - aimed at structural changes and damage to protein molecules involved in vital cellular processes (formation of cell complexes/organelles, metabolism, DNA synthesis, cell division or proliferation, etc.). Here we can add a third direction of terminal toxicity of metals (which is a consequence of the impact of the first two), associated

with the initiation of cell death processes - apoptosis or necrosis. Besides the OS pathway, the detrimental effects of heavy metal ions are also represented through various modes of interaction, for instance, by displacing essential metal ions in metalloproteins (MT); or by catalyzed the oxidation of amino acid side chains; including their bounding to free functional groups (thiol, carboxyl, or other groups) in the protein molecules [2]. Metallothioneins are a family of low molecular weight proteins (MW in the range of 500 to 14,000 Da) rich in cysteine residues (representing nearly 30% of their constituent amino acid residues), which causes them to bind many trace elements. They are localized in the cell cytoplasm, on the membrane of the Golgi apparatus, and can bind both physiological (as Zn, Cu, Fe, etc.) and xenobiotic heavy metals (as Pb, Cd, Hg, Ag, As, etc.) via the SH-groups of cysteine [57]. In this way, MT can bind up to 7 Zn atoms, an element that is preferred in the composition of these molecules. MT, like GSH, has an important protective role against metal toxicity (metal detoxification) and OS and is involved in the regulation of Zn and Cu [58]. In humans have been established four main isoforms of MT: MT1 (with all sub-types), MT2, MT3, and MT4. Their production in the human body (mainly in the liver and kidneys) depends on the presence of nutrition minerals (Zn, Cu, and selenium), as well as the amino acids histidine and cysteine. MT biosynthesis was increased several times during OS to protect cells against cytotoxicity and DNA damage. Also, this process could be induced by appropriate agents or factors, for instance, different hormones, medicaments, alcohols, and other biologically active substances [59]. Advanced studies have revealed an additional mode of metal action that targets both naturally folded proteins/enzymes and non-folded proteins (or re-folding proteins). *In vitro*, lead and Cd, as well as Hg, effectively inhibit the folding of 55 proteins, have been proved. [60,61]. Heavy metal ions proved to inhibit very efficiently the spontaneous refolding of chemically denatured proteins by forming high affinity multidentate complexes with thiol and other functional groups (IC50 in the nanomolar range). In addition, they are just as effective in inhibiting chaperone-assisted refolding of chemically or thermally denatured proteins [62]. Misfolded proteins are cytotoxic, as they may aggregate and/or interact inappropriately with other cellular components. Protein aggregation is a general mechanism of the metal action [5]. The interference of heavy metals and metalloids in the folding process extremely affects protein homeostasis and cellular viability [63].

Lead effects

Significant effects of Pb have been found on various fundamental cellular processes like intra-, and intercellular signaling, cellular adhesion, protein folding and maturation, apoptosis, ionic transportation, enzyme regulation, the release of neurotransmitters (choline, dopamine, and GABA), etc. [64]. These effects are related to the ionic mechanism of action of lead, mainly arises due to its ability to substitute other divalent cations like Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+} (which act as cofactors) and monovalent cations like Na+ (through bivalent cations are more readily substituted) [65]. The interaction between Pb and Na also seriously impairs the normal functioning of the sodium-dependent processes in cells [13]. Pb affects the sodium ion concentration, which is responsible for numerous vital biological activities like the generation of action potentials in the excitatory tissues for the cell to cell communication, uptake of neurotransmitters (choline, dopamine, and GABA), and regulation of uptake and retention of calcium by synaptosomes [66]. After the replacement of Ca^{2+}, the Pb could cross the blood-brain barrier, as also through the blood-testis barrier (BTB). Pb^{2+}, even at very low (picomolar) concentrations, replace Ca^{2+}, thereby affecting key neurotransmitters like protein kinase C, which regulates long-term neural excitation and memory storage. The mechanisms of Pb-induced toxicity in testes also include changes in zinc bioavailability as a result of the displacement of Zn in MT molecules, leading to interference and in calcium-mediated processes, involving disruption of BTB in the area of adhesion junctions. In addition, Pb interferes with the normal metabolism of Ca in cells and causes it to accumulate in them. Pb is considered a calcium mimic and may affect a variety of systems in the organism [67]. For instance, the interference of Pb in multiple isoforms of calcium and potassium channels in human testes and sperm may be involved in the early events of acrosomal reactions [14]. Another main reason for the toxicity of Pb is its interference in the activity of various enzymes, as it binds to the SH-groups contained in them. Some enzymes activities, such as alkaline phosphatase and sodium-potassium ATPase, have been shown to be reduced in the reproductive organs of lead-exposed rats [68,69]. Also, the decreased activity of other enzymes, as d-aminolevulinic acid dehydratase (ALAD, an indicator of long-term lead exposure), which are associated with decreased seminal plasma zinc levels, demonstrates the adverse effects of lead on prostate function [70]. Lead alters blood vessel permeability and collagen synthesis [71]. Specific targets of Pb include inhibition of enzymes involved in heme production, possibly due to its accumulation in erythrocytes, and induction of inflammation in vascular endothelial cells. Pb inhibits ALAD and causes an increased concentration of the substrate aminolevulinic acid (ALA, the first compound in the porphyrin synthesis and heme synthesis, respectively) in the blood, which leads to oxidation of hemoglobin and directly causes hemolysis of red blood cells (RBC) together with the generation of hydroxyl radicals [16]. Pb also inhibits the enzyme ferrochelatase, which catalyzes the binding of protoporphyrin and Fe^{2+} (necessary for heme formation), and thus leads to disruptions in heme synthesis and production [72]. Pb also interferes with enzymes that maintain cellular membrane integrity or aid in vitamin D synthesis and DNA transcription [73]. Along with these toxic effects, lead can cause excessive production of inflammatory proteins and the development of an inflammatory process in the testicular tissue and accessorial germ glands.

Arsenic effects

Arsenic can alter the functioning of about 200 proteins/enzymes, most notably those involved in cellular energy pathways and DNA replication and repair [74]. This metal affects the mitochondrial enzymes and interrupts the production of energy [75]. The toxicity of the non-organic compounds of as has been established to be higher than this of the organic and the toxicity of the trivalent arsenic (or arsenite - As^{3+} ion) is more toxic than the pentavalent (or arsenate - As^{5+}). Arsenic through various mechanisms leads to damage to cellular respiration and, accordingly, to reduced ATP formation in cells [76]. Both As3+ and As5+ inhibit the activity of many enzymes involved in cellular metabolic pathways as the processes of glycolysis or gluconeogenesis, the citric acid cycle, and lipid oxidation [77]. Arsenate (iAsV) can replace phosphate in several biochemical reactions, while arsenite (iAsIII) and the organic (trivalent - methylated) arsenicals react with SH-groups in proteins and inhibit their activity. As (III) interacts with many proteins and is supposed to interfere with their activity, e.g., binding to β-tubulin inhibits its polymerization [78,79]. Probably, in the same way, arsenic trioxide (As2O3, ATO) inhibits mammalian thioredoxin reductase (TrxR) by direct binding to the thiol groups of the enzyme. Inhibition of TrxR leads to thioredoxin oxidation, which is one of the main SH-dependent electron donor cellular systems, thereby affecting the cellular redox environment, as well as a wide range of cellular processes [80]. It was found that this form of arsenite which, is used in cancer therapy, directly bounds to cysteine residues in the PML-RARα oncoprotein that is expressed in patients suffering from acute promyelocytic leukemia (APL). ATO-binding induces oligomerization and subsequent degradation of the aberrant PML-RARα fusion protein, which, in turn, inhibits the growth of APL cells, thereby curing the disease [81,82]. Arsenic can directly modulate the activity of key enzymes and hormones, causing hormonal dysregulation and impaired TE production [83]. It was found also that arsenic affects the expression of some enzymes and proteins such as glutathione peroxidase 4 (GPX4), 11β -hydroxysteroid dehydrogenase (HSD11B1), nuclear autoantigenic sperm protein (NASP), and calcium-binding and spermatid-specific protein 1 (CABS1) increased, while for others it decreases critically, e.g., scaffolding factor B1 (SAFB1), transcriptional intermediary factor 1β (TIF1β), retinol-binding protein 1 (RBP1), DnaJ homolog subfamily A member 1 (DNAJA1), Y-box binding protein 3 (YBX3), and allopregnanolone, which causes abnormal spermatogenesis in the testis due to germ cell deficiency and low testosterone levels [84].

Mercury effects

Data published to date demonstrate that different species of mercury (Hg^{2+}, $HgCH_3$/MeHg) have a higher affinity for binding to SH-groups (of cysteine/Cys residues) compared to that of Cd, As, and Pb, which also shows the higher toxicity of Hg to thiol reactivity [85], causing enzyme inactivation [86]. The binding of Hg to Cys (Hg-Cys conjugation) mediates many toxic effects of Hg, especially inhibitory effects on endogenous/exogenous molecules - enzymes and other proteins including tubulin, GSH, ion channels, transporters and therefore potentially alter normal biological function. The level of Hg in the blood (after exposure to Hg) decreases rapidly, suggesting that it is likely that Hg^{2+} in plasma binds to SH-containing proteins, such as albumin and/or MT, and is thus easily absorbed by cells [87]. A study by Li et al. (2018) showed that hemoglobin, glutathione peroxidase 3 (GPX3), selenoprotein P (SELENOP), and albumin are major Hg-binding molecules/ligands after Hg^{2+} exposure as *in vivo* (139.7-778.4mg/l $HgCl_2$) and *in vitro* (100 -1000 mg/l Hg). Transferrin, ApoA-I, ApoA-IV, and ApoE have also appeared to bind to Hg at increased *in vitro* concentrations of $HgCl_2$ [88]. Additionally, cysteine, selenocysteine, GSH, albumin, haemoglobin, as well as MT, are major binding sites for Hg *in vivo*, and except for the last two ligands, the binding takes place both Hg^{2+} and CH_3Hg^+ through Cys thiols or selenol (-SeH) groups of selenocysteine - the active centers of selenoproteins. In this way, Hg damages the tertiary and quaternary protein structure and alters the cellular function, intervenes with the process of transcription and translation resulting in the disappearance of ribosomes and eradication of endoplasmic reticulum and the activity of natural killer cells [89]. On the other hand, the pathways for Me Hg transportation may include mimicking the amino acid - methionine with utilizing the large neutral amino acid transporter (LAT1) on the cellular membrane [90]. Except for albumin, Hg binds to multiple Cys-containing enzymes (including manganese-superoxide dismutase/Mn-SOD, arginase I, sorbitol dehydrogenase, δ-aminolevulinate dehydratase, etc.), involved in different biochemical processes. Other Cys-rich proteins (representatives of 21 protein families) also could be targets for Hg, including hepcidin, G-protein-coupled receptors-targeting proteins (as oxytocin, somatostatin, interleukin-8), enzyme-coupled receptor-targeting proteins (e.g., insulin-like growth factor, epidermal growth factor), extracellular enzyme inhibitors, and antimicrobial peptides [91]. According to other examples of enzyme inhibition, Hg may also affect the presence of cofactors, for example, by binding to thiol and amide groups of coenzyme A (CoA) with the formation of the Hg-CoA complex, leading to disruption of the enzymatic reaction and altering mitochondrial β-oxidation. Supposed that, Hg similarly affects other CoA-dependent processes [92]. The data demonstrate that Hg-induce covalent modification of enzymes may impair multiple processes, including DNA replication, carbohydrate and lipid metabolism, and heme synthesis. The majority of studies have demonstrated the impact of MeHg on the enzyme activity by covalent modifications of thiol-containing biomolecules by MeHg, known as "S-mercuration" [86]. There are studies that demonstrated the involvement of Hg-SH interaction in the alteration of ion channel function. Particularly, Na+-K+-ATPase is considered as the potential target of Hg toxicity due to Hg-thiol reactivity. Hg-induced thiol-mediated inhibition of Na+-K+-ATPase activity was observed in platelets, brain, and kidneys [93]. Thiol-dependent inactiva-

tion may also be involved in the Hg-induced reduction of myosin Ca^{2+}-ATPase activity in the ventricular myocardium of the rat [94]. In addition to Na+-K+-ATPase, Hg (II) ions have been shown to reduce mitochondrial NADH -O_2 oxidase activity by a concomitant increase of the F1FO-ATPase (F-ATPase) activity through a thiol-dependent mechanism, thus providing an additional link between Hg exposure and mitochondrial dysfunction [95]. Other studies have also demonstrated that canonical transient receptor potential channels (TRPCs), namely TRPC4 and TRPC5, may also be subjected to Hg-induced modulation through binding of extracellular Cys residues, resulting in their activation [96].

Cadmium effects

Compared to other heavy metals, Cd exhibits a stronger affinity for binding to SH-containing proteins (e.g. cysteine/Cys residues) after Hg. Cadmium could also bind to glutamate, histidine, and aspartate ligands and can lead to iron deficiency [97]. On the other hand, Cd may displace zinc and calcium ions from metalloproteins and zinc finger proteins (ZNFs) [98]. ZNFs, similarly to MT, are also a numerous group of proteins with a wide range of molecular functions. They can interact with DNA, RNA, PAR (poly-ADP-ribose), and other key proteins, thus participating in the regulation of many cellular processes. For instance, ZNF-containing proteins influence the processes of gene transcription, translation, DNA repair, mRNA trafficking, signal transduction, cytoskeleton organization, epithelial development, cellular adhesion, protein folding, chromatin remodeling, and numerous other vital processes [99]. These data have suggested an important biological role of the ZNFs in the development of the organism under normal physiological and pathological conditions. The damage caused by Cd is mainly due to its interference with zinc-mediated metabolic processes in cells, possibly by molecular mimicry of Zn [67]. It has also been found that cadmium and zinc are specifically coordinated with cysteine residues and that each metallothionein molecule can bind up to 7 Cd atoms instead of Zn. Cadmium transits easily into the sperm nucleus, adhering tightly to the free SH-groups of the protamines by displacing or competing with zinc, which is normally bound to Cys residues, has been found. Cd bound in this way prevents the formation of normal disulfide bonds between protamines during the final phase of gamete maturation. The formed Cd-SH bonds are very stable and prevent the necessary decondensation of chromatin immediately after fertilization [100]. Cadmium also inhibits human thiol transferases (glutathione reductase, thioredoxin reductase, thioredoxin) *in vitro*, again by binding to Cys residues in their active sites, causing cellular damages [101]. Additionally, Cd has been shown to cause DNA damages by targeting to DNA mismatch repair system [102]. Specifically, cadmium inhibits the ATPase activity of the Msh2p-Msh6p complex [103]. It is, however, not known whether Cd binds to a specific site or displaces a critical zinc ion. According to other data, there is evidence that Cd mediates functional changes in ion transport or ion channels associated with reproductive toxicity in men. Variations in genes, coding proteins that contain plasma membrane ion channels and transporters, could affect the sensitivity to Cd. While these proteins normally regulate calcium flow, other cations, such as cadmium and lead, have also been shown to use these channels [104]. For instance, L-type voltage-dependent ion channels (L-VDCC) usually provide cellular access for calcium, with the ion selectivity determined by binding sites in the pore area of the channel [105]. Several L-VDCC isoforms exist with variation in their performing units, one of which, a1C, is testes specific [106]. Benoff et al. found that two-thirds of men with varicocele contained a splice variant in the L-VDCC a1C region (responsible for ion channel activation) and at the same time significantly higher levels of cadmium in the testes than men without variation in the range of L-VDCC a1C. After varicocelectomy, the sperm counts of patients expressing the normal L-VDCC a1C transcripts have been significantly increased, while the sperm counts of men expressing the altered transcripts have not [107]. These data show that it is possible men with calcium channel variants may be at increased risk for the severity of varicocele and infertility associated with the higher cadmium levels of the testes. It is assumed that the basic mechanism of the toxic effect of Cd on the reproductive system of mammalians is due to morphological changes and dysfunction in the blood vessels of the testis and epididymis, which makes them more permeable [108]. Cd produces these effects by causing damages in the vascular endothelium integrity of the testicular capillaries and venules, including the BTB [109]. Cd has been shown to induce changes in the expression and function of vascular endothelial cadherin (VE-cadherin), which is a calcium-dependent cell adhesion molecule, involved in the reorganization of the actin cytoskeleton, and cell-cell contacts [110]. Another molecule, ZIP8 (a specific metal ion transporter), has also been identified to enhance Cd uptake by vascular endothelial cells in the testes of mice, and its expression supports Cd-induced testicular damage [111]. In this way, cadmium can cause specific injury of the internal spermatic artery, its testicular and epididymal branches, as well as the pampiniform plexus. This mechanism may also include the cytotoxic effect of Cd on vascular smooth muscle cells (VSMCs), which are involved in pathological changes occurring in the vessel wall, especially when the metal accumulates in these cells [112]. VSMCs perform a variety of physiological functions (both contractile and synthetic) that are characterized by changes in the morphology, proliferation, and rate of migration and expression of various marker proteins [113] relevant to the normal development of the germ cells [114].

Conclusion

The current review combines the literature data of two main mechanisms of the various toxic effects of heavy metals (Pb, Mg, As and Cd) on the human organism. According to these data, almost all functions of the living organism are influenced by these elements, even in lower concentrations. According to many findings, all heavy metals could be accepted as indicators of male infertility. They also disrupt the normal process of DNA transcription, interfere

with enzymes, influencing in this way their normal activities, cause mutations, mimic hormones, thus disrupting the endocrine and reproductive systems and hence, the male fertility. The main pathway of toxicity, induced by heavy metals, is probably by generation of ROS and OS, including by activated lipid peroxidation in the cells and tissues. In this way, the continuous exposition on the influence of heavy metals (as Pb, Mg, As and Cd) caused increased concentration of the lipid peroxides in the reproductive organs, which proposes the lipid peroxidation as an important molecular pathway, leading to injuries of the processes of embryonic cellular differentiation, in particular during the spermatogenesis. It is important to be underlined the dose-dependent effect of the caused by toxic metals OS, which suggests various reactions on different targets of the testicular tissue, including the insemination capability of the male germ cells. It is becoming increasingly clear that heavy metals/metalloids profoundly affect protein homeostasis and cellular viability by interference with protein/enzyme forming and/or folding processes in living cells. This mechanism of toxicity does not only affect individual proteins, but it also results information and accumulation of toxic protein aggregates in the cells. Another main mechanism of action of the heavy metals is by their participation in different ion mechanisms, mainly by mimicry and interference with essential ions as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} (co-factors), injuring in this way the normal functions of ion-dependent processes in the cells. The changes in the functions of the ion channels due to toxic action of heavy metals decrease the activity of the enzymes mitochondrial oxidases (which is thiol-dependent), which could lead to mitochondrial disfunction and ATP-ase (energy) deficiency. Investigations on the molecular mechanisms of the negative influence of heavy metals on the normal processes and functions, but also on the formation of normal structure organization in protein molecules and initiation of their normal aggregation (in *in vivo*-conditions), thus injuring the regular functions of the cells, are very important about the precise diagnostic of pathological processes, but also for development of appropriate therapeutic and preventive strategies against the heavy metals toxicity.

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