The Labile Side of Iron Supplementation in CKD

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ABSTRACT

The practice of intravenous iron supplementation has grown as nephrologists have gradually moved away from the liberal use of erythropoiesis-stimulating agents as the main treatment for the anemia of CKD. This approach, together with the introduction of large-dose iron preparations, raises the future specter of inadvertent iatrogenic iron toxicity. Concerns have been raised in original studies and reviews about cardiac complications and severe infections that result from long-term intravenous iron supplementation. Regarding the iron preparations specifically, even though all the currently available preparations appear to be relatively safe in the short term, little is known regarding their long-term safety. In this review we summarize current knowledge of iron metabolism with an emphasis on the sources and potentially harmful effects of labile iron, highlight the approaches to identifying labile iron in pharmaceutical preparations and body fluids and its potential toxic role as a pathogenic factor in the complications of CKD, and propose methods for its early detection in at-risk patients.


The anemia of CKD is routinely treated with erythropoiesis-stimulating agents (ESAs) and iron supplements, mostly via the intravenous route. In the last decade, several randomized controlled trials consistently indicated adverse cardiovascular outcomes with hemoglobin levels targeted above 13 g/dl.1–4 Therefore, current KDIGO guidelines for CKD recommend hemoglobin levels of 10 (or possibly less) to 11.5 g/dl.5 As a result of the renewed doubt regarding the long-term safety of ESAs, as well as the recently instituted ‘bundling’ of dialysis services to include both ESAs and intravenous iron (IVI), nephrologists have gradually moved away from the liberal use of ESA in CKD anemia toward more IVI supplementation. This policy, together with the introduction of large-dose iron preparations, has raised the future specter of potential inadvertent iatrogenic iron toxicity.

Although in the short term, all currently available iron preparations appear to be safe,6 little is known regarding the long-term safety of repeated IVI.7 The purpose of this review is to summarize current knowledge of iron biology, with an emphasis on the metal’s potentially harmful effects, highlight the role of iron toxicity as a pathogenic factor in the complications of CKD and propose methods for its early detection in at-risk patients. The role of iron in kidney injury will not be addressed and the reader is referred to an excellent comprehensive review of this subject.8

IRON BIOLOGY

The classic perception of iron in the biomedical field has been of a Janus-faced or double-edged sword element essential for life but life-threatening if not properly controlled.9 Thus, inherent to cell10,11 and system12 is the maintenance of a pool of iron available for biosynthetic purposes. In body fluids such a pool is associated with plasma transferrin (TF) molecules that safely carry the metal and supply it to cells ‘on demand’, i.e., as per the level of expression of cell TF receptors. In cells, most of the iron is stably associated with proteins or cofactors, but their manufacture or degradation also involve labile forms of the metal that are both redox-active and ligand-exchangeable. This poses a constant demand on cells to prevent labile Fe^{2+}/Fe^{3+} from promiscuous generation of toxically reactive O species (ROS) from reactive O intermediates (ROI, such as superoxide and hydrogen peroxide), that normally represent up to 1% of O_{2} consumed by the respiratory chain (Figure 1).13,14 To cope with such natural imperfections, mammalian cells rely on two complementary defense strategies: (1) control of labile cell iron (LCI) levels by coordinately balancing iron uptake versus utilization and/or storage, and (2) control of ROS formation by eliminating ROIs with superoxide dismutases and various peroxidases, as well as by antioxidant molecules. However, whenever...
Figure 1. Where and how does labile iron cause cell damage? Schematic representation of iron distribution among cell compartments (see number 1 in circle). Transferrin (Tf) bound iron (TBI) is the physiologic source of circulating Fe that is captured by cells via RME (see number 2 in circle), whereby the TBI is delivered into cells and the metal is released into the cytosol as LCI (see number 3 in circle) that is comprised of Fe (II and III). LCI can permeate into mitochondria (see number 4 in circle) where it serves as a metal source for heme and iron-sulfur cluster proteins. Labile iron in mitochondria (LMI) (see number 5 in circle) can catalyze formation of ROS (e.g., O_2^•– and H_2O_2, approximately 33 g/day from respiration) that are normally eliminated by cell enzymes such as superoxide dismutase (sod) and glutathione peroxidases (gpx) or catalase (see number 7 in circle). ROS can chemically damage membrane components such as lipids by peroxidation, proteins by oxidation of amino acids (AA) such as tyrosine (tyr), methionine (met), lysine (lys) and cysteine (cys), or via CO formation and nucleic acids by base modification and DNA breaks. Excess LCI is generally absorbed into ferritin iron shells (see number 8 in circle) that can then be dissolved by proteolytic cleavage of the protein in lysosomes (see number 9 in circle) whereby iron can be recycled into the LCI pool. In systemic iron overload, LPI forms (which are non-TBI, NTBI) (see number 10 in circle) can infiltrate cells, raise LCI and LMI and cause cell iron overload. Those protective measures fail or become insufficient, as in siderosis or inflammatory crisis, cell oxidative damage ensues, often leading to necrotic cell death or to more complex death paths such as ferroptosis or oxytosis, if resuable by iron chelators or antioxidants, respectively. Pathologic scenarios develop in the two main types of siderosis: systemic siderosis (inherited or iatrogenic), whereby cells are overwhelmed by excessive ingress of non-physiologic forms of plasma iron and in regional siderosis (inherited or acquired) where damage results from maldistribution of the metal within cell compartments.

Tissue Iron Accumulation and Siderotic Damage
The identification of tissue iron overload (siderosis) in experimental and clinical settings has largely relied on the detection of iron agglomerates by histochemical stains or magnetic resonance imaging (MRI). Although often perceived as deleterious, the mere presence of iron agglomerates in cells is not to be tacitly taken as indicating siderotic damage, insofar as the metal is chemically shielded within ferritin shells. The etiopathology of siderotic damage is most likely associated with ROS formation catalyzed by LCI. This relies largely on the fact that permeant iron chelators can confer direct protection from ROS-mediated oxidations by demonstrably complexing LCI and thereby rendering it non-labile. LCI can also be reduced by overexpression of ferritin molecules. These two features, redox activity and susceptibility to specific iron chelators, define LCI (also called labile iron pool, LIP) which is a physiologic component that is maintained homeostatically by most cell types.

The emerging issue is in which conditions does LCI attain levels that implicate it in siderotic damage or, conversely, in iron deprivation, which can also be deleterious. A related issue is how do different cell types cope when exposed to non-physiologic iron forms that appear in plasma of patients with systemic iron overload or with iatrogenic iron forms that are delivered parenterally as iron supplements?

Labile Cell Iron
LCI was hypothesized as a transitory pool of labile iron that is at the crossroads of cell metabolism. Experimentally, it was demonstrated in living cells with the aid of fluorescent probes that either sense labile iron per se or monitor its propensity to generate ROS when prompted by pro-oxidants but curbed by membrane permeant chelators. LCI is maintained in metabolically active cells at submicromolar level, representing 1%–2% of the total cell iron content. Those homeostatic levels reflect a balance between metal uptake and utilization versus the iron-absorptive capacity of cell ferritin, but also the cell metal ligand composition and the redox activity of the cell compartment in question. As mentioned above, the link between labile iron and biologic damage has largely leaned on the demonstrable ability of permeant iron chelators to protect or ameliorate cell functions affected by siderosis and, thereby, rescue cells from entrance into a death path.

That has provided a rationale for treating siderotic disorders of systemic or regional character with chelators, while taking into consideration that the benefits expected from regional iron detoxification by chelation need to be balanced...
against the possible systemic depletion of an essential metal.

**Labile Plasma Iron**

Unlike LCI, plasma normally has spare Tf iron binding capacity for effectively binding incoming labile iron (from absorption by the gut or recycling by the reticuloendothelial system (RES)) and thereby rendering it non-labile, i.e., non-redox-active and non-transferable to potential iron acceptors (e.g., citrate, nucleotides) and even to some chelators in pharmacologic use. Physiologically, after being taken up by cells via Tf receptor-mediated endocytosis, Tf will release the protein-occluded metal once exposed to the acidic milieu of the cell endosome. Under normal conditions, plasma Tf saturation (TSAT) is maintained in the 20%–40% range, rarely exceeding 60%, except in systemic iron overload,\(^1^8\) which results from hyperabsorption of iron (as in hereditary hemochromatosis), increased erythropagocytosis (as in transfusional siderosis), or hypotransferrinemia. In those conditions, an outpouring of iron that is not matched by sufficient local plasma unsaturated iron binding capacity, will lead to the formation of non-transferrin bound iron (NTBI), a group of iron forms initially found in plasma (serum) of patients with TSAT near or above 100% (i.e., approximately 0 unsaturated iron binding capacity).\(^2^7^–^2^9\) NTBI detection by colorimetric assays or by HPLC was made possible by extraction of sera with chelating/mobilizing agents followed by protein exclusion-filtration.\(^3^0\) The fact that NTBI was detected in high TSAT sera gave the parameter credibility as a genuine clinical indicator of systemic iron overload, although some overestimations caused by mobilization of iron from Tf-Fe by harsh extraction conditions have been noted.\(^3^1^\) Conceptually, the term NTBI, which ‘defines something just by what it is not’ (known classically as an apophasis) is rather misleading, particularly when used to define potentially toxic iron species in plasma and/or designate it as the source of tissue iron overload. Firstly, not all iron species in plasma that qualify *stricto sensu* as NTBI need to be toxic or even reflective of iron overload (e.g., plasma ferritin in inflammation, iron chelates in chelated individuals or polymeric iron particles (PIPs) in intravenous supplemented individuals). Secondly, studies attempting to demonstrate tissue iron overload associated with NTBI uptake have rarely been performed in pathophysiologically-relevant media or with genuine NTBI (from iron-overloaded patients). Thirdly, as plasma NTBI composition varies with the degree and/or the source of iron overload, its potential toxicity cannot be reliably estimated just from concentration. For example, in chronic diabetes, persistent plasma NTBI is detectable (even at TSAT as low as approximately 50%), but in the absence of overt tissue iron overload.\(^3^2^\) In chelated patients, some NTBI assays do not necessarily distinguish between genuine NTBI and iron chelates, leading to erroneous assessment of iron overload and of chelation efficacy. Last, but not least, the association of NTBI forms with plasma albumin might not only blunt the detection of NTBI but also affect it as a source of tissue siderosis.\(^3^3^\) A typical misuse of the term NTBI is found with PIPs that generate in plasma mM concentrations of NTBI. However, unlike the NTBI detected in systemic siderosis, iatrogenic NTBI derived from most PIP formulations is ‘mostly’ non-toxic, ‘safely’ acquired and processed by the RES macrophages.\(^3^4^,^3^5^\)

Thus, while the introduction of the term NTBI was a landmark in understanding the pathophysiology of systemic iron overload,\(^2^7^–^2^9^\) its indiscriminate use as an indicator of toxic forms of iron in plasma turned out to be a source of confusion. A more reliable indicator of pathologic forms of iron in biologic fluids is the catalytically-active component of NTBI, which can be revealed by physiologic reductants such as ascorbic acid. In early studies, bleomycin was used in conjunction with high concentrations of ascorbate to trigger the oxidation of sugars (free or as part of polymers such as DNA) and, thereby, rendering them detectable with thiobarbituric acid.\(^3^6^\) However, the detection of seemingly catalytic metal in iron chelates and in plasma of patients with TSAT <50%–60% apparently indicates a tendency for the bleomycin assay to overestimate levels of genuine redox-active iron in biologic samples. A parameter that defines redox-active components specifically associated with iron but also chelatable is labile plasma iron (LPI).\(^2^1^,^2^8^\) This parameter is assayable in biologic fluids and provides a clinically-relevant indicator of iron chelation efficacy or adequacy.\(^2^1^,^3^7^,^3^8^\) This is of particular importance for evaluating treatments that can confer round-the-clock prevention of LPI appearance as, by daily containment of LPI, it is feasible to reduce and possibly eliminate impending tissue iron overload. Moreover, LPI can also be used as a diagnostic indicator for initiating chelation therapy in naïve polytransfused thalassemia children.\(^3^8^\)

**IRON IN THE PATHOGENESIS OF CKD COMPLICATIONS**

On a backdrop of CKD, a condition of underlying oxidative stress, there is clear scope for excess iron-associated adverse outcomes via ROS-induced damage which could contribute to endothelial dysfunction, inflammation, and immune dysfunction.\(^1^8^,^3^9^\) Also, iron is required for bacterial growth and part of the human antibacterial armamentarium relies on iron mobilization from plasma into sanctuaries associated with the RES. However, iron-laden RES cells become vulnerable to intracellular pathogens.\(^4^0^\) Antibacterial host defenses also depend on iron-catalyzed formation of ROS, which is critical for normal phagocytic function. Conversely, excess iron has been linked to impaired neutrophil and T-cell function, thereby promoting bacterial growth both *in vivo* and *in vitro*, at least in short term (2–3 days) studies.\(^4^1^\) In addition there is evidence from the pre-ESA period for impaired neutrophil function in dialysis patients with iron overload and, subsequently, for abnormal T-cell function in mice iron-overloaded by intraperitoneal injection of iron dextran. In the latter experiments, the mice failed to mount a Th1-mediated protective response to *C. albicans* infection, but were rescued with the iron chelator deferoxamine.\(^4^2^\)
Against this theoretical background, observational studies linking amounts of administered iron to adverse outcomes in CKD have steadily increased in recent years, but with inconsistent results. Because the link between administered iron and outcomes has been comprehensively reviewed in a recent JASN article,\textsuperscript{43} we briefly summarize earlier data, address in more detail studies that have appeared since that publication and offer our own views on the subject.

As reported, observational studies have shown a weak association between amounts of iron administered and increased mortality or cardiovascular events.\textsuperscript{44–52} incidence of bacterial infection\textsuperscript{53,54} and a somewhat stronger association between ferritin levels and mortality.\textsuperscript{48} These intriguing results are difficult to interpret in light of ferritin levels that would be considered frank iron deficiency in the Western world. Nevertheless, mortality in Japanese ESRD and other patient groups is persistently lower than in Western countries. Although tempting to invoke a contribution of minimalist IVI protocols to this phenomenon, Japanese and Western populations differ in many other respects, besides IVI protocols, including prolonged genetic isolation, different dietary intake, and body habitus. Therefore, we reserve judgement on this issue.

In the recently published Dialysis Outcomes and Practice Patterns Study, involving 32,435 patients from 32 countries, IVI doses >300 mg monthly, as compared with lower doses, were associated with higher all-cause, cardiovascular, and infectious mortality, as well as hospitalization. Moreover, this association was seen not only when hemoglobin was >12 g/dl, as observed previously,\textsuperscript{1,3,4} but even within the guideline-recommended range of 10–12 g/dl, although not with values <10 g/dl.\textsuperscript{49} These studies were performed on prevalent dialysis populations and, despite multiple adjustments for confounding, using sophisticated contemporary statistical techniques, could still suffer from un-detected residual confounding and be subject to survivor bias. The only study to date on incident hemodialysis patients, also just published, showed that administration of ≤1050 mg IVI in 3 months or 2100 mg in 6 months was not associated with all-cause, cardiovascular, or infection-related mortality. However, non-statistically significant findings suggested the possibility of infection-related mortality with receipt of >1050 mg in 3 months or >2100 mg in 6 months.\textsuperscript{50}

Only two randomized controlled trials (RCTs) have addressed the association between iron dosage and infection and then only as a secondary end point. In the Dialysis Patients’ Response to IVI with Elevated Ferritin (DRIVE) study,\textsuperscript{56} patients who were randomized to iron gluconate had a similar incidence of bacterial infections as the placebo group, both at the end of the 6-week study period and after a further 6-week observation period (DRIVE II).\textsuperscript{57} However, this trial was too short and the sample size insufficient for assessing the likelihood of infrequent events or medium-term safety. Similar results were seen in the second RCT, which used iron dextran.\textsuperscript{58}

Iron dosing method and polymer composition may also contribute to infection risk. Bolus dosing has been associated with a higher infection risk than maintenance dosing\textsuperscript{53} and iron sucrose with a greater risk than dextran or gluconate.\textsuperscript{54} In this regard, newer preparations, such as ferric carboxymaltose and the recently FDA-approved ferric citrate (oral preparation),\textsuperscript{59} have yet to be assessed. Oral ferric citrate is of particular interest because it is marketed as a phosphate binder, but some iron is also absorbed. As shown in a recently published study, patients receiving ferric citrate not only had good phosphate control, but also their requirements for IVI were reduced compared with placebo controls. In addition, the favorable safety data obtained from the 56-week follow-up period are reassuring.\textsuperscript{60} The potential benefit of orally delivered iron is that absorption may be regulated more physiologically, if hepcidin-induced resistance to iron absorption is sufficiently countered. Conversely, we cannot exclude un-regulated absorption and longer-term safety issues, including generation of ROS and its adverse consequences.

The discrepant results elucidated above may be explained by different follow-up times, variable underlying comorbidities, inflammatory status (as reflected, at least in part, by varying serum ferritin levels) and oxidative stress. Other biomarkers, such as hepcidin, also suffer from this limitation of reflecting inflammation as well as iron overload.\textsuperscript{61} Taken together, the epidemiologic evidence for iron-associated adverse effects in CKD is, at best, unconvincing, most probably due to the many confounding factors involved. Nevertheless, some experts believe that indiscriminate use of IVI to achieve guideline-recommended hemoglobin targets in ESA hyporesponsive patients is likely to be detrimental.\textsuperscript{62,63}

LPI, Tissue Iron Deposition, Oxidative Stress, and Damage in CKD

The generation of LPI in ESRD patients supplemented with parenteral PIPs has been proposed in various studies that dealt with the appearance of oxidized plasma components\textsuperscript{55,64–74} and demonstrated by direct measurements of LPI.\textsuperscript{35,75}

Both ours\textsuperscript{35} and other groups\textsuperscript{75,76} have reported LPI detection in approximately 10% of hemodialysis patients receiving IVI, but, by 48 h after administration, LPI was apparently no longer detectable. Although several abnormalities detected during or shortly after iron administration have been reported, they are apparently also of transient nature. However, the critical and still outstanding question is whether relatively short but repetitive exposures to LPI will over time pose the risk of oxidative tissue damage caused either by cumulative LPI infiltration into cells or via plasma oxidation products.\textsuperscript{68,77,78}

Several studies have reported high tissue iron levels in CKD detected either
histologically or by MRI.\textsuperscript{79,80} In the pre-ESA era, ferritin levels of >4000 ng/ml were reported to be associated with end-organ damage.\textsuperscript{81} In the ESA era, a dramatic example was demonstrated by T2* MRI of iron deposits in the livers of hemodialysis patients with ferritin levels >2000 ng/ml.\textsuperscript{82} Although this observation directly reflects iron deposition as hemosiderin, it gives no information regarding iron-mediated tissue damage. IVI has also been implicated in the propagation of renal tissue damage in animal models of CKD.\textsuperscript{83} Moreover, in a very recent JASN paper, the first evidence for direct IVI involvement in atherogenesis in CKD was reported. Using an \textit{in vitro} model of leukocyte–endothelium interactions and a mouse remnant kidney model, iron sucrose was shown to accelerate early atherogenesis by upregulating the expression of intracellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and vasculitis of renal tissue damage in animal models.\textsuperscript{83} Furthermore, IVI sucrose can induce with adequate long-term follow-up. The study should be sufficiently powered to avoid inferences in the type of anemia, degree of inflammation, dialysis modality where appropriate, and available iron preparations.\textsuperscript{63} We would also emphasize the need for hard end points in these trials and look to the ongoing UK Multicentre Open-label Randomized Controlled Trial Of IV Iron Therapy In Incident Hemodialysis Patients (Proactive IV irOn therapy for Haemodialysis patients trial) to provide important answers, at least with regard to ferric carboxymaltose versus small doses of iron sucrose (EudraCT Number: 2013-002267-25).

**Potential Contamination of Intravenous Iron Formulations with Labile Iron**

Most IVI formulations in clinical use are nanoparticles comprised of a polyiron oxide/hydroxide core coated with carbohydrate (Table 1).\textsuperscript{85} It is generally accepted that these formulations, prepared as PIPs, supply metabolic iron to circulating Tf by a mechanism that recapitulates physiologic iron recycling via erythrophagocytosis (Figure 2A). With few exceptions,\textsuperscript{86} PIPs are regarded as ‘relatively’ free of labile iron\textsuperscript{87} and stable in circulation until endocytosed and processed by macrophages by mechanisms that are only partly...
understood (Figure 2B). However, an impending issue is the possible contamination of the newer IVI formulations designed for intensive iron supplementation, ferric carboxymaltose, ferumoxytol and iron isomaltoside, with labile iron and the subsequent generation of LPI following massive intravenous administration of these polymers. This is of particular importance for quality control of the formulations themselves, as well as for their fate in plasma of patients with CKD and other chronic inflammatory diseases. With the wider and more intense application of chemically diverse IVI supplements, it would seem prudent to obtain more information about the safety of the various products in terms of: (1) propensity to generate labile iron under different storage conditions and (2) possible adverse reactions related to transient rises in LPI as a function of delivery rates and dosage. The latter is especially relevant for patients with high plasma levels of (1) pro-oxidants, e.g., chronic patients with diabetes; (2) hepcidin due to an inflammatory state or mutations affecting its expression; or (3) low ferroportin activity, induced either directly by elevated hepcidin levels or, independently of hepcidin, via toll-like receptors 2 and 6 signaling. These phenomena, in turn, could cause excessive splenic or hepatic retention of PIPs as such or as elevated ferritin but also perhaps as potentially toxic levels of LCI. The recent introduction of methods to measure the LPI potentially contained in IVI preparations will allow the systematic testing of our hypothesis that LPI could be formed in highly oxidized plasma or plasma with little antioxidant capacity and/or high redox-active groups (e.g., advanced-glycation end products) as found in patients with CKD.

CONCLUSIONS AND FUTURE DIRECTIONS

Iron-induced cell damage or death, resulting from the intrinsic inflammatory nature of CKD and, possibly, exacerbated by repeated infusions of IVI in our patients remains a relatively unexplored field. The recent upsurgence in the use of IVI following reports incriminating excessive ESA dosing as being associated with increased mortality, behooves nephrologists to take heed of the potential for replacing ESA toxicity with iron toxicity. This potential is highlighted by the intermittent detection of labile iron in both CKD patients’ plasma and currently available IVI preparations. In addition, the recent promotion of preparations containing up to 500 mg of iron per dose, although by the nature of their structure thought to be safe, further enhances the risk of iatrogenic iron toxicity. Given this combination of factors, we propose the following two-pronged strategy: (1) multi-center, international RCTs comparing different iron-dosing regimens in both CKD and ESRD patients, with adequate long-term follow-up; (2) the systematic monitoring of LPI during and post administration, which could serve as a promising biomarker of impending iron toxicity.

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