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# Is allopurinol beneficial in the prevention of renal ischaemia–reperfusion injury in the rat?: evaluation by near-infrared spectroscopy

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(Received 1 July/14 November 1994; accepted 21 November 1994)

1. The role of allopurinol in the protection of kidney function following ischaemia–reperfusion injury has been investigated using the novel technique of near-infrared spectroscopy.

2. An *in vivo* model of rat kidney ischaemia was used, with the expected falls in blood and tissue oxygenation seen and confirmed by near-infrared spectroscopy.

3. Allopurinol infusion increased the rate of reperfusion of oxygenated blood seen in control rats ( $P < 0.05$ ).

4. Allopurinol enhanced the rate of tissue oxygenation during early reperfusion ( $P < 0.01$ ).

5. This study provides further evidence for the proposed benefits of allopurinol in ischaemia–reperfusion injury. Furthermore, the potential of near-infrared spectroscopy as a technique of value in interventional studies of this nature is confirmed.

$a$ ,  $a_3$  (cyt  $aa_3$ ), and is able to monitor simultaneously blood and tissue oxygenation. Optical methods have previously revealed invaluable information regarding renal tissue oxygenation both *in vivo* and *in vitro* [5–8]. We have already, using NIRS, studied the oxygenation changes and evaluated the injury that occurs in the *in vivo* model used here [9, 10]. We now apply that model to evaluate further the putative beneficial effects of allopurinol as it is believed to protect the kidney against ischaemia–reperfusion in several ways, including inhibition of xanthine oxidase, which both reduces the release of reactive oxygen species during reperfusion and prevents the degradation of adenine nucleotides [11]. Allopurinol has also been shown to scavenge reactive oxygen species and enhance the electron transport chain [12, 13]. The aims of this study were to determine whether or not allopurinol alters tissue reperfusion at an early stage and to confirm the ability of NIRS to detect changes in tissue oxygenation.

## INTRODUCTION

Ischaemia–reperfusion injury is a major cause of acute renal failure [1] and of probable importance in transplantation. The kidney is highly susceptible to ischaemic injury, particularly the renal interstitium, where tissue oxygenation is often critical [2–5]. Ischaemia results in depletion of high-energy adenine nucleotides, mitochondrial damage and an inability to maintain cell membrane ion gradients. During reperfusion with oxygenated blood, further injuries occur in response to the release of reactive oxygen species.

This study uses the technique of near-infrared spectroscopy (NIRS) to investigate further blood and, more importantly, tissue oxygenation in an *in vivo* model of ischaemia–reperfusion. This methodology uses an optical technique dependent on the absorption properties of two biological chromophores, haemoglobin and mitochondrial cytochrome

## METHODS

### NIRS [14]

The NIR range is the most useful spectroscopic range with which to monitor light absorbance in biological tissue as blood has high absorbance at wavelengths less than 700 nm and greater light-scattering effects beyond 1000 nm [15]. Blood in its deoxygenated form (Hb) demonstrates a peak absorbance at 775 nm. The terminal member of the respiratory chain, cyt  $aa_3$ , shows a broad absorption peak [16–19] at 845 nm when fully oxidized in purified preparations [18, 19]. A third wavelength (904 nm) is also used for monitoring [20]. The optical absorbance changes of the chromophores, Hb, oxygenated haemoglobin (HbO<sub>2</sub>) and cyt  $aa_3$  provide the basis for the NIRS technique.

The relationship between optical absorbance and

**Key words:** ischaemia, kidney, near-infrared spectroscopy.

**Abbreviation:** NIRS, near-infrared spectroscopy.

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chromophore concentration can be expressed by a modified Beer–Lambert Law as

$$A = eCnL + B$$

where  $A$  = optical absorbance,  $C$  = concentration of either Hb, HbO<sub>2</sub>, Hb<sub>tot</sub> or cyt  $aa_3$  (mmol/l),  $e$  = absorption coefficient for either parameter (1/cm × mmol),  $L$  = physical pathlength (interprobe distance/cm),  $B$  = absorbance losses and  $n$  = pathlength multiplication factor.

Scattering of light by biological tissue makes the actual pathlength many times more than the physical pathlength. For example, the optical pathlength in the neonatal brain is approximately 4.3 times the interoptode distance [21]. In the rat kidney the interoptode distance was set at 1 cm. The pathlength multiplication factor for kidney tissue is not yet known, and thus the pathlength multiplication factor was taken to be 1 to allow comparison of data.

Changes in the absorbance at 775, 845 and 904 nm can be converted to changes in the concentration of HbO<sub>2</sub>, Hb and redox changes in cyt  $aa_3$  by using matrix inversion incorporating analysis absorption coefficients of Hb, HbO<sub>2</sub> and oxidized and reduced cyt  $aa_3$  at each of the above wavelengths used for this analysis. Further details of this analysis is given elsewhere [14, 22, 23]. The changes in concentration can be expressed as

$$\Delta\text{HbO}_2 \text{ concentration} = (-1.156 \Delta A_{775}) + (0.074 \Delta A_{845}) + (1.428 \Delta A_{904})$$

$$\Delta\text{Hb concentration} = (1.641 \Delta A_{775}) + (-0.935 \Delta A_{845}) + (-0.178 \Delta A_{904})$$

$$\Delta\text{Redox changes of cyt } aa_3 = (-0.014 \Delta A_{775}) + (0.582 \Delta A_{845}) + (-0.491 \Delta A_{904})$$

where  $\Delta$  refers to changes. The total haemoglobin (Hb<sub>tot</sub>) can be expressed as the sum of Hb and HbO<sub>2</sub>.

The NIRS technique of monitoring cerebral total haemoglobin, cerebral blood volume and cerebral blood flow has been validated against other techniques [14]. Validation of NIR multiplier coefficients shown above has also been done previously on *in vitro* preparations [24].

### Protocol

Two NIRS instruments (BEMP, Keele University, U.K.) with laser firing synchronized by software (Desqview, Quarterdeck Office Systems, U.S.A.) were used to monitor both left and right kidneys simultaneously. Each kidney was transilluminated with near-infrared light via an optical fibre placed around it. The light emerging after absorption was collected by a similar optical fibre bundle, detected by an avalanche photodiode and amplified. The overall absorbance at each of three wavelengths, 775, 845 and 904 nm, was detected and background light interference subtracted. Data from the instru-

ments were recorded every 4 s after initial calibration against a glass filter of known absorbance ( $A$ ). The calculated optical densities were stored and used in the algorithms.

### Preparation

Male Sprague–Dawley rats (Charles Rivers, U.K.) of 400–500 g were anaesthetized using continuous left femoral arterial infusions of Saffan (Alphaxalone/Alphadolone, Pitman-Moore, U.K.) and maintained at 37°C. After tracheostomy, rats breathed room air. Both kidneys were exposed through an abdominal incision and kept moist with 5% glucose/phosphate-buffered saline. Small glass fibre-optic probes were placed on either side of each kidney by means of a specially designed probe holder. Physical pathlength was maintained at 1 cm in all kidneys to enable comparison of data. Blood pressure was continuously monitored via a right femoral arterial cannula.

Rats ( $n=12$ ) were randomly allocated to 80 min left renal artery occlusion in the presence of either allopurinol (Wellcome Foundation, U.K.), or vehicle as controls. Eighty minutes of ischaemia has been shown previously to cause irreversible damage and was felt to be the time period where maximal benefit, if present, could be elicited. Changes in blood and tissue oxygenation were monitored for 30 min during which time allopurinol (50 mg/kg) was infused through the right femoral artery to be mixed with the aortic circulation. The left renal artery was occluded for 80 min and an additional bolus of allopurinol (50 mg/kg) administered 5 min before release, in order to ensure optimal response [25]. Control animals received pH-adjusted saline as both an infusion and a bolus. Both kidneys were monitored for a further 4.5 h of reperfusion and then excised for histological assessment of damage.

Results from experiments were calculated by taking two readings before occlusion, while during occlusion concentration changes were taken from the data every 5 min. During reperfusion, changes were determined every 15 min.

### Histological assessment

Kidney sections were prepared for light microscopy and assessed for ischaemic damage. A score using a scale of 0–5, where 5 corresponds to severe injury, was applied to changes in (1) glomeruli, (2) proximal tubules, (3) distal tubules and (4) congestion in vessels. Using this semiquantitative method, morphological changes were statistically compared [9].

### Statistical analysis

Data are reported as means  $\pm$  SEM, unless otherwise indicated, and  $n$  refers to the number of animals in each group. Differences were considered significant at the 5% level.

Data were analysed using repeated measures

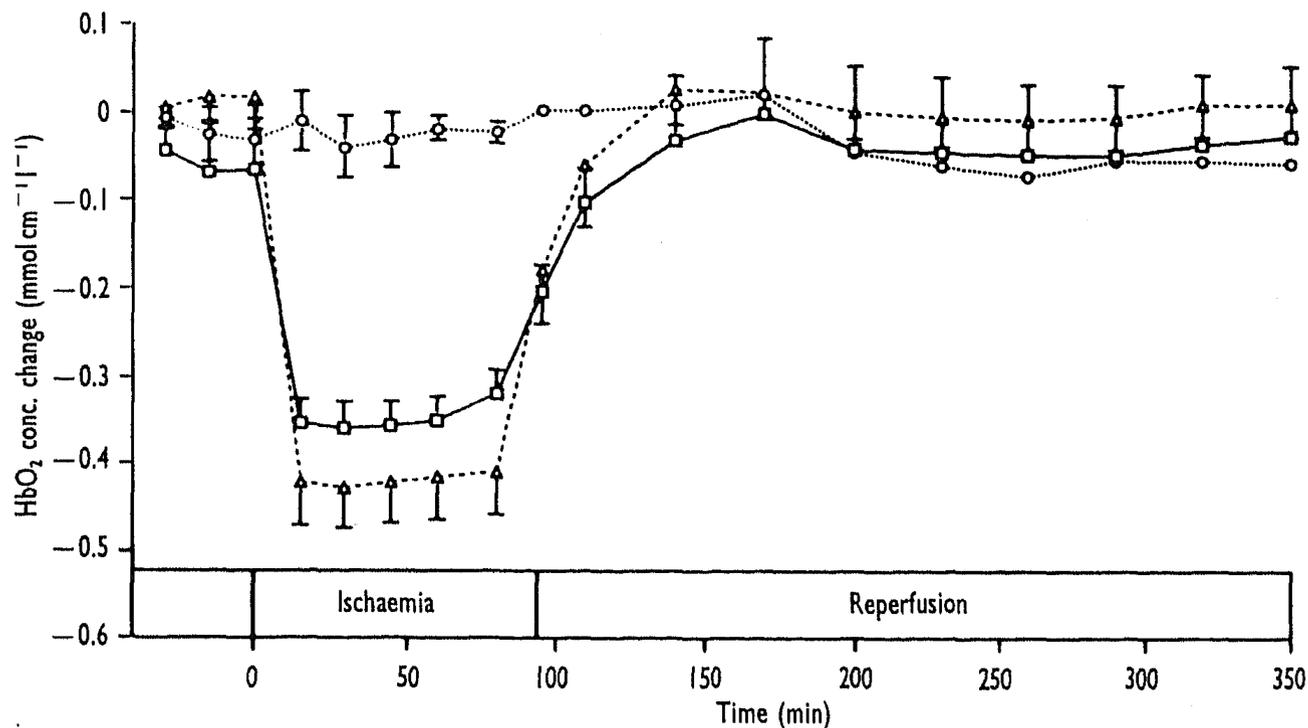


Fig. 1. Changes in HbO<sub>2</sub> concentration in response to 80 min left renal ischaemia in allopurinol-treated and control rats (means  $\pm$  SEM,  $n=6$ ). —□—, left control kidney; ···○···, right control kidney; --△--, left allopurinol-treated kidney.

analysis of variance, enabling the significance of differences between the control and allopurinol-treated group and differences between left and right kidneys with time to be tested together.

The scores obtained from the histological assessment were tested for significant differences between left and right kidneys using a Wilcoxon test.

The histology between the allopurinol-treated and control groups was tested using the Kruskal-Wallis test of multiple comparisons (NCSS, J. L. Hintze, UT, U.S.A.).

## RESULTS

### Ischaemia

Left renal ischaemia induced by renal artery occlusion resulted in a rapid decrease in both blood and tissue oxygenation.

The concentration change of HbO<sub>2</sub> fell from  $-0.01 \pm 0.06$  to  $-0.36 \pm 0.03$  mmol cm<sup>-1</sup> l<sup>-1</sup> in the left control kidneys (Fig. 1). The concentration change in the left kidney of the allopurinol-treated group fell from  $0.02 \pm 0.04$  to  $-0.04 \pm 0.05$  mmol cm<sup>-1</sup> l<sup>-1</sup>. Ischaemia significantly reduced the HbO<sub>2</sub> concentration change in the left kidney compared with the right in both the control and the allopurinol-treated groups ( $P < 0.05$ ). There were no significant differences in the HbO<sub>2</sub> concentration change in response to ischaemia between the two groups.

Similarly, Hb<sub>tot</sub> demonstrated a significant decrease in the concentration change in response to ischaemia in both control and allopurinol-treated groups ( $P < 0.05$ ). The concentration change in controls dropped from  $-0.06 \pm 0.03$  to  $-0.50 \pm 0.06$  mmol cm<sup>-1</sup> l<sup>-1</sup>, whereas in the allopurinol group the concentration change dropped from  $-0.01 \pm 0.04$  to  $-0.53 \pm 0.02$  mmol cm<sup>-1</sup> l<sup>-1</sup>. Although the degree of concentration change in Hb<sub>tot</sub>

in the two groups was not significantly different in response to ischaemia, the rate of concentration change during the ischaemic period was stable in the allopurinol group compared with the controls, which demonstrated a slow increase in Hb<sub>tot</sub> concentration change ( $P < 0.05$ ).

The concentration change in Hb fell slightly in response to ischaemia in both groups by up to  $0.14$  mmol cm<sup>-1</sup> l<sup>-1</sup>. This change was not significantly different from that in the right kidney as right kidney Hb concentration change also fell in the allopurinol group. Hb concentration change slowly increased ( $P < 0.01$ ) in the left kidney during ischaemia in the control group compared with the stable allopurinol group.

While there were obvious changes in the concentration of blood oxygenation due to ischaemia, a significant reduction in the concentration of oxidized cyt *aa*<sub>3</sub> was seen in the left kidneys of both groups compared with the right ( $P < 0.05$ ): from  $-0.009 \pm 0.004$  to  $-0.033 \pm 0.014$  mmol cm<sup>-1</sup> l<sup>-1</sup> in controls and from  $-0.006 \pm 0.002$  to  $-0.027 \pm 0.011$  mmol cm<sup>-1</sup> l<sup>-1</sup> in the allopurinol group (Fig. 2). Since redox changes of cyt *aa*<sub>3</sub> are monitored, an increase is interpreted as oxidation, while a decrease is interpreted as a reduction.

### Reperfusion

Reperfusion of the left kidney of both groups with oxygenated blood occurred rapidly on release of the left renal artery. The most pronounced changes were seen during the first 30 min of reperfusion. Although the concentration change of HbO<sub>2</sub> and Hb<sub>tot</sub> increased in control rats during this period, the rate of concentration change of HbO<sub>2</sub> ( $P < 0.05$ ) and Hb<sub>tot</sub> was significantly enhanced by allopurinol (Fig. 1). The concentration changes of HbO<sub>2</sub> and Hb<sub>tot</sub> reached preischaemic values after 4.5 h of reper-

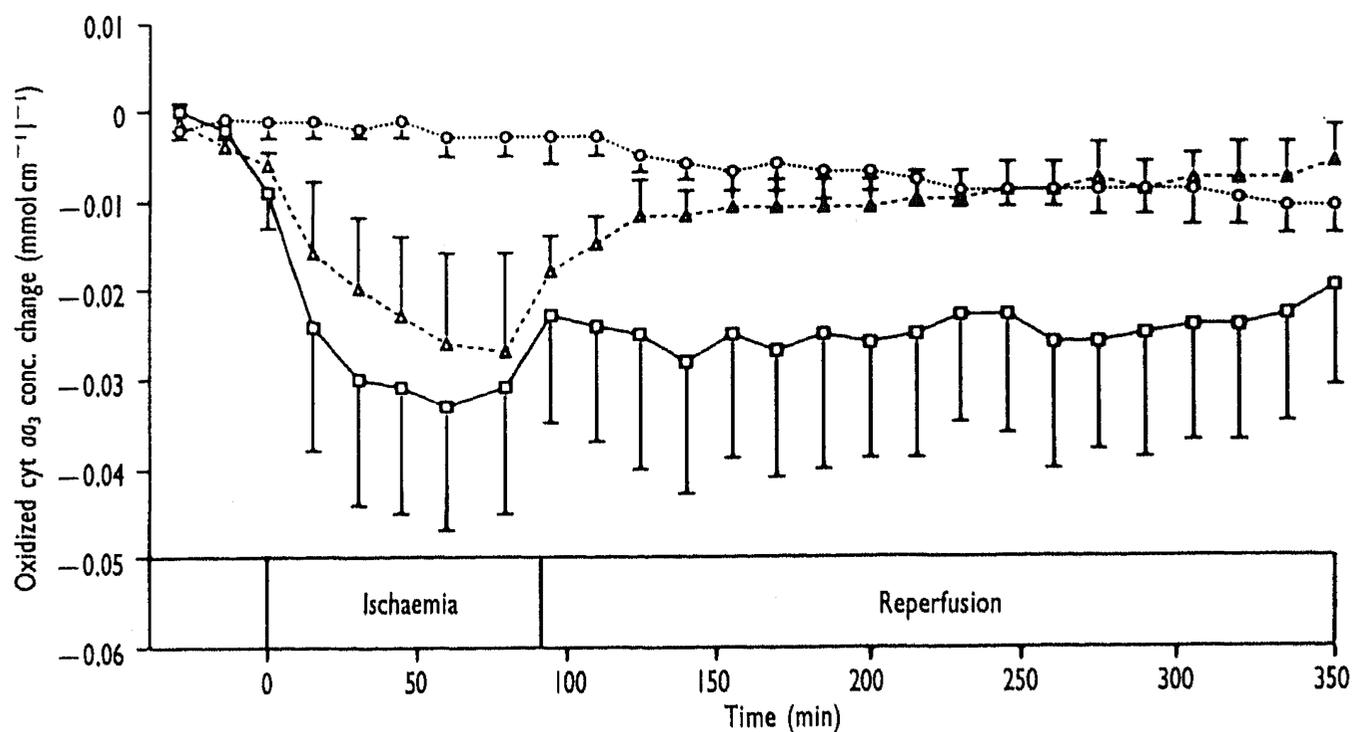


Fig. 2. Changes in oxidized cyt  $aa_3$  concentration in response to 80 min left renal ischaemia in allopurinol-treated and control rats (means  $\pm$  SEM,  $n=6$ ). Key as in Fig. 1.

fusion in both groups. There were no significant differences in the concentration change of  $HbO_2$  between left and right kidneys between 1 and 4.5 h of reperfusion.

The concentration change of Hb rose considerably compared with the right kidney ( $P < 0.01$ ) during the first 15 min of reperfusion in the control group, increasing from  $-0.1 \pm 0.05$  to  $0.15 \pm 0.04 \text{ mmol cm}^{-1} \text{ l}^{-1}$  and falling back to stable values after 45 min, which were not significantly different from the right kidney. Hb concentrations in the allopurinol group rose only slightly during the first 15 min of reperfusion to values not significantly different from those in the right kidney. The concentration changes of Hb during the last 3.5 h of reperfusion of the allopurinol group differed from those in the stable control group, in that the concentration change slowly increased in the left kidney compared with a slow decline in the right. Subsequently, the rate of change in the left kidney compared with the right of the allopurinol group was significantly different from that in the controls ( $P < 0.05$ ).

Cyt  $aa_3$ , slowly oxidized in the left kidney during the first 30 min of reperfusion ( $P < 0.01$ ) in both groups (Fig. 2). Allopurinol significantly increased the rate of oxidation of cyt  $aa_3$  in the left kidney during early reperfusion ( $P < 0.05$ ). During the entire 4.5 h of reperfusion the concentration change of oxidized cyt  $aa_3$  increased in the allopurinol group compared with the stable control group, which demonstrated large variations in concentration change. Because of these large variations, however, this was not found to be significant.

### Histology

Ischaemic damage was confirmed both in the left kidneys ( $16 \pm 4$ ) of the control group compared with

the right ( $7 \pm 4$ ) and in the left kidneys ( $15 \pm 3$ ) of the allopurinol group compared with the right ( $7 \pm 3$ ) ( $P < 0.01$ ) (mean scores  $\pm$  SD). Histologically, allopurinol showed little effect upon the morphological changes associated with ischaemic damage as assessed by the scoring method.

### DISCUSSION

This study demonstrates that allopurinol administered before both ischaemia and reperfusion improves the rate of renal perfusion with oxygenated blood and improves tissue oxygenation as assessed by the redox state of cyt  $aa_3$  in the reperfusion phase. As anticipated, no histological benefit was seen with allopurinol within this very early phase of reperfusion.

In ischaemic kidneys, red blood cells become trapped within the microvasculature of the outer medulla [26–32]. Reperfusion is clearly dependent upon the number of red blood cells trapped and their speed of clearance. The pretreatment of rats with allopurinol before ischaemia has been shown to elevate renal blood flow, lower renal vascular resistance and prevent erythrocyte accumulation in the medulla [33]. This finding would be supported by the demonstrated increase in the rate of reperfusion seen in this work.

The effect of allopurinol in tissue oxygenation is clear compared with controls. The rate of oxidation of cyt  $aa_3$  is significantly increased during early reperfusion. In the control group there was also considerably more variation in the redox state of cyt  $aa_3$  during the later stages of reperfusion. The increase in tissue oxygenation in the presence of allopurinol may be a result of increased reperfusion with oxygenated blood to areas which may otherwise remain hypoxic in the control group. NIRS in

this situation monitors whole-organ blood oxygenation so that the contribution of small areas of hypoxia may or may not be important compared with global kidney oxygenation.

The findings in tissue oxygenation seen in this study differ from our earlier studies designed to compare the severity of injury associated with 45 and 80 min of ischaemia followed by reperfusion [9] in two ways. Firstly, improvements to the monitoring system had been made after the said earlier study and most importantly, the controls in this study were not the same controls as described in the earlier study [9]. Secondly, the rats in the 45/80 min ischaemic groups did not receive an infusion of pH-adjusted saline for 30 min before ischaemia or a bolus before reperfusion, therefore the discrepancy between the two sets of data may reflect the effect of haemodilution.

Allopurinol is also known to have further complementary cellular effects in addition to its haemodynamic effects. Firstly it is an inhibitor of xanthine oxidase, thus enabling ATP to be regenerated from hypoxanthine and inhibiting the formation of reactive oxygen species, a product of xanthine oxidase activity [11]. Secondly, allopurinol has been shown to be a scavenger of hydroxyl radicals [12]. Finally, allopurinol has been shown to facilitate the transfer of electrons from the ferrous ion to ferric cytochrome *c*, which enhances the electron transport system during reperfusion [13]. If allopurinol is administered just before reperfusion, inhibition of xanthine oxidase enables hypoxanthine to be used to generate ATP, instead of being lost from the cell as uric acid [11]. Cell viability is related to its energy metabolism and importantly the ability to resynthesize ATP during reperfusion [34]. The improved rate of oxidation of cyt *aa*<sub>3</sub> in this study may reflect an improved cellular and mitochondrial viability in the allopurinol group, as allopurinol has also been shown to protect the structure and functional integrity of mitochondria [35]. As allopurinol can facilitate the transfer of electrons from the ferrous iron to ferric cytochrome *c* in the respiratory chain, electron transport may be enhanced during reperfusion irrespective of mitochondrial lipid membrane damage [12]. Allopurinol may therefore independently enhance the respiratory chain, increasing the rate of cyt *aa*<sub>3</sub> oxidation.

While the beneficial effects of allopurinol upon ischaemia-reperfusion injury were demonstrated using NIRS, no histological benefit was seen. This may suggest that monitoring subtle changes in blood and tissue oxygenation is a more sensitive method of revealing early beneficial properties of interventional therapies. The greatest morphological changes which occur as a result of these subtle changes in tissue oxygenation may be most evident not 4 h after reperfusion, as in this study, but days after the initial ischaemic injury. In addition, although the semiquantitative method of assessing the morphological damage in kidneys may allow statis-

tical comparison of the changes occurring, it is not fully quantitative, no measurements are actually performed and, with a limited scoring system, its sensitivity is comparatively low.

Thus, this study supports previous observations of the beneficial effects of allopurinol in this type of renal injury and demonstrates its effects in blood and tissue oxygenation. Whether allopurinol should be advocated in the treatment regimen for patients at risk from ischaemia-reperfusion injury is a question for further consideration. While the dose of allopurinol used in this study could be considered high in the context of treating patients with gout (maximum oral maintenance dose 900 mg/day; British National Formulary), it was administered directly into the circulation as a single preventative dose regimen to reduce the damage associated with ischaemia-reperfusion injury. This dose is most commonly quoted in experimental studies of ischaemia-reperfusion injury, i.e. 50 mg/kg as both an infusion and bolus in liver [22], 40 mg/kg in studies of renal failure [36] and 100 mg/kg as a 4-h infusion in aminonucleoside nephrosis [37]. Any adverse reactions accompanying a single dose administered over a short period may be different from those associated with a lower maintenance dose. Severe adverse reactions to a drug may be tolerated more readily than life-threatening acute renal failure resulting from the injuries associated with ischaemia-reperfusion injury. The purpose of this study was not, however, to propose immediately a treatment regimen for patients at risk from ischaemia-reperfusion injury, but to determine whether NIRS could be used to reveal the beneficial effects of a drug in reducing injuries associated with ischaemia-reperfusion. As this has never been demonstrated before, a drug regimen was selected to ensure the maximum protection against ischaemia-reperfusion injury to ensure maximum response in terms of the experimental set-up.

In conclusion, NIRS is a valuable approach to the investigation of haemodynamic and cellular effects of interventional strategies designed to minimize ischaemia-reperfusion injury.

## ACKNOWLEDGMENTS

We are financially indebted to West Midlands Regional Health Authority and North Staffordshire Medical Institute, U.K., for financial support. We thank the Wellcome Foundation, U.K., for the generous gift of allopurinol. We are also grateful to Mr John Belcher, Statistics Department, Keele University, U.K., for statistical advice.

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