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PARENTERIAL IRON SUCROSE- INDUCED RENAL PRECONDITIONING:

DIFFERENTIAL FERRITIN HEAVY AND LIGHT CHAIN EXPRESSION

IN PLASMA, URINE, AND INTERNAL ORGANS

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ABSTRACT

Experimental data suggest that iron sucrose (FeS) injection, used either alone or in combination with other pro-oxidants, can induce “renal preconditioning”, in part, by up-regulating cytoprotective ferritin levels. However, the rapidity, degree, composition (heavy vs. light chain), and renal ferritin changes following FeS administration in humans remain to be defined. To address these issues, healthy human volunteers (HVs; n,9) and stage 3-4 CKD patients (n,9) were injected once with FeS (120, 240, or 360 mg). Plasma ferritin was measured from 0-8 days post-injection as an overall index of ferritin generation. Urinary ferritin served as a ‘biomarker’ of renal ferritin production. FeS induced rapid (≤2hrs), dose-dependent, plasma ferritin increases in all subjects, peaking at ~3-5x baseline within 24-48 hrs. Significant urinary ferritin increases (~3x), without dose-dependent increases in albuminuria, NGAL, or NAG excretion, were observed. Western blotting with ferritin heavy chain (Fhc)- and light chain (Flc)-specific antibodies demonstrated that FeS raised plasma Flc, but not Fhc, levels. Conversely, FeS increased both Fhc and Flc in urine. To assess sites of FeS-induced ferritin generation, organs from FeS-treated mice were probed for Fhc, Flc, and their mRNAs. FeS predominantly raised hepatic Flc. Conversely, marked Fhc and Flc elevations developed in kidney and spleen. No cardio-pulmonary ferritin increases occurred. Ferritin mRNAs remained unchanged throughout, implying post-transcriptional ferritin production. We conclude that FeS induces rapid, dramatic, and differential Fhc and Flc up-regulation in organs. Renal Fhc and Flc increases, in the absence of nephrotoxicity, suggest potential FeS utility as a clinical renal “preconditioning” agent.

INTRODUCTION

Over 250,000 patients undergo 'on pump' heart surgeries per year (9). Approximately 50% of these cases are coronary artery bypass grafts (CABGs), with the remaining ~40% being aortic or mitral valve replacements or repair (9). Acute kidney injury (AKI) develops in up to 30% of these patients, with ~1-5% requiring renal replacement therapy (RRT) (28). Cardiovascular surgery- induced AKI (CVS-AKI) is a serious adverse event, leading to increased hospital length of stay, post-operative complications (27,32), and the potential onset of progressive renal disease (2,11,21). RRT, if required, portends a ~50% mortality rate (14,25).

Given these considerations, there has been a major interest in developing pharmacologic agents to either prevent CVS-AKI or attenuate its severity. The former approach is more promising, given that it is easier to prevent AKI than to reverse it once injury has occurred. A potentially useful approach for AKI prevention is so called "renal preconditioning". With this strategy, a mild renal 'stressor' is administered which triggers the up-regulation of diverse renal cytoprotective proteins over the ensuing 24 hrs (e.g. ref. 5, 33,36-40). As a result, renal resistance to diverse forms of AKI emerges (33,36,37). Although it has been recognized for >100 years that mild renal stressors, e.g. nephrotoxin exposure, can evoke experimental renal 'preconditioning' (13), a safe and effective way to clinically recapitulate this state has not yet emerged.

Towards this goal, our laboratory has explored the concept of so called "oxidant preconditioning" in several experimental studies (15-17, 36-40). The strategy has been to activate redox sensitive signaling pathways within the kidney by administering pro-oxidant agents, most notably intravenous iron (iron sucrose; FeS), given either with or without a heme oxygenase inhibitor (tin protoporphyrin) (36-40). With the induction of mild, transient, oxidant stress, the redox sensitive Nrf2 cytoprotective pathway is activated, causing marked up-regulation of diverse anti-oxidant / cytoprotective proteins (16). Over the ensuing 24 hrs, striking protection against diverse forms of experimental AKI emerges.

Ferritin up-regulation is a critical component of this oxidant preconditioning state (15,26,31). This 450,000 dalton macromolecule is typically made up of 12 "light" chains (Flc) and 12 "heavy" chains

(Fhc). However, the relative proportion of Fhc vs. Flc within the ferritin molecule can vary substantially, depending on the organ, cell type, and the nature of the inducing stimulus (6,12,29). Ferritin's cytoprotective properties stem from at least four actions. *First*, intact ferritin can sequester, and hence neutralize, large amounts of 'catalytic' (pro-oxidant) Fe as it is released from intracellular heme proteins (mitochondria and cytochromes) during evolving cell injury (46). *Second*, Fhc possesses ferroxidase activity which converts pro-oxidant Fe2+ to its less reactive Fe3+ form (46). *Third*, because catalytic Fe can activate an inflammatory cascade (41,43), ferritin-induced Fe binding exerts an anti-inflammatory effect; and *fourth*, it has recently been posited that ferritin can exert a variety of protective actions that are independent of iron sequestration (8,23,47). Supporting the *clinical* relevance of ferritin's protective actions comes from two recent studies which reported that: *i*) pre or peri-operative plasma ferritin levels inversely correlate with the frequency of CVS-induced AKI (7); and *ii)* elevated plasma ferritin levels are associated with improved clinical renal transplant outcomes (30).

We have previously documented that FeS has utility as an experimental "oxidant preconditioning" agent, in part by up-regulating ferritin expression (15-17,36-40). However, translation of these prior experimental findings to the clinical arena rests on several important assumptions. *First*, FeS must be able to rapidly (e.g., with 12-24 hrs) and robustly increase ferritin levels in humans if it is to confer rapid protection against impending renal insults, e.g., cardiac surgery. Although FeS is well known to increase ferritin levels in humans, this has classically been documented within weeks, not hours, of its administration (4,19). *Second*, it is unknown whether FeS predominantly up-regulates Fhc vs. Flc expression. Given that ferritin's protective actions are in large part Fhc dependent (46), FeS-mediated Fhc up-regulation seems essential if it is to induce a protective effect. *Third*, although the liver is the dominant site of ferritin production, its large size (450,000 daltons) precludes glomerular filtration, thereby preventing direct tubular access. Thus, if FeS is to induce renal cytoprotection in humans, an increase in intra-renal ferritin / Fhc production would seemingly be required. Finally, FeS "preconditioning" must be well tolerated by the human kidney despite its inducing transient renal oxidant stress (15-17, 36-40). Were FeS to exert overt nephrotoxicity, this would preclude its clinical

acceptance as a preconditioning agent. Hence, to gain insights into each of these issues, the present clinical study, complemented with a few supporting mouse experiments, was undertaken.

METHODS

Clinical studies: Nine healthy volunteers (HVs) and nine patients with stage 3-4 CKD were recruited for this investigation. The study received IRB approval from Advarra IRB, Columbia, MD, and informed consent was obtained from each subject. IRB approval from Fred Hutchinson Cancer Center was waived because only laboratory analysis of de-identified samples was performed at this site. Study exclusion criteria included pregnancy, any significant medical illness other than the presence of CKD, iron administration in the prior 30 days, or a plasma ferritin concentration of >500 ng/ml. Specific demographic data, screening eGFR (CKD-EPI equation), BUNs, serum creatinines, and blood pressures are presented in Table 1. This study was undertaken as part of a larger study which was enrolled in Clinicaltrials.gov (NCT03630029). The specific aspects of this sub-trial was also Clinicaltrials.gov enrolled (NCT04072432).

The HV and CKD groups were each divided into 3 equal cohorts (n,3 each), each cohort to receive either 120, 240, or 360 mg of FeS (12 mg/ml stock solution; Cascade Custom Chemistry; Portland, OR). The FeS dose (10, 20, or 30 ml of stock solution) was infused IV with 100 ml of saline over 1 hr. The subjects remained overnight at the study site (Riverside Clinical Research, Edgewater, FL) to screen for potential adverse events and to obtain timed blood and urine samples. Blood and urine samples were collected at baseline (0) and at 2, 4, 8, 18, 24, 48, 96, and 192 hrs after FeS infusion. Plasma ferritin concentrations were measured by Halifax Laboratory Services, Daytona Beach, FL (Vista 1500 autoanalyzer; Siemens; K6440 –10445136). Urine ferritin was measured by ELISA (ELH ferritin, RayBiotech, Norwich, GA). Selected plasma and urine samples were specifically probed for ferritin heavy chain (Fhc) and ferritin light chain (Flc) by Western blotting, as described later in the Methods section.

Assessments of potential FeS mediated nephrotoxicity in human subjects. To gauge whether FeS evoked nephrotoxicity, three independent, well established, AKI biomarkers were employed: 1)

urinary albumin concentrations (reflecting both potential changes in glomerular perm-selectivity and/or decreased proximal tubule reabsorption; ref. 18,35); 2) urinary NGAL (a marker of stress gene up-regulation; ref. 22,24); and 3) urinary NAG, a marker of direct tubular injury as reflected by tubular release of this lysosomal enzyme (10,22). Albumin was measured with a fluorometric assay (BioVision - #K550-100, Milpitas, CA). NGAL was determined by ELISA (R&D Systems, Minneapolis, MN; # DY1857); and NAG was quantified by a colorimetric assay (Bioassay Systems; #DNAG-100). All values were factored by the urinary creatinine concentration (BioChain; Newark, CA; #Z5030020) in each test urine sample. The assessed time points were as follows: baseline, and 8, 24, and 48 hrs following 120, 240 and 360 mg FeS administration. BUN and plasma creatinine concentrations at these three time points were also assessed (Halifax Laboratories).

Mouse studies.

Organ-specific responses to FeS injection. The following experiment was undertaken to determine: i) organ sites of FeS-mediated ferritin generation; ii) whether Fhc vs.Flc was produced; and iii) whether any ferritin up-regulation occurred via increased Fhc / Flc gene transcription, as assessed by potential increases in Fhc and Flc mRNAs. Male CD-1 mice (35-40 gms; Charles River Labs, Wilmington, MA) were used for all animal studies which were approved by the institution's IACUC. Ten mice were injected via the tail vein with either 1 mg of FeS or vehicle (n, 5 each). Four hrs later, they were deeply anesthetized with pentobarbital (40-50 mg/Kg), the abdominal and thoracic cavities were opened through midline incisions, and the heart, lung, kidneys, spleen, and liver were resected. The organs were iced, and total RNA and protein were extracted (15). Renal cortical, hepatic, splenic, cardiac, and lung samples were assayed for Fhc and Flc mRNAs by RT-PCR using the primer pairs presented in Table 2. Values were factored by simultaneously determined GAPDH product. Fhc and Flc protein levels were probed by Western blotting as described below. To determine the durability of the changes observed at 4 hrs post FeS injection, mice were injected with FeS or vehicle (n,3 each), and 18 hrs later, Fhc and Flc protein and mRNA assessments in kidney and liver were made.

Western blotting of Flc and Fhc in human plasma/urine and in mouse tissue samples. To assess the degrees to which Fhc and Flc ferritin are induced by FeS, human plasma and urine, and mouse

kidney, liver, heart, lung and spleen tissue homogenates, were evaluated by Western blotting. Normalized, denatured protein, under reduced conditions, was electrophoresed on Criterion XT Precast gels (4-12% Bis-Tris; BIO-RAD #3450124, Hercules, CA) and transferred onto 0.45μm nitrocellulose using the semi-dry method. To prevent non-specific binding, each blot was blocked with 5% non-fat milk prior to primary antibody incubation. The following Fhc antibodies were used: mouse tissue, Abcam ab183781, Cambridge, MA; human plasma or urine, Abcam ab75972. For Flc detection the following primary antibodies were used: mouse tissue, Abcam ab69090; human plasma or urine, Abcam ab243096. A goat-α rabbit secondary antibody was used for all blots (Abcam ab97051) and binding was assessed by chemiluminescence.

Statistics: The primary endpoints of the clinical portion of this study were change in plasma ferritin level and change in urine ferritin level from baseline (before FeS administration) to 24 and 48 hrs post FeS administration. These time points were selected prior to study initiation because this is the time frame during which the height of the experimental renal preconditioning state is expressed as shown previously (15-17,39,40). Statistical comparisons of change from baseline at these two time points were made using the one-sample t-test. Fold-change was considered to be of primary interest, so the change from baseline was estimated as a ratio. These ratios were then transformed with a logarithm function of base 2, resulting in a difference in logs as our primary outcome. In other words, testing if $\log_2$(ferritin at 24/48 hours) – $\log_2$(ferritin at baseline) is zero is equivalent to testing if the ratio (ferritin at 24 hours)/(ferritin at baseline) equals 1. Since the post-FeS ferritin level and the pre-FeS level are paired, a one-sample t-test was used to test the null hypothesis that the mean of the difference in log-transformed values (post-FeS plasma level minus pre-FeS plasma level) is equal to zero (equivalent to testing if the ratio is equal to 1). If the value of difference in log-transformed values is 1, this corresponds to a doubling of plasma ferritin; if the difference is 2, this corresponds to a 4-fold change; in general, if the difference in log-transformed values is n, this corresponds to a 2^n-fold change. For purposes of interpretability, the difference in the logarithms were exponentiated (with a base of 2) to get a fold-change, and the median fold-change is presented (the median is used as the summary measure for these purposes as fold-changes are inherently non-symmetric). Sensitivity analyses were

conducted to assess the impact of CKD (vs. HV), sex (male vs. female), and FeS dose (120 vs. 240 vs. 360 mg) post FeS administration on the change from baseline (at 24 hours, 48 hours) using simple linear regression. Secondary analyses were conducted by utilizing all post-FeS plasma values and, essentially, asking if the average fold-change among these values is different from 1. Since the various ferritin levels within a subject are correlated, we used repeated-measures analysis of variance (ANOVA) to assess the change from baseline, this allowing us to consider the within-subject variability of ferritin levels. As with change from baseline at 24 and 48 hours, change from baseline was modeled as the difference in the log-transformed ferritin values, and this change was regarded as the outcome in the repeated-measures ANOVA. Similar sensitivity analyses as described above were conducted for these secondary analyses, in addition to assessing the impact of time post FeS administration (2, 4, 8, 12, 18, 24, 48, 72, 96, 192 hours) on change from baseline. Additional secondary analyses assessed the change in urine albumin, urine NAG/creatinine ratio, and urine NGAL/creatinine ratio from baseline to 24 and 48 hours, these changes being analyzed as detailed above. Other secondary analyses looked at change in plasma ferritin from baseline to the earliest time (2 hours) and the latest time (192 hours) to assess the rapidity of increase from baseline as well as the persistence of the change. Since our primary endpoint of change in plasma and urine ferritin level from baseline to 24 and 48 hours consisted of four outcomes, in addition to nominal (unadjusted) p-values we also present Bonferroni-adjusted p-values accounting for these 4 comparisons. This adjustment is very conservative, however, as these 4 outcomes are clearly highly correlated. Only nominal p-values are provided for secondary analyses, as well as selected sensitivity analyses described previously.

RESULTS

Human subject baseline information. Selected demographics and baseline information for each of the study subjects are presented in Table 1. Baseline eGFR for the CKD group was 39.2±9.5 ml/min. The eGFR for each member of the HV group was reported as >70 ml/min. Baseline serum creatinines were 1.54±0.5 mg/dl and 1.0±0.2 mg/dl for the CKD and HV subjects, respectively. Six of the 9 CKD patients, but none of the HVs, were classified as having diabetes. Blood pressures were comparable between the HV and CKD groups (mean values, HVs: 127/79; CKDs: 130/75). All subjects received

the full scheduled FeS dosage. No adverse events that could be ascribed to Fe infusion were noted (1 case of “nasal congestion”; 1 case of “fatigue”).

Plasma ferritin responses to IV iron. Individual plasma ferritin levels for each of the subjects at each of the assessed time points and with each FeS dose are presented in Supplementary Table 1 https://figshare.com/s/3d8243ff5e03c1af8281. Baseline ferritin values varied widely amongst the subjects (HVs, 62±76 ng/ml; SD, CKD, 99±101). The mean difference from baseline in log-transformed ferritin values are shown by dose in Figure 1, and by disease (CKD vs. HV) in Figure 2.

For the primary endpoints of change in plasma ferritin from baseline to 24 and 48 hours, the mean change in the log-transformed values was 1.93 (nominal and Bonferroni-adjusted $p<.0001$, Table 3). The corresponding median fold-increase was 3.35. At 48 hours, similar qualitative conclusions were reached, with a mean change in log-transformed values of 2.25 (nominal and Bonferroni-adjusted $p<.0001$, Table 3). The corresponding median fold-increase was 4.17. All 18 subjects had an increase in plasma ferritin level from baseline to 24 and 48 hours.

Because each subject had an increase from baseline to both 24 and 48 hours, sensitivity analyses showed an increase, on average, from baseline in every subset of subjects based on presence of CKD, sex, and dose of FeS. In particular, the mean change in log-transformed values at 24 hours was 1.98 in females and 1.87 in males, respectively; the mean change in HV subjects was 2.26 and 1.60 in CKD subjects, respectively. Results were qualitatively similar for change from baseline to 48 hours, with a mean change of 2.31 in females, 2.18 in males, 2.52 in HV subjects, and 1.98 in CKD subjects. Further sensitivity analyses suggested a dose-dependent effect, as the mean change in log-transformed values from baseline to 24 hours was 1.22, 2.13, and 2.45 for doses of 120 mg, 240 mg, and 360 mg, respectively. For change at 48 hours, the mean changes were 1.36, 2.47, and 2.92, respectively.

Secondary analyses that assessed change from baseline at all times, not just at 24 and 48 hours, yielded qualitatively similar results. Among all subjects (HV and CKD at all doses), there were 180 plasma ferritin values at all time points following FeS administration. Of these, 178 were increased compared to the corresponding baseline value, and the mean change in log-transformed values was

1.40 (p<.0001 from repeated-measures ANOVA), with a range of -1.35 to 5.29. The increases in ferritin level from baseline did not show obvious differences between CKD and HV subjects (p=.23) nor based on sex (p=.79). There was little suggestion of a statistical interaction between sex and presence of CKD (p=.79), although the number of subjects precludes a reasonable assessment of any interactions (n=2 males with CKD, n=6 HV males, n=7 females with CKD, n=3 HV females). The ferritin response increased as the FeS dose increased (mean difference in log-transformed values of 0.82, 1.60, 1.77 for doses of 120 mg, 240 mg, 360 mg, respectively, p=.03).

An increase, albeit modest, in plasma ferritin was seen at the earliest time point of 2 hours. The mean change in log-transformed values was 0.34 (p<.0001) (corresponding median 1.25-fold increase). Of note, each of the 18 patients had an increase from baseline to 2 hours. The mean change at 2 hours was similar in HV and CKD (0.36, 0.33, respectively). At 192 hours, the mean change in log-transformed values was 1.54 (p<.0001) (corresponding median 2.76-fold increase). Of note, 17 of the 18 patients had a higher plasma ferritin level at 192 hours than at baseline. The mean increase at 192 hours was 2.00 in the HV group, 1.08 in the CKD group.

Western blotting of human plasma ferritin. It is noteworthy that clinically deployed ferritin auto analyzer assays do not provide information as to the relative amounts of heavy chain vs light chain that are present. Thus, to gain specific information in this regard, baseline plasma samples and urine samples obtained 24-48 hrs after 240-360 mg Fe infusion were probed by Western blotting using either heavy chain or light chain specific antibodies. As shown in lanes A and B of Fig. 3, FeS markedly increased plasma light chains in the absence of discernible heavy chain increases. To determine whether the observed light chain increases reflected free light chains within the circulation (i.e., not incorporated into the intact 450 kDa ferritin molecule), 48 hr post FeS plasma samples were filtered through a 50kDa molecular weight exclusion filter and the recovered (low molecular weight) fractions were assayed by ferritin ELISA. No ferritin was detected, implying that the plasma light chain increases were not free within the circulation. The retained fraction contained ferritin concentrations that approximated the auto analyzer results (data not shown).

Human Urine ferritin. Because of its large size (450kDa), and the absence of potentially filterable free Flc or Fhc within the circulation (as determined above), the presence of ferritin in urine is presumed to reflect a renal origin (20). Hence, we measured urinary ferritin and expressed the results as ng/mg creatinine. At baseline, the CKD patients had higher urine ferritin levels than did the HVs (32.88 vs. 2.26 ng/mg). At 24 hrs following FeS injection, the mean difference in log-transformed urine ferritin values was 2.22 (nominal p=.0001, Bonferroni-adjusted p=.0006, Table 3) with a median fold-change of 3.93, and mean increases in log-transformed values in both HV (2.46) and CKD (1.97) groups. Of note, all 18 patients had an increase from baseline in urine ferritin at 24 hours. Similar results were seen at 48 hours, with a mean difference in log-transformed values of 1.74 (nominal p=.006, Bonferroni-adjusted p=.02, Table 3; mean differences of 1.51 and 1.92 in HV and CKD, respectively) and a median fold-change of 2.62. Seventeen of 18 subjects had an increase from baseline to 192 hours. The mean change from baseline in log-transformed values depicted by dose in Fig. 4. Absolute urine ferritin/creatinine values at baseline, 24, and 48 hrs post FeS injection are presented in Supplemental Table 2. https://figshare.com/s/3d8243ff5e03c1af8281.

Human urine ferritin Western blotting. The above noted increase in urinary ferritin, as determined by ELISA, reflected increases in both light and heavy chains (see Fig. 3, lanes C and D). This stands in contrast to the previously noted plasma Western blot analyses which demonstrated only a light chain increase in response to FeS injection.

Assessment of potential FeS nephrotoxicity in human subjects.

Urinary albumin concentrations**.** Baseline urine albumin/Cr ratios were higher in the CKD vs the HV groups (0.73 mg/gm vs. 0.11 mg/gm creatinine, respectively). Consistent with the findings of Agarwal et al (1), occasional subjects (10 of 18) manifested minor increases in urine albumin concentrations within 8 hrs of Fe administration (supplemental Table 3; https://figshare.com/s/3d8243ff5e03c1af8281). These increases occurred even less frequently at 24 and 48 hours; the mean change in log-transformed values was -0.08 (increase in 9 of 18 subjects) and -0.31 (increase in 6 of 18 subjects), respectively. There was no suggestion of a dose-response association with mean change in log-transformed values from baseline to 24 hours (means of 0.42, -0.64, -0.03 for 120 mg, 240 mg, 360 mg,

295 respectively) or 48 hours (0.02, -0.42, -0.52, respectively), implying a possible infusion effect, rather
296 than direct FeS toxicity.

297 *Urinary NAG concentrations.* Baseline urinary NAG/Cr values were approximately twice as high in the
298 CKD (4.66) vs. the HV group (2.26). There were modest increases from baseline to 24 hours (12 of 18
299 subjects) and 48 hours (11 of 18 subjects), with a mean change in log-transformed values of 0.45
300 (p=.004) and 0.32 (p=.02), respectively. These increases were similar in HV and CKD subjects (0.45
301 vs. 0.44, respectively, at 24 hours; 0.30 vs. 0.33, respectively, at 48 hours) (supplemental Table 4
302 https://figshare.com/s/3d8243ff5e03c1af8281). There was no suggestion of a dose-response association
303 with mean change in log-transformed values to 24 hours (mean changes of 0.47, 0.49, 0.38 for 120 mg,
304 240 mg, 360 mg, respectively) or 48 hours (0.23, 0.48, 0.24), again suggesting a possible infusion
305 effect, rather than direct FeS toxicity.

306 *Urinary NGAL concentrations.* Individual urinary NGAL/Cr ratios are presented in supplemental table 5
307 https://figshare.com/s/3d8243ff5e03c1af8281). Baseline urinary values were quite variable, but were, on
308 average, higher in the CKD compared to the HV subjects (110.9 ng/mg vs. 10.1 ng/mg). The changes
309 from baseline were quite variable; there were some increases from baseline to 24 hours (10 of 18
310 subjects) and to 48 hours (7 of 18 subjects), with a mean change in log-transformed values of 0.63 and
311 0.09, respectively, but these increases were not convincingly real and/or were, at most, modest (p=.25,
312 p=.80, respectively). The mean changes in log-transformed values in HV and CKD subjects were 0.88
313 and 0.39, respectively, at 24 hours; -0.41 vs. 0.59, respectively, at 48 hours. There was no suggestion
314 of a dose-response association with change from baseline to 24 hours (mean change in log-
315 transformed values of 0.38, 1.22, 0.30 for 120 mg, 240 mg, 360 mg, respectively) or 48 hours (-0.06,
316 0.43, -0.10).

317 **Mouse experiments: Probing mouse organs for heavy and light chain**.

318 *Kidney.* As discussed above, it is assumed that the increase in urine ferritin heavy and light chains
319 seen in HVs and CKD patients reflected renal production, rather than ferritin filtration due to ferritin's
320 large size (450kDa). However, to provide experimental proof that the kidney produces both Fhc and
321 Flc, we probed mouse renal cortical extracts at 4 and 18 hrs post FeS administration. At baseline, Fhc,

but not Flc, Western blot bands were observed (Fig 3). Following FeS administration, marked Flc and Fhc increases were noted at both the 4 and 18 hr time points. Representative baseline plots and 18 hr blots are depicted in Fig. 3, lanes E and F.

Extra-renal organs: Baseline liver samples demonstrated both Flc and Fhc expression. FeS induced marked Flc, but only small Fhc, hepatic increases (Fig. 3. lanes G and H). Spleen demonstrated substantial Fe induced Flc and Fhc increases (not shown), recapitulating the kidney pattern as noted above. FeS failed to alter either Flc or Fhc in heart or lung (not shown).

Mouse ferritin mRNA assessments. FeS induced no significant increases in Flc or Fhc mRNA in any of the tested organs (Table 4), implying that translational, rather than transcriptional events were responsible for the above noted FeS- mediated ferritin increases.

DISCUSSION

A classic experimental maneuver for up-regulating ferritin, and thereby triggering its cytoprotective properties, is the administration of heme proteins (most notably hemoglobin or myoglobin; e.g., ref. 26,31,36). Within 24 hrs of their injection, striking increases in renal ferritin levels, and renal resistance to AKI, result. However, given the propensity of heme proteins to co-precipitate with Tamm Horsfall protein (i.e., uromodulin) in the distal nephron under aciduric conditions (34), the potential for obstructive cast formation precludes heme protein use as clinical preconditioning agents. Given this consideration, our laboratory previously tested whether FeS can recapitulate heme protein-mediated ferritin up-regulation, and thus have potential utility as a renal protective agent (15-17; 36-40). Our prior experimental results strongly support this possibility, given that within 18-24 hr of FeS administration, significant ferritin up-regulation and partial protection against diverse forms of AKI develop (15-17, 36-40).

For FeS to have clinical utility as a renal preconditioning agent, a rapid ferritin response (e.g., within 12-24 hrs prior to cardiovascular surgery), including an increase in *renal* ferritin levels, is presumably required. Hence, the present clinical study was undertaken to assess the time course of FeS- induced ferritin up-regulation using plasma and urine ferritin levels as biomarkers of this response. Surprisingly, within just 2 hrs of FeS administration to either healthy volunteers or CKD patients,

significant, likely dose-dependent, plasma ferritin elevations were observed. These increases were progressive with time, rising ~3-5 fold above baseline values in both the HV and CKD cohorts within just 24-48 hrs. It is notable that the plasma ferritin increases were well maintained throughout the 8-day study period. Thus, the above data suggest that the FeS- mediated ferritin response is sufficiently rapid, robust, and sustained to have potential utility as a clinical pre-operative renal preconditioning agent.

Although we previously demonstrated that FeS induces a marked increase in *renal* ferritin levels in mice (15-17; 36-40), we had no evidence that the same holds true in humans. Thus, to gain insight into this matter, we have now used urinary ferritin concentrations as a biomarker of human kidney ferritin expression (20). By 24-48 hours post FeS administration, ~3-5 fold urinary ferritin elevations were observed. Three factors support the concept that these urinary ferritin increases do, indeed, reflect renal ferritin induction. *First*, given its large size (450 kDa), circulating ferritin is far too large to pass through the glomerular filter, particularly in healthy volunteers with an intact glomerular barrier. This implies that increased renal ferritin production, with partial ferritin egress into tubular lumina, occurs. *Second*, if one were to contend that FeS damages the glomerulus, thereby facilitating ferritin filtration, one would expect a corresponding (and even larger) increase in urinary albumin excretion given its much smaller size (69 kDa). However, no matching urinary albumin increases were observed. This precludes increased glomerular filtration as the reason for increased urinary ferritin excretion. *Third*, we considered the possibility that the urinary ferritin increases could theoretically result from the filtration of low molecular weight *free heavy or free light chains (23 kDa and 21 kDa respectively)* into urine. However, when we filtered post-iron treated human plasma samples through a 50 kDa exclusion filter, no low molecular weight ferritin moieties were detected in the filtrate. Thus, these three findings support the concept that the detected urine ferritin increases after FeS treatment reflected increased renal ferritin production. Of interest, it has previously been documented that clinical hemoglobinuria also increases urinary ferritin excretion (so called “ferritinuria”; ref. 20). This further implies that an up-regulation of renal ferritin expression is marked by increased urinary ferritin excretion. Of note, the post

375 Fe urinary ferritin levels varied substantially amongst tested subjects. Whether relatively high vs. low
376 urinary ferritin responders might predict degrees of renal protection is an intriguing consideration.

377 When interpreting plasma or urine ferritin ELISA results, it is important to recognize that
378 clinically deployed ferritin assays are neither Fhc- nor Flc- specific. This is because there is ~50%
379 homology between the two ferritin chains and ELISA- based assays do not distinguish between them.
380 Depending on the type of tissue assayed, the ratio of heavy chains to light chains can substantially
381 differ (e.g., liver and spleen: Flc>>Fhc; kidney and heart: Fhc>Flc) (6,12,29). Furthermore, the relative
382 proportions of Flc and Fhc can vary under different physiologic / pathophysiologic conditions (12).
383 Given these considerations,and given that FeS evoked marked plasma and urine ferritin increases as
384 assessed by ELISA, we sought to determine whether FeS preferentially evoked Fhc vs Flc expression.
385 To this end, we performed Western blotting of clinical plasma and urine samples using Fhc- and Flc-
386 specific antibodies that were raised against synthetic non-homologous Fhc and Flc amino acid
387 sequences. FeS evoked dramatic plasma Flc, but not Fhc, increases. Conversely, comparable
388 urinary Fhc and Flc increases were observed. To gain more specific insights into organ- specific
389 patterns of Fhc vs. Flc production in response to FeS, we probed mouse organ samples. Liver
390 manifested a marked Flc, but a lesser Fhc, response to FeS injection, consistent with the pattern
391 observed in human plasma. Conversely, comparable Fhc and Flc increases were seen in kidney, again
392 consistent with the human urine pattern. Of note, the spleen recapitulated these renal results, whereas
393 neither heart nor lung manifested FeS- induced ferritin increments. In concert, these findings indicate
394 that different organs demonstrate different ferritin responses to FeS, and that the kidney and spleen are
395 the dominant (tested) organs to mount Fhc increases. Finally, it is well established that both
396 transcription and translation can increase ferritin production (29). That FeS failed to substantially
397 increase either Fhc or Flc mRNA in any of the tested organ suggests that FeS predominantly stimulated
398 ferritin production via translational, rather than transcriptional, events.

399 For FeS to be a clinically acceptable renal preconditioning agent, it must induce this response in
400 the absence of a significant nephrotoxic effect. In this regard, we have previously reported that when
401 FeS was administered to mice in supra-pharmacologic doses, mild tubular toxicity was, indeed,

observed (42,44). Thus, we have now explored whether the currently employed FeS doses (120, 240, 360 mg) would be well tolerated by both the normal and diseased (stage 3-4 CKD) human kidney. This appears to be the case, given that BUN and creatinine levels remained stable post FeS treatment, and that no significant dose dependent increases in urinary NAG, NGAL, or albumin concentrations were observed. Of note, all three of these urinary biomarkers appeared elevated at baseline in the CKD vs. the HV cohorts, underscoring their role as renal injury biomarkers.

The ultimate clinical utility of FeS- induced preconditioning remains to be defined, and the present study has a number of limitations in this regard. *First*, although it is clear that ferritin possesses potent cytoprotective properties, the degree to which they are expressed is presumably dependent on cellular, not plasma or urinary, ferritin concentrations. In this regard, it remains to be determined how accurately plasma and urine ferritin assays reflect intracellular ferritin pools. *Second,* while the present mouse results clearly indicate that FeS increased renal ferritin heavy and light chain levels, only direct human tissue ferritin analysis, currently not feasible, can provide definitive data. *Third*, although FeS can clearly evoke oxidant preconditioning, it should be emphasized that many other redox sensitive proteins in addition to ferritin contribute to the emergence of a post-oxidant renal cytoprotective state. Notable in this regard are heme oxygenase 1, haptoglobin, and hemopexin which can also be up-regulated by Fe mediated preconditioning (3, 15-17, 36-40, 45). Thus, the potential efficacy of FeS as a preconditioning agent cannot be gauged solely by ferritin assessments; and *fourth*, in our prior experimental work, we have documented that the co-administration of a transient heme oxygenase inhibitor, tin protoporphyrin, dramatically enhances the FeS- induced preconditioning state (15-17; 39,40). Hence, combination agent administration is likely to have greater potential for inducing clinical success. Each of the above issues will obviously necessitate further clinical studies, some of which are currently in the planning stage.

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589 **FIGURE LEGENDS**

590 **Figure 1. Change in plasma ferritin concentrations from baseline over time following Fe**
591 **sucrose administration by dose (120 mg, 240 mg, 360 mg) for all subjects combined.** The data
592 represent the mean change from baseline in log-transformed values. Individual vales for each subject
593 and at each time point are presented in Supplemental Table 1
594 (https://figshare.com/s/3d8243ff5e03c1af8281). The 95% confidence intervals at the primary endpoints, 24
595 and 48 hrs post FeS, are presented.

596 **Figure 2. Changes in plasma ferritin concentrations following Fe sucrose administration for**
597 **health volunteers vs. CKD patients.** The data represent the mean difference from baseline in log-
598 transformed values. Individual vales for each subject and at each time point are presented in
599 Supplemental Table 1 (https://figshare.com/s/3d8243ff5e03c1af8281). The 95% confidence intervals at the
600 primary endpoints, 24 and 48 hrs post FeS, are presented.

601 **Figure 3. Western blots of human plasma, human urine, and mouse kidney and liver samples**
602 **using ferritin heavy chain (HC) and ferritin light chain (LC) specific antibodies.** Lane samples,
603 representative of overall Western blot probes, are presented. As depicted, at 48 hrs post 240 mg of
604 FeS administration, human plasma manifested a marked increase in light chain (LC) (Lane A), but not
605 in heavy chain (HC) ferritin (Lane B), compared to baseline (BL) levels. Of note, the maximal increase
606 in plasma ferritin, as detected by ELISA, was at 48 hrs; thus, this time was selected for Western
607 blotting. Human urine showed both light chain (Lane C) and heavy chain (Lane D) responses to FeS
608 treatment. (Samples from both CKD and HV subjects revealed comparable changes; not shown).
609 In the lower half of the figure, mouse kidney and liver blots are depicted at 18 hrs post FeS
610 administration and compared to blots obtained using control (C) kidney and liver tissues. 1) Mouse
611 kidney showed virtually no light chain (LC) expression in the absence of FeS treatment; however, by 18
612 hrs post FeS, marked renal light chain accumulation was observed. 2) Mouse kidney revealed heavy
613 chain (HC) expression under control conditions, and marked heavy chain increases resulted from FeS

614 treatment. 3) In liver, both heavy and light chains were expressed under control (C)conditions
615 (Lanes G and F). FeS most prominently up-regulated hepatic light chain expression.
616

617 **Figure 4. Change in urine ferritin concentrations (as assessed by ELISA) from baseline to 24**
618 **and 48 hrs following Fe sucrose administration by dose (120 mg, 240 mg, 360 mg).** The bar
619 heights represent the mean change from baseline in log-transformed values. Individual urine ferritin
620 values, factored by urine creatinine at baseline, and at 24 and 48 hrs post iron administration are
621 presented in Supplemental Table 2 https://figshare.com/s/3d8243ff5e03c1af8281). The vertical lines
622 represent the 95% confidence limits.

623

624

Table 1. Subject demographics.

Dose, 120 mg	AGE	SEX	WEIGHT	BP (S-D)	EGFR	BUN	SCr	DM
HV	59	Female	64.9	127-88	>70	9.0	0.69	N
HV	51	Male	93.6	130-78	>70	16.0	0.90	N
HV	55	Female	83.0	117-78	>70	12.0	0.90	N
CKD	72	Male	103.0	140-71	29	42.0	2.20	Y
CKD	59	Female	111.2	121-88	47	22.0	1.26	N
CKD	64	Female	82.5	133-78	34	36	1.47	Y
Dose, 240 mg								
HV	62	Male	111.5	140-79	>70	11.0	1.04	N
HV	50	Male	100.7	124-84	>70	18.0	1.18	N
HV	31	Female	93.0	110-77	>70	12.0	0.75	N
CKD	66	Female	71.4	154-84	30	26.0	1.74	Y
CKD	66	Female	101.2	104-72	52	16.0	1.10	N
CKD	71	Female	88.8	146-80	40	20.0	1.54	Y
Dose, 360 mg								
HV	50	Male	111.2	115-77	>70	12.0	1.17	N
HV	59	Male	73.3	135-79	>70	13.0	1.13	N
HV	51	Male	93.4	127-80	>70	27.0	1.27	N
CKD	74	Male	103.9	125-53	27	37.0	2.28	Y
CKD	68	Female	79.1	112-72	50	18.0	1.12	N
CKD	78	Female	109.4	136-77	44	12.0	1.19	Y

Table 1 Legend. Demographics and biologic data pertaining to the nine healthy volunteers (HVs) and nine patients with CKD, are presented according to the Fe sucrose dose. Body weight, in Kg. Blood pressure, systolic (S) – diastolic (D). eGFR, estimated GFR (ml/min) by CKD-EPI equation. HV eGFRs are given as >70 ml/minute. BUN and serum creatinine (SCr), mg/dl. DM= diabetes mellitus, yes or no (y/n).

Table 2: Mouse Primers for RT-PCR

mRNA	Primer Sequences	Product Size
Heavy Chain Ferritin	5'- CGG TTT CCT GCT TCA ACA GTG CTT -3' 5'- ATG CAC TGC CTC AGT GAC CAG TAA -3'	741 bp
Light Chain Ferritin	5'- GGG CCT CCT ACA CCT ACC TC -3' 5'- AGA TCC AAG AGG GCC TGA TT -3'	265 bp
GAPDH	5'- CTG CCA TTT GCA GTG GCA AAG TGG - 3' 5'-TTG TCA TGG ATG ACC TTG GCC AGG - 3'	437 bp

Table 2 legend. Primer pairs used for detection of mouse ferritin heavy chain and ferritin light chain mRNAs by competitive RT-PCR. GAPDH product served as the housekeeping gene.

Table 3. Changes in plasma and urine ferritin values (ng/mg creatinine) from baseline to 24 and 48 hours following Fe sucrose administration.

	Plasma ferritin		Urine ferritin	
	24 hours	48 hours	24 hours	48 hours
Mean (standard deviation) change from baseline in log-transformed values	1.93 (1.12) (p<.0001, p<.0001)	2.25 (1.12) (p<.0001, p<.0001)	2.22 (1.93) (p=.0001, p=.0006)	1.74 (2.33) (p=.006, p=.02)
Median fold-change from baseline	3.35	4.17	3.93	2.62

Table 3 legend. Mean values of difference in the $\log_2$-transformed values are provided (p-values from one-sample t-test, unadjusted and Bonferroni-adjusted). For ease of interpretation, the median fold-change from baseline is also presented.

647 **Table 4. Ferritin heavy chain (Fhc), ferritin light chain (Flc), and NGAL mRNA levels in mouse**
648 **test organs either under control conditions or 4 hrs post FeS injection.**

	Fhc mRNA (4 hr)	Flc mRNA (4 hr)	Fhc mRNA (18 hr)	Flc mRNA (18 hr)
Kidney				
FeS	1.49±0.06	1.47±0.09	1.40±07	1.35±0.17
Control	1.38±0.01	1.10±0.09	1.39±0.09	1.41±0.17
Liver				
FeS	1.71±0.14	3.63±0.13	1.31±0.08	2.32±0.21
Control	1.77±0.11	3.65±0.15	1.75±0.12	2.8±0.17
Heart				
FeS	0.58±0.03	0.09±0.01 (6:1)	ND	ND
Control	0.55±0.04	0.09±0.01	ND	ND
Lung				
FeS	1.80±.0.05	1.53±0.1	ND	ND
Control	1.72±0.12	1.82±0.12	ND	ND
Spleen				
FeS	2.99±0.51	1.97±0.23	ND	ND
Control	2.72±0.68	1.87±0.22	ND	ND

649

650 **Table 4 legend.** All organs were tested at 4 hrs post injection (n,5 each). Kidney and liver mRNAs
651 were also assessed at 18 hrs post injection (n, 3 each). Differences between FeS injected mice and
652 control mice were similar. ND, not done.

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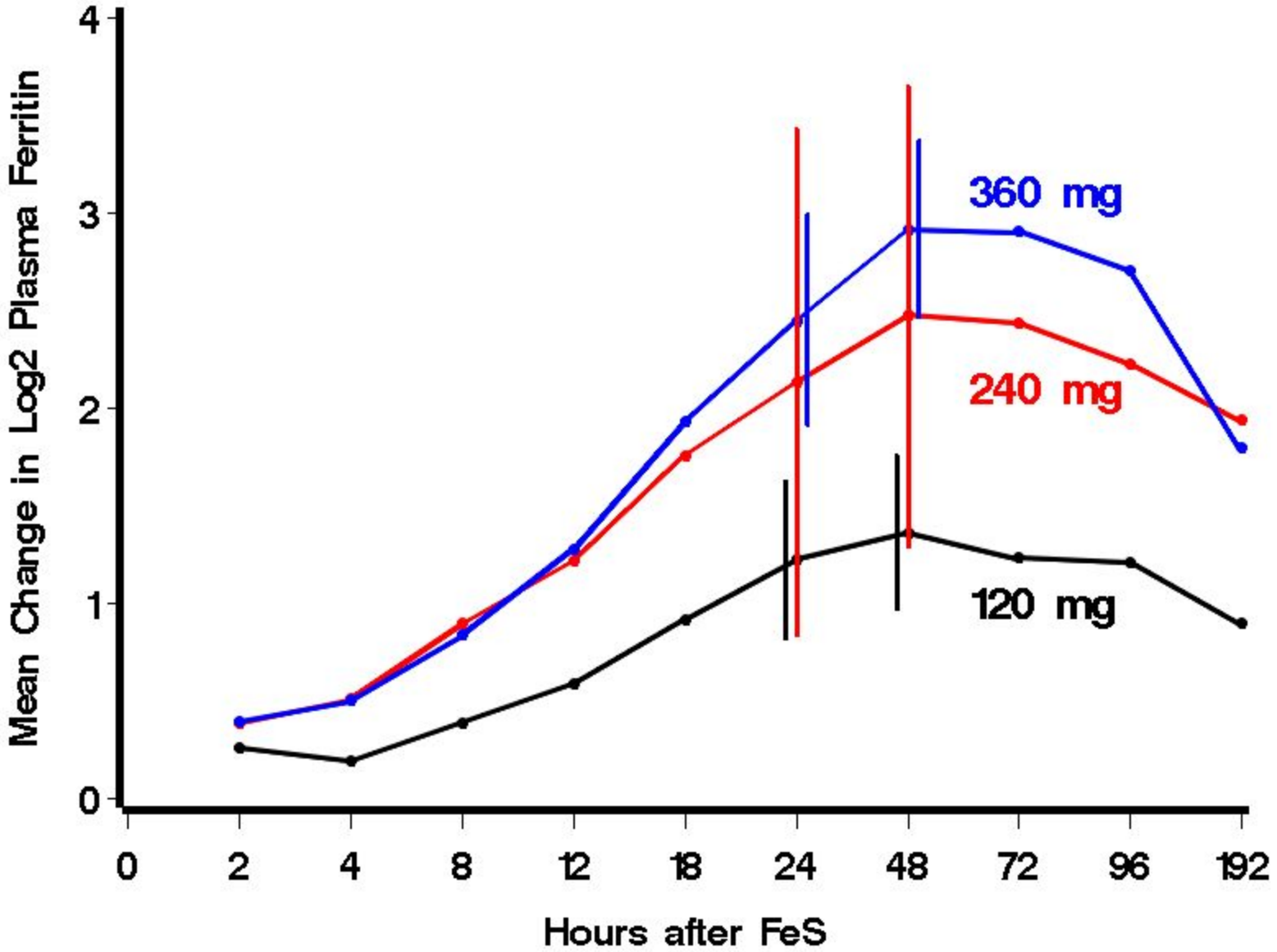

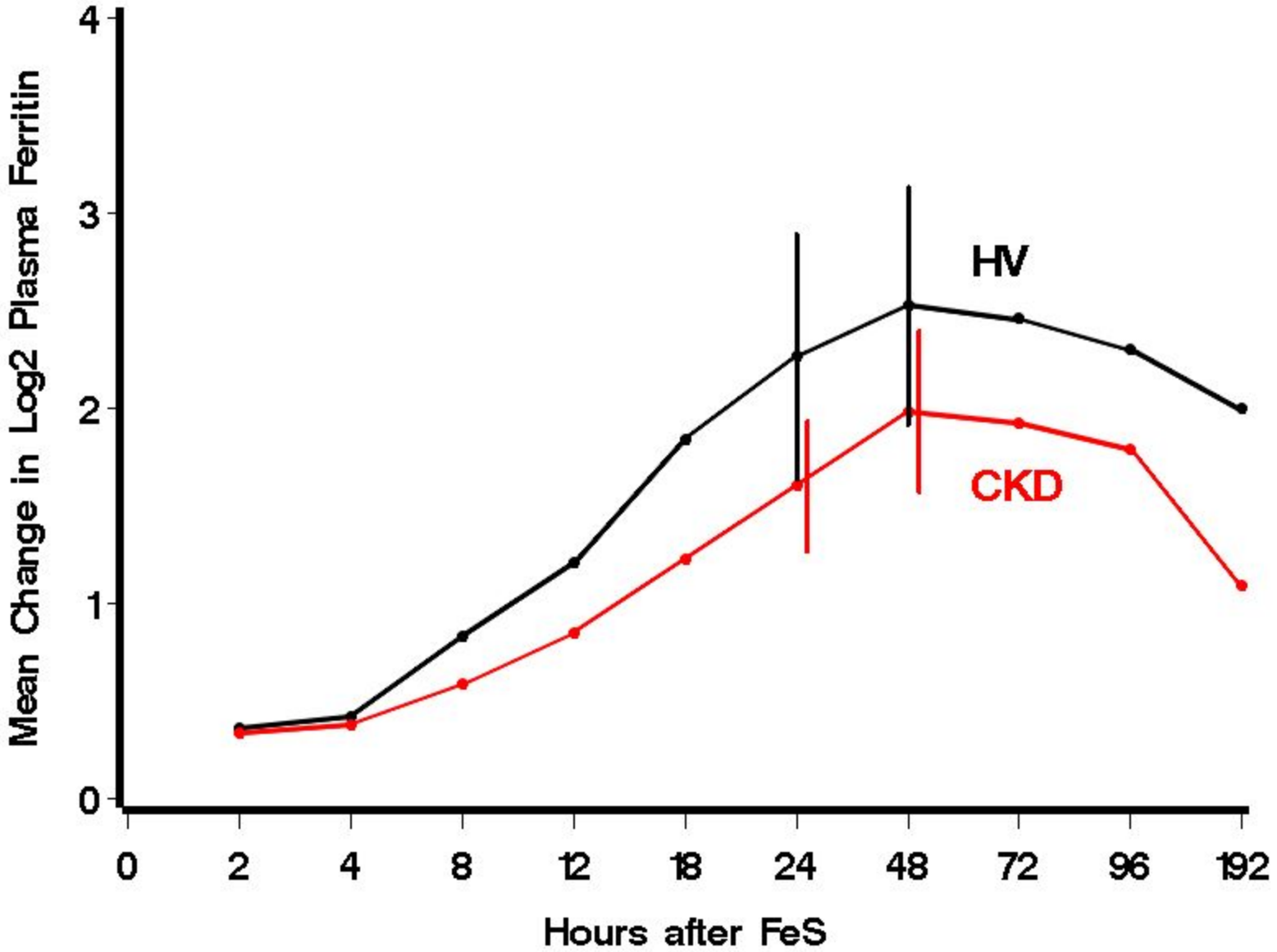

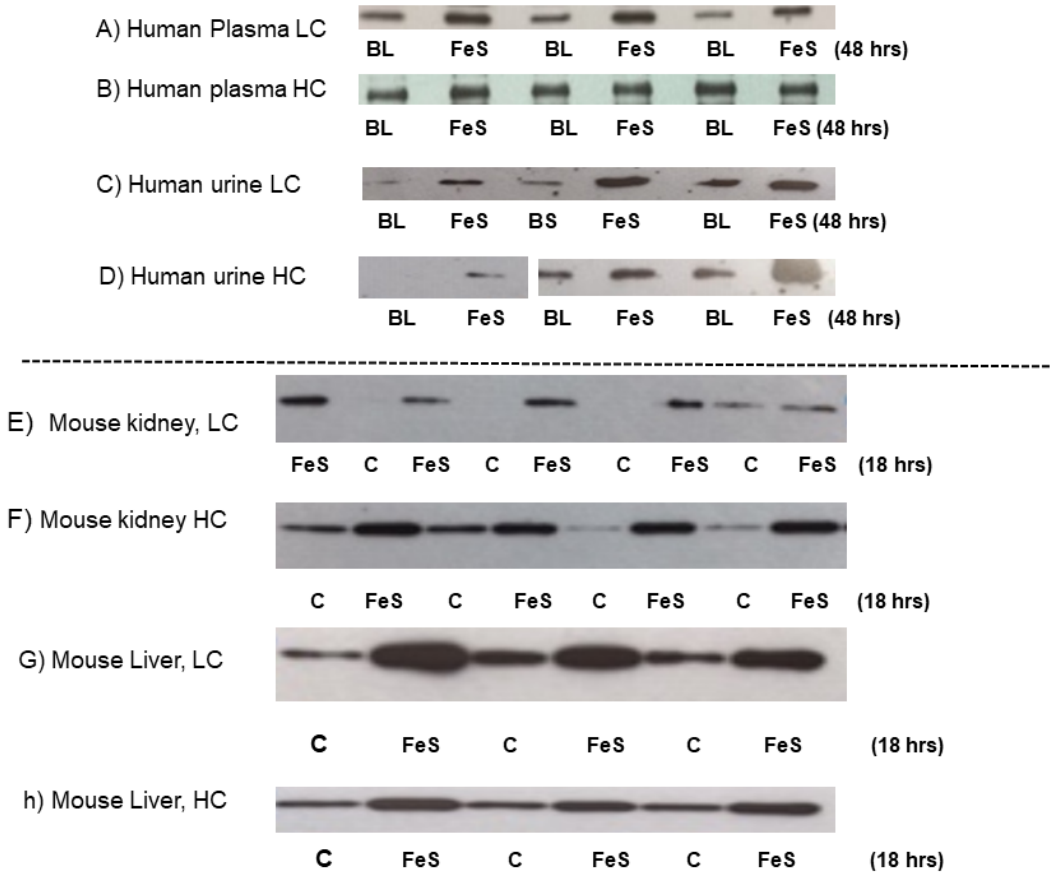

Fig. 3