Original Research

Impact of intravenous iron on cardiac and skeletal oxidative stress and cardiac mitochondrial function in experimental uraemia chronic kidney disease

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1. Abstract

Introduction: Uraemia leads to changes in cardiac structure, metabolic remodeling and anaemia, key factors in the development of heart failure in patients with chronic kidney disease. Previous studies have identified abnormalities in mitochondrial function, potentially impairing energy provision and enhancing oxidative stress. This study characterised oxidant status and changes in mitochondrial function in uraemia and the impact of correcting anaemia via intravenous iron therapy.

Methods: Experimental uraemia was induced in male Sprague-Dawley rats via a subtotal nephrectomy and parenteral iron administration given 6 weeks post-surgery. Oxidative stress from tissue samples was evaluated by measuring pro-oxidant activities and anti-oxidant capacities in both sham and uraemic animals with and without iron supplementation. Thiobarbituric acid-reactive substances (TBARS), aconitase activity and cardiolipin were measured. Mitochondrial function was assessed using the Seahorse XFp analyser on isolated mitochondria excised from cardiac tissue. Results: Oxidative stress in this uraemic model was increased in cardiac tissue (increased GSSG/GSH ratio, TBARS and increased activities of pro-oxidant enzymes). There was no impact on skeletal tissue. Parenteral iron ameliorated oxidative stress by enhancing the anti-oxidant defense system in cardiac tissue and skeletal tissue. Examination of respiratory reserve in cardiac mitochondria demonstrated that parenteral iron restored mitochondrial function. This experimental model of uraemia demonstrated a specific oxidative stress on the heart muscle without significant changes in skeletal oxidant status. Iron therapy improved anti-oxidant defence system, consequently reducing oxidative stress in the heart and skeletal tissue. There was an improvement in cardiac mitochondrial function. Conclusions: This experimental evidence indicates that iron therapy could reduce vulnerability to oxidative stress and potentially improve both cardiac and skeletal functional capacity from improvements in mitochondrial function.

2. Introduction

Patients with chronic kidney disease (CKD) are at increased risk of cardiovascular related death in comparison to the general population [1, 2]. Forty-four to fifty percent of deaths in patients with renal failure (RF) are as a result of cardiovascular events [3, 4], a mortality that is 15–30 times higher than age adjusted general population [5]. The high adverse cardiovascular outcomes in this group of patients is...
partly accounted for by the existence of a “unique” uraemic cardiomyopathy (UCM) which is characterised by left ventricular hypertrophy (LVH), systolic and diastolic dysfunction, and left ventricular dilation. Left ventricular hypertrophy in UCM is accompanied by compromised energetics and alterations in mitochondrial function which impair energy provision and lead to an increased susceptibility of the uraemic heart to injury [6–8]. Other factors contributing to UCM include chronic hypertension, volume overload, hyperparathyroidism and iron deficiency anaemia [9], the latter which is important in clinical outcomes for patients with CKD or requiring dialysis therapy [10, 11].

Chronic kidney disease associated anaemia is characterised as normocytic and normochromic and arises from erythropoietin deficiency, chronic inflammation and iron deficiency amongst other causes [12]. This anaemia affects about 60–80% of patients with CKD and contributes towards the development of cardiac hypertrophy (left ventricular hypertrophy (LVH)) [13]. A prospective study found that for each 0.5 g/dL decrease in haemoglobin concentration there was a 32% increased risk of LVH [14]. Despite this observation, correction of anaemia does not lead to complete regression of LVH [15]. A small cohort study and two larger randomised studies have examined the effects of intravenous (IV) iron in patients with heart failure and shown an increased haemoglobin, better renal function, and lower N-terminal Pro Brain Natriuretic peptide (NT pro BNP) [16–18]. The FAIR-HF study [18] demonstrated improved functional capacity (6-minute walking distance), quality-of-life and New York Heart Association (NYHA) functional class. CONFIRM-HF verified these results in a larger randomised controlled trial [19]. Taken together these studies suggest that IV iron may have beneficial effects on the heart which may impact on reduced cardiovascular events, improved functional capacity and reduced symptoms if present. Finally, the EFFECT-HF trial in 172 patients with systolic heart failure demonstrated improved functional status and well-being [20]. A 4 trial meta-analysis also suggested treatment with intravenous ferric carboxymaltose was associated with a reduction in recurrent cardiovascular hospitalisations in systolic heart failure patients [21]. Also, in iron deficient non anaemic chronic kidney disease patients a recent randomised controlled trial suggested a trend to an improved quality of life and reduction in brain natriuretic peptide (BNP) [22]. Anaemia significantly increases the risk of morbidity and mortality in CKD [12]. Iron deficiency anaemia in CKD is associated with diminished cytochrome c oxidase activity, decreased mitochondrial oxidative capacity and reduced total antioxidant capacity resulting in mitochondrial oxidative stress [23]. Therefore, anaemia correction by erythropoietin and iron therapy is an integral component of CKD management. Iron therapy via intravenous (IV) administration of an iron complex such as ferric carboxymaltose, iron dextran, iron isomaltoside, iron sucrose, ferric gluconate and ferumoxytol has proven to be effective in correcting iron deficiency anaemia in CKD and more recently reducing cardiovascular events and mortality in dialysis patients [10, 24, 25].

Iron replacement therapy is also associated with the induction of oxidative stress evidenced by increased lipid [26, 27] and protein [28] oxidation following iron sucrose administration [29]. This effect may be attributed to “free” iron [30]. “Free” or labile iron readily cycles between ferrous and ferric oxidation states that enables it to participate in the generation of reactive oxygen species (ROS) such as hydroxyl radicals, ferryl and perferryl ions [31]. Hydroxyl radicals from the iron-catalysed Haber-Weiss reaction contribute to the initiation and propagation of mitochondrial lipid peroxidation. Iron induced lipid peroxidation is associated with intense mitochondrial damage [32] that could lead to a decline oxidative capacity. Studies have found increased evidence of systemic oxidative stress in CKD following IV iron with limited research work on cardiac oxidant status [33]. However, as we have previously noted mitochondrial enzymes such as those the Krebs cycle and the electron transport chain require iron for optimal function and potentially minimisation of oxidative stress [34].

Despite the central role of iron in key mitochondrial proteins (aconitase, complex I, II or III), the impact of parenteral IV iron therapy as employed in clinical practice on mitochondrial function and oxidative stress in cardiac and skeletal muscle in CKD has not been sufficiently characterised. There is a paucity of data in patients with CKD [35]. We have previously confirmed the phenotype of the uraemic model with iron deficiency anaemia and the impact of IV iron therapy on oxidative stress and mitochondrial function in renal tissue [33]. The aim of this study was to further examine the cardiac oxidative response to IV iron administration and test the hypothesis that supplementation with intravenous iron “normalises” mitochondrial function, improves cardiolipin content and mitochondrial bioenergetics (increases the mitochondrial efficiency of respiration) and thus reduces the vulnerability of the uraemic heart and uraemic skeletal muscle to oxidative stress and may improve oxidant capacity (via restoration of systemic anti-oxidant activity). In addition, we examined the impact of uraemia and iron treatment on cardiolipin (CL) content and changes in mitochondrial bioenergetics in the uraemic heart and skeletal tissues. These changes thus may lead to clinical improvements in cardiac and skeletal function.

3. Materials and methods

3.1 Induction of experimental uraemic model

We have detailed the methods previously [7, 8, 33] but confirm once again that all experiments using animals were in accordance with the UK Animals (Scientific Procedure) Act 1986 and were approved by the University of
Hull Ethical Review Process (PPI number 70/7966). Male Sprague-Dawley rats (180–200 g) (Charles River Laboratories, Kent, UK) were used to create a uraemic model via a one-stage subtotal nephrectomy, as described previously [8, 33, 36]. In brief, anaesthetic induction of animals was achieved with 3% isoflurane in 3 L/min oxygen (O2) and maintenance anesthesia with 2.5% isoflurane in 1 L/min oxygen. During this surgery consisted of a midline abdominal incision for exposure and decapsulation of the left kidney. The renal vasculature was clamped and 50% of the kidney removed. Any bleeding was treated using Surgicel® (Johnson & Johnson, Maidenhead, Berkshire, UK) before the remnant kidney was replaced. The right kidney was subsequently decapsulated before closure of the abdominal muscular and dermal layers. Sham animals were subjected to exposure and decapsulation of both kidneys only. Animals were kept in individual cages for twelve weeks post-surgery (six weeks after the IV iron) and pair fed (sham and uraemic) with standard chow diet and had free access to water.

3.2 Intravenous iron therapy

A single intravenous injection via the tail vein of iron (Ferumoxytol supplied by Takaeda UK Ltd., Holborn, London, UK) at a dose of 10 mg/kg body weight was given 6 weeks post-surgery. The protocol for use of Ferumoxytol therapy (one of several forms of available parenteral iron in clinical practice) used in this study was an equivalent weight adjusted bolus injection clinical practice, which uses a dose of 510 mg in adults (i.e., 8–10 mg/kg body weight).

3.3 Oxidative stress measurements

Oxidative stress was investigated by assessing markers of oxidative stress, pro-oxidative activities, and antioxidant capacity in four experimental groups 12 weeks post-surgery. These experimental groups were:

1. sham operated rats;
2. uraemic rats (following 5/6 nephrectomy);
3. sham operated rats receiving IV iron (ferumoxytol) at week 6 post-surgery;
4. uraemic rats receiving IV iron (ferumoxytol) at week 6 post-surgery.

Enzyme assays were performed using supernatant from 200 mg of cardiac and skeletal tissue extracted in 1 mL 50 mM Tris hydrochloric acid (HCL) buffer (pH 7.2).

Anti-oxidant marker samples were prepared and analysed for reduced glutathione (GSH) and oxidised glutathione (GSSG) using the method of Nuhu et al. [37]. Data analysis was performed on Agilent Chem station software version 2.30 (Agilent Technologies, Waldbronn, Baden-Württemberg, Germany).

3.4 Lipid peroxidation markers

Markers of lipid peroxidation included peroxide lipids (LOOH), thiobarbituric acid (TBA) and TBA reactive substance (TBARS). TBARS was analysed by high performance liquid chromatography (HPLC) according to modified method of Seljeskog et al. [38]. TBARS peaks in samples were identified by comparison with the retention times of 1,1,3,3-tetraethoxypropane (TEP) standard and a TEP standard curve was used to quantify TBARS levels in samples.

3.5 Pro-Oxidant activities

Pro-oxidant activity in the uraemic heart was evaluated through the activity of NADPH oxidase using modified method of Reusch and Burger [39]. NADPH oxidase activity was calculated using an extinction coefficient of 6.22 mM−1 cm−1.

3.6 Antioxidant capacity

The antioxidant capacity of the uraemic heart and skeletal muscle were assessed via measurement of the activities of superoxide dismutase (SOD), glutathione peroxidase (GPxs), glutathione reductase (GR) and catalase.

Total SOD activity was determined using the modified method of Marklund and Marklund [40]. The activity of SOD was recorded by comparing % inhibition of samples to that of authentic SOD standard (Randox laboratories, Crumlin, UK) and enzyme activity expressed as U/mg protein. One unit of SOD is the amount of the enzyme which causes a 50% inhibition under conditions of the assay.

The endogenous GPx activity was measured using commercially available Ransel kit (Randox laboratories, Crumlin, UK) according to the method of Paglia and Valentine [41]. This method is based on the oxidation of glutathione by cumene hydroxide under GPx catalysis. The decrease in absorbance following the concomitant oxidation of NADPH to NADP+ is measured at a wavelength of 340 nm. The GPx activity expressed as U/mg protein in samples was determined by comparing the change of absorbance over 2 minutes to that of authentic GPx standard (Sigma Aldrich, UK).

Glutathione reductase activity was measured using a commercially available kit (Glut Red, Randox Laboratories, UK) which is based on the reduction of GSSG in the presence of nicotinamide adenine dinucleotide hydrogen phosphate (NADPH) by GR. The GR in samples was determined by comparing the change of absorbance (at 340 nm) of authentic GR standard (Sigma Aldrich, UK) over 4 minutes following 1 minute incubation at 37 °C and activity was expressed as U/mg protein.

Catalase activity was measured by modified method of Aebi [42]. 3 mL of assay mixture containing 50 mM KH2PO4 (monopotassium phosphate) and 10.4 mM H2O2 (hydrogen peroxide) was pre-incubated at 30 °C. The reaction was activated by the addition of tissue and the change of absorbance over 2 minutes at a wavelength of 240 nm was monitored and recorded. The final rate was corrected following subtraction of blank rate derived from the
spontaneous decomposition of hydrogen peroxide (H$_2$O$_2$) without cardiac tissue and enzyme activity calculated using the extinction coefficient of H$_2$O$_2$ (43.6 M$^{-1}$ cm$^{-1}$).

### 3.7 Aconitase

Aconitase, found both in the cytosol and mitochondria, is an iron-sulphur enzyme that catalyses the isomerisation of citrate to isocitrate via the intermediate isocitronate. This isomerisation is necessary both in the tricarboxylic acid and glyoxylate cycles. During oxidative stress, exposure to oxidants (such as O$_2^-$ and H$_2$O$_2$) renders aconitase inactive. Thus, loss of aconitase activity can be interpreted as a measure of oxidative damage. The activity of aconitase was measured spectrophotometrically in accordance with Fransler and Lowenstein [43]. The enzyme activity was calculated using extinction coefficient of 3.6 mM$^{-1}$ cm$^{-1}$.

### 3.8 Mitochondrial function in isolated cardiac cells

Mitochondrial function was studied using the Seahorse XFp analyser (Agilent Technologies, Santa Clara, US). The Seahorse analyser measures real-time oxygen consumption ratio (OCR) of mitochondria in a microplate. Oxygen consumption (respiration) causes rapid, easily measurable changes to the concentrations of dissolved oxygen in a transient microchamber which are measured by sensor probes residing 200 microns above a mitochondrial monolayer. An integrated drug delivery system allows sequential addition of up to four compounds per well at adjustable intervals.

Cardiac tissue was excised, minced separately and mitochondria isolated as previously described [7] in mitochondrial isolation buffer (containing 70 mM sucrose, 210 mM mannitol, 5 mM 4- (2-hydroxyethyl)-1-piperazineethanosulphonic acid) (HEPES), 1 mM ethyle glycolbis (tetraaminoethylene) NN’N’-tetra-acetic acid (EGTA) and 0.5% (w/v) fatty acid-free BSA, pH 7.2). The protein content was determined using the Bio-Rad protein assay. Mitochondrial coupling or electron flow experiments were carried out at 37 °C according to Roger et al. [44] and results were analysed on the Wave 2.3.0 software (Agilent Technologies, Santa Clara, CA, USA).

### 3.9 Cardiolipin

Cardiolipin (CL) is a tetra-acyl phospholipid almost exclusively found in the inner mitochondrial membrane where it constitutes 20% of the total lipid. It comprises of two phosphatidyl head groups connected on a glycerol backbone, and four fatty acyl chains. CL is essential for maintaining the structural integrity of the mitochondria and optimal function of a number of enzymes involved in mitochondrial energy metabolism. Changes in CL structure and composition have been associated with mitochondrial respiratory dysfunction and increased susceptibility to oxidative stress.

Total lipid from 3 mg freeze-thawed cardiac mitochondrial suspension or 50 mg freeze-dried tissue was spiked with 60 nmol of tetramyristic cardiolipin internal standard (IS) (Advanti Polar Lipids, USA) and extracted. Cardiolipin (CL) was then separated using thin layer chromatography (TLC). 200 μL re-suspended lipid extract was loaded onto a 20 cm × 20 cm × 0.25 mm silica gel glass plate (DC-Fertigplatten SIL G-25, by Macherey-Nagel, Germany) and eluted with chloroform : methanol : glacial acetic acid : water (40 : 10 : 2 : 1, v/v) mobile phase. The plate was stained in iodine and the CL band was determined by comparison of the relative mobility to a cardiolipin standard (tetra linoleic cardiolipin from bovine heart, Sigma-Aldrich UK, tetraoleic cardiolipin and tetramyristic cardiolipin, Advanti Polar Lipids, USA). The CL band was scrapped from the TLC plate and extracted five times using chloroform : methanol (1 : 2, v/v), dried under nitrogen gas.

For gas chromatography mass spectrometry (GCMS) analysis of total CL and side chain constitution, dried samples were resuspended in 2 mL of chloroform and then methylated using anhydrous methanolic HCl using Agilent GC/MS 6890N Gas Chromatograph with 7683 autosampler which uses splitless inlet with a 5973 MSD mass selector detector system, a 30 m × 0.25 mm × 0.25 μm HP-5MS column (Agilent Technologies) and helium as the carrier gas. The relative proportion of each acyl chain was expressed as a percentage of the most prominent acyl chains to give a measure of remodeling.

LCMS (liquid chromatography mass spectrometry) measurement of cardiolipin molecular species was performed on a Dionex Ultimate 3000 system, the column used was Symmetry® C18 5 μm, 150 × 3.0 mm (Waters); guard, SecurityGuard C18 4.0 × 2.0 mm (Phenomenex), a mobile phase consisting of solvent A (90% (v/v) acetonitrile, 10% water containing 0.5% (v/v) triethylamine and 0.5% (v/v) acetic acid (v/v)) and solvent B (90% propan-2-ol, 10% water containing 0.5% (v/v) triethylamine and 0.5% (v/v) acetic acid (v/v)) at a flow rate 0.3 mL/min and temperature of 35 °C. The LCMS method was optimised to detect the major CL species (CL(18:1)$_4$, CL(18:2)$_3$(18:1)$_2$, CL(18:2)$_2$(18:1), CL(18:2)$_4$ and CL(18:2)$_3$(16:1)) in addition to other minor species such as CL(18:3)$_2$(18:2)(20:2). Remodeling in uraemic cardiac mitochondria and the impact of iron therapy were investigated.

### 3.10 Mitochondrial coupling and electron flow

Mitochondrial coupling or electron flow experiments was initiated at 37 °C and results were analysed on the Wave 2.3.0 software of the Seahorse (Agilent Technologies LDA UK Limited, Stockport, Cheshire, UK, Fig. 1A,B).
4.1 Oxidative stress

To characterise vulnerability to oxidative stress, the pro-oxidant activity and antioxidant capacity of cardiac and skeletal tissue were evaluated by studying alterations in the activities of glutathione, TBARS, superoxide dismutase, aconitase and catalase.

3.11 Numbers of animals used

Ideally power calculations should be carried out for studies, including the potential level of animal attrition. In this pilot/exploratory set of studies examining underlying potential mechanisms which is in part hypothesis generating, the number of animals used was determined by the investigator’s experience and personal judgment, and hence are typically small. There are different numbers of animals used in experiments due to the related number of studies carried out where there was access to the tissue (cardiac and skeletal tissue) to measure the various biomarkers. This approach was used to maximise as much data as possible to ensure the data was more robust. Therefore, we included all data with was available from multiple experiments with the same protocol and collection and storage of tissue.

3.12 Statistical analysis

Data was recorded as means and Standard error of the means (SEM). Difference between the control (sham) and experimental (uraemic) groups was calculated using the unpaired student t test and comparisons between control untreated and experimental treated groups was performed using one way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test when comparing greater than two experimental groups on IBM SPSS statistics software version 21 (online download, IBM, Portsmouth, Hampshire, UK). A p value less than 0.05 was considered statistically significant.
4.2 Cardiac oxidative stress

The uraemic heart exhibited a net increase in pro-oxidative activity as evidenced by increased cardiac oxidised glutathione (GSSG) relative to the reduced form (GSH) (69% increase in GSSG/GSH ratio, Fig. 2A,B; \( p < 0.01 \)), enhanced lipid peroxidation indicated by a 38% rise in thiobarbituric acid-reactive substances (TBARS) levels (Fig. 2C; \( p < 0.01 \)) and a significantly (79%) upregulated
Fig. 3. Cardiac tissue glutathione peroxidase and glutathione reductase activities in uraemic and sham animals and after exposure to a single dose of intravenous iron at 6 weeks. (A, B) Each of these figures has four groups Sham (circles) and uraemic (squares) animals untreated (n = 11 for each group) and sham (circles) and uraemic (squares) animals treated (n = 6 for each group). Uraemic increased antioxidant activity, while intravenous iron led to a further increase in both sham and uraemic animals (p < 0.01). (C) SOD activity in the cardiac tissue from uraemic animals (p = NS). (D) Catalase activity in cardiac tissue from uraemic animals (p = NS). Results are presented as mean ± SEM (*p < 0.01; **p < 0.05).

NADPH oxidase activity (Fig. 2D; p < 0.01) in this model of UCM. This was associated with limited changes in the anti-oxidant systems to cope with the stress. Cardiac glutathione peroxidase (GPx) and glutathione reductase (GR) activities were significantly increased by 30% (Fig. 3A; p < 0.01) and 88% (Fig. 3B; p < 0.01) respectively without changes in superoxide dismutase (SOD) (Fig. 3C) and catalase (Fig. 3D). These adaptations may function to replenish the levels of GSH and rid the heart of potentially toxic GSSG. Despite the evidence of increased pro-oxidative activities, aconitase activity was not altered in the uraemic heart relative to sham in both untreated and IV iron treated animals (Fig. 4).

4.3 Impact of intravenous single dose iron therapy on cardiac oxidative stress

Parenteral iron (ferumoxytol) therapy ameliorated the oxidative stress in the uraemic heart indicated by the re-
Table 1. Glutathione content in skeletal tissue from sham and uraemic animals.

<table>
<thead>
<tr>
<th>Glutathione (nmol/mg of tissue)</th>
<th>Untreated</th>
<th>Treated (IV iron)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>Uraemic</td>
</tr>
<tr>
<td>Oxidised (GSSG)</td>
<td>0.031 ± 0.00</td>
<td>0.041 ± 0.00*</td>
</tr>
<tr>
<td>Reduced (GSH)</td>
<td>0.30 ± 0.01</td>
<td>0.34 ± 0.01</td>
</tr>
<tr>
<td>Total glutathione</td>
<td>0.33 ± 0.01</td>
<td>0.38 ± 0.01*</td>
</tr>
<tr>
<td>GSSG/GSH ratio</td>
<td>0.11 ± 0.01</td>
<td>0.12 ± 0.01</td>
</tr>
</tbody>
</table>

Results are presented as mean ± SEM (n = 4 in each group) (*p < 0.05, sham vs uraemic).

4.4 Skeletal oxidative stress

There was an increase in oxidised glutathione (GSSG) in skeletal tissue in uraemic animals (Table 1; p < 0.05), reflective of enhanced pro-oxidative activity. This was associated with changes to reduced glutathione (GSH) (Table 1) to maintain the glutathione balance (GSSH/GSH ratio; Table 1; p < 0.05) and restore oxidant status. Uraemic animals demonstrated significantly reduced activities of both GPx and GR in skeletal muscle from uraemic animals (Fig. 5A,B). This was accompanied by a 26% reduction in the activity of the pro-oxidant marker NADPH oxidase (Fig. 5C; p < 0.01) in skeletal tissue of uraemic compared animals leading to maintenance of the status quo in the GSSG : GSH ratio (Table 1) and TBARS levels (Fig. 5D).

4.5 Impact of intravenous single dose iron therapy on skeletal oxidative stress

Single dose parenteral iron therapy resulted in a significant 68% reduction of TBARS (Fig. 5D; p < 0.01) in accordance with the improved skeletal antioxidant GR activity in the skeletal muscles of uraemic animals. A two-fold decrease in glutathione reductase (GR) activity (p < 0.01) (Fig. 5B) was observed in iron treated sham operated animals in line with 41% decrease in the activity NADPH oxidase (Fig. 5C; p < 0.01) in sham animals. Iron did not impact NADPH activity in uraemic animals. Superoxide dismutase (SOD) activity was increased 17% with IV iron in skeletal muscle (Fig. 6; p < 0.05).

4.6 Cardiac cell mitochondrial function and impact of uraemia and IV iron on mitochondrial cardiolipin

Cardiolipin (CL) was increased in the uraemic cardiac mitochondria relative to sham animals. This accounts for the increased absolute quantities of the prominent acyl chains (linoleate, palmitate, oleate and stearate) (Fig. 7A; p < 0.05). Untreated groups demonstrated an 18% increase in overall CL content and between 26–63% CL molecular species in the uraemic cardiac mitochondria compared to sham animals (Fig. 8A; p = NS).
4.7 Impact of iron therapy on mitochondrial membrane remodeling

Iron therapy was associated with an increase in all major acyl chain constituents of CL in the uraemic heart (Fig. 7B; \( p < 0.05 \)) and an overall trend to an increase in total cardiolipin content (Fig. 7C; \( p = \text{NS} \)). There was a modest 19% increase in overall CL content (Fig. 7C). Intravenous iron (Ferumoxytol) treatment modulated CL remodeling in the uraemic heart with between a 7% reduced and 38% increase in molecular species; CL(18:2)\(_2\)(18:1)\(_2\)CL(18:2)\(_2\)(18:0), CL(18:2)\(_3\)(18:1) and CL(18:2)\(_4\) were found to be increased by 37%, 38% and 14% respectively whilst CL(18:2)\(_3\)(16:1) decreased by 7% compared to untreated uraemic animals (Fig. 8A–D; \( p = \text{NS} \)).
Fig. 6. Pro-oxidant activity (measured via superoxide dismutase (SOD) activity) in skeletal tissue from sham and uraemic animals and after exposure to intravenous iron. Intravenous Iron produced a significant increase in SOD ($p < 0.05$). Results presented as mean ± SEM; *$p < 0.05$.

### 4.7.1 Impact of uraemia and iron treatment on cardiac mitochondrial respiration

Uraemia led to an increase in isolated cardiac mitochondrial State 3 respiration in the presence of various substrates of energy source (Fig. 9A; $p < 0.05$ for SR) Glutamate and Malate (GM); Succinate and Rotenone (SR); and Malate and Palmitoyl Carnitine (PC). State 4 respiration was enhanced though not significantly (Fig. 9B) with SR and PC as substrate. Respiratory control ratio, a measure of mitochondrial respiration efficiency was unchanged in the presence of GM and SR but was depressed in the presence of PC suggesting reduced efficiency in the uraemic heart (Fig. 9C; $p < 0.05$) as previously observed [7].

Iron therapy had no impact on mitochondrial State 3 respiration in either group following treatment irrespective of substrate used (Fig. 10A). However, it did significantly lower State 4 in uraemia in the presence of PC (Fig. 10B; $p < 0.05$) thus overcoming the inefficiency (Fig. 10C; $p < 0.05$) observed in the untreated group (Fig. 9C).

Iron treatment reduced mitochondrial uncoupling (as reflected in the State 4 respiration) in the uraemic heart indicating improved efficiency without impact on State 3 with GM as substrate (Fig. 11A,B; $p < 0.01$). This resulted in increased respiratory control rate (RCR) in response to iron therapy (Fig. 11C; $p < 0.01$). No change was observed in State 3 and 4 respiration in the presence of SR and thus RCR. However, both State 3 and State 4 respiration were reduced in the presence of PC and consequently increased RCR following therapy (Fig. 11C).

There were no observed changes in State 3 and 4 respiration in the presence of SR (Fig. 12A,B) and thus RCR (Fig. 12C). However, both State 3 and State 4 were reduced by 39% and 43% respectively (Fig. 13A,B) in the presence of PC and consequently increased RCR following therapy (Fig. 13C). Therefore, the respiratory data suggests improved efficiency of mitochondrial respiration following iron therapy in the presence of GM and PC.

### 4.7.2 Mitochondrial bioenergetics

Uraemic animals demonstrated cardiac mitochondrial respiratory insufficiency evidenced by diminished maximal respiration and respiratory reserve capacity albeit with reduced proton leak in the presence of GM (Fig. 14A) and SR (Fig. 14B) as substrates. Iron treatment restored mitochondrial proton leak in uraemic hearts to the level of baseline sham with GM as substrate (Fig. 14D) and also respiratory capacity while iron therapy with SR, despite no significant change in proton leak did also improve, respiratory reserve capacity indicative of improved cardiac mitochondrial oxidative function in the uraemic animals (Fig. 14C,D).

There was a “compensatory/adaptive increase” in complex I, complex II and complex IV respiration in the uraemic animals relative to sham animals (Fig. 15A) and this change was absent following iron therapy, further highlighting restoration of mitochondrial (Fig. 15B).

### 5. Discussion

In this study on cardiac and skeletal tissue in an experimental model of uraemia we showed that changes in oxidative stress as a result of uraemia are improved with intravenous iron therapy as a result of increased antioxidant activity compared to pro-oxidant activity. These effects in turn have shown that mitochondrial respiration as a result of uraemia had increased uncoupling of mitochondria through enhanced State 4 respiration but little change in State 3 (ADP dependent respiration). Cardiolipin (CL), the major phospholipid component of the inner mitochondrial membrane (IMM), was increased in uraemic cardiac mitochondria with evidence of changes in fatty acid constituents, indicative of mitochondrial biogenesis. Intravenous iron (Ferumoxytol) enhanced this remodeling and reduced the extent of respiratory uncoupling with improved cardiac mitochondrial function. Overall, these data emphasise the complex metabolic interplay with oxidative stress and mitochondrial function and intravenous iron leading to overall benefit in cardiac mitochondria.
Fig. 7. (A) Total cardiolipin (CL) and fatty acyl chain composition content. (B) Variation of fatty acyl chain composition and CL content following iron treatment. (C) Cardiolipin (CL) content of mitochondria impact of iron therapy. (A) Baseline data for the total cardiolipin (CL) and its fatty acyl chain composition content of hearts for untreated sham and uraemic animals. Results are presented as mean ± SEM (*p < 0.05; uraemic vs sham). Data show an increased CL in the uraemic heart relative to control sham animals which accounted for the increased absolute quantities of the prominent acyl chains (linoleate, palmitate, oleate and stearate). (B) Variation of fatty acyl chain composition and CL content in uraemic hearts following iron (ferumoxytol—IV iron) treatment. Results are presented as mean ± SEM (*p < 0.05; uraemia vs sham). Iron therapy (Ferumoxytol) was associated with an increase in all major acyl chain constituents of CL in the uraemic heart. (C) Cardiolipin (CL) content (derived from summation of molecular species from LCMS) in uraemic heart and impact of iron therapy (shown as speckled bars). Results are presented as mean ± SEM (*p < 0.05; uraemic vs sham).

5.1 Impact of uraemia on cardiac and skeletal oxidant status

In the normal physiological state, reactive oxygen species (ROS) generated during oxidative phosphorylation (OXPHOS) and/or from enzymatic sources [45, 46] are cleared by antioxidant systems [47, 48]. Perturbations
in the balance between the antioxidant defence system and pro-oxidative activities result in oxidative stress as seen in various cardiac pathologies \([49, 50]\) including UCM \([51]\).

This model of experimental uraemia was associated with a significantly enhanced tissue NADPH oxidase activity in the uraemic heart. The mechanism of NADPH oxidase activation here is unknown, but there is evidence that accumulation of uraemic toxins in renal disease activates NADPH oxidase and enhances oxidative stress \([52, 53]\). The observed increased NADPH oxidase activity reflecting increased generation of \(O_2^-\) was associated with a significant increase in markers of oxidative stress (GSSG/GSH ratio and TBARS). GSH is a potent intracellular scavenger which is preferentially oxidised to GSSG thus detoxifying (ROS) in the process \([54]\). The observation of net increased GSSG over GSH in the uraemic heart can be interpreted as increasing oxidative stress. Elevated ROS induces cellular alterations such as DNA damage, protein and lipid peroxidation, thus explaining the observation of increased TBARS levels in the uraemic heart. The enhanced pro-oxidant activity in the uraemic heart was associated with changes in the antioxidant systems to handle the stress. GPx activity was enhanced in line with the increased accumulation of GSSG. Remodeling of the antioxidant system in the uraemic heart is further supported by a pronounced rise in GR activity which may replenish some of the depleted GSH.

An effective antioxidant defence system depends upon the cohesive and coordinated action of all components. Perturbations of one or more components signifi-
Fig. 9. (A) Isolated Mitochondrial state 3 respiration. (B) Isolated mitochondria State 4 respiration. (C) Respiratory control ratio. (A–C) Isolated Mitochondrial Respiration (A) State 3 (B) State 4 and (C) respiratory control rate in the presence of 5 mM glutamate and 1 mM malate (GM); 5 mM succinate and 1 mM rotenone (SR); and 5 mM malate and 40 µM palmitoyl carnitine as substrates (PC). Results are presented as mean ± SEM (*p < 0.05 vs sham).

Significantly lower the ability to maintain efficient ROS detoxification. Hence, the reduced SOD and catalase activity (though not significant at this stage) could culminate in the increased oxidative stress observed in the uraemic heart. Several studies, both clinical and experimental [55–58] have illustrated increased oxidative activities in CKD. Increases in GSSG/GSH ratio have been found in patients with renal dysfunction [59]. Increased oxidative activities in the uraemic heart were not accompanied by changes in aconitase, despite increased evidence of oxidative damage (TBARS). This indicated that the changes in cardiac antioxidant status observed here are early events that could explain the increased vulnerability of uraemic cardiomyocytes to oxidant induced stress indicated by a 30% reduction in cell viability following exposure to 200 µM H₂O₂ [7].

The aetiology of oxidative stress in CKD is complex and not fully elucidated. Oxidative stress in CKD can result from several factors including increased uraemic toxins [60], inflammation, iron deficiency [14], and decreased antioxidant capacity [59]. The observed increase in cardiac antioxidant markers in this model of UCM could be due to the stage of renal dysfunction as different stages of CKD are associated with varying antioxidant activities [29, 59–61]. Taken together the increased NADPH oxidase with parallel
increased GSSG/GSH and TBARS, it is conceivable that the uraemic heart is in a pro-oxidant state and hence predisposed to the cardiovascular complications associated with oxidative stress.

The induction of uraemia resulted in increased GSSG concentration in skeletal tissue with evidence of decreased antioxidant capacity. The parallel reduction of pro-oxidant NADPH oxidase activity (26%) could be an adaptive response to maintain pro oxidant-antioxidant balance, leading to no changes in either GSSG/GSH ratio or TBARS in skeletal tissue from uraemic animals. The reduction of GR and GPx antioxidant enzymatic activities in muscle from uraemic animals could increase the vulnerability to oxidative stress as uraemia progresses. A recent report by Avin et al. [62] demonstrated evidence of oxidative stress in the skeletal muscle of CKD rats with high level of Nox4 expression. The authors also reported increased expression of antioxidant SOD1 and SOD2 as an adaptive response to offset the increased Nox4 [62].
5.2 Impact of intravenous iron on oxidant status in cardiac and skeletal tissues from uraemic animals

5.2.1 Cardiac muscle

Iron therapy ameliorated oxidative stress in the uraemic heart. NADPH oxidases activity was not significantly altered following therapy in agreement with the report of Lim and Vaziri [63]. The authors reported a limited change in cardiac NADPH $p^{22\text{phox}}$ and aortic $p^{67\text{phox}}$ subunits. However, they also demonstrated increased expression of cardiac $p^{67\text{phox}}$ subunit and reduction in aortic $p^{67\text{phox}}$ subunit. Indeed, the use of gene and protein expression by Lim and Vaziri is limited as their changes may not represent alteration in functional activity of the NADPH oxidase complexes. The current study measured the biochemical activity of NADPH oxidase rather than protein expression, which could explain some of the discrepancies between the two studies.

Iron treatment significantly increased antioxidant capacity in the hearts from uraemic animals illustrated by elevated activity of SOD, catalase, GPx and GR. Enhance antioxidant defence coupled with little alteration of NADPH oxidases could underpin the observed decrease in markers of oxidative stress (TBARS and GSSG/GSH). This experimental model of UCM demonstrated compensatory antioxidant response evidenced by up-regulation of GPx and GR in the untreated uraemic heart at week 12. Previous studies have indicated compensated cardiac remodeling at week 6 [7, 8] in this model of UCM. It is conceivable that this cardiac remodeling could include up-regulation of the antioxidant system. It was concluded that the hearts from uraemic animals at week 6 had an extra capability to deal with potential acute oxidative toxicity of IV iron allowing them to respond beneficially to iron treatment compared to sham. This capacity is certainly absent in the sham operated animals showing evidence of cardiac oxidative stress following therapy. One might speculate that the presence of uraemia primes the oxidant system to better react to further potential insults on the pro-oxidant system such as iron in this model.

The lack of agreement in the observation here of decreased oxidative stress (following iron therapy in uraemic animals) with some published reports [33, 64], could be attributed to different stages of cardiac dysfunction, type, and dose of iron injection and time of oxidative stress studies post injection of iron. The study by Lim and Vaziri [63] employed iron dextran at a dose of 500 mg/kg body weight at time zero. In contrast, the current
Fig. 12. (A) Isolated Mitochondrial state 3 respiration with succinate and rotenone. (B) Isolated mitochondria State 4 respiration with succinate and rotenone. (C) Respiratory control ratio with succinate and rotenone. Isolated Mitochondrial Respiratory rates (A: State 3; B: State 4 and C: respiratory control rate) in the presence of 5 mM succinate and 1 mM rotenone (SR). Results are presented as mean ± SEM). Results and mean and SEM for n = 10 untreated (sham and uraemic) and n = 5 for treated animals (sham and uraemic) in each group.

Iron therapy had a beneficial impact on cardiac aconitase activity (2-fold increase) in uraemic animals in agreement with literature report [71]. Mitochondrial iron homeostasis modulates the expression and thus activity of aconitase by changing the interaction between iron regulatory proteins (IRP) and iron responsive elements (IRE) of aconitase mRNA [72, 73]. The presence of iron reduces the binding affinity of IRE to IRPs. Conversely, iron deficiency activates IRPs binding to IRE resulting in translational inhibition of aconitase expression [74]. Aconitase exists in two forms, active (4Fe-4S) and inactive forms (3Fe-4S). Mitochondrial iron concentration determines F-S cluster formation, by the mitochondrial protein frataxin [75] necessary for post-translational stabilisation of aconitase active site (4Fe-4S).

The present model of UCM demonstrated modulation of cardiac iron content with evidence of reduced myocardial unbound iron following iron therapy in uraemic hearts. A recent study [75] demonstrated loss of cardiac aconitase activity in HF, a phenomenon that was compounded in the presence of comorbid myocardial iron deficiency. This reinforces the importance of cellular iron in cardiac mitochondrial function and supports the observation of increased aconitase activity following iron therapy at this stage of UCM. The Fe-S clusters in aconitase are vulnerable to ROS-induce oxidative damage [68]. The improvement of cardiac oxidant status (GSSG/GSH ratio and TBARS through increased GPx, GR, SOD and catalase) could reduce the risk of damage leading to the improved aconitase activity following iron treatment. However, the observation of increased aconitase in sham operated ani-
Fig. 13. (A) Isolated Mitochondrial state 3 respiration with palmitoyl carnitine. (B) Isolated mitochondria State 4 respiration with palmitoyl carnitine. (C) Respiratory control ratio with palmitoyl carnitine. Isolated mitochondrial respiratory control ratio (A: State 3; B: State 4 and C: respiratory control rate) in the presence of 5 mM malate and 40 µM palmitoyl carnitine (PC). Results and mean ± SEM for n = 10 untreated (sham and uraemic) and n = 5 for treated animals (sham and uraemic) in each group (*p < 0.05 vs untreated).

Mals treated with iron did not mirror the evidence of enhanced oxidative stress with little alteration in cardiac or liver iron levels and therefore remained unexplained.

5.2.2 Skeletal muscle

Similar to the heart, iron treatment was beneficial in improving oxidative stress in skeletal muscle. TBARS level was reduced by 68% in skeletal tissue in uraemic animals, though no change in glutathione (reduced, oxidised or total) was observed. Therapy modulated skeletal oxidant status in the uraemic animals by enhancing antioxidant capacity indicated by increased GR (p < 0.05), GPx (p < 0.05) and SOD (p < 0.05) activities. Increased GR without changes in pro-oxidant NADPH oxidase favours excess antioxidant capacity to deal with uraemic associated ROS. The beneficial impact of therapy on oxidative stress was not specific to uraemic animals; skeletal TBARS was also reduced in sham operated animals consistent with the reduction of NADPH oxidase activity. This might explain some of the benefits seen in clinical trials of intravenous iron in heart failure patients [76–78] of which a proportion had CKD. Indeed, recent data from Charles-Edwards et al. [79] on mitochondrial function strengthens this hypothesis. Lipid peroxidation which was markedly enhanced in untreated uraemic cardiac tissue was normalised after iron treatment indicating amelioration of oxidative stress. This beneficial impact of IV iron on cardiac oxidant status was mediated through further increase in the anti-oxidant capacity. Lipid peroxidation experiments in skeletal tissue were unsuccessful with no meaningful data produced. Also, IV iron therapy was associated with improved antioxidant capacity in skeletal tissue. These findings imply that IV iron treatment in early stages of CKD could reduce oxidative stress in the heart and potentially lessen the adverse cardiac outcomes associated with more severe CKD.

5.3 Impact of uraemia on cardiac mitochondria

Mitochondria represent the main source of energy for all cells. Mitochondria are commonly called the “powerhouse of the cell”, generating energy in the form of adenosine triphosphate (ATP) and are thus critical for normal function of cardiac and skeletal cells. Inflammation and oxidative stress are common in patients with CKD [80–83]. Dysfunctional mitochondria are one of the sources of oxidative stress and inflammation. Conversely, inflammation and oxidative stress can lead to mitochondrial dysfunction
Fig. 14. (A) Isolated cardiac Mitochondrial Respiration in untreated animals in presence of glutamate and malate. (B) Isolated cardiac Mitochondrial Respiration in treated animals in presence of glutamate and malate. (C) Isolated cardiac Mitochondrial Respiration in untreated animals in presence of succinate and rotenone. (D) Isolated cardiac Mitochondrial Respiration in treated animals in presence of succinate and rotenone. (A, B) Isolated Mitochondrial Respiratory rates in the presence of 10 mM glutamate and 10 mM malate (GM). (C, D) Isolated Mitochondrial Respiratory rates in the presence of 0.6 µg mitochondria, 10 mM succinate and 2 µM rotenone (SR). Proton leak = (minimum rate measured after Oligomycin injection) – (non-mitochondrial respiration rate or minimum rate measured after injection of Antimycin A); Maximal respiration = (maximal rate measured after FCCP injection) – (non-mitochondrial respiration rate); Respiratory reserve capacity = (maximal respiration – basal respiration).
Fig. 15. (A) Isolated Mitochondrial Respiratory rates (complex 1, II and IV) in untreated animals. (B) Isolated Mitochondrial Respiratory rates (complex 1, II and IV) in iron treated animals. Isolated Mitochondrial Respiratory rates (complex 1, II and IV) in the presence of 10 mM pyruvate, 2 mM malate and 4 µM FCCP. Results are presented as mean ± SEM (n = 3 or 2, untreated; n = 3 treated). (A) Untreated animals (sham in blue and uraemic in orange) and (B) Treated animals with Intravenous iron sand uraemic.

by damaging the mitochondria. Removal of damaged mitochondria relies on mitophagy and mitochondrial fission, a process of mitochondrial dynamics that segregates damaged mitochondria [84–86]. Mitochondria offer the common oxidative pathway of metabolism. Some inner mitochondrial carrier proteins called uncoupling proteins cause a proton leak across the inner mitochondrial membrane. Uncoupling is associated with mitochondrial dysfunction and this mitochondrial dysfunction is implicated in skeletal and cardiac abnormalities present in uraemic patients. The detailed functional role of the mitochondrial membrane phospholipid cardiolipin (CL) remains unclear, though several studies have demonstrated the unique ability of CL to interact with numerous proteins in the inner mitochondrial membrane [87].

CL is required for the structural organisation of mitochondrial complexes involved in oxidative phosphorylation (OXPHOS). CL is also required for their optimal functioning of the complexes and enzyme complexes. There is increased evidence that loss of CL is important in oncotic and apoptotic cell death in cardiac tissue [88] indicating a potential role in metabolic remodeling/alteration in heart failure. Uraemia in this study led to increased oxidation and a decrease in CL content and change in mitochondrial bioenergetics. Iron treatment modulated CL remodeling in the uraemic heart and skeletal tissues leading to stabilisation of the mitochondrial and increased mitochondrial ATP production possibly to cope with the increased uraemic induced uncoupling. In this regard, targeting of CL may be a prudent therapeutic strategy for new drug discovery.

Mitochondrial dysfunction is considered an important contributory factor in heart failure and skeletal muscle function [89]. Eventually there is a series of maladaptive events which leads to cardiac dysfunction and heart failure due to the depletion of energy reserves and a reduction in respiratory efficiency in part related to this metabolic remodeling which is beneficial acutely but not chronically. This leads to mitochondrial transition pore (mPTP) opening which subsequently leads to mitochondrial swelling, rupture and apoptosis. These processes of energy deprivation, because there are limited reserves in all organs, culminate in impaired contractile function/cellular remodeling and tissue and organ dysfunction. Taken together, these observations would suggest a central role for mitochondrial dysfunction in the uraemic heart [90].

Collapse of the mitochondrial membrane potential (MMP) is a characteristic of cell death, thought to coincide with the opening of the mitochondrial permeability transition pore (MPTP). Under conditions of stress, the pore opens, increasing mitochondrial permeability. Consequently, the MMP is dissipated with loss of energy, generation of ROS and release of pro-apoptotic proteins [91].

Uraemic animals demonstrated that in cardiac tissue mitochondria, there was a significant increase in inefficiency (enhanced proton leak and complex I dysfunction) (Figs. 14,15). Iron therapy produced a mixed and complex result with no change in the protein leak but an increased maximal respiration and respiratory reserve capacity suggesting improved mitochondrial oxidative capacity. However, there was a reduction of complex II and complex IV driven respiration (Fig. 15), which would suggest a degree of mitochondrial dysfunction.

Studies here on mitochondrial respiration showed increased uncoupling of mitochondria through enhanced State 4 respiration but little change in State 3 (ADP dependent respiration). Cardiolipin (CL), the major phospholipid
component of the IMM, was increased in uraemic cardiac mitochondria with evidence of changes in fatty acid constituents, indicative of mitochondrial biogenesis.

The end effect of this oxidative stress was a reduction in mitochondrial function (Fig. 14) and altered mitochondrial morphology perhaps similar to that seen in renal mitochondria. There were also potential changes in mitochondrial membrane remodeling. Increased glycolytic flux, reduced oxidative capacity, increased uncoupling are indicative of impaired bioenergetics and mitochondrial dysfunction seen here as a result of uraemia. The sequential measurement of electron flow through different complexes of the electron transport chain confirmed impaired cardiac mitochondrial bioenergetics (Fig. 15) which was ameliorated with iron therapy (Fig. 15).

6. Conclusions

In conclusion, within the limitations of this series of experiments which including the number of experimental animals used, the use of only one iron preparation in consideration of the the potential differences in physicochemical structures with iron preparation [91], the lack of dosing changes which may impact on outcomes as demonstrated in the PIVOTAL and FIND-CKD studies and the more recent FERWON study [92], this model of uremia demonstrated a specific oxidative stress on the cardiac and skeletal tissues. Parenteral single dose iron therapy ameliorated the oxidative stress in cardiac tissue and improved oxidant status in skeletal tissues. In addition, this produced an improve in mitochondrial function. The evidence indicates that iron therapy during the early stages of CKD could reduce vulnerability to oxidative stress and lessen its associated adverse cardiovascular outcome. Further larger sample size experiments are required to validate these findings.

7. Author contributions

SB, conceptualisation, methodology and design of research project for PhD in collaboration. SB drafted the manuscript from the work carried out in the lab, edited and revised the manuscript, and approved final version of manuscript, including adjusting figures and tables as required.

8. Ethics approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and that all experiments using animals were in accordance with the UK Animals (Scientific Procedure) Act 1986 and were approved by the University of Hull Ethical Review Process (PPI number 70/7966).

9. Acknowledgment

I thank Anne-Marie Seymour who retired over 2 years ago for her work in collaboration with this research. I also thank Faisal Nuhu the research fellow who while in the lab performed the experiments, sample preparations, and sample analysis under supervision. He has now moved abroad but the work has formed part of his Finally thanks to Mrs Kath Bulmer, Laura Goodlass and Danielle Webster for their technical support and dedication, and Roger Sturmey for assistance with oxidative measures.

10. Funding

Prof Bhandari received funding from Takeda UK Ltd and The Hull & East Riding Cardiac Trust Fund and Renal Research Fund (Hull University Teaching Hospitals NHS Trust) to allow the lab to complete this research project. SB is the grant holder.

11. Conflict of interest

The author declares no conflict of interest.

12. References


Keywords: Anaemia; Cardiac; Chronic kidney disease; Iron; Mitochondria; Oxidative stress

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