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Non-invasive measurement of respiratory chain dysfunction following hypothermic renal storage and transplantation

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Non-invasive measurement of respiratory chain dysfunction following hypothermic renal storage and transplantation. Ischemia/reperfusion (IR) damage is a major cause of dysfunction in transplanted organs. The objective of the present study was to correlate *in vivo* measurements of respiratory chain (RC) function with histological and physiological parameters. Non-invasive *in situ* (surface fluorescence) measurements of mitochondrial NADH and near infrared spectroscopic measurements of cyt aa_3 were made in unstored (Group 1) and 72 hour stored (1 to 2°C) (Group 2) autografted rabbit kidneys. The effect of sodium pentobarbitone on NADH levels was investigated. In Group 1, there was a significant change in the redox state of cyt aa_3 in all ($N = 6$) kidneys on reperfusion which correlated with organ viability and increased NADH oxidation and minimal edema on histological examination. In Group 2 there was no significant change in cyt aa_3 compared to baseline, and this correlated with poor long term organ viability, slower NADH oxidation, and severe cortical edema. Pentobarbitone inhibition of the RC resulted in rapid and complete reduction of NAD^+ in Group 1, but none or only a slight reduction in Group 2. The results demonstrate that it might be possible in future to predict organ viability and histological changes by non-invasive measurements of RC dysfunction in the clinical transplant situation.

Damage incurred during ischemia and reperfusion (IR) is one of the major causes of dysfunction in transplanted organs. Identification of key biochemical changes associated with IR which correlate with tissue dysfunction might lead to the development of novel pharmacological strategies to increase an organ's ischemic tolerance.

During ischemia there is a rapid inhibition of mitochondrial electron transfer, resulting in depletion of ATP, a net breakdown of adenine nucleotides to non-phosphorylated metabolites, accumulation of reduced pyridine nucleotides [1], loss of homeostasis involving a fall in intracellular pH [2, 3], mitochondrial calcium loading and cellular swelling [4].

In irreversibly injured cells, respiratory control is lost and is accompanied by oxidation of cytochromes a and a_3 and NADH [5], the latter attributed originally to substrate deficiency [6]. More recent studies, however, indicate that this may be an enzymological defect resulting in an inability to metabolize NADH-linked substrates [5, 7]. In the transplant situation it has been shown that mitochondrial phosphorylative activity is a prerequisite for recovery to restore cellular energy charge [8]. Return of function may therefore relate to preservation of inner mitochondrial membrane integrity, and the structure and activities of the RC complexes I to IV [4, 5]. In essence, the integrity of oxidative metabolic pathways and capacity to resynthesize ATP rather than the immediate post-ischemic ATP levels appears to determine the return of function [5].

In recent studies [9] the activities of RC complexes I to IV in mitochondria from perfused rat hearts subjected to global ischemia or anoxic perfusion prior to ischemia were investigated. The results indicated that differences in the sensitivity of these complexes to ischemic damage were dependent upon the duration of ischemic episode and the presence of oxygen. The most sensitive site was complex I [4, 9]. The demonstration that complex I is a major defective site during ischemia depended upon isolation of mitochondria from homogenates of the tissue, using *in vitro* methods. However, for a method to be of predictive value in assessing organ viability either before or immediately after transplantation it will be necessary to develop non-invasive methods for measuring mitochondrial dysfunction. These could provide an early indication of subsequent IR damage in the whole organ. When Chance and his co-workers [10–13] showed that direct *in vivo* measurements of fluorescence could be made from intracellular pyridine nucleotides in brain and kidney, the possible importance of these measurements as indicators of intracellular hypoxia was emphasized. It was shown that fluorescent signals from cytosolic NADH are negligible compared to those from the mitochondrial pool owing to quenching of fluorescence by cytosolic glyceraldehyde 3-phosphate dehydrogenase [14]. Recently, the use of pyridine nucleotide fluorescence measurement to determine changes in mitochondrial and cytosolic redox status in perfused rat livers has been reported [8], and was found to correlate closely with energy charge and mitochondrial phosphorylative activity [8, 15]. We have extended these whole organ measurements further by using the non-invasive method of near infrared spectroscopy (NIRS). The principle depends upon the fact that NIR light (700 to 1000 nm) penetrates tissue and allows absorption measurement of oxygen-dependent chromophores. Information is therefore obtained about the supply of oxygen to the tissue and intracellular oxygen availability as well as RC function [16–22]. This enables monitoring of changes in tissue concentrations of HbO_2, Hb and cyt aa_3, the final RC component responsible for reduction of molecular oxygen [16]. Near infrared spectroscopy provides information complementary to that

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obtained by magnetic resonance spectroscopy [17]. The use of ^{31}P magnetic resonance spectroscopy has shown a correlation between impaired oxidative metabolism (low PCR/Pi ratios) in neonates and poor prognosis [23–25]; in particular abnormal ^{31}P spectra have been associated with periventricular leukomalacia [25]. However, near infrared spectroscopy can provide information about hemodynamic events that precede and accompany secondary energy failure in newborn infants [17].

In this study we have investigated the effect of IR injury associated with storage and transplantation of rabbit kidneys, by correlating histological parameters with the RC components NADH and cyt aa_3. Measurements were made on two groups of rabbit kidneys in which the organ had been autografted either after harvesting from the donor with no storage period or after a 72-hour cold storage period. From previous work in this experimental model, we expect 95 to 100% viability in the recipients of unstored renal autografts, but only between 20 to 30% of rabbits receiving 72-hour stored kidneys survive with good renal function [26]. To further identify the site of IR damage, the effect of sodium pentobarbitone inhibition of NADH dehydrogenase (complex 1) was determined on the rate of NADH production [27].

Methods

Anaesthesia/physiological monitoring/surgical procedures

Female NZW rabbits (2.5 kg) were anesthetized with an i.m. injection of ketamine (50 mg/kg) and xylazine (8 mg/kg), tracheotomized and artificially ventilated with a 50:50 oxygen:nitrous oxide mixture for periods of up to eight hours. Surgical anesthesia was maintained by continuous i.v. infusion of ketamine and xylazine (50 mg:8 mg/kg/hr).

The left femoral artery was carefully dissected and cannulated with an 18 G plastic cannula, which was inserted for a distance of 5 cm until the tip reached the base of the aorta. The cannula provided protective housing for a Continucath oxygen electrode (Biomedical Sensors Ltd., High Wycombe, UK) coupled via a three-way tap to a pressure transducer. Blood pressure (BP) and PO_2 were thus continuously monitored throughout the study period. BP was maintained during reperfusion by continuous i.v. infusion of Haemaccel (30 ml/hr). Intermittent blood samples were taken for PCO_2, pH and Hct determination. Core temperature was monitored using an esophageal probe and was maintained between 37 and 38°C with a heated pad (37°C). In addition, the ECG, FiO_2 and $EtCO_2$ were continuously monitored. The transplant procedures have been described previously [26]. Essentially, the left kidney was harvested on as long a renal pedicle as possible via a midline laparotomy. At the time of transplantation, the right kidney was removed leaving the renal pedicle as long as possible together with the ureter divided close to the kidney. The left kidney was then autografted onto the right pedicle by microsurgical techniques.

Experimental groups

Group 1. Freshly nephrectomized left kidneys were flushed with 30 ml of cold (1 to 2°C) HCA via the renal artery and autografted immediately into the right renal bursa using standard microsurgical techniques.

Group 2. Kidneys were flushed as in Group 1 and then stored in HCA surrounded by ice to maintain a temperature of 1 to 2°C for 72 hours before autografting.

Histological examination

Lateral slices were taken from kidneys soon after termination of reperfusion and fixed in 10% formal saline for processing to paraffin wax. Sections were stained with hematoxylin and eosin (H&E). IR damage was assessed by several histological features: edema, congestion, hemorrhage, inflammation and necrosis in the cortex, cortico-medullary junction and medulla.

Surface fluorometric measurements of mitochondrial NADH

Surface fluorometric (SF) measurements were made using a Perkin Elmer LS 50 with a fiberoptic attachment. An SF probe was placed gently touching the surface of the *ex vivo* or *in situ* kidney and fixed in position by attachment to a retort stand. Emission spectra of NADH from 366 to 600 nm were obtained by excitation at a fixed wavelength of 340 or 366 nm (slit widths 10/10 nm). These wavelengths were chosen to make: (a) comparisons with published data; and (b) compensatory changes for the effect of scattering which is dependant upon changes in blood volume and hemoglobin deoxygenation [10, 28, 29]. In agreement with Koybashi et al [29] and Frank, Barlow and Chance [10], we observed that changes in reflectance in the perfused organ were small; hence, uncorrected fluorescence readings could be used, except under extreme conditions, for example if the renal artery was clamped in which case a large reflected signal was measured at 366 nm. To assess the effect of movement and normal cyclical variations in Hb oxygenation levels on NADH fluorescence emission, each spectrum was performed at least in duplicate, within one minute, and repeatedly scanned up to 10 times in the early reperfusion stages. The values thus obtained agreed within 5 to 10% at most. The results are presented in terms of percent change of relative fluorescence from the pre-reperfusion value.

Experimental protocol

(a) The intensity of NADH fluorescent emission, and spectral properties, were measured in:

(i) *Ex vivo* control freshly harvested flushed kidneys, stored in HCA at 0° to 2°C on ice for up to 90 minutes (Group 1 control) and after 72 hours of storage (Group 2 control).

(ii) *In situ* Group 1 kidneys, prior to transplantation and reperfusion with blood.

(iii) *In situ* Group 2, 72 hours stored (1 to 2°C) kidneys prior to transplantation and reperfusion with blood.

(b) The change in the intensity of the NADH emission was measured in both experimental Groups in response to:

(i) Alterations in the inspired FiO_2.

(ii) Reperfusion measured in both Groups up to six hours post-transplant at 5, 15, 45, 60 minutes and at two, four and six hours.

(iii) Reperfusion in which continuous measurements of NADH fluorescence intensity were made by scanning up to 90 minutes at 1000 nm/min between 366 and 600 nm in an additional three unstored and stored transplants to ensure that any variation in fluorescence intensity was not due to movement

artifact (positional) or heterogeneity of NADH due to mitochondrial clustering [10].

(iv) Rapid infusion of sodium pentobarbitone: 200 mg/kg (i.v.) (<5 sec) [27] whereafter NADH fluorescence was monitored for a further 20 minutes.

NIRS measurements of cyt aa_3

These were made using a NIRO-500 monitor (Hamamatsu). The technique of near infrared spectroscopy (NIRS) has been used for the measurement of cerebral oxygenation for many years [20–22, 31–33]; however, its use in renal physiology has been limited [30]. NIR transmission spectroscopy through tissues up to 10 cm is possible because of the relative transparency of biological tissues to NIR light (700 to 1000 nm). Near infrared spectroscopy is dependent upon the light absorption properties of key biochemical components, hemoglobin and myoglobin. The importance of using NIRS to assess the oxygenation status of hemoglobin and myoglobin is a virtue of their behavior in acting as intrinsic probes for oxygen. Absorption due to oxyhemoglobin (HbO_2) and deoxyhemoglobin (Hb) can be quantitated using a modified form of the Beer-Lambert Law. The Beer-Lambert Law is applicable to a homogenous scattering medium in which absorption changes are linearly related to concentration.

$$\text{Absorbtion (in absorbance units)} = \Sigma.c.L.B. + G.$$

In which Σ is the extinction coefficient ($mM^{-1}cm^{-1}$) of the chromophore of interest (light absorbing compound) and its concentration c (mM), B is the pathlength factor which compensates for the increased pathlength due to scattering, and G is a geometry-dependent factor. Hence, providing Σ, l and B are known, the change in concentration can be obtained from change in absorbance.

$$\Delta C = \frac{\Delta A}{c.L.B.}$$

An algorithm has been determined for the quantification of oxy- and deoxyhemoglobin following measurements on infants and experimental animals [17, 34]. The extinction coefficents of HbO_2 and Hb were obtained from studies of lysed human blood and estimates for B (for cerebral use) were made using time of flight studies in live rats and postmortem babies [17]. It has been reported that the optical pathlength (and differential pathlength factor) has been shown to be constant within the NIR region despite gross changes in oxygenation and perfusion, before and after death (obtained from time resolved spectroscopic studies) [33, 35, 36]. The maximum variation was shown to be <9%. A linear relationship between hemoglobin measured spectroscopically and biochemically in a scattering medium has been found experimentally over a wide range of hemoglobin concentrations [32, 33, 36].

The multiplier coefficients used in the estimation of concentration changes of cyt aa_3 were obtained from experimental procedures involving flourocarbon transfusion studies in anesthetized animals [34, 37]. These studies were performed in order to remove optical interference from hemoglobin, and similar experiments were performed to remove the optical effect of bone by removing small sections of the skull in anesthetized rats and performing oxidation-reduction experiments [21, 34].

Table 1. Multiplying factors

Wavelength *nm*	772	830	842	909
Hb	1.2209	−0.8437	−0.7215	0.6859
HbO_2	−0.6945	−0.4851	−0.1382	1.9579
$cytaa_3$	−0.0871	0.7550	0.5309	−1.1466

Changes in the concentrations of HbO_2, HbR and $cytaa_3$ were calculated by linear summation of absorption changes (measured in absorbance units) multiplied by the following factors at each wavelength (Table 1) [37].

The calculated concentration change is expressed in mmol/liter multiplied by pathlength in cm.

One indication of reliability of these algorithms is the independence of the cyt aa_3 response compared to oxyhemoglobin, that is, if changes in the cyt aa_3 oxidation do not mirror the HbO_2 changes (and they do not). It has been reported that in damaged tissues in which the mitochondria are uncoupled, cyt aa_3 can become oxidized despite no measurable change in the HbO_2 level [38–41].

NIR monitoring facilitates measurements several centimeters into tissues thereby providing more information than other optical methods, namely NADH fluorometry and visible spectroscopy which penetrates only surface layers. NIR optrodes were placed on either side of the kidney (immobilized as above) to give an indication of the oxygenation status across the medulla and cortex. Concentration changes of HbO_2, Hb and of cyt aa_3 were continuously measured in *in situ* kidneys (pretransplantation to assess normal changes in parameters) and pre- and post-reperfusion (following release of clamps after completion of the anastamoses) in both groups for up to six hours, with SF measurements made as described previously.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance between groups was tested by an unpaired Student's *t*-test.

Results

In vivo surface fluorescence measurements of NADH in the rabbit kidney

A broad fluorescence emission maximum of NADH (excitation wavelength 340 nm) was measured in the *ex vivo* and *in situ* kidney which shifted to lower wavelengths with sodium pentobarbitone infusion consistent with an altered NADH fluorophore (Figs. 1 and 2). An increase in fluorescence emission of NADH (470 nm) from the surface of the perfused rabbit kidney, excited at 366 nm, was obtained in response to decreasing the systemic pO_2 from 21 to 5 Kpa.

In vivo surface fluorescence measurements of NADH in harvested kidneys pre- and post-transplantation

The fluorescence emission of control kidneys which had been removed and flushed with HCA and stored on ice for up to 90 minutes did not significantly change after storage (3.03 ± 0.09 (mean ± SEM, N = 19 estimates), and there wa.. no significant difference between the relative fluorescence intensity of the two

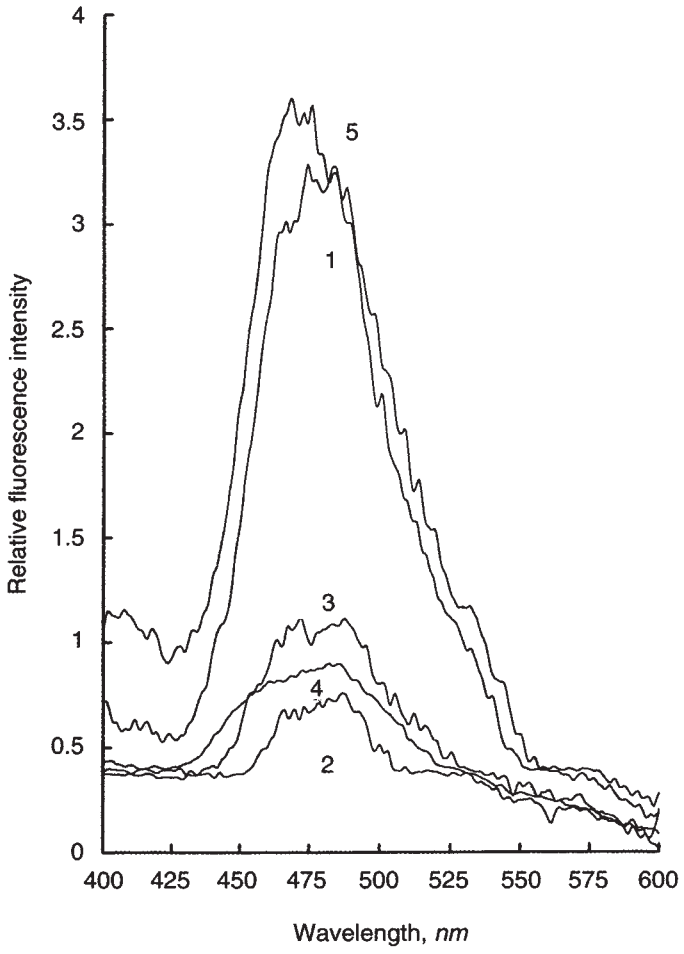

Fig. 1. In vivo *surface fluorimetric measurements of NADH in a transplanted fresh kidney*. **1.** Pre-reperfusion; **2.** 1 minute after reperfusion; **3.** 12 minutes after reperfusion; **4.** 60 minutes after reperfusion; **5.** 5 minutes after sodium pentobarbitone infusion (200 mg/kg). Emission spectra from continuous measurements of NADH pre- and post-reperfusion in an unstored kidney are shown.

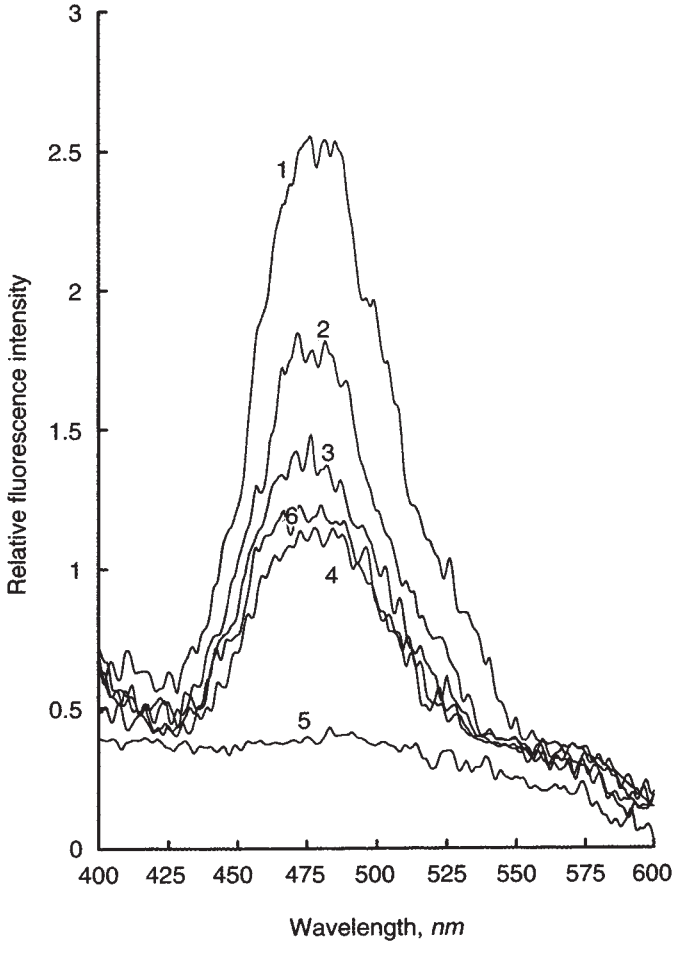

Fig. 2. In vivo *surface fluorimetric measurements of NADH in a transplanted 72 hour-stored kidney*. **1.** Pre-reperfusion, **2.** 1 minute after reperfusion, **3.** 6 minutes after reperfusion, **4.** 10 minutes after reperfusion, **5.** 20 minutes after reperfusion and **6.** 5 minutes after sodium pentobarbitone infusion and remained constant until the end of monitoring, (20 min). This figure shows emission spectra from continuous measurements of NADH pre- and post-reperfusion in a stored (72 hr) kidney.

groups *in situ* (pre-reperfusion): 2.85 ± 0.196 (Group 1, $N = 9$ kidneys) and 2.94 ± 0.26 (Group 2, $N = 7$ kidneys).

Table 2 shows the percent change in fluorescence from the pre-reperfusion values during the initial phase (5 to 20 min) post-reperfusion of Group 1 and Group 2 kidneys. There was no significant difference between the percent oxidation observed in the two Groups: 82.85 ± 11.29 (Group 1) compared to 89.85 ± 11.92 (Group 2). Figure 1 shows emission spectra obtained by continuous scanning of NADH fluorescence pre- and post-reperfusion in a Group 1 kidney. Immediate oxidation of NADH between 70% and 100% occurred within one minute (Table 2) and values oscillated within this range. In comparison in a Group 2 kidney, the magnitude and rate of oxidation of NADH was less (Fig. 2). However, 20 minutes following reperfusion, 100% of the NADH had become oxidized.

Effect of sodium pentobarbitone infusion

In Group 1 kidneys, infusion of sodium pentobarbitone 200 mg/kg (< 5 sec) resulted in rapid production of NADH (in all 8) kidneys within one minute as expected for the inhibition of NADH dehydrogenase [27] (Table 2). In Group 2 kidneys, sodium pentobarbitone resulted in a decreased rate and magnitude or no NAD^+ reduction in five out of seven kidneys within the monitoring period of 20 minutes (Table 2). In one kidney, sodium pentobarbitone resulted in an NADH emission to a maximum of 37% of the pre-reperfusion value over five minutes and remained constant. In the final kidney the NADH level reached values as observed in Group 1. This kidney had minimal edema and normal cyt aa_3 responses.

In vivo measurements of cyt aa_3 during reperfusion using NIRS

Figure 3 shows a representative plot of change in concentration of HbO_2, Hb and cyt aa_3 following reperfusion of a Group 1 kidney. The initial stages of reperfusion resulted in a large increase in $[HbO_2]$ and in [Hb], a net increase in the oxygenation index $[[HbO_2] - [Hb]]$ (as defined by Delpy et al [35]), and no measurable change in the redox state of cyt aa_3. However, as

Table 2. Correlation of respiratory chain function and morphology

Group no.	Maximal redox change cyt aa_3 μM[a]	% NADH oxidized upon reperfusion	% Of pre-reperfusion NADH formed upon pentobarbitone infusion	Severity of cortical edema
1	−58.25 ± 0.848	90	95	mild-moderate
1	−60.0 ± 0.5135	72	76	mild
1	−49.7 ± 0.218	96	69	absent
1	−62.0 ± 0.34	70	70	absent
1	−103.05 ± 1.43	87	70	mild
1	−65.25 ± 0.206	NIRS only	NIRS only	NIRS only
1	SF only	72	70	SF only
1	SF only	88	100	SF only
1	SF only	95	80	SF only
2	nsd	96	0	severe
2	−16.5 ± 0.238	67	82	moderate
2	nsd	96	0	severe
2	nsd	84	8	severe
2	nsd	86	0	moderate
2	SF only	100	0	SF only
2	SF only	100	37.5	SF only

Abbreviation nsd, no significant difference from baseline (usual baseline variation 0.75 ± 0.197 (mean ± SEM) in the *in situ* reperfusing kidney).

[a] Maximal change measured in cyt aa_3 in the 10 minutes post-perfusion (mean ± SEM of 6 time points).

the HbO_2 level fell at the end of reactive hyperemia phase, cyt aa_3 became reduced. In all the unstored transplants, cyt aa_3 became steadily oxidized over several hours (6 hr of reperfusion) to approximately 50% of the change observed in the initial stages of reperfusion. However, this was not observed in Group 2 (in 4 out of 5 kidneys; Table 2).

In Group 1, the reduction of cyt aa_3 (in all 6 kidneys) correlated with minimal edema and with the expected response to sodium pentobarbitone, NAD^+ reduction. In Group 2 there was no significant change in the cyt aa_3 level compared to baseline values (in 4 out of 5 kidneys). There was a very significant difference in the cyt aa_3 response. This correlated with dysfunction of the RC as judged by an inability to reduce NAD^+ which should occur in an undamaged NADH dehydrogenase in the presence of sodium pentobarbitone. In one Group 2 kidney oxidation of cyt aa_3 occurred slowly over two to three hours and was accompanied by reduction of NAD^+ in the presence of sodium pentobarbitone and with more normal histological appearance (Table 2). In summary, in Group 2 there was a decreased rate and magnitude of NAD^+ reduction with sodium pentobarbitone, in contrast to a rapid and complete reduction of NAD^+ in Group 1.

Histological and functional examination of kidneys

In Group 1 there was very little edema (Table 2). In isolated instances there was focal mild to moderate intracellular edema, especially of the proximal tubular cells. There was no congestion, hemorrhage nor an inflammatory infiltrate (such as neutrophils) seen. In Group 2, there was severe cortical intracellular edema in almost every instance but no congestion. In the medulla, there was moderate interstitial edema and congestion. Estimations of renal function following 72 hour storage and transplantation are obscured by purely mechanical factors. While it is true that the glomerular filtration rate correlates with functional viability, severe medullary congestion can entirely abolish urine production even if tubular or glomerular function is unimpaired. Thus, after 72 hours of storage and transplantation, urine production was negligible for the first three to four hour of reperfusion, and so estimations of GFR were not possible and must be assumed to be zero. Urine production tended to increase after this period despite no apparent change in RC function. These changes correlated with the clearance of medullary congestion as observed by NIRS and by barium sulphate angiography.

Discussion

The measured emission spectra in the rabbit kidney cortex were similar to the characteristic NADH fluorescent emission when excited at 340 nm and at 366 nm previously observed elsewhere *in vitro* and *in vivo* in rat brain and kidney [10–13]. Surface measurements of NADH were responsive to fluctuations in the systemic PO_2. These findings are in agreement with Frank, Barlow and Chance [10] who correlated surface fluorescence monitoring of reduced pyridine nucleotide with physiological function of perfused *ex vivo* rat kidneys when subjected to various hypoxic insults.

Our pre-reperfusion, *in situ* NADH measurements were not significantly different between the two groups; hence, it is unlikely that fluorescence of NADH was altered as a result of enhancement or quenching during storage, as distinct from changes in the pyridine nucleotide levels. Although we have not measured nucleotide levels in tissue homogenates we believe that there are several factors which support the view that the fluorescent emission can be attributed to NADH. Most important, there are few other fluorophores whose emission characteristics are identical to that of NADH (whose emission is oxygen dependent), and which are responsive to sodium pentobarbitone whose mode of action is to inhibit NADH-dehydrogenase resulting in an increase in NADH [27].

Our results demonstrate that it is possible to make both discrete and continuous measurements of NADH fluorescence in the *in situ* transplanted kidney. In all kidneys there was a large NADH peak before reperfusion which disappeared in each case within 20 minutes of reperfusion. However, the rate of oxidation was slower in Group 2 kidneys than in Group 1 in continuously monitored kidneys. Moreover, in Group 2 there was no oscillation of NADH levels in response to minor alterations in PO_2 or vascular changes, which also suggests damage to the RC.

The effect of sodium pentobarbitone administration was quite distinct in the two groups. In Group 1 kidneys there was a rapid increase in the relative fluorescence intensity of NADH to pre-reperfusion levels within one minute as expected. In Group 2 that there was little or no response to sodium pentobarbitone infusion. The maximum NADH emission measured in the revascularized transplanted fresh kidney after either low pO_2 or sodium pentobarbitone was not significantly different from the NADH emission measured during storage (in the absence of hemoglobin). This supports the view that (in these experiments) the presence of blood did not affect the maximum measured NADH signal. These results are in agreement with Koyboshi et al [29] and Frank et al [10]. It has been shown that superficial glomerular blood flow is relatively unaffected by a severe

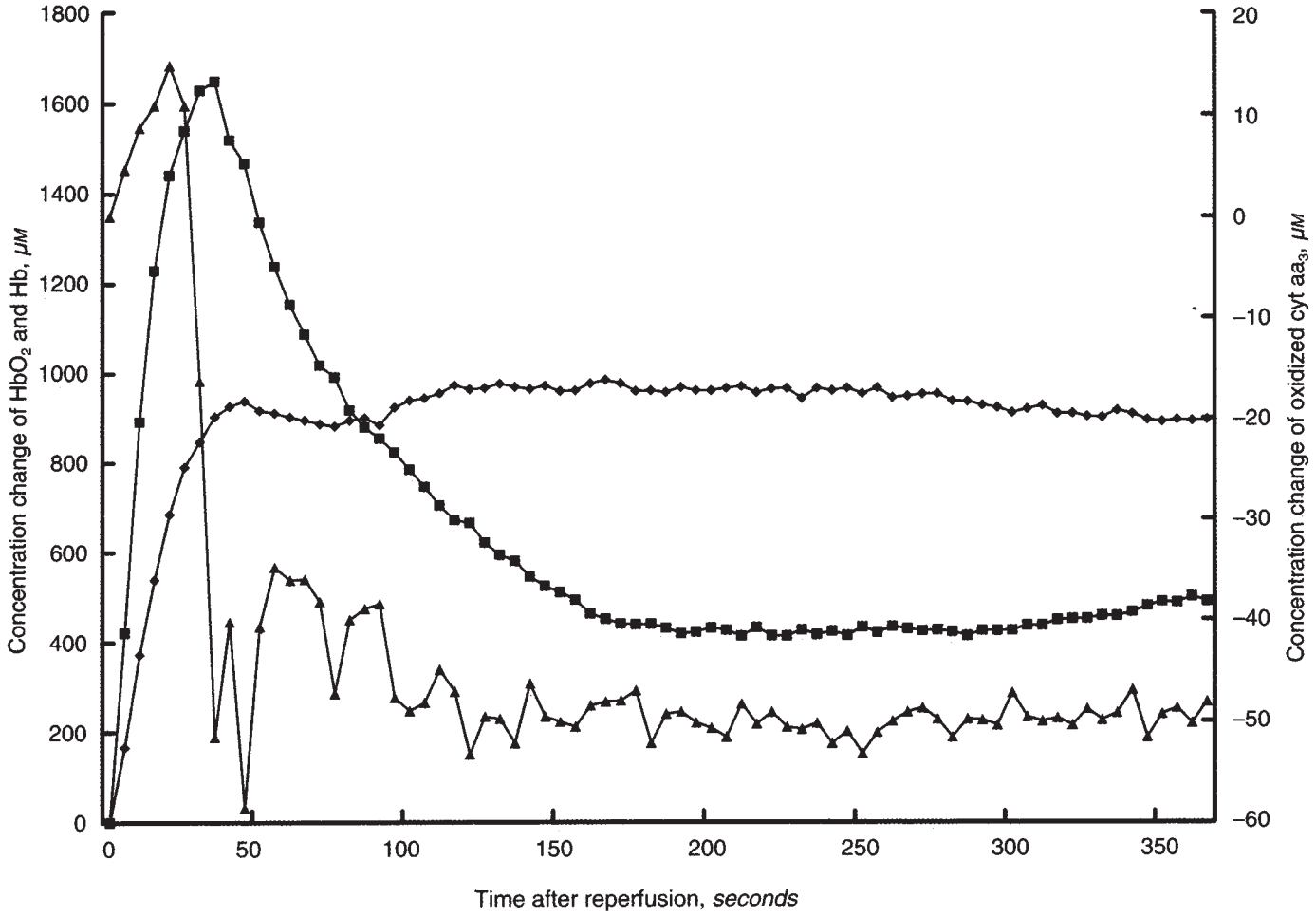

Fig. 3. *Change in concentration of HbO_2 (■), Hb (-◆-) and cyt aa_3 (-▲-) upon reperfusion in a Group 1, unstored kidney.*

ischemic episode [42]; hence, the failure of Group 2 kidneys to respond to sodium pentobarbitone is unlikely to be due to circulatory defects. In Group 1 kidneys, NADH oxidation upon reperfusion correlated with minimal histological changes and consistent variations in cyt aa_3. In contrast, Group 2 kidneys displayed no cyt aa_3 change in kidneys (4 out of 5). In Group 1 kidneys the reduction of cyt aa_3 correlated with the end of a hyperemic phase (Fig. 3). The results agree with the view that cyt aa_3 could have become hyperoxidized and hence, we were observing the change reverting to baseline. This rapid oxidation is in agreement with the known kinetics of oxidation of cyt aa_3. Similar patterns have been measured in anesthetized rats in response to release of carotid occlusion [43] and in cat skeletal muscle [44]. In both cases return to normal baseline conditions-post-hyperemia was associated with a reduction of cyt aa_3. Injection of indocyanine green in tissues in order to determine the extent of perfusion has also been performed to assess the correlation between blood volume and the redox state of cyt aa_3 [38]. It is well established that the oxygen affinity by cyt aa_3 is far greater than that of Hb in the *in vitro* situation and is almost completely oxidized in the steady state [6]. However, the *in vivo* situation is still debateable. Many groups have reported that changes in the redox state of cyt aa_3 occur in response to several factors including: epileptic seizures, hemodilution, in addition to asphyxia [31, 38, 40–42, 45–51]. Similarly it has been reported that cyt aa_3 is not maximally oxidized *in vivo* and values between 20 and 50% reduced have been reported [18].

It is relatively well known that in the kidney oxygen concentration gradients occur and substantial portions of the kidney may normally operate on the brink of hypoxia despite high arterial and venous pO_2 values [52]. This may explain the observations that mitochondrial cytochromes are partially reduced under normoxia [30]. The oxygen supply to support mitochondrial function is determined by mitochondrial clustering in renal tissue [52]. The clustering could cause a steady state reduction of mitochondrial cytochromes even during normoxia. Mills and Jobsis [53] also obtained evidence that the reduction of cyt aa_3 is O_2 dependent at normoxic O_2 concentrations. The single Group 2 kidney which exhibited a similar cyt aa_3 response to Group 1 kidneys was also inhibited by sodium pentobarbitone and was only moderately edematous. In other Group 2 kidneys there was moderate to severe edema but no evidence of neutrophil infiltration.

We have found that severe cortical edema combined with medullary congestion in the early hours of reperfusion are associated with developing renal failure (unpublished results). There was a consistent correlation between the NADH and cyt aa_3 response and histological appearance. Taken together,

these data strongly suggest that 72 hour storage resulted in mitochondrial dysfunction. The fact that sodium pentobarbitone inhibits NADH dehydrogenase suggests that the damage (lack of response to sodium pentobarbitone) may be at complex I or substrate deficiency leading to complex 1. Similarly, it has been observed that mitochondria isolated from perfused rat hearts subjected to global ischemia and reperfusion were unable to metabolize NAD^+ linked substrates and the site of damage was shown to be complex I [4, 10]. It is probably not coincidental that the two main sites of mitochondrial damage during ischemia, complex I and III, are also the major sources of oxygen- derived free radical production during normal electron transfer in mitochondria *in vitro* [54].

Tokunga et al [15] reported a decrease in the rate of NAD^+ reduction in perfused stored rat livers in response to ischemia (inhibition of flow) on an *ex vivo* circuit following storage. It was also suggested that the NAD^+ reduction to NADH can be considered to be a function of the reverse direction of the RC [8, 15] which is energetically unfavorable, and would therefore be more critically dependent upon integrity of the respiratory chain, hence, more sensitive to damage. In our study in both Group 1 and Group 2 kidneys the NADH was rapidly oxidized to NAD^+, and this is consistent with the energetics of the RC. However, the magnitude and rate of the NAD^+ to NADH reaction were considerably reduced in Group 2 compared to Group 1 kidneys.

From these experiments, we are encouraged to believe that SF measurements could be useful in the clinical transplant situation in which damage may have occurred during storage. We have presented data showing that changes in the redox level of RC components correlate with changes in morphology and viability. As far as we are aware, this is the first report correlating two non-invasive methods with organ function and morphology following storage and transplantation. Our findings suggest that this defect is extremely important in the failure of reperfused ischemic tissue. Non-invasive measurements of NADH and cyt aa_3 could provide a valuable indication of organ viability and function if measured on an *ex vivo* circuit prior to transplantation.

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