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Original Article

Labile iron in parenteral iron formulations: a quantitative and comparative study

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Abstract

Background. Evidence of iron-mediated oxidative stress, neutrophil dysfunction and enhanced bacterial growth after intravenous (IV) iron administration has been ascribed to a labile or bioactive iron fraction present in all IV iron agents.

Methods. To quantify and compare the size of the labile fraction in several classes of IV iron agents, we examined iron donation to transferrin (Tf) *in vitro*. We added dilutions of ferric gluconate, iron sucrose and each of two iron dextran preparations to serum *in vitro*, passed the resulting samples through alumina columns to remove iron agent and free organic iron, and measured Tf-bound iron in the resulting eluates. Comparing results to serum samples without added iron, we calculated delta Tf-bound iron for each agent at each concentration. Finally, we compared delta Tf-bound iron to the concentration of added agent and calculated the percent iron donation to Tf.

Results. We found that Tf-bound iron increased with added iron concentration for each agent: delta Tf-bound iron was directly related to the concentration and type of iron agent ($P < 0.001$). Mean percent iron donation to Tf ranged from 2.5 to 5.8% with the following progression: iron dextran-Dexferrum® < iron dextran-INFeD® < iron sucrose < ferric gluconate. Pairwise differences between agents for percent iron donation were statistically significant ($P < 0.05$) only between ferric gluconate and both iron dextran agents, and between iron sucrose and iron dextran-Dexferrum®.

Conclusions. Approximately 2–6% of total iron in commonly used IV iron compounds is available for *in vitro* iron donation to Tf. This fraction may contribute to evidence of bioactive iron in patients after IV iron administration.

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Introduction

Oxidant stress, atherogenesis, infection and inflammation are hallmarks of the dialytic milieu [1]. Each process holds a plausible pathogenic role for biologically active iron. Intravenous (IV) iron therapy, commonly administered to dialysis patients as an adjunct to managing anaemia, provides a potentially rich source for intradialytic bioactive iron [2]. All IV iron agents tested, including iron dextran [3], iron polymaltose [4], iron sucrose [3–5] and ferric gluconate [3,4,6], show evidence of bioactive iron release *in vitro* and *in vivo*. IV iron agents have been found to induce oxidative stress [3,4,6], boost bacterial growth *in vitro* [2,5] and disturb neutrophil function [2].

Bioavailability of IV iron agents (iron sucrose [7], ferric gluconate [8], iron polymaltose [9] and iron dextran [10]) stems primarily from intracellular release of low-molecular-weight iron after clearance from plasma and uptake by cells of RES. However, *in vitro* evidence of iron-mediated biological activity [3,4] suggests the presence of a labile iron fraction in IV iron agents capable of exerting biological impact prior to cellular uptake.

Debate over the clinical implications of labile iron in IV iron agents is vigorous. Some recommendations have been quite explicit, including caution to avoid use of 125 mg doses of ferric gluconate [6] or 300 mg doses of iron sucrose in dialysis patients [2]. Other commentators have been more general, suggesting that IV iron agents contain 'free iron' and that classes of IV iron agents differ in their capacity to release free iron [11]. Though the debate promotes alarm and confusion, it resists resolution in part because quantitative and comparative information is lacking. Specifically, the fraction of total iron represented by labile iron in IV iron agents has not

been quantified and comparative data on labile iron generation has not been obtained.

A substantial impediment to quantifying the labile iron fraction arises from one of its intrinsic biochemical effects: in standard serum iron assays, labile iron is difficult to distinguish from transferrin-bound (Tf-bound) iron. Indeed, false elevation of serum iron determinations by IV iron agents was likely the first reported manifestation of labile iron [12,13]. Since standard serum iron assays erroneously detect 2–60% of added iron agent as serum iron [12], yet, according to preliminary reports [4], iron agents may also donate IV iron directly to Tf, quantifying the relative contribution of labile iron to Tf-bound iron in serum has heretofore not been possible.

In the current studies, we sought to quantify and compare labile iron fractions in commonly used IV iron agents. We used direct *in vitro* donation of Tf-bound iron as a marker of labile iron. To measure iron donation to Tf but exclude interference in the iron assay, we first added, then removed, iron agent from serum. By comparing Tf-bound iron before and after adding iron agents to serum, we determined the relationship between the concentration of parenteral iron and the degree of *in vitro* iron donation to Tf. We thereby derived an estimate of a labile iron fraction in each iron agent formulation.

Materials and methods

To determine the magnitude of direct donation of iron from iron agents to Tf and to explore the potential role of this process in the saturation of Tf after IV iron administration, we added dilutions of each IV iron agent to fresh serum over a range of concentrations, passed the resulting samples through an alumina column to remove intact iron agent, and assayed the resulting eluate for Tf-bound iron. This assay has been shown to reliably exclude both iron agent and inorganic iron from interfering with the colorimetric assay of Tf-bound iron in serum [13].

Parenteral iron formulations

We examined ferric gluconate (sodium ferric gluconate in sucrose; Ferrlecit®, 12.5 mg/ml in 5 ml ampules; Watson Pharmaceuticals, Inc., Corona, CA, USA), iron sucrose (iron sucrose injection, USP; Venofer®, 20 mg/ml in 5 ml vials; American Regent, Inc., Shirley, NY, USA) and both available formulations of iron dextran (INFeD®; Watson Pharmaceuticals; and Dexferrum®; American Regent; both 50 mg/ml in 2 ml vials). For each experiment, we examined all agents at all experimental concentrations on the same day. For each concentration of iron agent studied, we prepared equimolar stock solutions of each of four agents on the day of use, employing successive dilutions ($\leq$1:10) in 0.9% NaCl. All agents were used before lot expiration dates.

Experimental iron concentrations

We examined concentrations of iron formulations from 859 to 6875 μg/dl (153–1228 μmol/l), a range expected to include the maximum plasma concentration of agent after IV push injection (C_{max}) of 125 mg of ferric gluconate [1900 μg/dl (339 μmo/l)], 100 mg of iron sucrose [3000 μg/dl (536 μmol/l)] or 100 mg of iron dextran [3080–3396 μg/dl (550–606 μmol/l)], according to data from the respective product package inserts.

Determination of transferrin-bound iron

Using a previous method [13], we prepared final experimental samples by adding 0.1 ml of stock solution to 1.5 ml of fresh pooled serum [average TIBC 370 μg/dl (66.1 μmol/l)] and incubating for 5 min. We then passed 1.5 ml of the resulting sample over a 2.0 g alumina column to absorb inorganic and drug-bound iron, collected the eluate, reconstituted the eluate to a total volume of 1.5 ml, and determined the final iron concentration on a Hitachi 717 chemistry analyser (Boehringer Mannheim Corporation, Indianapolis, IN, USA) using Hitachi-specified ferrozine reagents (Boehringer Mannheim) which include detergent, buffers of citric acid and thiourea, ascorbate and ferrozine. Briefly, this is a non-deproteinizing method in which detergent serves to clarify lipaemic samples, buffers lower pH to $<$2.0 to free iron as Fe^{3+} from Tf, ascorbate reduces Fe^{3+} to Fe^{2+} and ferrozine reacts with Fe^{2+} to form a coloured complex measured spectrophotometrically at 560 nm. We processed blank serum samples (0.1 ml of 0.9% NaCl plus 1.5 ml of serum, no added iron agent) in a similar manner. To determine the rise, if any, in serum iron (delta iron, μg/dl), we subtracted the serum blank value from those obtained after iron agent addition and column extraction. To determine percent iron donation to Tf, we divided delta iron by the concentration of iron agent and multiplied by 100.

Statistical analysis

We used two-way analysis of variance (ANOVA) to determine the effect of the agent and concentration on delta iron (SigmaStat Version 2.03; SPSS Science, Chicago, IL, USA) and one-way repeated measures ANOVA on ranks (Friedman) with pairwise multiple comparison procedures (Tukey test) to determine effect of the agent on percent iron donation.

Results

To determine the reliability of the method to exclude iron agent or inorganic iron from the measurement of Tf-bound iron, we prepared plasma-free (Tf-free) solutions of each iron agent at a high concentration (6875 μg/dl) in 0.9% sodium chloride, passed the solutions through alumina columns, and assayed the eluate. Results (Table 1) showed that this method of sample preparation and assay detects $<$1% of added iron agent, regardless of the agent assayed.

To further evaluate the reliability of the method, we next determined the within-test variability of Tf-bound iron results in serum with iron agent added. We assayed 12 samples of serum after adding ferric gluconate to a final concentration of 1719 μg/dl, a concentration approximating the maximum expected after administering 125 mg of ferric gluconate IV over

Table 1. Evaluation of reliability of the method to exclude contaminating iron agent

Iron agent	Detected iron (μg/dl, μmol/l)	Iron agent detected (%)
Ferric gluconate	42 (7.3)	0.61
Iron sucrose	30 (5.4)	0.44
Iron dextran-INFeD®	36 (6.4)	0.52
Iron dextran-Dexferrum®	27 (4.8)	0.39

Serum-free (Tf-free) solutions of each iron agent at 6875 μg/dl (1228 μmol/l) concentration in 0.9% sodium chloride were prepared, the solutions were passed through alumina columns, and the eluate was assayed. In the absence of serum, the assay detected <1% of the added iron.

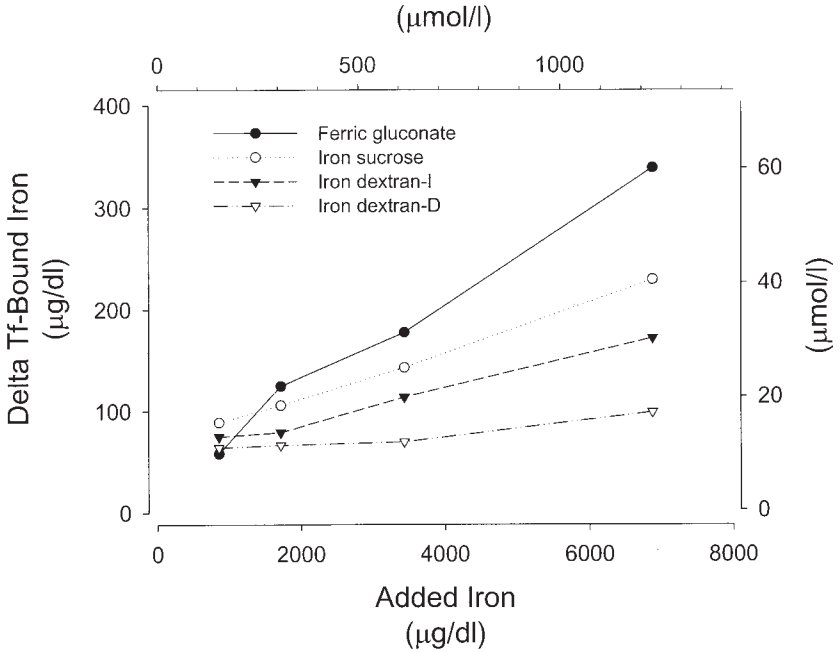

Fig. 1. Relationship between the change in Tf-bound iron (Delta Tf-Bound Iron) and concentration of added iron for each of four iron formulations. The two iron dextrans examined include iron dextran-I (INFeD®) and iron dextran-D (Dexferrum®). Each data point represents the mean of six replicate experiments.

10 min. The 12 replicate samples processed with the test method yielded a within-test coefficient of variation of 9.0%.

We then used the column separation method to determine the relationship between the concentration of added iron agent and change, if any, in serum Tf iron concentration. Results, expressed as delta Tf-bound iron, are shown in Figure 1. At low levels of added iron agent, delta iron results were low regardless of the class of agent. Delta iron increased with added iron concentration for all agents. The degree of increase in delta iron differed according to class and identity of agent. The effect of concentration and class of agent were each significant ($P = < 0.001$). There was a statistically significant interaction between concentration and agent ($P = 0.002$).

We then calculated the delta Tf-bound iron as a percent of the total concentration of iron agent added. At concentrations of iron agent (1719–6875 μg/dl) expected to be achieved after USFDA-recommended doses are administered IV push in adults, the median increase in Tf-bound iron represented ~2.5–5.8% of added iron depending on the agent added (Figure 2). In general, the effect of agent on percent iron donation was highly significant ($P = < 0.001$), with means progressing as follows: ferric gluconate > iron sucrose > iron dextran-INFeD® > iron dextran-Dexferrum®. Differences between agents for percent iron donation were statistically significant ($P < 0.05$) only between ferric gluconate and both iron dextran agents, and between iron sucrose and iron dextran-Dexferrum®.

Discussion

These results are, to our knowledge, the first to demonstrate that IV iron formulations donate iron directly to Tf *in vitro*, to show that the degree of iron donation is concentration-dependent, to estimate the size of the labile iron fraction, and to compare the size of labile iron fractions among commonly used IV iron agents. We found that the biologically available or labile iron fraction estimated by our methods represents 2.5–5.8% of total iron in IV iron agents

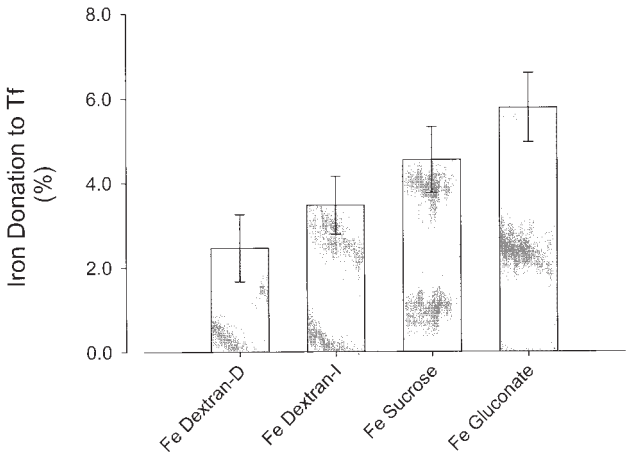

Fig. 2. Percent iron donation to Tf by parenteral iron agent (mean ± 95% CI). Differences in iron donation between agents reached significance ($P < 0.05$) as follows: ferric gluconate greater than either iron dextran-I (INFeD®) or iron dextran-D (Dexferrum®); iron sucrose greater than iron dextran-D only.

and varies according to the sequence iron dextran-Dexferrum® < iron dextran-INFeD® < iron sucrose < ferric gluconate.

Our findings are consistent with the hypothesis that iron bioavailability among commonly used IV iron agents is not determined solely by intracellular metabolism and may be manifested prior to RES uptake of the IV iron compound. The manifestation of labile iron we examined in the current assay was an increase in Tf-bound iron. Previous studies suggesting a labile iron fraction revealed induction of oxidative stress [3,4], generation of bleomycin-detectable or redox-active iron [5], promotion of bacterial growth [5] and impairment of neutrophil phagocytic function [2]. A recent unreviewed report, using sophisticated fluorescent methods to measure *in vitro* Tf-iron binding, showed that uptake of iron by apotransferrin from IV iron agents is rapid in the presence of ascorbate [4]. Quantitative determinations were not made and qualitative comparisons were not offered, but the degree of iron donation appeared to follow the sequence we observed, i.e. ferric gluconate > iron sucrose ≥ iron polymaltose. Iron dextran was not examined.

The acute effect of IV iron on Tf saturation has previously been examined in patients. Administration of ferric gluconate at a rate of 62.5–125 mg over 30 min or 125 mg over 240 min or 100 mg of iron saccharate (Ferrivenin; Laevosan, Austria; this was not iron sucrose) over 1 min are associated with a Tf saturation >100% [2]. That is, measured serum iron levels after IV iron injection exceeded serum iron-binding capacity. These results, however, have been difficult to interpret because of potential interference of drug-bound iron with the assay for serum Tf-bound iron [12]. Thus, the possibility that drug iron interference accounted for all or part of the rise in serum iron immediately after IV iron infusion could not be excluded. As others have demonstrated [13], even high concentrations of iron formulations do not significantly interfere with results of the assay we used to determine Tf-bound iron. In our assay, the magnitude of the increase in serum Tf-bound iron we observed, at drug iron concentrations likely to have been achieved after doses used in the *in vivo* studies, is 10-fold higher than the expected level of drug iron interference. This finding prompts two conclusions about the delta Tf-bound iron results we observed. First, our results do not arise from contamination by iron agents passing through the column. Secondly, the absolute values of delta Tf-bound iron we observed are theoretically sufficient to predict Tf over-saturation after IV iron administration in patients if iron doses are high and given rapidly and pretreatment unbound iron-binding capacity (UIBC) is low.

The role of the UIBC of plasma in preventing consequences of labile iron release should be considered crucial. Tf binds iron at either or both of two binding sites. At physiological pH, the binding of iron to Tf is sufficiently stable (stability constant 10^{24}) to assure that it would take nearly 10 000 years for one atom of iron to dissociate spontaneously. The avidity of Tf for ferric (Fe^{3+}) iron renders unbound (apo) Tf a potent endogenous iron chelator. *In vitro* addition of apotransferrin effectively blocks the oxidant damage [4], bacterial growth enhancement [5] and anti-phagocytic effects [14] of labile iron associated with IV iron agents. *In vivo* administration of apotransferrin binds free iron and removes redox-active non-Tf-bound iron in patients after chemotherapy for haematological malignancies [15].

If IV iron agents indeed include a labile iron fraction, as our data and others demonstrate, and if adverse clinical outcomes are shown to result, then care should be taken in patients to assure sufficient UIBC to accommodate the delta iron expected after rapid IV administration of iron agents.

That labile iron is not free iron is an important distinction. Dialysable free iron has not been detected in any formulation of IV iron agent thus far examined, including iron dextran, iron sucrose or ferric gluconate. Labile iron lacks biochemical characterization and is therefore functionally described as that portion of the total IV iron agent that exerts a disproportionate early biological activity. Furthermore, recent results suggest that the bioactivity of labile iron in the extracellular space extends to the intracellular compartment. The response of human hepatoma cells to the addition of IV iron agents to culture media suggests early release of bioactive iron from iron preparations [16]. As anticipated by our current results, intracellular iron effects follow the progression ferric gluconate ≥ iron sucrose > iron dextran.

Despite the abundant and longstanding albeit indirect evidence of labile iron in all iron agents examined here, and despite widespread therapeutic use of these agents for more than 50 years, no adverse patient outcomes attributable to labile IV iron have been demonstrated in patients when the agents are given in recommended doses and within recommended laboratory iron indices. Prospective multicentre cohort

studies in haemodialysis patients found no relationship between the incidence of bacteraemia and either the serum ferritin or the total dose of IV iron [17]. In a retrospective study, although an increased risk of mortality was reported in haemodialysis patients receiving more than 10 vials (equivalent to 1.0 g) of iron dextran in a 6 month period [18], this conclusion was not sustained when similar data were assessed using more sophisticated statistical techniques (H.I. Feldman, submitted for publication). Higher doses of IV iron dextran were described among non-survivors compared with survivors of the Normal Hematocrit Heart Trial, but the significance of this effect is controversial [2]. More recently, iron sucrose was administered to patients in the Scandinavian Hemoglobin Normalization trial [19]. Despite the need for high IV iron doses among haemodialysis patients randomized to the normal compared with the low haemoglobin treatment group, there was no difference in mortality or morbidity between treatment groups and no difference in iron sucrose dose between survivors and non-survivors [19]. Since iron sucrose doses averaged ~80 mg per week, these latest findings are particularly reassuring.

Taken together with the results of the foregoing clinical trials, our findings confirming and quantifying a labile iron fraction in IV iron agents, support the overall safety of IV iron therapy. Our results predict, however, that Tf super-saturation is theoretically possible after IV iron infusion. If so, occasionally, patients with low serum UIBC may experience super-saturation, manifesting a labile iron reaction (hypotension, cramping, diarrhoea or chest pain) at the recommended upper limits of IV iron infusion rates: 200 mg of iron sucrose over 5 min or 125 mg of ferric gluconate over 10 min. In such patients, caution, lower doses and slower infusion rates should accompany subsequent IV iron administration.

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Conflict of interest statement. D. Van Wyck is a consultant to American Regent, Inc., Amgen Inc., Gambro Healthcare and Shire Pharmaceuticals. He serves on the speakers boards for American Regent and Amgen.

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