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INTRACELLULAR IRON REDISTRIBUTION

AN IMPORTANT DETERMINANT OF REPERFUSION DAMAGE TO RABBIT KIDNEYS

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Abstract—These studies were designed to examine the possible role of low molecular weight intracellular iron chelates (desferrioxamine-available (DFX-A) iron) in the damage which occurs during cold storage and subsequent reperfusion of kidneys. The level of DFX-A iron increased significantly ($P < 0.005$) in the cortex of rabbit kidneys rendered cold ischaemic (CI) for 24 hr and the amount of iron available for DFX chelation increased significantly ($P < 0.05$) in both the cortex and medulla of kidneys stored for 48 or 72 hr compared with fresh non-ischaemic controls. During *ex vivo* reperfusion of the organs with an oxygenated asanguinous perfusate, DFX-A iron returned rapidly to pre-ischaemic levels in 24 hr CI kidneys, but remained elevated following 48 and 72 hr CI ($P < 0.05$ compared with 24 hr CI kidneys after 5 min reperfusion), returning to control levels only after 30 min reperfusion. There was no concurrent increase in total iron levels, indicating that a redistribution of iron to more accessible pools had occurred within the tissue. We suggest that decompartmentalization of intracellular iron during ischaemia and raised DFX-A iron levels over an extended period during subsequent reperfusion are responsible for increased catalysis of oxygen-derived free radical-mediated lipid peroxidation, and are an important factor in the deterioration of physiological function observed in rabbit kidneys following extended periods of cold storage.

Kidneys can currently be stored for up to 48 hr whilst awaiting a recipient, but shorter periods are still preferable for optimum post-transplant renal function. Current storage techniques are effective to a degree [1–3], but deterioration of kidney function during cold storage still occurs. Although the underlying reasons for this remain unclear, many of the pathological events have been well documented [4–7]. Improving renal preservation would therefore not only benefit post-transplant viability, but ultimately also provide a larger pool of organs available for transplantation.

There is now considerable evidence that the production of oxygen-derived free radicals may be a primary cause of the tissue damage which accompanies ischaemia [8, 9] and post-ischaemic reperfusion [10–12], not only in the kidney, but also in heart [13], intestine [14], liver [15] and skin flaps [16]. Probably the most damaging of these species are hydroxyl ($OH^{\cdot}$) and iron complexed radicals [17]. The production of $OH^{\cdot}$ is catalysed by the presence of transition metals, and in particular iron, via the iron-catalysed Haber–Weiss reaction [18]. These radicals damage proteins, DNA and many other biomolecules, and initiate peroxidation of membrane polyunsaturated fatty acids.

Iron is stored safely in the cell as ferric hydroxide micelles within ferritin molecules [19]. However, this iron must be available for various metabolic requirements such as haem synthesis and activation of iron-dependent enzymes. To facilitate this, it has been proposed that a small intracellular "transit" iron pool exists in the cytosol, which would not only enable the exchange of iron between ferritin and transferrin, but could provide a source of iron in a form capable of catalysing free radical formation. The iron in this pool is thought to be bound to low molecular weight (LMW§) chelates such as nucleotides, citrate, glycine and glucose [20–22]. As many of these LMW species are known to catalyse free radical reactions *in vitro* [23], the size of this intracellular pool may be important in determining the level of oxidative stress placed upon the cell.

A progressive release of LMW iron has been shown to occur following administration of allyl alcohol [24] and after ethanol intoxication [25]. Iron release has also been implicated in the pathogenesis of inflammatory diseases [26], and the iron chelator desferrioxamine (DFX) has been demonstrated to inhibit the resulting lipid peroxidation in these systems [25, 27]. DFX is a clinically-approved iron chelator [28] which binds one atom of Fe^{3+} with a very high stability constant (10^{31}), but has relatively little affinity for other metal ions [29]. Previous work in this laboratory has demonstrated that administration of DFX very effectively inhibits the raised levels of lipid peroxidation which occur in rabbit kidneys following both warm [30] and cold [31–33] ischaemia, thus indirectly suggesting an important role for catalytic iron in the pathogenesis of ischaemic and reperfusion injury in this organ.

Accurate quantitation of catalytic iron is difficult

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§ Abbreviations used: CI, cold ischaemia; DFX, desferrioxamine; DFX-A, desferrioxamine-available; FX, ferrioxamine; HCA, hypertonic citrate solution; HPLC, high pressure liquid chromatography; LMWC, low molecular weight chelatable; PUFA, polyunsaturated fatty acid.

as it constitutes only a very small fraction of the total iron present. A novel method was therefore devised to enable the measurement of micromolar levels of iron in biological samples which is available for chelation by DFX [34, 35]. This involves quantitation of DFX and its iron-bound form ferrioxamine (FX) by reversed-phase high pressure liquid chromatography (HPLC).

Using this method, we have previously demonstrated a significant increase in the levels of desferrioxamine-available (DFX-A) iron during periods of both warm and cold ischaemia (CI), without any alteration in total iron [36, 37]. In the present investigation, those studies have been extended to examine the effect of longer and more physiologically-damaging periods of CI. In addition, by use of a continuous *ex vivo* perfusion apparatus, the effect of reperfusion on DFX-A iron levels was monitored. These experiments, which provide an understanding of the kinetics of LMW iron release following ischaemia and reperfusion, were designed with a view to improving, by pharmacological intervention, the physiological function of stored organs.

MATERIALS AND METHODS

Materials. Desferrioxamine was obtained from Ciba-Geigy Ltd (Basle, Switzerland). All other chemicals were analytical grade and obtained from the Sigma Chemical Co. (Poole, U.K.). Polyamide tubing was obtained from Portex Ltd (Kent, U.K.) and filter units from Millipore Ltd (Bedford, U.K.).

Surgical procedures. New Zealand White rabbits of approximately 3 kg were anaesthetized by i.m. injection of fentanyl-fluanisone at 0.2 mL/kg, followed 5 min later by slow i.v. injection of diazepam (1.0 mg/kg). They were then supplied via an open face mask with oxygen flowing at 2 L/min for the duration of the operation.

The right kidney was exposed through a midline abdominal incision and removed after careful dissection and ligation of the renal vessels and ureter under magnification. A cannula was carefully passed into the renal artery and tied in place to provide both a means of flushing the kidney and to enable its later attachment to the perfusion circuit. The kidneys were then flushed with 30 mL of sterile hypertonic citrate solution (HCA) [2] which had been previously cooled to 4° and placed in a sterile beaker containing 50 mL of the flush solution, which was surrounded by ice within a closed polystyrene container and kept in a refrigerator. The storage temperature was thus maintained at a constant 0°. Kidneys were stored for 24, 48 to 72 hr before determination of DFX-A iron and total iron levels.

Kidney perfusion. The perfusion apparatus used was based on that previously described by Fuller *et al.* [38] (Fig. 1). This has been shown to allow controlled perfusion of isolated rabbit kidneys at 37° and results in the maintenance of a range of functional characteristics including glomerular filtration rate (GFR), glucose and sodium reabsorption within the expected physiological range [38]. Other authors have used the system to investigate renal function under a variety of conditions including ischaemia (e.g. Ref. 39). Temperature control was

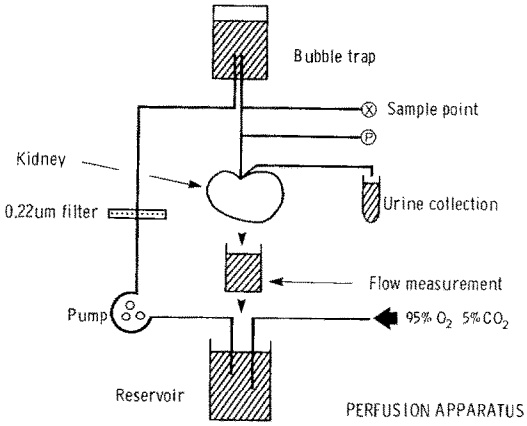

Fig. 1. The kidney perfusion apparatus, enclosed within a thermostatically-controlled room maintained at 37°.

achieved by maintaining the equipment in a constant temperature room at 37° and by immersing the reservoir in a water bath also at 37°. The perfusate was pumped from a reservoir through a sterile vented filter unit (76 mm diameter; 0.22 μm pore size) and a bubble trap to the arterial cannula. The venous effluent was allowed to return by gravity to the reservoir, where it was continuously bubbled with 95% O_2: 5% CO_2 gas. The tubing used for all connections was of polyamide (nylon) because of its low permeability to oxygen and carbon dioxide. Arterial pressure was continuously monitored from a T-connection immediately above the arterial cannula and was maintained at 110 mm Hg by varying the pump speed.

The perfusate (25 mM $NaHCO_3$, 0.6 mM $MgCl_2$, 0.3 mM KH_2PO_4, 0.465 mM Na_2HPO_4, 5 mM glucose, 5 mM hydroxybutyric acid, 5 mM hypoxanthine) was freshly prepared and contained polygeline (25.75 g/L) to provide colloid pressure [40] and carbenicillin (100 mg/mL) to provide antibacterial protection.

Before each kidney was perfused, the apparatus was flushed with EDTA (4 mM) followed by distilled water to remove any trace iron contamination. After being subjected to periods of CI, the kidneys were reperfused on the circuit for either 5, 15 or 30 min before determination of DFX-A iron and total iron.

Determination of DFX-A iron. Kidneys were bisected longitudinally, the cortex and medulla differentiated by colour, rapidly separated by gross dissection and homogenized in 0.1 M Tris–HCl buffer (pH 7.4) (25% w/v). After centrifugation, triplicate aliquots (1.0 mL) of supernatant were mixed with DFX (100 μL) (2 mM final concentration) and incubated at 37° for 60 min to allow the reaction between chelatable iron and DFX.

DFX and its iron-bound form, ferrioxamine (FX) were extracted from the incubation mixture [35] and the extracts dried under vacuum in a rotary evaporator at 45° before storage at −70° prior to analysis. FX and DFX were quantitated by HPLC as described previously [35], the FX:DFX ratio calculated, and the amount of DFX-A iron present determined by

Table 1. The level of desferrioxamine-available iron in rabbit kidneys stored at 0° in hypertonic citrate solution

Group	nmol Fe/g tissue Cortex	Medulla
Fresh kidneys	15.94 ± 5.19	12.08 ± 6.70
24 hr cold ischaemia	33.82 ± 5.77†	25.38 ± 8.94
48 hr cold ischaemia	45.95 ± 9.27†	40.00 ± 12.62*
72 hr cold ischaemia	41.73 ± 17.75*	39.42 ± 14.94*

Values represent the mean ± SD of six determinations performed in duplicate.

* $P < 0.05$ ($t > 3.28$, Bonferroni adjustment) compared to fresh kidneys.

† $P < 0.005$ ($t > 4.71$, Bonferroni adjustment) compared to fresh kidneys.

comparison with a standard curve constructed on the same day.

Trace iron contamination was removed from all buffers and solutions by the method of Gutteridge [41] and all glassware, pipette tips and tubes were washed with EDTA (4 mM) followed by distilled water.

Determination of total iron. The total iron content of the kidney homogenates was determined at 249 nm on a Perkin–Elmer 2380 atomic absorption spectrometer [42].

Statistical analysis. Statistical analysis was performed by Student's *t*-test using the Bonferroni adjustment for multiple group comparisons with a single control [43].

RESULTS

There were measurable levels of DFX-A iron present in both cortex and medulla of fresh control kidneys prior to any ischaemic insult (Table 1). When kidneys were subjected to 24 hr cold storage, there was a significant ($P < 0.005$) increase in the level of DFX-A iron in the cortex compared with the fresh control group and an increase in the medulla which failed to attain significance as determined by Student's *t*-test with Bonferroni adjustment (Table 1). Following storage for 48 and 72 hr, DFX-A iron levels were significantly elevated in both regions of the kidney compared to the fresh controls. The levels observed after 48 and 72 hr CI were both higher than those following 24 hr CI, but these differences did not attain significance.

On reperfusion at 37°, all kidneys (control and stored for 24, 48 and 72 hr) exhibited perfusion flow rates of between 6–8 mL/min/g tissue at a constant pressure of 110 mm Hg during the time of observation (30 min). This range is similar to that of a previous report [39] which also demonstrated that perfusion flow rates did not significantly alter when rabbit kidneys were perfused on this apparatus following extended periods (up to 90 min) of warm ischaemia.

Dramatic differences were observed in the levels of DFX-A iron between the ischaemic groups when the stored organs were reperfused. After 24 hr CI, the levels of DFX-A iron immediately decreased and returned rapidly to control levels in both the cortex and medulla within 5 min (Fig. 2). In contrast, following the more physiologically damaging 48 hr period of CI, DFX-A iron levels remained elevated in both cortex and medulla during the first 5 min of reperfusion and returned to control levels only after 30 min (Fig. 3). A similar response was observed in the medulla of kidneys stored for 72 hr, however in the cortex of these organs, DFX-A iron levels actually increased during the first 5 min of reperfusion before returning to control levels after 30 min (Fig. 4). Thus, 5 min after commencing reperfusion, DFX-A iron levels in 48 and 72 hr stored kidneys were significantly higher, by a factor of 3 to 4, than the levels in either fresh controls or after the less physiologically damaging 24 hr storage period (Table 2).

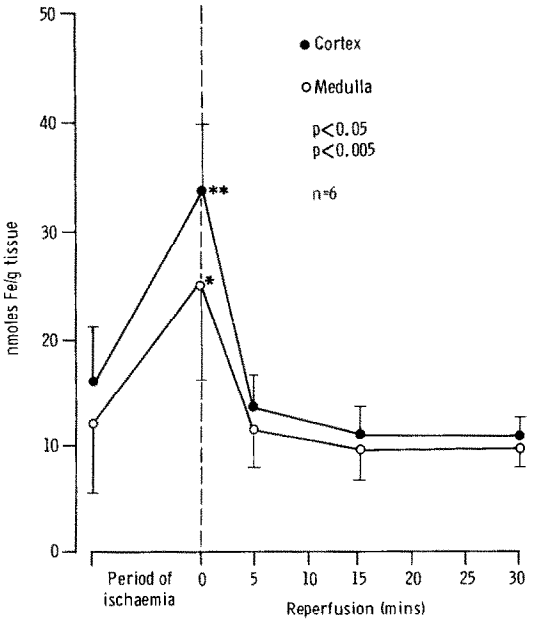

Fig. 2. The level of desferrioxamine-available iron in cortex and medulla of kidneys stored for 24 hr at 0° in hypertonic citrate solution, followed by 30 min reperfusion with an asanguinous perfusate.

In order to obtain a measure of both the extent and duration of iron decompartmentalization on reperfusion of the organs, the area under each reperfusion curve (Figs 2–4) was integrated between 0 min (i.e. the start of the reperfusion period) and 30 min of reperfusion taking the amount of DFX-A iron found in fresh kidneys as the basal level (Table 3). This clearly showed that while 24 hr cold storage induced some iron release, storage periods of 48 hr or longer resulted in much more substantial changes in iron homeostasis in both regions of the kidney.

Total iron contents of cortex and medulla were measured by atomic absorption spectroscopy and no significant differences ($P > 0.05$) were found between control kidneys, the groups rendered ischaemic or the groups subjected to subsequent reperfusion. All group means fell within 15% of the

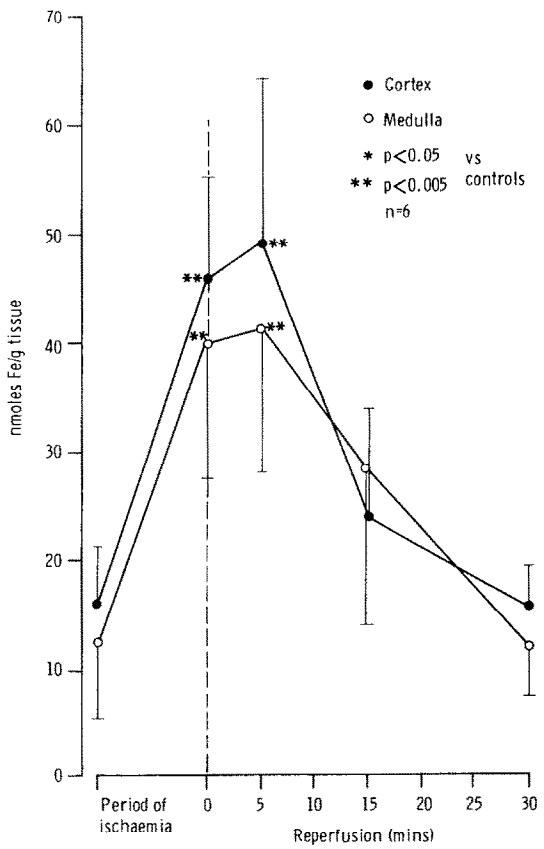

Fig. 3. The level of desferrioxamine-available iron in cortex and medulla of kidneys stored for 48 hr at 0° in hypertonic citrate solution, followed by 30 min reperfusion with an asanguinous perfusate.

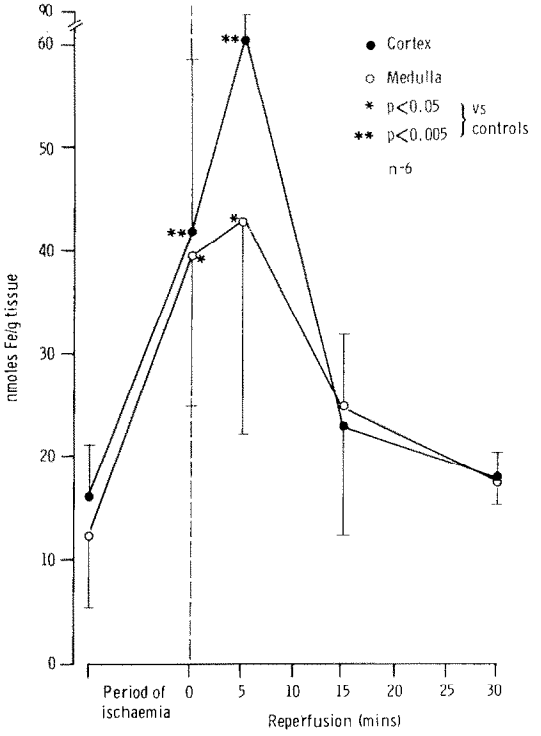

Fig. 4. The level of desferrioxamine-available iron in cortex and medulla of kidneys stored for 72 hr at 0° in hypertonic citrate solution, followed by 30 min reperfusion with an asanguinous perfusate.

mean level in fresh cortex (980 nmol/g tissue; mean of six determinations) and the mean level in fresh medulla (530 nmol/g tissue; mean of six determinations). In addition, the perfusate was analysed for DFX-A iron by the same sensitive method as used for determining tissue DFX-A iron levels. No detectable iron could be detected in perfusate from control kidneys or those rendered ischaemic for the longest time period (72 hr), thus no significant amounts of iron were released from the organs during the 30 min perfusion period. These results therefore indicated that the differences in the levels of DFX-A iron observed between the groups reflected a redistribution of iron occurring within the tissue.

Table 2. The level of desferrioxamine-available iron in rabbit kidneys stored at 0° in hypertonic citrate solution followed by 5 min reperfusion

Group	nmol Fe/g tissue Cortex	Medulla
Fresh kidneys	15.94 ± 5.19	12.08 ± 6.70
24 hr CI + rep	13.32 ± 3.22	11.36 ± 3.42
48 hr CI + rep	49.39 ± 15.15†	41.59 ± 13.48†
72 hr CI + rep	60.68 ± 22.82*	43.11 ± 21.72*

Values represent the mean ± SD of six determinations performed in duplicate.

* $P < 0.05$ ($t > 3.28$, Bonferroni adjustment) compared to fresh and 24 hr CI + rep kidneys.

† $P < 0.005$ ($t > 4.71$, Bonferroni adjustment) compared to fresh and 24 hr CI + rep kidneys.

CI, cold ischaemia; rep, reperfusion.

DISCUSSION

The rabbit kidney model was employed to investigate possible pharmacological strategies for improving renal function following damaging periods of ischaemia and reperfusion. We have previously shown in this laboratory that chelation of potentially catalytic iron by DFX resulted in significant prevention of ischaemic and reperfusion damage, as measured by a range of markers of lipid peroxidation [30–33]. We subsequently demonstrated, by use of a novel method for measuring the level of intracellular iron available for chelation by DFX [34, 35], that DFX-A iron levels increased during both warm and cold ischaemia [36, 37]. In the present study, these investigations were extended to investigate DFX-A

Table 3. The extent of iron decompartmentalization in rabbit kidneys stored at 0° in hypertonic citrate solution followed by reperfusion for 30 min

Storage period	nmol/g tissue min Cortex	Medulla
24 hr CI + rep	77.5	53.7
48 hr CI + rep	437.5	486.8
72 hr CI + rep	512.5	491.4

Values represent the extent of iron release above control (fresh tissue) levels between 0 and 30 min reperfusion as determined by integration of the areas under the curves shown in Figs 2–4.

CI, cold ischaemia; rep, reperfusion.

iron levels during longer and more physiologically-damaging CI periods, and in addition to examine the effects of reperfusion.

Rabbit kidneys stored at 0° for 24 hr in HCA, a clinically-used storage medium, will all recover normal physiological function following autotransplantation. However, 50% of kidneys stored for 48 hr in HCA will not be viable, and only very limited function is observed after 72 hr storage [44]. These three storage periods were therefore selected to investigate the hypothesis that levels of DFX-A iron present are an important factor in the cellular damage occurring as a result of ischaemia and reperfusion.

The results of this study demonstrate that there is a significant increase in DFX-A iron levels during CI compared to fresh kidneys, and that these levels tended to be higher in the organs subjected to the longer and more damaging periods of storage. However, more dramatic differences between the ischaemic groups occurred during reperfusion, since DFX-A iron levels rapidly returned to control levels on reperfusion of the 24 hr CI kidneys but remained elevated for extended periods and decreased more slowly in the organs stored for 48 and 72 hr. It is interesting to note that increased DFX-A iron levels were more pronounced in the cortex and we have previously shown [45] that this region is more susceptible to iron-catalysed lipid peroxidation following cold storage than the medulla. Thus, after 5 min reperfusion, three to four times as much iron was available for chelation by DFX after 48 and 72 hr storage than after 24 hr CI. Integration of the areas under the reperfusion curves from 0 to 30 min, which gives a measure of both the extent and duration of iron decompartmentalization, clearly showed a six to seven-fold increase in the extent of iron decompartmentalization following the two longer and more physiologically damaging storage periods, than after 24 hr CI, where the kidneys remain viable. These differences in intracellular iron homeostasis could not have been due to differences in the vascular resistance of the organs as all kidneys exhibited perfusion rates of between 6 and 8 mL/min/g tissue at constant pressure over the 30 min period. However, the procedures of storage and reperfusion may have affected the distribution of perfusate flow within regions of the kidneys. This could influence the levels of DFX-A iron in different regions because some may be relatively hypoperfused and thus a period of hypoxia at 37° may be imposed on the damage seen after cold storage alone.

As no significant changes in the total iron content of the organs was observed and no iron was lost to the perfusate throughout ischaemia and reperfusion, a redistribution of iron must have occurred within the tissue leading to an increase in the percentage available for chelation by DFX. This increase as a result of CI indicates an increase in the iron flux through the transit pool in the cytosol. One possibility is that the source of this extra iron was transferrin molecules, but this is unlikely as any transferrin present would have been removed from the kidneys through the flushing procedure. A more likely source is the intracellular iron-storage protein ferritin, which stores iron in the ferric form, but releases it in the ferrous state after reduction [46]. This reduction could be facilitated by the low oxygen tension encountered as a result of ischaemia and by the accompanying fall in pH [5]. Such an environment would favour increased levels of reducing agents which may release iron from ferritin. However, the nature of such agents has yet to be established.

On reperfusion it would appear that alterations in the balance between DFX-A iron uptake and release mechanisms might be responsible for the differences in DFX-A iron levels observed between kidneys stored for varying periods. Thus, on reperfusion of the better preserved 24 hr CI organs, the uptake mechanism is still fully functional and so dominates, quickly removing DFX-A iron released during ischaemia and preventing any further release of iron. However, following storage periods of 48 hr or longer, this uptake mechanism appears to be impaired, and in addition the results suggest that further iron release may take place in the cortex during the first 5 min of reperfusion of 72 hr stored kidneys. The combination of these events thus leads to the presence of elevated DFX-A iron levels during 30 min of reperfusion.

We can only postulate the mechanisms of DFX-A iron uptake and release. It seems likely that the iron is taken back up into ferritin since total iron levels remain constant and no detectable iron is lost into the perfusate, although it is possible that some other, as yet unidentified system is responsible for the reduction in iron levels. The mechanism of iron incorporation into ferritin is still poorly understood, but it is believed to involve oxidation of LMW iron chelates, and may therefore require molecular oxygen [46].

Further release of iron into the DFX-A pool on reperfusion of the 48 and 72 hr stored kidneys may be a result of iron release from ferritin by superoxide anions [47]. These free radical species have been shown to release iron from ferritin *in vitro* [26, 48], and there is evidence for the production of significant levels of superoxide anion during reperfusion of ischaemic tissue, as discussed below.

It is likely that, in stored kidneys, the increased levels of iron in the LMW transit pool which are over and above the normal metabolic needs of the cell would, if not removed or utilized rapidly, catalyse free radical-mediated cell damage. The importance

of DFX-A or LMW chelatable (LMWC) iron in ischaemia and reperfusion injury has been suggested in several organs [49, 50]. LMWC iron exerts its effects by catalyzing a number of adverse reactions. Transition metals can convert superoxide anions ($O_2^{\cdot-}$) to hydroxyl radicals (OH$^{\cdot}$) via the Haber–Weiss reaction [18]:

$$2O_2^{\cdot-} + 2H^+ \rightarrow H_2O_2 + O_2. \quad (1)$$

$$Fe^{3+} + O_2^{\cdot-} \rightarrow Fe^{2+} + O_2. \quad (2)$$

$$Fe^{2+} + H_2O_2 \rightarrow OH^{\cdot} + OH^- + Fe^{3+}. \quad (3)$$

Superoxide anions may be formed during reperfusion either by the conversion of xanthine dehydrogenase to xanthine oxidase, which utilizes hypoxanthine formed by the catabolism of ATP during ischaemia to produce $O_2^{\cdot-}$ from O_2 [47], or through the accumulation of reducing agents from the electron transport chain, which spontaneously react with molecular oxygen once the tissue is reperfused. The OH$^{\cdot}$ radical is highly reactive towards all biomolecules, and will initiate lipid peroxidation by abstracting hydrogen atoms from polyunsaturated fatty acids (PUFAs). In addition, there is evidence that this damaging process may be initiated directly by iron-centred species [17]. Once initiated, a chain reaction ensues in which alkoxy and peroxy radicals attack further molecules of PUFA which degenerate to lipid hydroperoxides. Iron can further propagate this cycle by catalysing the breakdown of lipid hydroperoxides back to reactive alkoxy and peroxy radicals [51].

The low levels of DFX-A iron present in fresh control kidneys constitutes LMW iron chelates moving through the cytosol which are necessary for the normal metabolic requirements of the cell, and is likely to be responsible for the low level of lipid peroxidation observed in this tissue. The two- to three-fold rise in these levels on reperfusion immediately following 24–72 hr ischaemia would be expected to initiate free radical damage to all of the stored kidneys by interaction with incoming oxygen. Indeed, a small but statistically non-significant rise in the level of lipid peroxidation is seen in kidneys stored for only 24 hr [52]. However, kidneys stored for this short period have been shown to be functionally viable. Thus it appears that the extra DFX-A iron is not present for long enough on reperfusion to pose a level of oxidative stress which cannot be sufficiently countered by the antioxidant defence mechanisms of the cell, and extensive cellular damage is prevented. In contrast, after the longer storage periods, the further rise and continued elevation of DFX-A iron levels might result in extensive and prolonged propagation of the damage initiated on reperfusion, thus causing more widespread cellular injury, with the result that these kidneys are less physiologically viable than those stored for 24 hr. We conclude that it is this continued elevation in DFX-A iron after reperfusion that is important in determining the degree of tissue damage rather than the levels present at the end of the CI period itself.

That the iron measured in this study is definitely in a form capable of catalysing oxygen-derived free radical-mediated lipid peroxidation cannot be conclusively proved. However, the fact that the iron is available for chelation by DFX makes it highly likely that it is able to catalyse these damaging reactions. Indeed, it has been shown that ferritin can donate the necessary iron for microsomal lipid peroxidation induced by NADPH or xanthine oxidase [53], and can stimulate the peroxidation of lipids [54]. It is therefore probable that increased levels of DFX-A iron, especially those in the immediate post-ischaemic period, are important in determining the degree of reperfusion damage to rabbit kidneys, and that iron chelation, particularly prior to reperfusion, would be a worthwhile therapeutic approach to preventing this damage, with the ultimate goal of improving the viability of transplanted human organs.

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