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Review Articles

Free Radicals and Oxidative Stress as the Main Mechanism of Heavy Metal Toxicity in the Male Reproductive System

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Heavy metals and metalloids such as lead, arsenic, mercury, and cadmium are dense elements with potential toxicity, widespread in the environment. One of the ways of toxic actions of these metals is related to their ability to generate reactive oxygen species (ROS) and oxidative stress (OS), causing cell damage, inflammatory processes, and apoptosis in various biological systems. The purpose of this review is to discuss ROS/OS, as one of the main factors and mechanisms by which metals/metalloids influence or contribute to the appearance and development of pathological processes in the male reproductive system. Studies on the mechanisms of heavy metals associated with augmentation ROS production, as well as their complex effect on the male reproductive system (in particular on spermatozoa), are essential in elucidating male infertility.

Keywords: Heavy metals/ Pb, Cd, Hg, As, reactive oxygen species, oxidative stress, male infertility

Introduction

The production of reactive oxygen species (ROS) in the organism can be accelerated by various exogenous factors, such as radiation, heavy metals, bacteria, and their toxins, viruses, or xenobiotics (including some drugs). 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. 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 [39]. Toxic heavy metals are difficult to metabolize, respectively they can accumulate in the body, as well as combine and inhibit vital cellular functions [4]. At the cellular level, heavy metals/metalloids interfere with membrane function and nutrient assimilation, perturb protein function and activity, cause DNA damage, and/or impair DNA repairs mechanisms [39]. On the one hand, heavy metals can directly cause oxidative damage to biomolecules, but on the other indirectly, through the action of the intracellular ROS induced by them, which through changes in signaling pathways and epigenetic modifications mediate multiple abnormal changes in cellular behavior.

The most often circulating forms of ROS are these, which contain active oxygen atoms, as superoxide anions ($O_2^{\cdot}$), hydroxyl radicals ($OH^{\cdot}$), hydroperoxyl/peroxyl radicals ($HOO^{\cdot}/ROO^{\cdot}$), hydrogen peroxide (H_2O_2), etc [27]. 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 [59]. Another type is 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. All cells in the organism maintain metal homeostasis within physiological or sub-toxic levels, respectively, and utilize metal detoxification mechanisms. Glutathione is the most common circulating source of non-protein SH-groups in mammalian cells [45]. The reduced form of 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 [22]. GSH is one of the main cellular factors that act on the first line of defense against oxidative stress (OS). Normal GSH content of a cell that is imperative to maintain a balance between depletion and synthesis ranges from 1 to 10 mM [8].

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 damage in a variety of biological systems [30, 50]. 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. ROS not only damages nucleic acids and inhibits DNA repair, and/or initiates membrane lipid peroxidation, but ROS can also inhibit the production of sulfhydryl antioxidants, as well as cause inflammatory processes in many organs, including the testes [27]. 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 [2]. 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 generates more new free radicals) is a biomarker for OS since the free radicals collect electrons from lipid molecules present inside the cell membrane [23], which ultimately destroys the plasmalemma and other membrane structures. Malondialdehyde (MDA) 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.

Our previous reviews have shown a lot of literature data about the negative influence of toxic heavy metals on human health particularly, on the male reproductive system [27, 28]. 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 ROS/OS, as one of the main factors and mechanisms by which metals/metalloids influence or contribute to the appearance and development of pathological processes in the male reproductive system.

Lead toxicity. The variety of adverse effects due to increased tissue ROS levels is mainly related to Pb exposure [50]. 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. [47, 61]. Generally, proteins are not easily damaged by H_2O_2 and other simple oxidants unless transition metals are available. Thus, protein damage 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 [19]. Epidemiological studies on the male reproductive system show a positive correlation between Pb levels in seminal plasma (ejaculate) and ROS in germ cells [36], leading to a premature course of capacitation and acrosome reaction, processes related to the fertilizing ability of spermatozoa [26, 32, 36]. 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 [26]. 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 [44]. 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 [26]. Other experiments reported an increase in lipid peroxide concentration in the reproductive organs in rats chronically exposed to Pb [44]. 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 is 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% [23]. 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 [32]. 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, and 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 [57]. 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.

Cadmium toxicity. The main manifestation of toxicity through which Cd causes tissue damage in the organism is its ability to produce ROS to a very large extent (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. Through the mechanism of accumulation of free radicals and OS, cadmium can be the cause of male infertility associated with damaged testicular tissue (including Sertoli cells/SCs and blood-testis barrier/BTB) and spermatogenesis, decreased cell proliferation, morphological abnormalities in germ cells, decreased number and motility of spermatozoa, and damage to Leydig cells (LCs) with reduced TE synthesis [28]. For example, a characteristic feature of Cd intoxication is increasing OS through activating processes of peroxidation and NO• formation, leading to lower levels of antioxidants in the testis, including SOD, peroxidase, catalase, GR, and GPx [2, 10, 21, 49]. On the other hand, studies by Elmallah et al. (2017) show that nuclear factor erythroid-derived 2-like 2 (NFE2L2 or NRF2) is significantly reduced, while heme oxygenase (HMOX1) is significantly increased in the testis tissue of intoxicated with Cd rats [21]. NFE2L2 is known to be a transcription factor that can regulate the expression and activity of antioxidant proteins (or elements of the antioxidant response) that protect cells from oxidative damage. HMOX1 is also a protein with a cytoprotective effect against induced OS and probably its increased expression, in this case, is related to the adaptive response of cells to the toxic action of Cd. These authors have also established increased levels of tumor necrosis factor-α (TNF-α) together with the noted decreased content of proliferating cell nuclear antigen (PCNA) in germ cells on the influence of Cd. PCNA is a nuclear protein that is involved in replication and DNA repair mechanisms and is involved in the process of cell proliferation. In addition, PCNA in the testes is used as a proliferative marker to quantify the effectiveness of spermatogenesis. Moreover, through the mechanism of OS, Cd indirectly reduces cell proliferation by increasing the expression of the proapoptotic BCL-2-associated-X-protein/Bax and tumor necrosis factor-α (TNF-α) but decreases the expression of the antiapoptotic gene/Bcl2 in the testis. Thus, Cd-induced oxidative damage to testicular tissue is accompanied by depletion of active DNA content in dividing spermatogenic cells [21, 15]. Furthermore, ascorbic acid (Vit. C) content is also significantly declined in the testes of Cd-exposed mice [2,10]. The injured prooxidant-antioxidant balance leads to injuries in the membrane structures (plasmalemma, cell-cell contacts, etc.) and cellular organelles (mitochondria or microsomes), but also in the spermatogenic epithelium of the seminiferous tubules, abnormal function of SCs and germ cells with decreased sperm count and increased sperm abnormality [2]. Mahmoudi et al., (2018) have also confirmed the availability of increased ROS production and significantly increased levels of malondialdehyde (MDA) in the testicular tissue with decreased numbers of spermatogonia, SCs, and LCs, but also decreased sperm motility and count, as well as inhibition of TE synthesis in rats treated with cadmium chloride. In fact, Cd exposure causes atrophy and swelling of the tubules, the height of the germ layer decreases (respectively reduces the diameter of the seminal tubule), and many spermatogonia cells

are lost. The authors have also revealed ameliorated sperm defects in cadmium chloride intoxicated rats after experimental application of green tea and reduced effects of ROS by the protection mechanism of green tea [43]. According to another study, generated ROS and increased MDA, but decreased testicular antioxidant activity (SOD, catalase, and ascorbic acid) lead to BTB disruption. However, the results of this study show that high doses of ascorbic acid can protect BTB destruction via suppressed Cd-induced OS (by decreasing the levels of ROS and MDA) and by inhibiting the TGF-β3/p38 MAPK signaling pathway in the testis of Cd-exposed rats [10].

Through the mechanism of OS, cadmium could cause degeneration of Leydig cells and to inhibit the testicular steroidogenesis [38, 49]. In the investigation of OS in the testis of adult male rats exposed to Cd-acetate is established that Cd generated ROS by elevating testicular MDA and decreased activities of the antioxidant enzymes SOD, catalase, glucose 6 phosphate dehydrogenase, and glutathione-S-transferase in the mitochondrial and/or post-mitochondrial fractions. Thus, the activities of LC steroidogenic enzymes 3β and 17β-hydroxysteroid dehydrogenase (Hsd3b1 and Hsd17b3) are also remarkably reduced, leading to altered TE production [49]. In other experiments with rats, tumors were found in Leydig cells as a result of a significant increase in lipid peroxidation and H_2O_2 formation and a decrease in the activity of antioxidant enzymes (catalase and GR) in LCs, after prolonged Cd treatment [38]. According to the results of Khanna et al, (2016), LCs of rats, *in vitro*-exposed to Cd, have had a simultaneous increase in the intracellular calcium (Ca^{2+}) and reduced mitochondrial membrane polarization, followed by significant induction of ROS and MAPK–extracellular-regulated kinases with concurrent GSH depletion and cell death (both necrotic and apoptotic), as well as decreased transcription of Hsd3b1 [34]. A similar *in vitro* study of SC-germ cell co-culture found that free radicals produced by Cd reduced GSH and caused cytochrome c release, caspase-3 activation, and SC apoptosis [34].

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 [63]. Arsenic induces the formation of singlet oxygen ($^1O_2$), $O_2^{-\bullet}$, H_2O_2, $^{\bullet}OH$, and $ROO^{\bullet}$ in different cell lines during the reduction of the molecular oxygen [54]. Under physiological conditions, the formation of ROS by arsenic lay on the oxidation of inorganic arsenite (iAs III) to arsenate (iAs V) [16]. 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 [1]. Intermediate As forms, such as dimethyl arsenic peroxyl radicals, could be generated during the metabolic processing of DMA. 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 [20]. 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 the production of O_2^{-}• (which gives rise to other forms of ROS) [13], and/or by activation of enzyme nicotine adenine disphosphonucleotide oxidase/Nox, which also contributes to O_2^{-}• generation [20]. The endoplasmic reticulum is also thought to be a source of ROS caused by DMA III [46].

Arsenic (like Pb) has been shown to bind to glutathione and several antioxidant enzymes, thus decreasing the protective capacity of cells and inducing OS [59]. According to several studies, the interference of As with cellular antioxidants such as GSH, SOD, catalase and other GSH-related enzymes [11,48] indirectly results in increased ROS levels. Furthermore, As can alter signal transduction pathways (for example, the influence of extra- and/or intracellular signaling molecules on 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 [52]. Many studies have shown that the major ROS-affected pathways in response to As including signaling pathways, 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) [17, 60].

These results suggest that oxidative stress generated by As and As compounds (like many other heavy metals) can damage testicular tubules, leading to reduced cell proliferation, and/or could cause gonadal dysfunction through reduced testosterone synthesis, apoptosis, and/or necrosis. Monomethylated and dimethylated arsenicals increase the production of ROS and OS associated with many cytotoxic and genotoxic effects, including oxidative DNA damage and chromosomal aberrations [37, 18]. Methylated trivalent metabolites are highly reactive and are more potent inhibitors of GSH reductase and thioredoxin reductase compared with arsenite or pentavalent metabolites [58]. In the Japanese eel, low doses (0.1 ~ 1 μM) of arsenic have been shown to inhibit spermatogenesis by suppressing steroidogenesis, and at high doses (100 μM), As mediates OS and induces germ cell apoptosis [12]. At 10 μM arsenic trioxide (As_2O_3) effectively induced ROS cytotoxicity and apoptotic cell death in murine TM4 Sertoli cells. *In vivo* studies have shown that oral exposure to inorganic As causes dysfunctions in spermatogenesis, decreased testosterone and gonadotropins, but also impaired steroidogenesis [35]. Additionally, in male rats, exposed to sodium arsenite, have been assessed decreased weights of the testes and of the accessory sex glands, but also reduced epididymal sperm counts [31]. Experiments with mice exposed to As have also shown dose-dependent gradual reductions in seminiferous tubular diameter and various gametogenic cell populations, such as resting spermatocytes, pachytene spermatocytes, and elongated spermatids [55]. It has also been found that the lack of transcriptional intermediary factor 1β, a key molecule associated with heterochromatin structures in Sertoli cells and round spermatids, as well as the formation of meiotic chromosomes [62], leads to a significant defect in spermatogenesis associated with failure to release spermatids and testicular degeneration [25, 29]. Other studies complete the toxic effects of arsenic on male fertility concerning inhibition of steroidogenesis and sperm maturation [35].

Mercury toxicity. Mercury (both organic and inorganic) generated ROS and affects the antioxidant defense system of the cells by connection with thiol groups (or SH-containing residues). The main mechanism of biochemical action of Hg^{2+} is connected with the strong affinity of this ion to SH-groups (with high stability constants), which are the 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 [51]. 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 [42]. Hg can cause disruption to the mitochondrial membrane potential and interrupt with intracellular calcium homeostasis. Besides that, the binding of Hg may also occur in other sites - e.g., ligands containing amino or carboxyl groups are generally less favorable (with almost 10 times lower Hg binding constant) than to SH-groups. Mercury generates mainly H_2O_2 and $O_2\bullet^-$, which in the presence of redox-active transition metals are converted into highly reactive OH• radical (by the reactions of Fenton and Haber-Weiss) [59]. Hg has been shown to induce cellular malignant growth through the generation of free radicals, inducing OS, as well as through disruption of DNA molecular structure, or the repair and maintenance system [14]. 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 in 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 of the influence of Hg and the chemical nature of the Hg, accepted in the organism, have been proved. Methylmercury (MeHg, 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 [7]. 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+} [41], which hampers mitochondrial function. These mechanisms cause membrane lipid peroxidation, leading to dysfunction and abnormal morphology of spermatozoa, but also to oxidative damages in their DNA, decreased mobility, and abnormal acrosome reaction [5]. The injured membrane integrity of the cell could also lead to increased membrane permeability and decreased compatibility to regulate the intracellular concentrations of ions, which are responsible for the control of the male germ cell's movement [6]. 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 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 [40]. The data have shown a disbalance between the prooxidant and antioxidant systems, which could cause a significant reduction of the activity of two main antioxidant enzymes in the testis – SOD and catalase, as well as

increased lipid peroxidation, leading to OS in exposure to Hg and/or its compounds in animals (mice, rats, etc.), [53, 56]. Hg has also been proved to cause strong interstitial oedema and vasodilatation in the testes of the individuals, exposed to its influence, which could lead to suppressed spermatogonia differentiation or induce apoptosis of these cells, but also to spermatocyte degeneration and degradation of the embryonic epithelium in the seminiferous tubules in rats [3, 9].

According to many review publications, the intoxication with Hg and/or its compounds, together with the induced OS besides affecting the spermatogenesis, also leads to abnormal steroidogenic functions (androgens deficiency) in the testes of male experimental animals and men [33]. Because LCs are the main testosterone-producing source in the testes, Hg intoxication modulates the functions of these cells by reducing the production of steroid hormones in the treated animals [24].

Conclusion

The data presented in the current review show that the main pathway of heavy metal toxicity is the induction of ROS, increased lipid peroxidation, and induction of OS in the reproductive organs. Together with their general properties, various types of free radicals influence various specific mechanisms, which distinguishes them from one another, and hence, their investigation could be useful for the development of easily-applying methods for laboratory analyzes and technologies for assessing these metabolic products in different biological materials (tissue probes, body fluids, etc.). Oxidative stress is a powerful mechanism that can lead to DNA damage in the germ cell lines during spermatogenesis, to the production of dysfunctional germ cells, and ultimately to male infertility. The diagnosis of abnormalities in the spermatogenesis process as a result of excessive ROS production is an important step in determining the etiology of male infertility and the development of appropriate therapeutic strategies.

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