A comparative study of the protection offered by Vitamin E and Captopril, when used as additives in kidney storage solutions

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Summary

The demand for organ transplantation has stimulated a search for optimal methods of cadaveric kidney preservation techniques. The basis of development of these methods depends on an understanding of cellular metabolic function during the period of storage and the changes confronting the organ during reperfusion. Free radicals are implicated in both these phenomena. Consequently agents that are capable of combating free radical attack (free radical scavengers/antioxidants) are now being investigated more extensively, with the hope of providing better post-transplant organ function.

This study has attempted to answer the question whether better organ preservation and post transplant viability could be achieved with kidney storage solutions containing the antioxidants vitamin E or angiotensin converting enzyme (ACE) inhibitors. Post-storage viability of kidney tissue was assessed by assessing renal functions in isolated perfused animal kidneys. The isolated perfused kidney (IPK) model was used to test renal function such as glomerular filtration rate, sodium and glucose reabsorption and protein excretion after the organs were stored in solutions containing vitamin E or captopril. These results were also correlated with the ultrastructural appearances of the kidney tissue.

The findings of this study suggest that inclusion of vitamin E into the storage solutions offered protection to the renal tissue. This was shown by improved renal function of the kidneys stored in solutions containing vitamin E. Electron microscopic studies further strengthened these findings. However the study using ACE inhibitor captopril failed to show similar results.

Key words: Free radical scavengers, vitamin E, captopril.

Introduction

Oxygen free radicals (OFR) have been implicated in tissue damage that occurs during cold storage and re-perfusion of donor organs (1,2). Recently developed methods of preserving organ viability during cold storage include incorporation of OFR scavengers into the storage solutions (3,4). For example, glutathione and glycine have been shown to protect liver tissue during cold ischaemia, and improve post-storage viability by OFR scavenging (4). In a previous study (5), we have also demonstrated that significant protection against changes in renal tissue metabolism (as assessed by the gluconeogenic potential) and ultrastructure, that occur during cold storage of rat kidneys in Marshall's citrate (MC) can be achieved by including vitamin E in the storage solution.

Angiotensin converting enzyme (ACE) inhibitors such as captopril that have thiol side groups have been reported to possess free radical scavenging properties (6) independent of their ACE inhibiting activity. The present investigation was carried out to compare the abilities of vitamin E and captopril when they are used as additives in kidney storage solutions, to protect rabbit kidneys against changes in renal function that occur during storage prior to transplant.

Material and Methods

Preparation of kidney storage solutions

(a) Marshall's Citrate solution (MC)

Marshall's Citrate solution was prepared according to the method described by Ross *et al* (7). The final composition contained the following in

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mmol/L: trisodium citrate 27.5, tripotassium citrate, 27.3, magnesium sulphate, 40.0 and mannitol 100.0.

(b) MC + vitamin E 1

Vitamin E was added to MC at a concentration of 50% of that of its LD 50, the dose that would result in the death of 50% of the animals under study.

(c) MC + Captopril

This solution contained MC to which captopril had been added at a dose of 10% of that of its LD 50. Since captopril is expensive, only 10% of its LD 50 was used in preparation of this solution.

Isolation of kidneys

Adult cross-bred albino rabbits (1.5 - 2.0 kg body weight) were used in the study.

After induction of anesthesia by using an ether mask and injection of pentobarbitone sodium (0.5 mL/kg), the left kidney of each rabbit was exposed following a mid-line incision. The ureter was cleared and severed after leaving a length of about 5 cm from the kidney. The left renal artery and vein were ligated and divided and the kidney excised. The kidney weight ranged from 4-6 g. The kidney was immediately immersed in ice-cold MC, MC + E1 or MC + captopril solution. The renal artery was cannulated with a 23G butterfly needle and the vasculature was perfused with 50 ml of sterile solution of the respective group at 4° C, according to the solution tested. Fresh kidneys were used as controls. These were flushed with only MC. The right kidney was also removed in a similar manner and the kidney weight estimated. After flushing, the kidneys were stored for 24 hours at 0° C in ice, in the three different storage solutions that were being tested, and reperfused on the isolated perfusion kidney (IPK) circuit.

Reperfusion of kidneys in IPK model

The IPK model used in the present investigation was set up according to the technique described by Fuller and Pegg (9). The perfusate medium used was a modification of that used by Toffa *et al* (10). The modifications concern a higher concentration of creatinine and the replacement of hydroxyethyl starch by mannitol. The perfusate used in this study contained the following in mmol/mL: Na^+ 155, K^+ 6.8, Ca^{2+} 2.0, Cl^- 139, HCO_3^- 25, H_2PO_4 1.5, glucose 5, creatinine. 0.2, and mannitol 100.0. Bovine serum albumin was included as a tracer for protein excretion assessment, at a concentration of 1g/L. The osmolality of the perfusate was 400 mosm/kg. Prior to use in the IPK machine, the above solution was filtered through a series of filters and transferred to sterile containers. Control kidneys (freshly isolated) were attached to the isolated perfusion circuit immediately after they were harvested. The other experimental kidneys were attached after they were flushed and cold stored for 24 hours in the respective storage solutions at 0° C in ice. The kidneys were subject to normothermic, bloodless perfusion on this circuit for 30 – 45 minutes after connecting to the IPK apparatus. Freshly prepared and filtered perfusate was then passed through. The perfusate was aspirated using a Watson-Marlow peristaltic pump (101 U: output 8 mL/min), from the reservoir and sent through a bubble trap to the renal arterial cannula. The arterial pressure was measured with a pressure transducer (Datascope 2002 A) situated on a side-arm close to the cannula.

The pressure was maintained between 100 and 120 mm Hg by the adjustment of the flow rate of the perfusate delivered to the kidney. The perfusate medium in the reservoir was constantly, vigorously gassed with a mixture of 95% O_2: 5% CO_2. Samples of perfusate were collected at 15 minute intervals. Samples of urine were collected at 15 minute intervals. Samples of urine were collected at the end of every 15 minutes from the graduated tube that contains the ureter (i.e. samples were collected at 15 and 30 minute intervals). The first collection of urine was done after allowing the machine to run for about 10 minutes, as the urine flow may be irregular at the beginning of the cycle.

Assessment of renal function

During reperfusion, urine was collected and renal function assessed as described by Ross (7). The variables estimated for assessment of renal function were: (i) creatinine clearance (Ccr), (ii) tubular glucose reabsorption, (iii) tubular Na^+ reabsorption, (iv) urinary protein excretion. At the end of renal function assessment, the kidneys stored in the different storage solutions and fresh kidneys were examined by electron microscopy to detect any ultrastructural changes.

Creatinine clearance was measured by adding creatinine to the perfusate at a concentration of 0.2 mmol/L. The creatinine concentration in samples of urine and perfusate collected at 15 minute intervals was estimated by the method described Toffa (10). Commercially available reagent kits from Randox Laboratories Ltd, UK was used for these estimations. All estimations were done in duplicate and average values were used in all calculations.

Ccr was expressed in mL/min per gram weight.

$$Ccr = \frac{\text{Urine creatinine (mmol/L) X Urine flow (mL/min)}}{\text{Perfusate creatinine (mmol/L)}} \text{ mL/min}$$

Tubular glocose reabsorption was measured by adding glucose to the perfusate at a concentration of 5.0 mmol/L. The glucose levels in the perfusate and urine at 15 minute intervals was measured and the percentage tubular glucose reabsorption was calculated as follows:

$$\% \text{ reabsorption} = \frac{\text{Filtered load - Excreted load}}{\text{Filtered load}} \text{ X } 100$$

where the,

Filtered load = Perfusate sodium X creatinine clearence

Excreted load = Urine sodium X Urine flow

Tubular sodium reabsorption was measured by the flame photometry method (10) Sodium levels were measured in each experiment in the perfusate and urine samples collected at 15 minute intervals. Sodium was added to the perfusate at a concentration of 155 mmol/L. The percentage tubular sodium reabsorption was calculated using the same principle as for the calculation of glucose reabsorption. Urinary protein concentration was measured using the method used by Lowry *et al* (11). This was expressed as a percentage of the perfusate protein concentration. This estimation was determined by the biuret method using Randox protein kits. The glucose reabsorption, sodium reabsorption and the proteinnuria estimated in the above experiments gives an indication of the tubular function of the kidneys.

Statistical analysis

Mean and standard deviations (SD) were calculated for each group of solutions tested for the measurement of creatinine clearance (Ccr), glucose reabsorption, sodium reabsorption and protein excretion.

Analysis of variance (ANOVA) was used when simultaneous comparisons were made with measurements from more than two samples (12). The means between more than two samples were compared by ANOVA.

Students "t" test was used in the comparison of means when there were only two samples. A probability (p) value of <0.05 was taken to be statistically significant.

In this study where kidneys stored in different solutions (MC, MC+EI, MC + captopril) and the control group (fresh kidney) were analysed, the mean values of the Ccr, sodium reabsorption, glucose reabsorption, and protein excretion between each group was compared by the ANOVA. If the ANOVA showed that there was a significant difference of means between the groups ($p<0.05$), then the Tukey's Honestly Significant Difference (HSD) test was done (12). The calculated value for HSD signifies that in order to be statistically significant at a probability of 0.05, the difference be-

tween any pair of means of the particular test must be at least equal to the HSD or more than it. This method also helps to determine which group has the best mean which does not differ significantly from the control mean.

Results

Creatinine clearance

The results are shown in Table 1.

Table 1

Creatinine clearance (Ccr) of rabbit kidneys at 15 minutes of perfusion (mL/min/g wet)

Group	mean ± SD
Fresh kidneys (control)	0.21 ± 0.02
MC only	0.12 ± 0.01
MC + E1	0.14 ± 0.01
MC + captopril	0.11 ± 0.01

ANOVA (F value) 4.833; $P < 0.05$

HSD	0.081
Control and MC	$P < 0.05$
Control and MC + E1	$P > 0.05$
Control and MC + captopril	$P < 0.05$

(MC – Marshalls citrate, E1 – vitamin E at a dose of 50% of LD 50)

SD = Standard deviation
n = 5 for each group
HSD = Honestly significant difference

The HSD test showed that there was no statistically significant difference between the mean Ccr of the control (fresh) group and the mean Ccr of the kidneys stored in MC+E1 ($p>0.05$). The results of Ccr of kidneys stored in MC+E1 was next best to the results of the fresh (control) sample. However the HSD showed that there was statistically significant difference between the mean Ccr of fresh kidneys and kidneys stored in MC ($p<0.05$).

The HSD test also show that the difference of significance had occured at the level of MC and MC+ captopril and not with MC+E1. Results of the present investigation show that of the stored kidneys the mean Ccr was highest in the kidneys stored in MC+E1 at 15 minutes of perfusion (0.14 + 0.01).

Tubular glucose reabsorption

The results are shown in Table 2.

Table 2

Percentage of glucose reabsorption of rabbit kidneys at 15 minutes of perfusion

Group	mean ± SD
Fresh kidneys (control)	63.1 ± 7.8
MC only	42.7 ± 5.3
MC + E1	52.6 ± 5.8
MC + captopril	36.8 ± 5.2

ANOVA (F value) 2.053; $P > 0.05$

n = 5 for each group
Abbreviations same as in Table 1.

A decline in glucose reabsorption at 15 minutes of perfusion was observed in the kidneys during storage as compared to the kidneys of the control group.

Of the stored kidneys glucose reabsorption was highest in the kidneys stored in MC+E1 (52.6 + 5.8). However a comparison of the mean glucose reabsorption values of the groups showed that there was no statistically significant difference between the means ($p>0.05$).

Tubular sodium reabsorption

The results are shown in Table 3.

Table 3

Perecentage of sodium reabsorption of rabbit kidneys at 15 minutes of perfusion

Group	mean ± SD
Fresh kidneys (control)	56.4 ± 9.4
MC only	29.1 ± 3.6
MC + E1	47.5 ± 6.7
MC + captopril	22.9 ± 2.5

ANOVA (F value) 4.033; $P < 0.05$

HSD	31.492
Control and MC	$P > 0.05$
Control and MC + E1	$P > 0.05$
Control and MC + captopril	$P < 0.05$

n = 5 in each group

Abbreviations same as in Table 1.

When the percentage mean sodium reabsorption values between the four groups (fresh, MC, MC+E1, and MC+captopril) were compared with each other (by ANOVA) there was a statistically significant difference between them ($F=4.003$, $p < 0.05$). Since there was a significant difference of the means between the groups as shown above, the Tukey's HSD test was done between the percentage mean sodium reabsorption of the four groups, to assess the best percentage mean sodium reabsorption close to the control group percentage mean. The HSD test showed that there was no statistically significant difference between the percentage mean sodium reabsorption of the control (fresh) group and the percentage mean sodium reabsorption of the kidneys stored in MC+E1 ($p>0.05$). This is to say the sodium reabsorption of kidneys stored in MC+E1 was next best to the results of the control (fresh) sample. However the HSD showed that there was a statistically significant difference between the percentage mean sodium reabsorption of fresh kidneys and kidneys stored in MC+captopril ($p< 0.05$). Results also showed that the difference between the percentage mean sodium reabsorption of kidneys stored in MC, was not significantly different ($p>0.05$) to those of fresh kidneys. These results show that the percentage mean sodium reabsorption values of kidneys stored in MC+ captopril were significantly less than that of fresh ones. The HSD test also shows that the difference of significance had occurred at the level of MC+ captopril and not with MC+E1 or MC.

Effects on urinary protein excretion

Urinary proteins were measured using commercially available protein estimation kits (supplied by Randox laboratories Ltd.) using the biuret method (11) and expressed as a percentage of the perfusate protein concentration as done by Toffa *et al* (10). When the glomerular protein leakage was assayed by the above method it was found that protein leakage was high during the early period of perfusion (15 minutes). Kidneys stored for 24 hours in all the groups showed increased mean proteinuria when compared to the fresh (control) kidneys at 15 minutes of perfusion (Table 4). At 15 minutes of perfusion, the stored kidneys also showed a mean proteinuria which was higher than the perfusate concentration of protein, indicating a net loss of protein. Results obtained indicate that of the stored kidneys, the least amount of protein was excreted by kidneys stored in MC+E1 (Table 4). At 15 minutes of perfusion when the mean proteinuria values between the four groups (fresh, MC, MC+E1, and MC+captopril), were compared there was no statistically significant difference between them ($p>0.05$).

Table 4

Proteinuria of rabbit kidneys at 15 minutes of perfusion (expressed as percentage of perfusate protein)

Group	mean ± SD
Fresh kidneys (control)	91.2 ± 3.9
MC only	105 ± 8.7
MC + E1	102.8 ± 7.3
MC +captopril	108.2 ± 7.2

ANOVA (F value) 1.346; $P > 0.05$

n = 5 for each group

Abbreviations same as in Table 1.

Effects on kidney ultrastructure

Light microscopy did not show any morphological changes in the kidney slices in any of the four groups that were examined (fresh, MC+E1, MC, and MC+captopril). There were no changes either in the fresh (control) sample or on the samples of kidneys stored for 24 hours.

The glomeruli appeared normal and the lobular architecture was well maintained. Proximal tubular cells were normal. The nuclei in these cells were clearly seen and the cytoplasm was eosinophilic. Interstitial oedema was not seen. No abnormalities could be detected on light microscopy in the proximal convoluted tubules.

However electron microscopy (EM) showed changes especially in the mitochondria of the proximal convoluted tubules which are active sites of glucose production. In fresh unstored kidney slices the mitochondria were normal and cylindrical shaped and sequentially arranged close to one another. The membranes of the mitochondria were clearly demarcated and the cristae were seen clearly.

The electron microscopic appearance of the kidneys stored in MC+E1 for 24 hours showed very little change when compared to the control group. The mitochondria were cylindrical and arranged close to one another as in the fresh kidney. Most of the mitochondria were of normal size and shape. Mitochondrial membranes and the cristae were clearly seen although some mitochondria showed cellular swelling. In contrast, kidneys stored in MC and MC+captoril for 24 hours showed marked changes in the mitochondria of the proximal tubules when compared to fresh (control) ones. In the kidneys stored in MC the size and shape of the mitochondria were not uniform. The cylindrical shape was lost and the cells showed swelling. The cristae were not clear and different stages of damage to the mitochondria could be seen. The process of cellular disintegration had begun. The kidneys stored in MC+captopril showed the most extensive ultrastructural changes. Here the mitochondria had completely lost their normal architectural pattern; they were hardly identifiable as mitochondria. Gross mitochondrial swelling and disintegration which leads to cellular destruction were seen. Cristae pattern was not seen at all. Thus the EM appearances of the kidney tissue stored in MC+E1 showed minimal changes when compared to the changes observed in those stored in MC and MC+captopril. The ultrastructure of the kidney was well preserved in kidneys stored in solutions containing vitamin E1.

Discussion

Results of the present investigation demonstrates that in stored kidneys, the mean values obtained with respect to creatinine clearance, sodium reabsorption, glucose reabsorption, were lower than the corresponding values in the fresh control kidneys, while the values for urinary protein excretion were higher than in the control kidneys. These results support the theory that there is a decrease in glomerular and tubular function in the kidney during ischaemia and reperfusion. These changes are probably due to the release of free radicals because a certain amount of protection against the above changes in renal function, could be achieved by including the free radical scavenger vitamin E into the kidney storage solution.

That vitamin E can help to protect against alterations in glomerular filtration that occurs during ischaemia and reperfusion has also been demonstrated by other researchers. An investigation with dog kidneys have shown that inclusion of vitamin E in Euro Collins flush storage solution results in a higher glomerular filtration rate (assessed by the creatinine clearance) and a reduction in lipid peroxidation, compared to kidneys stored in Euro Collins solution containing no vitamin E (13). It has also been demonstrated in rats, that a diet deficient in vitamin E can exacerbate the reperfusion injury due to the loss of oxidant scavenging activity in the kidneys (14). Results of the present investigation demonstrate that inclusion of vitamin E in storage can help to protect against alterations in glomerular filtration and tubular reabsorption that occur in kidneys during storage. Captopril, although considered to be a free radical scavenger (6), could offer little or no protection against renal function alterations in rabbit kidneys during storage and reperfusion. These results are also supported by observations in the morphological study.

There were only minimal changes in the electron microscopic appearance of kidneys stored for 24 hours in MC containing vitamin E as an additive, when compared to the control kidneys. The mitochondria of the proximal tubules (which are the active gluconeogenic sites), show close similarity in appearance and arrangement, to those in the fresh kidneys. In contrast, in kidneys stored for 24 hours in MC or MC containing captopril, there were maked changes in the shape and size as well as loss of the normal architecture of tubular mitochondria. The kidneys stored in MC+ captopril showed the most extensive ultrastructural changes. Whether the differences in the protective effects of vitamin E and captopril were due to the differences in the amounts of these added to the storage solution is not clear. However, the fact that captopril is not an effective free radical scavenger is also demonstrated by results of other investigators (15,16). Results of the investigation by Westlin *et al* show that ability of captopril to protect ischaemic-perfused myocardium may be due to inhibition of compliment activation and other cardioprotective effects rather than due to free radical scavenging (15). Kukrije *et al* have shown that captopril is an ineffective superoxide radical scavenger (16). They also conclude that captopril, by virtue of its thiol (-SH) group, acts as a non-specific antioxidant.

From these results it can be concluded that vitamin E is a better free radical scavenger than captopril for use as an additive to flush-storage solutions, to protect against alterations in renal function that occur during ischaemia and reperfusion.

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