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Mechanisms of Action of Heavy Metals, Related with Abnormal Protein and Enzyme Activity in Male Infertility Aspect

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Abstract

There is the main agreement that proteins are key targets for heavy metals. Apart from the oxidative stress (OS) pathway, the harmful effects of heavy metal ions are also represented by different modes of interaction with protein molecules. For example, by changing the functions of proteins (such as displacement of the main metal ions in enzymes and metalloproteins/MT, or oxidation of amino acids in side residues of peptide molecules), including their attachment to free functional groups (thiol, carboxyl or other groups). In addition, heavy metals can interfere with the synthesis and spatial structuring of forming proteins (by inhibiting the processes of protein folding or refolding upon transfer through cell membranes, or denaturation), causing aggregation of nascent proteins in living cells. The current review aims to discuss some of the possible biochemical and physiological mechanisms related to the protein/enzyme structure and functional activity through which metals influence or contribute to the disruption of male reproductive processes.

Key words: heavy metals, peptide molecules, protein/enzyme structure and functions, protein folding, oxidative stress, male infertility

Introduction

Proteins are the most varying molecules in the living systems, involved in all cellular processes. Each of them performs a specific biological role. Their importance in the preservation and realization of genetic information determines them among the main substances in living matter, together with nucleic acids, lipids, and carbohydrates.

About their important biological role, the abnormalities in their structure and functions underline many diseases, some of which could be fatal or connected with reproductive problems. The spatial structure of the protein molecule (three-dimensional or 3D structure) is related to the formation of the active and allosteric centers that determine the chemical reactivity and biological function of proteins [31]. It is known that for the normal catalytic activity of many enzymes is necessary metal ions for the correct folding of the peptide chain and formation of their active center. Disturbed homeostasis of the main metal ions - zinc, iron, copper, and others could be the reason for the misfolding and aggregation of proteins. Structurally changed proteins are already cytotoxic, as they can participate in the course of pathological processes in cells [28, 81, 85]. For instance, different variations are possible in the structure of the proteins' diluted forms (in which they are normally present in the living cells), as they are subjected to various effects by different other substances (from microelements to bio-active substances). At all stages of structural and functional organization, the protein molecules could be influenced by changes in pH, temperature, pressure, the concentration of certain substances (heavy metals, detergents, etc.), or ionizing radiation. Proteins subjected to similar change undergo various levels of denaturation. When homeostasis is restored, the reverse renaturation process is possible, in which the native physiologically active conformation of the protein molecule is restored [9]. In the living cell, this process is helped by other additional proteins, which are known as chaperons and chaperonins. In this aspect, metal toxicity can occur in two main directions: one is related to the inhibition and/or blocking of the physiological activity of specific, naturally folded proteins/enzymes (associated with increased free radicals or OS generation), and the other is aimed at structural changes and damages in the 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 first two), associated with the initiation of cellular death processes – apoptosis or necrosis. Besides the OS pathway, the detrimental effects of heavy metal ions are also represented through various mechanisms of interaction, for instance, by displacing essential metal ions in enzymes or metalloproteins (MPs) or by modification of some amino acids (oxidation of amino acid residues), including their binding to free functional groups (thiol, carboxyl, etc.) in the peptide molecules [54]. MTs, like glutathione (GSH), have an important protective role against metal toxicity (metal detoxification) and OS and are involved in the regulation of the balance between Zn and Cu [78]. MT biosynthesis depends on the presence of both essential trace elements and amino acids histidine and cysteine. Their production increases several times during OS to protect cells against cytotoxicity and DNA damage. This process could be induced by appropriate agents or factors, such as different hormones, medicaments, alcohols, or other biologically active substances [86]. Advanced studies have revealed an additional mode of metal action that targets both naturally folded proteins/enzymes and non-folded proteins [42, 81]. For instance, *in vitro* experiments have shown that Pb and Cd, as well as As and Hg suppress the proteins' folding [75]. 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. Furthermore, the last is just as effective in the inhibition of chaperone-assisted refolding of chemically or thermally denatured proteins [32]. In the living cells, chaperons ensure the return of

the protein conformation after damage, as well as the creation and/or degradation of protein complex structures. Most of the chaperons belong to the group of heat shock proteins (HSP) and the increased environmental temperature has been assessed as a significant increase in their intracellular concentrations. From a medical point of view, both denaturation and renaturation of the proteins are pivotal in the processes of immune protection. The non-specific immune response of the organism is expressed with increasing body temperature, and in this way is possible denaturation of the proteins in the composition of the virus particles, which causes the degradation of the last. Presently, there is ample evidence that metals may increase the tendency to aggregate disease-related proteins and promote the progression of some neurodegenerative diseases [3, 28]. During evolution, mechanisms are formed to control the quality of proteins, which protect cells against the harmful accumulation of protein aggregates. The malfunction of these quality-control systems may result in disease or cell death [32, 82]. **Figure 1** suggests some heavy metal-induced pathological mechanisms of cell damage and reprotoxicity, respectively.

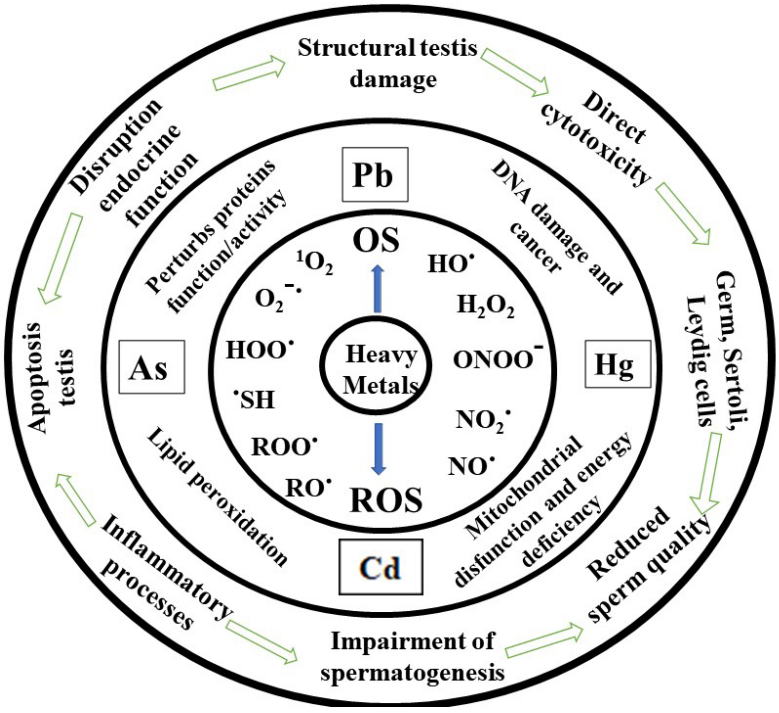

Fig. 1. Schematic review of the proposed pathogenic mechanisms of metal-induced cellular damage and reprotoxicity.

The effect of heavy metals on proteins can significantly affect protein homeostasis and cell viability. Independently of the mechanisms of influence of these metals on many proteins, which are of key importance in the germ cells development (such as β-tubulin, thioredoxin reductase (TrxR), insulin-like growth factors/IGFs, EGF, mitochondrial

enzymes, transporters, etc.), leading to the appearance of cytotoxicity, inflammatory processes and damage in the testes, serious disturbances in the endocrine functions and spermatogenesis, with a reduction in sperm quality (**Fig. 1**). Our previous reviews of heavy metals bring together abundant literature proving their negative impact on the male reproductive system and general human health [38-40]. 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 the current review is to be discussed some of the possible biochemical and physiological mechanisms related to the protein/enzyme structure and functional activity, by which metals influence or contribute to the disruption of the male reproductive processes.

Arsenic toxicity. Arsenic can alter the functioning of about 200 proteins/enzymes, most notably those involved in cellular energy pathways and DNA replication and repair [70]. This metal affects the mitochondrial enzymes and interrupts the production of energy [91]. Arsenic through various mechanisms damages cellular respiration and, accordingly, to reduced ATP formation in cells [29]. Both As^{3+} and As^{5+} 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 [37, 40]. 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 [47]. Probably, in the same way, arsenic trioxide (ATO, As_2O_3) 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 [58]. Arsenic can directly modulate the activity of key enzymes and hormones, causing hormonal dysregulation and impaired androgens (testosterone) production [40, 44]. It was found also that arsenic increased the expression of some enzymes and proteins such as selenoproteins (e.g., glutathione peroxidase 4 and selenoprotein P), 11β–hydroxysteroid dehydrogenase (HSD11B1), nuclear autoantigenic sperm protein (NASP), and calcium-binding and spermatid-specific protein 1 (CABS1), while others decreased 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 testes due to germ cell deficiency and low testosterone levels [36]. Of all the selenoproteins, glutathione peroxidase 4 (GPX4) and selenoprotein P (SELENOP) have the most significant role in male reproductive functions [6,19,23,69,73]. Moreover, GPX6 is the lone exception and a selenoprotein in men [19]. *GPX4* is distinctly expressed in testes and has both an antioxidant as well as a structural role [23]. It is believed that in the early stages of spermatogenesis, GPX4 protects developing germ cells from DNA damage caused by oxidative stress, but in the later phase ensures the integrity of the middle part of the sperm, becoming a structural component of the mitochondrial membrane, enveloping the flagellum, which is the basis of the stability and motility of sperm [6,74]. It has been also found that in humans GPX4 is abundantly distributed in late spermatocytes, and spermatids, and localized in the sperm midpiece, particularly

in the mitochondria [41]. SELENOP serves as a transport protein for Se and is also expressed in vesicle-like structures in the basal region of the Sertoli cells (SCs), and also *Selenop* mRNA was expressed in Leydig cells (LCs) of rats [66]. In addition, selenogins (including GPX1 and GPX3) are found in the epididymal epithelial and sperm [65]. Decreased levels and/or inactivation of these proteins be able to lead to severe disorders of spermatogenesis.

Lead toxicity. Literature data show that lead has significant effects on various vital cellular processes such as folding and maturation of proteins, enzyme regulation, ion transport, internal and intercellular signaling, cell adhesion, apoptosis, neurotransmitters release, and others [24]. These effects are related to the ionic mechanism of action of lead, with its ability to replace other bivalent cations such as Mg^{2+}, Fe^{2+}, Ca^{2+}, and Zn^{2+} (which act as cofactors), and monovalent cations such as Na^{+}, despite their more difficult replacement [22, 57]. The interaction between Pb and Na also seriously impairs the normal functioning of the sodium-dependent processes in cells [11]. The effect of Pb on the concentration of Na^{+} can lead to the disruption of important biological processes, such as the generation of action potentials in the excitatory tissues for the cell to cell communication, including the uptake of neurotransmitters (choline, dopamine, and GABA) and regulation of uptake and calcium retention by synaptosomes [11]. After the replacement of Ca^{2+}, Pb could cross the blood-brain barrier, as also through the blood-testis barrier (BTB). Pb^{2+}, even at very low (picomolar) concentrations, replaces Ca^{2+}, thereby affecting key neurotransmitters like protein kinase C, which regulates long-term neural excitation and memory storage [23]. 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 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 [12]. 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 [7]. 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. The influence of lead, in the reproductive organs of rats, have been reduced the activities of some enzymes such as alkaline phosphatase and sodium-potassium ATPase [4, 83]. Also, the decreased activity of other enzymes, such as δ-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 [60].

Lead alters blood vessel permeability and collagen synthesis [62]. 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 [83]. 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 [1,67]. 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 [45, 75]. Pb also interferes with enzymes that maintain cellular membrane integrity or aid in vitamin D synthesis and DNA transcription [87]. 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.

Mercury toxicity. Different forms of mercury (Hg^{2+}, CH3Hg/MeHg) have shown higher affinity to SH-groups (of cysteine/Cys residues) compared to Cd, As, and Pb, which suggests higher toxicity of Hg to thiol reactivity, causing enzyme inactivation [2, 18, 43, 56, 88]. Hg-Cys conjugation mediates many toxic effects on various endogenous/exogenous peptide molecules, potentially altering their normal biological function. For example, the inactivation of manganese superoxide dismutase/Mn-SOD, arginase I, sorbitol dehydrogenase, δ-aminolevulinate dehydratase, etc. [2]. Many studies have demonstrated the impact of MeHg on enzyme activity by covalent modifications of thiol-containing biomolecules, known as "S-mercuration" [88]. On the other hand, pathways of MeHg transport through the cell membrane may involve mimicking the amino acid methionine by the sodium-independent exchanger large neutral amino acid transporter (LAT-1) [18]. LAT-1 is found in the brain, testes, and placenta, and mediates the transport of large neutral amino acids (such as tyrosine) and thyroid hormones (triiodothyronine) through the cell membrane [25]. There is evidence that glutathione peroxidase 3 (GPX3), selenoprotein P, albumin, and hemoglobin are the primary Hg-binding molecules/ligands after Hg exposure *in vitro* (100-1000 mg/l Hg) and *in vivo* (139.7-778.4 mg/l HgCl2) [56]. Transferrin, as well as ApoE, ApoA-I, and ApoA-IV have also appeared to bind to Hg at increased concentrations of $HgCl_2$ *in vitro*. Transferrin (synthesized by SCs) is involved in the transport of iron ions (iron shuttle system) necessary for the normal development of germ cells, and inactivation of this transporter may be the cause of impaired spermatogenesis. The levels of seminal transferrin, are proportional to sperm production in humans and may be an effective indicator of Sertoli cell function [80]. Additionally, albumin, cysteine, selenocysteine, GSH, hemoglobin, as well as MT, are major binding sites for Hg *in vivo*, and except for the last two ligands, both Hg^{2+} and CH_3Hg^+ bind through Cys thiols or selenol (–SeH) groups of selenocysteine - the active centers of selenoproteins [43]. Other Cys-rich proteins like G-protein-coupled receptors-targeting proteins (interleukin-8, somatostatin, oxytocin), enzyme-coupled receptor-targeting proteins (insulin-like growth factors, epidermal growth factor), extracellular enzyme inhibitors and antimicrobial peptides could also be targets for Hg [53]. For instance, the insulin growth factor family (insulin, insulin-like growth factors - IGF1 and IGF2, and their insulin receptors/IGF1R), provide essential signals for the control of growth, metabolism, and reproductive functions (during embryogenesis, SC proliferation, germ cell proliferation/differentiation and steroidogenesis) [14,30]. In the testis, IGFs act in an autocrine-paracrine manner [30], and IGF1R mediates the effects of follicle-stimulating hormone (FSH) via the PI3K/AKT pathway [14].

Mercury may also influence the cofactors, including by binding to thiol groups of coenzyme A (CoA) with the formation of the Hg-CoA complex, leading to altering mitochondrial β-oxidation and enzyme reactions disruption [27]. CoA is a metabolite of pantothenic acid/Vit. B5, which is essential for many key cellular processes, including energy production, and lipid and amino acid metabolism [79].

In experiments with mice, enzyme acyl-coenzyme A (CoA) synthetase (ACSL) 6 preferentially converts long-chain polyunsaturated fatty acids/LCPUFAs into LCPUFA-CoA, thus contributing to the local accumulation of LCPUFA-containing phospholipids in spermatids, which is important about the normal spermatogenesis [76]. The involvement of the Hg-SH interaction in altering the function of the Na^+-K^+-ATPase ion channel, which appears to be a potential target of Hg toxicity, has been demonstrated [50]. Na^+-K^+-ATPase is a ubiquitous plasma membrane enzyme that uses ATP hydrolysis to regulate cellular Na^+ and K^+ levels and fluid volume. This enzyme is also proved in the seminiferous and epididymal epithelium of rats of various ages, and it has been associated particularly with the borders of SCs (on the apical and lateral SC membrane and of junctional specializations), and this distribution continues until spermatids present in the epithelium. Furthermore, Na^+-K^+-ATPase is found in the excurrent and efferent ducts in the testes of immature and mature rats [13]. As the Na^+-K^+-ATPase-bound Hg(II) ions cause a decreased activity of mitochondrial NADH-O_2 oxidase accompanied by F1FO-ATPase/F-ATPase activation, the thiol-dependent mechanism could also confirm the link between Hg exposure and mitochondrial dysfunction [63]. This defines Na^+-K^+-ATPase as a potential biomarker for male infertility [55]. Thiol-dependent inactivation may also be involved in the Hg-induced reduction of different Ca^{2+}-ATPase isoforms [61]. Ion homeostasis is determined by Na^+-K^+-ATPase, and Ca^{2+}-ATPase, which are the integral enzymes found within the plasma membrane of most cells, including sperm [84]. A considerable variety of tissue-specific functions of the plasma membrane Ca^{2+}-ATPase (PMCA) [77] (and of different tissue-specific subtypes/isoforms) associated with the regulation of normal physiological cell functions have been assessed. This enzyme has been established to be localized in sperm flagella membranes [89] and interacts with the sperm-activating and attracting factor/SAAF, which amplifies the ATPase activity of PMCA. The impairment of Na^+ K^+-ATPase, PMCA4, and their isoforms has been proven to lead to decreased sperm motility. Ca^{2+}-ATPase has also been proven as a calcium pump, which is responsible for the support of Ca homeostasis and in the initiation of spermatozoa motility and acrosome reaction [55]. Hg exposure is also proved to inhibit the action and/or activation of other important ion channels as transient receptor potential channels (TRPCs) by Hg ions binding to their extracellular Cys residues [72]. TRPCs are integral membrane proteins, performing the role of membrane ion channels, important in the mediation of spermatozoa Ca^{2+} transport, thus regulating Ca homeostasis and supporting vital sperm functions (motility, chemotaxis, thermotaxis, capacitation, acrosome reaction, etc.) [52]. In the spermatozoa of patients with asthenozoospermia (associated with varicocele), altered activity of the TRPC5 was found, accompanied by reduced SOD activity and cellular motility [90].

Cadmium toxicity. Cadmium could also bind to glutamate, histidine, and aspartate ligands, thus leading to iron deficiency [16]. On the other hand, Cd may displace Zn and Ca^{2+} from metalloproteins and Zn finger proteins (ZNFs) [21, 33]. ZNFs, similarly to MPs, are a numerous group with very diverse functions. They can interact with DNA, RNA, and other key molecules, thus influencing the regulation of many cellular processes as for instance, gene transcription, translation, DNA repair, mRNA trafficking, signal transduction, cytoskeleton organization,

epithelial development, cellular adhesion, protein folding, chromatin remodeling and numerous other vital processes [15, 52]. These data suggest important biological roles of the ZNFs in the development of the organism under normal physiological and pathological conditions. The damages caused by Cd are mainly due to its interference with Zn-mediated metabolic processes in cells, probably by molecular mimicry of Zn [12]. It has also been found that Cd and Zn are specifically coordinated with cysteine residues and that each MPs molecule can bind up to 7 Cd atoms instead of Zn [68]. Cd has been found to transit easily into the sperm nucleus, adhering tightly to the free SH groups in the protamines by displacing or competing with Zn which is normally bound to Cys residues. 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 [37]. Cd also inhibits *in vitro* human thiol transferases (thioredoxin reductase, thioredoxin, glutathione reductase), again by binding to Cys residues in their active sites, causing cellular damage [17]. Furthermore, Cd has been shown to cause DNA damage by influencing the DNA mismatch repair system that is, by inhibiting the ATPase activity of the Msh2p-Msh6p complex. It is, however, not known whether Cd binds to a specific site or displaces a critical Zn ion [5, 46]. 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 containing plasma membrane ion channels and transporters, could affect the sensitivity to Cd. While these proteins normally regulate Ca^{2+} flow, other cations, such as Cd and Pb, have also been shown to use them [48]. For instance, L-type voltage-dependent ion channels (L-VDCC) usually provide cellular access for Ca, and the ion selectivity is determined by binding sites in the pore area of the channel [35]. Several L-VDCC isoforms exist with variations in their performing units, one of which, a1C, is testes specific [26]. According to Benoff et al. (2005), 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 Cd in the testes than men without variations in the range of L-VDCC a1C [8]. These data suggested the possibility men with Cd-channel variants are at increased risk for severe varicocele and infertility, associated with the higher Cd 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 [59]. Cd produces these effects by causing damage to the vascular endothelium integrity of the testicular capillaries and venules, including the BTB [64]. 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 [49]. 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 [10, 34]. In this way, Cd can cause specific injury to 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. VSMCs perform a variety of physiological functions (both contractile and synthetic) that are characterized by changes in the morphology, proliferation, rate of migration, and expression of various marker proteins [20, 71] relevant to the normal development of the germ cells.

Conclusion

The current review combines the literature data about the mechanisms of influence of metal ions on the protein molecules, but several concrete mechanisms are particularly underlined: the metal ions either replace Zn and other essential metal ions in metal-dependent proteins or bind to free thiol and other functional groups of certain native proteins. These mechanisms not only affect individual proteins but also lead to the formation and accumulation of toxic protein aggregates in the cells. Possible consequences of protein folding inhibition by heavy metal ions could be expressed in deficiencies in the amounts of the affected proteins, in disruption of the normal activity of enzymes, as well as of the normal process of DNA transcription, in the initiation of mutations, in imitation of hormones, thus disrupting the endocrine and reproductive system, and leading to male infertility. In such conditions the homeostasis of the cellular proteins could be affected, including some key proteins, participating in many subtle long-term changes in the germ cells during spermatogenesis. Another main mode of action of the heavy metals is by their participation in different ion mechanisms, mainly by mimicry and interference with essential ions such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, and Cu^{2+} (co-factors), injuring in this way the normal functions of ion-dependent processes in the cells. So, the toxic action of heavy metals decreases the activity of the enzymes mitochondrial oxidases (which are thiol-dependent), which could lead to mitochondrial dysfunction and ATP-ase (energy) deficiency. Studies on the induced by heavy metals mechanisms leading to cytotoxic effects in the organism are crucial not only for the development of appropriate therapeutic strategies but also for clarifying the pathological process diagnosis.

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