Common Mechanisms in Nephropathy Induced by Toxic Metals

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Key Words

Apoptosis • Cadmium • Cisplatin • Heavy metals • Lead • Mercury • Necrosis • Nephrotoxicity

Abstract

Various metals of unknown function in the body (Cd, Cr, Hg, Pb, U), trace elements in excessive concentrations (Co, Cu, Fe, Zn), or metals used in cancer therapy (Pt, V), accumulate in the mammalian kidney, largely in the proximal tubule (PT) cells, and cause functional and structural damage that results in reabsorptive and secretory defects. The intracellular mechanisms of their toxicity in the PT cells are not well known. Recent studies have indicated an oxidative stress with associated lipid peroxidation, apoptosis, and necrosis as common phenomena in the course of nephrotoxicity of these metals. However, a number of other phenomena, such as the selective inhibition and/or loss of various membrane transporters, enhancement of ion conductances, increased cytoplasmic concentration of calcium, deranged cytoskeleton and cell polarity, impaired endocytosis, swelling and fragmentation of mitochondria, increased expression of metallothionein, heat-shock and multidrug resistance proteins, loss of cell membrane integrity, as well as the damage of mitochondrial and genomic DNAs have been fragmentarily demonstrated for the action of some toxic metals, but their importance for the course of nephrotoxicity and the sequence of events in relation to oxidative stress, apoptosis, and necrosis have not been clearly established. Recent studies of metal toxicity in various tissues and cells of non-renal and renal origin enable us to estimate 'causes and conse-

quences' of various phenomena in the metal-induced nephrotoxicity, and to assemble them in a possible common, time-related sequence.

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Introduction

The mammalian kidney is a structurally and functionally complex organ that plays an important role in control and regulation of homeostasis with various reabsorptive, secretory, metabolic and endocrine functions. Failure to perform these functions is manifested in reabsorptive and secretory defects along the nephron, which in cases of limited malfunctions result in a small molecular weight proteinuria, in more severe cases exhibit also polyuria, glucosuria, aminoaciduria, phosphaturia, and increased excretion of electrolytes, as well as an elevated blood urea nitrogen and creatinine, while in most severe forms, a generalized damage to the kidney functions manifests as the Fanconi syndrome [reviewed in 1]. Whereas some of the functional defects of the nephron are related to the inherited malfunctions of specific genes and/or proteins, a significant number of such cases, particularly in the adult human population, are induced by toxic damages to the nephron following acute (rare event) or chronic (widespread) exposure to various environmental and/or occupational (toxic metals, mycotoxins, pesticides, aristolochic acid, herbicides, etc.) or chemotherapeutic substances (some toxic metals, various drugs used in medicine).

A variety of metals is nephrotoxic. Some, such as cadmium (Cd), hexavalent chromium (Cr), mercury (Hg), lead (Pb), and uranium (U), have no known functions in the body, whereas some others, like copper (Cu), cobalt (Co), and iron (Fe), represent trace elements that are necessary for the function of various enzymes and other cellular proteins, and become nephrotoxic in case of excessive absorption in the gastrointestinal tract and intracellular accumulation due to inborn errors and deranged metabolism [reviewed in 2]. Here also belongs zinc (Zn), which in normal concentrations antagonizes the toxicity of other metals, but becomes nephrotoxic in case of overloading. Platinum, usually in form of cisplatin (cisPt), and vanadium (V) have been used in human medicine to treat cancers [3, 4], while V has also been experimentally tested as a promising drug to control diabetes [5].

According to the urinary data, proximal tubules (PT) seem to be the major site of metal-induced nephrotoxicity. Dependent on the severity of intoxication, functional defects are often accompanied with a plethora of structural damages in the PT epithelium, including loss of cell-cell contacts and detachment of cells from the basement membrane, blebbing, shortening and loss of microvilli, loss of basolateral invaginations, vesiculation of the cytoplasm, derangement of the cytoskeleton, swelling, vacuolation and fragmentation of mitochondria, swelling of lysosomes and whole cells, etc. [reviewed in 2, 6, 7].

The mechanisms of metal entry into the PT cells are generally poorly known, and may be different for different metals. For example, the circulated Cd-metallothionein (Cd-MT) and similar metal-protein complexes are filtered in the glomeruli, reabsorbed largely in the proximal convoluted tubules (S1 and S2 segments) by megalin/cubilin receptor-mediated endocytosis, degraded in the endolysosomal compartment, and the liberated metals are somehow translocated into the cytoplasm, possibly via proton-coupled divalent metal transporter 1 (DMT1) in the endolysosomal membranes [2, 8, 9, and references therein, 10]. Recent studies have indicated that a significant uptake of Cd, Hg, Pb, and cisPt in PT and some other mammalian cells may proceed by ionic and/or molecular homology or 'mimicry', e.g., transported as free ions through calcium (Ca^{2+}) channels or ion-transporting proteins (Ca^{2+} -ATPase, DMT1, Mn and Zn transporter ZIP8), or bound to thiol (SH)-containing amino acids and organic anions and cations (methionine, cysteine, homocysteine, N-acetylcysteine (NAC), glutathione (GSH)), that are carried by specific transporters located in the basolateral (BLM) and/or brush-border membranes (BBM) [8, 11–14]. The nephrotoxicity following

molecular mimicry of metal uptake via organic anion and cation transporters develops predominantly in the straight (S3) portion of PT, reflecting the segment-specific localization of the transporters [13].

After reaching the cytoplasm, most of the toxic metals seem to act by directly hitting various intracellular targets, whereas cisPt has to be metabolically transformed before becoming a nephrotoxin [15]. However, it is assumed that an immediate toxicity, especially during chronic intoxication with small doses of metals, is precluded by the presence of a limited abundance of pre-existing intracellular MT and other SH-rich compounds that bind and sequester some (but not all) toxic metals, and that toxicity starts after this pool of detoxifying substances becomes overwhelmed by the free metal [reviewed in 8, 11]. Although various nephrotoxic metals can directly damage the integrity of isolated renal PT cell membranes in vitro [16], it is disputable whether they can act similarly in vivo, before entering the cell.

Mechanisms of Metal Nephrotoxicity

The mechanisms of nephrotoxicity at the cellular level of various toxic metals have been studied for decades, and are still known only in fragments; some more details have been collected for the actions of Cd, Hg, and cisPt [reviewed in 3, 8, 11]. Recent studies in vivo (experimental animals) and in vitro (renal cortical slices, various cell lines of the renal origin, isolated mitochondria) have indicated oxidative stress, apoptosis, and necrosis as common phenomena in the intracellular action of all toxic metals studied thus far. A number of other phenomena, such as the inhibition of ion channels, ATPases and other transporters, enhancement of ion conductances, increase in intracellular (cytoplasmic) concentration of Ca^{2+} ($[\text{Ca}^{2+}]_i$), deranged metabolism, cytoskeleton and cell polarity, impaired endocytosis and intracellular vesicle recycling, increased synthesis of MT, upregulation of heat-shock proteins in the cell cytoplasm and multidrug resistance proteins (MDR) in the BBM, selective loss of transporters from the BBM and BLM, destabilization and/or loss of the cell membrane integrity, and distinct structural and functional damage in mitochondria have been demonstrated in fragments in the course of action of Cd, Hg, and cisPt, but their appearance with other toxic metals have not yet been reported. Also, their importance for the onset and progress of nephrotoxicity, and the sequence of these events in relation to the oxidative stress, apoptosis, and necrosis, have not been clearly

Fig. 1. A simplified scheme of the common mechanisms in metal-induced nephrotoxicity. The toxic metal (TM) can enter the proximal tubule cell via endolysosomal compartment (ELC) at the brush-border membrane (BBM) or via organic anion and cation transporters (OAT/OCT) at the basolateral membrane (BLM), or via some other, less identified mechanisms (?). In the cell, free TM directly inhibit various transport proteins in the plasma membrane (T) and endoplasmic reticulum (ER), polymerization of cytoskeleton (CS), oxidative phosphorylation in mitochondria (M), metabolic processes in peroxisomes (P) and the cytoplasm (MP), and activity of superoxide dismutase (SOD), and deplete reduced glutathione (GSH). The oxidative stress due to diminished elimination and increased production of free radicals leads to the accumulation of reactive oxygen species (ROS). These highly reactive molecules directly or indirectly (via lipid peroxidation (LP)) induce further damage to the function/permeability of various

intracellular organelles, leading to the cytoplasmic accumulation of Ca^{2+} . The elevated Ca^{2+} promotes a variety of intracellular reactions, including depolymerization of the cytoskeleton, thus contributing to the diminished endocytosis and intracellular vesicle recycling (ICVR). Synergistic action of ROS and Ca^{2+} leads to the damage of mitochondrial (M) function, organelle swelling and release of cytochrome c (CytC) and other proteins via ruptured membranes into the cytoplasm, where it promotes transformation of inactive procaspases (PROCASP) into caspases. The final, executive caspase 3 (CASP3) translocates the 'cell death message' into the nucleus (N). The cell death may proceed by apoptosis, if the mitochondrial ATP synthesis is still active (+ATP), or by necrosis, if ATP is heavily depleted (-ATP). For more details, see the text. Effects labeled with '-' indicate inhibition, whereas effects labeled with '+' indicate stimulation of the activity/process.

established. However, by comparing the data obtained in recent studies of metal toxicity in various tissues and cells of non-renal and renal origin enable us to estimate 'causes and consequences' of various phenomena that occur in the metal-induced nephrotoxicity, and to assemble them in a possible common, time-related pattern (fig. 1).

An initial, common event in the action of all toxic metals in the PT cells studied thus far seems to be generation of oxidative stress, that is manifested by: (a) depletion of intracellular antioxidants (largely GSH) and free radical scavengers (vitamins E and C), (b) inhibition of the activity of various enzymes that contribute to the metabolism and detoxication of free radicals (reactive oxygen species (ROS)), such as GSH-peroxidase, GSH-reductase, GSH-transferase, catalase and superoxide dismutase (SOD), and (c) increased production of ROS (superoxide anion radical, hydrogen peroxide, peroxy radical, hydroxyl radical, nitric oxide, peroxynitrite radical, etc.). In intact cells, a limited amount of ROS is produced in normal metabolic processes in the cytoplasm and peroxisomes, whereas a bulk of these substances is generated as a side product during oxidative phosphorylation in mitochondria [reviewed in 17–19]. In subtoxic concentrations these products may act as second messengers in some of the intracellular signal transduction pathways [reviewed in 20] or are buffered by GSH, the main scavenger of ROS in the cell cytoplasm and mitochondria, and by other intracellular antioxidants, or further metabolized by metalloenzyme SOD into less damaging molecules. However, having a high affinity for SH groups, free toxic metals can directly bind to cellular antioxidants and related enzymes, and inhibit buffering and detoxication of ROS. Some redox-active metals, such as Fe, Cu, and Cr, directly catalyze generation of hydroxyl radical from hydrogen peroxide (Fenton reaction) and other ROS [17, 18, and references therein], whereas the redox-inactive metals, such as Cd, Hg, and cisPt, diminish the ROS-scavenging capacity in the cells, but may also stimulate the generation of ROS indirectly – by displacing Fe and Cu from MT and other metal-containing cellular proteins, which then may accelerate production of ROS via Fenton reaction [21–23].

Numerous studies in non-renal experimental models of metal toxicity [18, and references therein, 24], and in various models of nephrotoxicity induced with Cd, Hg, cisPt, Cr, Co, and V [reviewed in 3, 8, 11, 25, 26], showed that the elevated concentrations of ROS affect a number of cellular ion transport pathways via: (a) modulation of the activity of various ion transport proteins by changing

(oxidizing) the redox state of intracellular space and/or directly oxidizing their functionally-important SH groups, (b) peroxidation of membrane phospholipids that could change the microenvironment necessary for optimal function of the transporters and increase membrane permeability for Ca^{2+} and other ions, (c) altering transmembrane signaling by inhibiting regulatory enzymes, and (d) decreasing cellular ATP levels by inhibiting oxidative phosphorylation in mitochondria. While some of these effects may be caused by direct binding of toxic metals to the respective proteins and phospholipids, in many models of metal (nephro)toxicity the induction of oxidative stress was thus far demonstrated only indirectly, by finding an increased abundance of peroxidized lipids, as estimated from an enhanced production of malondialdehyde and thiobarbituric acid-reactive substances in tissues and cells [3, 8, 11, and references therein]. However, recent studies have shown that mitochondria from the mammalian kidney and other organs are a direct target for Cd and some other toxic metals. Metals rapidly accumulate in this organelle, possibly via Ca^{2+} uniporter in the inner mitochondrial membrane, bind to the electron transfer proteins and inhibit oxidative phosphorylation, causing hyperproduction of ROS [24, 27–29]. Studies have also shown that in toxic conditions due to exposure to some toxic metals and other xenobiotics mitochondria are not only the major producer of, but are also the major target for ROS [reviewed in 19, 30–33]. The locally generated oxidative radicals further damage the mitochondrial function and structure by: (a) depleting the pool of matrix GSH and thus shifting the redox status of the matrix into more oxidized direction, (b) oxidizing respiratory enzymes and inhibiting their activity, (c) peroxidizing cardiolipin, a major lipid and cytochrome c-anchoring component of the inner mitochondrial membrane, (d) dissipating transmembrane potential and ionic gradients via triggering Ca^{2+} -assisted formation of mitochondrial permeability transition pore (see later), and (e) causing mitochondrial DNA mutations, fragmentations, and deletions. In most of these studies the symptoms of oxidative stress, ROS production, peroxidation of lipids, and their deleterious consequences to the cell and mitochondrial structure and function were ameliorated or prevented by treating animals/tissue slices/cell lines/isolated mitochondria with one or more free radical scavengers and/or antioxidants (GSH, NAC, melatonin, vitamins A, E and C, selenium, mannitol, aspirin, glycine, glutamine, L-carnitine, dimethylthiourea, pyruvate, lipoic or caffeic acid, Trolox, various bioflavonoids (catechin, quercetin, curcumin,

erdosteine, phytoestrogens, tannin, G-rutin)), thus indicating ROS as the crucial, initial trigger of all further damaging processes.

Although a number of ion channels, pumps, cotransporters and exchangers in the kidney tissue/cells/isolated membranes can be directly inhibited by various toxic metal or ROS, or diminished by lysosomal and proteasomal degradation [reviewed in 9, 11, 18, 26, 34], or perturbed in activity due to oxidative stress-mediated shift of the cellular redox status to a more oxidized state, an effect of these compounds upon mechanisms that control intracellular Ca^{2+} homeostasis seems to be of paramount importance to the further changes in cell function and structure. Studies have shown that the dysfunction of intracellular Ca^{2+} homeostasis is manifested as a dramatic increase in $[\text{Ca}^{2+}]_i$ caused by: (a) opening of Ca^{2+} channels in endoplasmic reticulum and plasma membranes via activation of inositol-1,4,5-triphosphate (InsP_3)-mediated cell signaling, (b) direct inhibition and/or loss of Ca^{2+} transporters (Ca^{2+} -ATPases, $\text{Na}^+/\text{Ca}^{2+}$ exchanger) in the plasma membrane and sarcoplasmic reticulum, (c) displacement of Ca^{2+} from the intracellular binding proteins, and (d) release of Ca^{2+} from the intracellular stores (various organelles including mitochondria) and its influx from the extracellular space *via* ROS-, peroxidized lipid-, and/or metal-induced increased permeability of intracellular organelles and the plasma membrane [20, 32, 35–37, and references therein]. A rapid mobilization of $[\text{Ca}^{2+}]_i$ via the InsP_3 -mediated signaling mechanism was shown for Cd and other divalent metals in cultured fibroblasts [38], while a significant mobilization of $[\text{Ca}^{2+}]_i$ in the renal tissue and cells has thus far been demonstrated for Cd, Hg, and cisPt [11, 26, and references therein, 39].

Studies in different experimental models have shown that elevation of $[\text{Ca}^{2+}]_i$ has most serious consequences for the integrity of cytoskeleton, intracellular vesicle recycling, cell polarity, and for further processes that affect mitochondrial structure and function, and in ultima linea initiate cell death via apoptosis or necrosis [30–32, 35, and references therein]. Sustained increase in $[\text{Ca}^{2+}]_i$ disrupts the cytoskeletal network and activates various Ca^{2+} -dependent catabolic enzymes, such as phospholipase A_2 and other phospholipases, proteases (calpains), and endonucleases. Ca^{2+} itself, or via Ca^{2+} -activated proteases, strongly promotes disassembly of microtubules and dissociation of actin microfilaments from their anchoring proteins in the plasma membrane. In addition, studies in cell-free systems and in various non-renal and renal cell lines have shown that Cd, Hg, Pb, Cr, Cu, and

cisPt can directly interfere with polymerization of microtubules and actin filaments, probably by binding to the SH groups in the monomeric proteins. The breakdown of the cytoskeleton network causes blebbing of the plasma membrane [40], and, as shown in our recent studies with CdMT in rats *in vivo* [6], in PT cells it causes impaired intracellular vesicle recycling, selective removal of some brush-border transporters and their translocation into the basolateral membrane, and loss of cell polarity. Similar Ca^{2+} -dependent processes may also account for the increased molecular mobility in the lipid phase and strongly increased K^+ conductance in the renal cortical BBM from Cd-treated rats [41].

Recent studies have shown that in intact cells the mitochondrial respiration and maintenance of the membrane potential are tightly regulated by the InsP_3 signaling-mediated pulsatile nature of $[\text{Ca}^{2+}]_i$ [reviewed in 42, 43]. Under physiological conditions, Ca^{2+} is accumulated in the mitochondrial matrix via a coordinated action of voltage-dependent Ca^{2+} channel in the outer membrane and the membrane potential-driven Ca^{2+} uniporter in the inner membrane, whereas Ca^{2+} efflux is largely mediated by the $\text{Na}^+/\text{Ca}^{2+}$ antiport in the inner membrane. Inside the matrix, Ca^{2+} is a positive effector of ATP synthesis via the Krebs cycle and oxidative phosphorylation; a limited increase in the matrix Ca^{2+} concentration accelerates respiration and ATP production. However, in various toxic conditions the massive and sustained overloading with Ca^{2+} becomes detrimental to the mitochondrial function and structure because it promotes additional ROS formation by structural alterations of the inner mitochondrial membrane via disorganizing the assembly of lipids (largely cardiolipin) and respiratory chain proteins, and stimulates the ROS-mediated oxidation of the SH-rich inner and outer mitochondrial membrane proteins, causing their cross-linkage and aggregation, thus accelerating formation of the non-specific, cyclophylin-mediated and cyclosporin A-inhibitable mitochondrial permeability transition pore. This pore is highly permeable to protons, water, and other ions and small molecules of $<1,500$ Da. A possible role of water channel AQP8, that is present in the inner mitochondrial membrane [44] and found to be upregulated by Cd treatment in mitochondria from the rat renal cortex [29], in these processes has not been clarified. The loss of ionic/osmotic gradients leads to dissipation of mitochondrial transmembrane potential, heavily disrupted oxidative phosphorylation and ATP synthesis, organelle swelling and rupture of its membranes, fragmentation, detachment of cytochrome c from cardiolipin and a release of

this and other mitochondrial proteins into the cytoplasm [29, 32, 34, 42, 43, and references therein].

In a variety of experimental models of metal-induced nephrotoxicity, oxidative stress and its immediate intracellular consequences described above can lead to the PT cell death by apoptosis or necrosis. Dependent on the concentration of applied toxic metal and/or extent and duration of the injury (elevation of ROS and cytoplasmic/mitochondrial Ca^{2+} concentration), the mitochondrial ATP-producing machinery may determine the apoptotic or necrotic fate of the cell; small concentrations of Cd or some other toxic metal, which produce less ROS and incompletely inhibit oxidative phosphorylation, favor apoptosis, whereas sudden exposition to high concentrations of toxic metals, or their significant intracellular accumulation after a long-term exposition to lower concentrations, may induce an oxidative injury with massive breakdown of cellular metabolism, strong increase in cytoplasmic and mitochondrial Ca^{2+} , dramatic inhibition of mitochondrial function and ATP depletion, that result in necrosis [reviewed in 9, 30–32].

Apoptosis, or programmed cells death, is initiated by the (ROS + Ca^{2+})-mediated release of cytochrome c and several other mitochondrial proteins into the cytoplasm, where in complex reactions they activate a cascade of specific proteases (caspases), including the caspase 3, that executes the final apoptotic processes in the nucleus that result in DNA and cell fragmentation [reviewed in 30–32]. Apoptosis is an energy-dependent and gene-regulated process, morphologically characterized by cell membrane blebbing, nuclear condensation and fragmentation, chromatin aggregation, coarse degradation of the genomic DNA, cell shrinkage and fragmentation into spherical apoptotic bodies, that are finally digested by adjacent cells and macrophages, without inflammation. Although apoptosis can be triggered by the processes in mitochondria (intrinsic pathway) and/or receptor-mediated signaling through death receptors on the cell membrane (extrinsic pathway), and both of them were demonstrated in some models of metal-induced nephrotoxicity (Cd, cisPt), the oxidative stress-related mechanisms that trigger mitochondrial processes and are inhibited or prevented by free radical scavengers and antioxidants may be the predominant mode of inducing apoptosis in various models of metal nephrotoxicity [9, 25, 30–32, 35, and references therein]. Necrosis seems to use the biochemical pathways identical to those in apoptosis which, however, have lost regulations due to heavy depletion of the energy. In its developed stage, necrosis is morphologically characterized by the loss of cell membrane integrity, cytoplas-

mic swelling, vacuolation of mitochondria, nuclear pyknosis, release of cell content and lysosomal enzymes in the surrounding extracellular space, causing local inflammation [reviewed in 30, 31]. In experimental animals treated with usual nephrotoxic doses of Cd, Hg, or cisPt, both apoptotic and necrotic events can be detected in the same kidney tissue.

Mechanisms of Protection from Cell Death in Metal Nephrotoxicity

If not immediately killed by apoptosis or necrosis, in case of metal intoxication the PT cells express a battery of short- and long-term effects that can be activated, favoring the cell survival. Short-term effects include: (a) release of Zn, displaced by the toxic metal from the preexisting intracellular pool of Zn-MT; the liberated Zn can transiently inhibit the activity of metabolic enzymes, protect free SH groups from oxidation, stabilize membranes, protect various Zn-dependent proteins in the cytoplasm and Zn-finger proteins in the nucleus, and potentially inhibit caspase 3 and Ca^{2+} -dependent endonucleases, both critical for finishing apoptosis [reviewed in 45]; (b) inhibition/loss of H^+ -ATPase and Na^+/H^+ -exchanger NHE₃ from the BBM in PT cells, as detected in Cd- and cisPt-treated rats [reviewed in 32, 46], may result in impaired extrusion of protons and intracellular acidosis, which diminishes apoptosis by inhibiting the activity of degradative enzymes and permeability of the mitochondrial membrane [47]; (c) mobilization of antiapoptotic protein Bcl-2 from intracellular membranes, which inhibits release of cytochrome c from mitochondria, and thus interrupts the apoptotic process [30, 31, 47, and references therein]. Long-term effects include: (a) increased synthesis of MT via metal-response and antioxidant-response elements present in the MT gene promoter [48]; MT acts as a multifunctional stress protein induced by various toxic metals that restricts their toxicity by binding them, and also serves as an efficient scavenger of hydroxyl radicals, (b) increased abundance of GSH and SOD after prolonged oxidative stress induced with some toxic metals in rat kidney [reviewed in 49], (c) induction of heme oxidase in the kidney, associated with increased renal content of heme and ferritin in cisPT nephrotoxicity [50], which leads to the binding and detoxication of the redox-active Fe and thus decreases the Fenton reaction-mediated production of ROS, (d) upregulation of MDR proteins in the BBM of the PT cell line by Cd [9, and references therein] and of the kidney PT in rats treat-

ed with Cd, Hg, Pb, and cisPt [pers. unpubl. data]; these ATP-driven drug efflux pumps may extrude peroxidized lipids and various other toxic compounds generated during oxidative stress, and (e) induction of various protective proteins (p38, heat-shock proteins) that interfere with the process of apoptosis and/or contribute to amelioration of various, oxidative stress-induced intracellular injuries [31, 32]. In most cases, these protective mechanisms were also more or less attenuated or prevented by free radical scavengers and antioxidants, indicating oxidative stress and ROS as major causes of their induction.

Conclusion

Oxidative stress, apoptosis and necrosis appear as common mechanisms in various in vivo and in vitro models of nephrotoxicity induced by toxic metals. These phenomena, that take place largely in PT, can be more or less corrected or prevented by free radical scavengers and antioxidants, indicating oxidative stress and ROS as 'the

mother of most (if not all) problems'. Most of these phenomena resemble those in the hypoxia-induced PT damages, which can also be corrected with antioxidants and free radical scavengers [51]. These facts point to the possible strategy to common therapy of such renal injuries where the intracellular uptake of toxic metals or other toxic substances would be adequately prevented by the relevant inhibitors (chelators), whereas the ROS generation and ROS-mediated processes would be prevented or ameliorated by relevant antioxidants and scavengers of free radicals and Fe.

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