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ORIGINAL PAPER

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Impact of different combined preservation modalities on warm ischemic kidneys: effect on oxidative stress, hydrostatic perfusion characteristics and tissue damage

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Abstract Hemodynamic disorders in brain dead organ donors induce hypoxia, warm ischemia and finally tissue damage. A cold preservation period also induces tissue and cellular lesions. The two major modes of preservation are cold storage (CS) and hypothermic pulsatile perfusion (HPP). We aimed to compare the influence of each mode of preservation and their combination on oxidative stress, perfusion characteristics and tissue damage, after a period of warm ischemia. Rat kidneys which had undergone ischemia (0, 30, 60 min) were preserved either by CS (12, 24 h), or by HPP (12 h), or by a combination of both (HPP + CS, CS + HPP), in University of Wisconsin cold storage solution (UWCSS) at +4°C. During HPP, renal vascular pressure decreased then increased to reach 90 mmHg after perfusion for 7 h. If HPP followed CS, the mean pressure reached 200 mmHg, showing successive high amplitude peaks. HPP had a deleterious effects on tissue structure with tubular necrosis, and induced an increase in catalase (Cat) and a decrease in manganese superoxide dismutase (Mn SOD) and gluthatione peroxidase (GPx) activity. Copper zinc superoxide dismutase (Cu/Zn SOD) activity was not reduced except with CS + HPP. During CS, we observed an increase in GPx, Cu/Zn SOD and Cat activity, a decrease in Mn SOD activity and no histological alterations in the kidney. CS induces a slight oxidative stress which is not important enough to induce major tissue damage. HPP with UWCSS induces a stronger stress, which overpowers the antioxidant defences, inducing tissue damage. The reperfusion of HPP with UWCSS emphasises the stress initiated by CS. In addition an increase in damage occurred in the CS + HPP group.

Keywords Renal preservation · Pulsatile perfusion · Antioxidant enzyme · Ischemia-reperfusion · Cold storage · Peroxidation

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Introduction

Expanded criteria for donor kidneys have been proposed for improving access to cadaveric kidneys and to reduce organ shortage. As a consequence there has been greater emphasis on non-heart-beating donor kidney sources [1]. The mechanisms of injuries sustained by the non-heart-beating donor kidney are not fully understood. There is ample evidence to suggest that part of the injury mechanisms involve reactive oxygen species (ROS), particularly the superoxide anion. ROS induces lipid, protein and nucleic acid degradation leading to cell damage and death [23]. Superoxide anions are further metabolised by the superoxide dismutase reaction to hydrogen peroxide, which must be detoxified by catalase and glutathione peroxidase.

Recent investigations have demonstrated the benefit of the hypothermic pulsatile perfusion (HPP) of ischemic kidneys from non-heart-beating donors on the post-transplant acute tubular necrosis (ATN) level and primary non-function. The use of pulsatile perfusion machines have been clearly associated with improved transplant function, shorter lengths of stay in hospital and decreased hospital costs. The improved initial function of cadaveric kidneys has led to lower rates of antibody induction, the earlier use of anticalcineurin and

significantly fewer episodes of transplant rejection. Advances in machine perfusion technology and techniques have made hypothermic pulsatile perfusion a safe and effective method of preserving kidneys for transplantation. Nevertheless, according to our policy, simple cold storage (CS) is the first method used for kidney preservation in France. Secondarily, the perfusion of previously CS kidneys may lead to improved metabolic integrity. The use of hypothermic pulsatile perfusion as an initial method for renal preservation, with secondary simple cold storage for transportation, may also be a useful method for ischemic kidney preservation. Our aim was to compare the influence of each mode of preservation as well as a combination of both modes on oxidative stress, perfusion characteristics and tissue damage to previously ischemic kidneys.

Materials and methods

Animals

Male Sprague-Dawley rats (200–220 g), obtained from Laboratoire Janvier, (Saint Doulchard, France), were housed under standard laboratory conditions with free access to food and water.

Operative procedures

The rats were anaesthetised with a single intraperitoneal dose of ketamine (200 mg/kg, Ketalar, Parke-Davis, USA). The kidneys were exposed through a midline abdominal incision. The aorta was canulated and heparinized 5 min before the induction of warm ischemia. This was induced by aorta clamping for different periods of time (0 min group A, 30 min group B, 60 min group C). Before harvesting the kidney, the abdominal cavity was cooled to 4°C with 0.9% saline buffer and flushed with University of Wisconsin cold storage solution (UW CSS) (15 ml at 4°C) through an aortic catheter.

Renal preservation procedures

Cold storage

The kidneys were stored in UW CSS at +4°C.

Hypothermic pulsatile perfusion

The kidneys were perfused with 200 ml of recirculating UW CSS at +4°C in a hypothermic pulsatile perfusion machine as previously described [11]. The flow was adapted to obtain an initial perfusion pressure of 60 mmHg and kept constant thereafter. The pressure could vary according to the renal vascular resistance. The renal perfusion pressure (mmHg) was continuously monitored and data were recorded every 5 min.

Experimental groups

The rat kidneys were randomly divided into seven groups of eighteen kidneys each, depending on the mode of preservation: G I, control group, surgical procedure without preservation; G II, preserved 12 h by CS; G III, preserved 24 h by CS; G IV, preserved 12 h by HPP; G V, preserved 12 h by HPP and 12 h by CS; G VI, preserved 12 h by CS and 12 h by HPP; G VII, preserved 12 h by CS and 12 h by HPP with 10^{-5} M Verapamil (Verapamil chlorhydrate, Bayer) initially added to the perfusate.

Each group was divided into three subgroups (six rats per group) depending on the duration of warm ischemia: group A = 0 min, group B = 30 min, group C = 60 min.

Biochemical analyses

At the end of each experiment, tissue samples were immediately frozen and stored in liquid nitrogen until they were assayed. The samples were homogenized while still frozen in a 10 mM KH_2PO_4 solution, with 1 mM EDTA, pH 7.8.

Antioxidant enzymes

The activities of catalase (Cat), glutathione peroxidase (GPx), copper zinc superoxide dismutase (Cu/Zn SOD), and manganese superoxide dismutase (Mn SOD) were determined at the end of each preservation modality: Cat activity was measured by using a spectrophotometric method as previously described by Johanson [17]; GPx activity was measured using a Ransel kit (Randox, Hardmore, Irland). The homogenates were incubated in KH_2PO_4 buffer (pH 7.8), with NADPH, GSH, glutathione reductase and t-butylhydroperoxide. The rate of disappearance of NADPH was measured at 340 nm; Cu/Zn SOD, and Mn SOD were measured using a Ransod kit (Randox). The homogenates were incubated in KH_2PO_4 buffer, xanthine, xanthine oxidase, and tetrazolium blue (INT). The INT reduction kinetics was measured at 550 nm. Total SOD activity was measured at pH 7.8, and Cu/Zn SOD at pH 10.2. Mn SOD was expressed as the difference between total SOD and Cu/Zn SOD.

Lipid peroxidation breakdown products

The extent of lipid peroxidation was determined by measuring malonaldehyde (MDA): the thiobarbituric acid reaction was used as described previously by Conti [6]. In 50 mM KH_2PO_4 buffer, 1 mM EDTA, (pH 3), MDA and 1 mM diethylthiobarbituric acid (DETBA) react to produce a fluorescent compound. After extraction with butanol, the assay is performed using synchronous fluorometry.

Pathological analyses

Two samples of each kidney were fixed in Bouin solution. The tissues were paraffin embedded and stained with hematein and eosin. The evaluation was made by an observer who had no knowledge of the sampling procedure. Acute tubular necrosis (ATN) and congestion were assessed using a semi quantitative grading scale (0: no lesion; 1: mild intensity; 2: moderate intensity; 3: severe intensity).

Statistical analysis

For the perfusion characteristics, the results were expressed as means ± SD, and compared using ANOVA. The biochemical results were expressed as means ± SD, and also compared using ANOVA. The Kruskall Wallis test was used for the histological grading scale comparisons. $P < 0.05$ was considered to be statistically significant.

Results

Perfusion pressure

HPP (G IV) and HPP-CS (G V) preserved kidneys had identical perfusion pressure characteristics so only the

HPP-CS group (G V) was used for statistical analysis and compared with the CS-HPP group (G VI).

The length of warm ischemia had no significant effect on perfusion pressure.

The perfusion pressure kinetics showed three successive phases (Fig. 1). During the first phase, the pressure decreased for 25 min for G V (HPP-CS) and 10 min for G VI (CS-HPP) to a mean level of 44 mmHg and 54.7 mmHg, respectively. The second phase was characterized by increasing pressure for a mean duration of 5 h (1–7 h). During this phase, mean pressures were significantly higher in G VI (CS-HPP) than in G V (HPP-CS). After this higher-pressure period, G VI (CS-HPP) pressure became unstable, showing successive and variable spikes. The pressure briefly increased (1–2 min) and then decreased for 5–20 min. The spike amplitudes and frequencies varied between different kidneys. During the third phase, G V (HPP-CS) pressure stabilized at 90 mmHg while G VI (CS-HPP) amplitude and spike frequency pressures increased to 200 mmHg.

In G VII, for which the perfusate was initially supplemented with verapamil, we did not observe such spikes and the perfusion pressures were identical to those obtained with HPP-CS.

G IV (HPP) and G V (HPP-CS) preserved kidneys, which had identical perfusion pressure characteristics, were compared with G VI (CS-HPP group). For each group, the perfusion pressure variation over time during preservation was statistically significant ($P < 0.001$).

The mean pressures observed during the experiment (Table 1) were significantly higher in G VI (CS-HPP) compared to G V (HPP-CS) in each warm ischemia group: A, $P = 0.01$; B, $P = 0.005$: C: $P = 0.02$.

However, the mean pressures were not significantly different between G V (HPP-CS) and G VII (CS-HPP Verapamil) (G V Vs G VII: $P = 0.4$). Nevertheless, the curve pattern reflected by the dispersion of the pressure values from the mean, (the peak value and spikes) were significantly different. In other words, the value of the pressure point by point was highly different when the different groups were compared.

The pressure variations between G V (HPP-CS) and G VI (CS-HPP) were significantly different for each warm ischemia group A: $P = 0.0002$; B: $P < 0.001$; C: $P = 0.02$). There were no differences between G V (HPP-CS) and G VII (CS-HPP Verapamil) (G V Vs G VI $P = 0.7$).

Histology

Histological investigation of G I sham renal tissues, G II (CS-12 h), and G III (CS-24 h), showed minimal acute tubular necrosis (Fig. 2). However, red blood cell trapping and congestion throughout the renal cortex and medulla were observed in the glomeruli and preglomeruli arterioles. No significant differences were found between G I (control), G II (CS-12 h), and G III (CS-24 h).

Conversely, G IV (HPP), G V (HPP-CS), and G VI (CS-HPP) preserved kidneys showed medullary and massive cortical tubular necrosis ($P < 0.0001$ Vs I, II,

Table 1. Perfusion pressure (mmHg, means ± SD) after 9.5 h of perfusion according to the different groups and the duration of the ischemia

	Duration of ischemia		
	A (0 min)	B (30 min)	C (60 min)
G IV	104.71 ± 57.9	77.5 ± 54.12	62.67 ± 15.59
G V	87.0 ± 28.26	103.2 ± 54.44	88.0 ± 32.72
G VI	173.0 ± 60.14	222.25 ± 34.06	157.0 ± 58.57
G VII		61.19 ± 23.98	

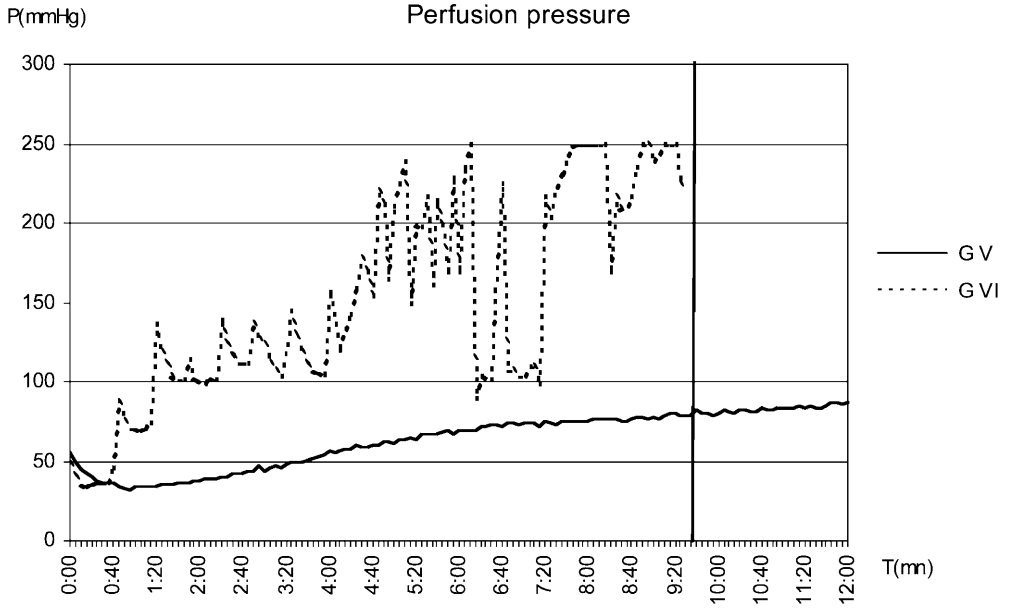

Fig. 1. Perfusion pressure kinetics. Representative curves of G V and G VI. Pressure in G V showed an evolution in three phases with relatively stable pressure. In G VI we observed the same three phases, but in the third phase the pressure was highly unstable and always higher than in G V

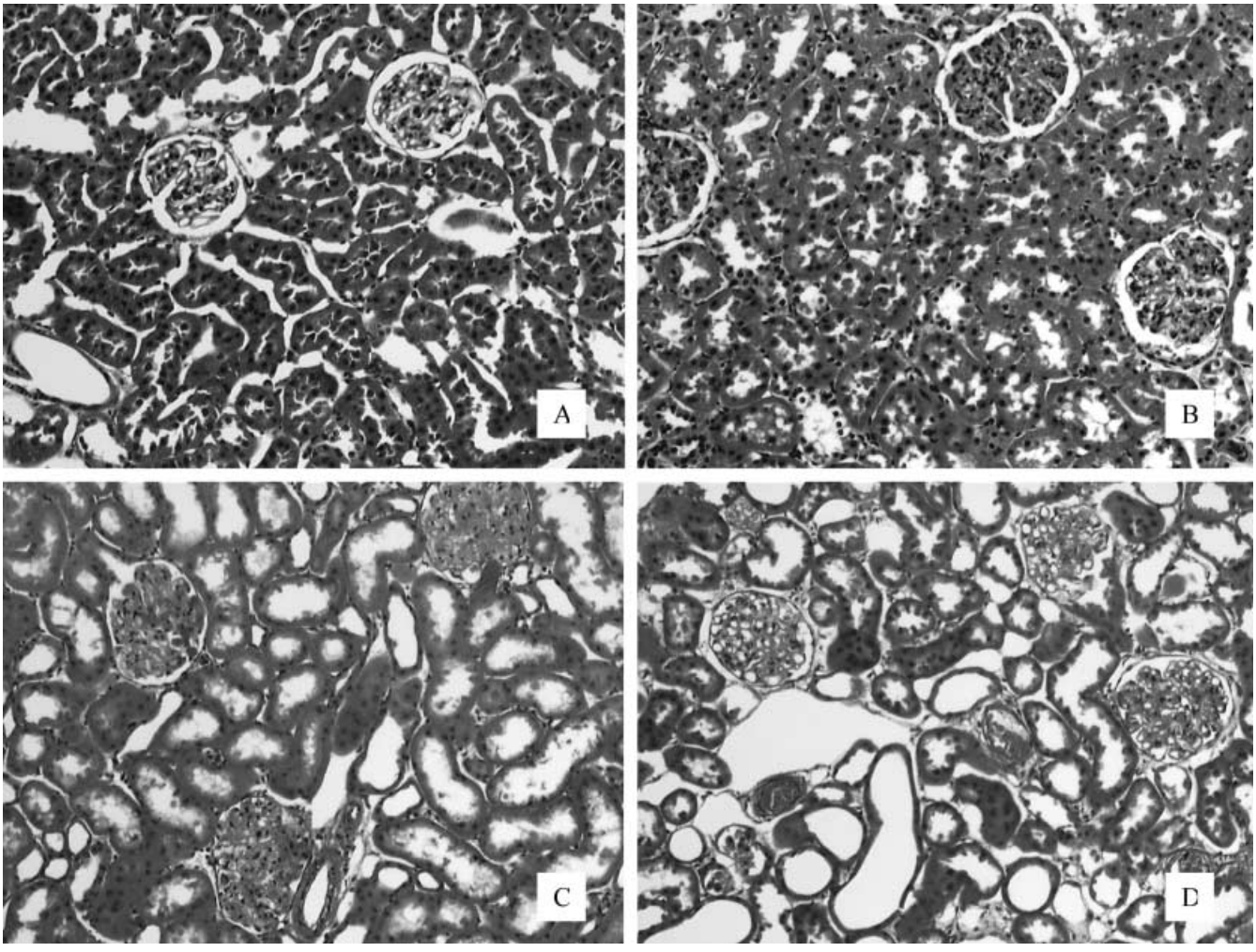

Fig. 2A–D. Histological analysis of renal tissue obtained from group I (sham, **A**), group III (cold storage, 24 h, **B**), group V (HPPS-CS, **C**) and group VI (CS-HPP, **D**). **A** No kidney damage, **B** congestion in the glomeruli without any significant tubular necrosis, **C, D** massive cortical tubular necrosis characterized by dilated and flattened tubules

III), associated with less red blood cell trapping and congestion ($P < 0.0001$). There were no statistical differences between G IV (HPP), G V (HPP-CS) and G VI (CS-HPP) preserved kidneys.

Regardless of the mode of preservation or the duration of warm ischemia, the kidneys never showed any edema or cellular swelling.

Biochemistry

The biochemical results are summarized in Fig. 3.

Biochemical data shows that the anti-oxidant enzymes were influenced by the length of warm ischemia.

Cat activity decreased after 30 and 60 min warm ischemia ($P = 0.04$, $P = 0.005$, respectively). Cu/Zn SOD activity decreased after 60 min of warm ischemia ($P = 0.005$). However, Mn SOD and GPx activities were not influenced by the duration of warm ischemia.

G II (CS 12 h) – G III (CS 24 h): Mn SOD activity decreased after 12 and 24 h of CS (35% and 25%, respectively), GPx and Cu/Zn SOD activity increased after 24 h of CS after treatment with 60 min of warm ischemia (40%, and 28.5%, respectively), Cat activity increased after 12 and 24 h of CS (about 96% and 90%, respectively) and MDA increased after 12 and 24 h of CS (140% and 360%, respectively).

G IV (HPP): When HPP was used alone, Mn SOD activity decreased (about 50%) and GPx activity decreased after 0 and 60 min of warm ischemia (27% and 40%, respectively). Cu/Zn SOD activity was not affected and Cat activity increased (56%). MDA also increased (105%).

G V (HPP-CS): Mn SOD activity decreased after HPP-CS (60%). GPx decreased after 0 min warm ischemia (20.6%). Cu/Zn SOD activity was not significantly modified and Cat activity increased (65%). MDA increased (225%).

G VI (CS-HPP): Mn SOD activity decreased (55%), GPx and Cu/Zn SOD activity decreased respectively after 0 min, and 0 min or 30 min of warm ischemia

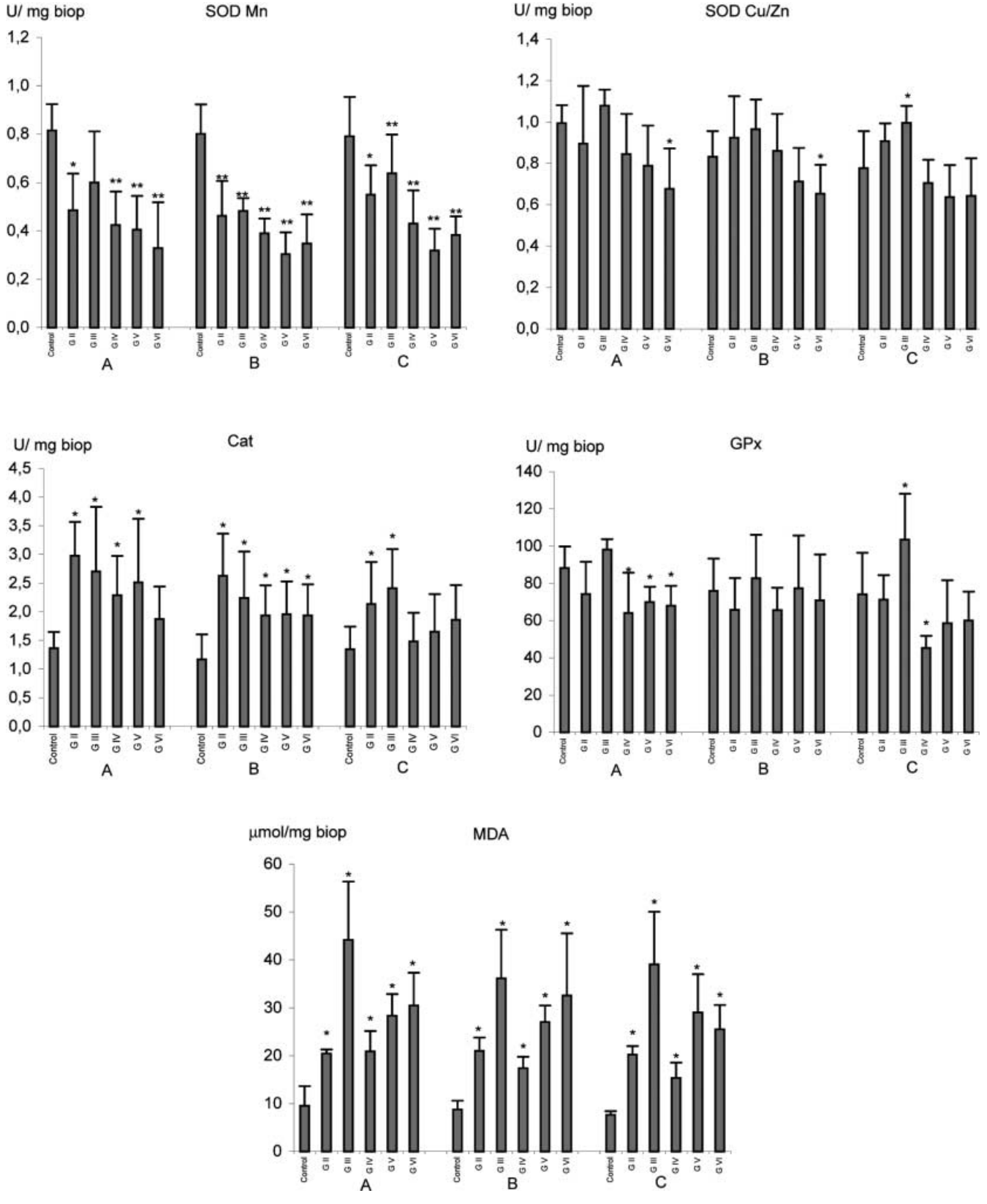

Fig. 3. Evolution of antioxidant enzyme activities and MDA concentration (mean ± SD) as a function of the duration of ischemia (a = 0 min; b = 30 min; C = 60 min) and preservation conditions (*control* = G I; CS 12 h = *G II*; CS 24 h = *G III*; HPP 12 h = *G IV*; HPP CS = *G V*; CS HPP = *G VI*). Significance is given in comparison to the control of each group (**P* < 0.05, ***P* < 0.01)

(23%, and 32% and 21%, respectively). Cat activity increased after 30 min of warm ischemia (65%). MDA also increased (+240%).

Discussion

Recent studies [5, 7, 21, 22, 23] indicate that HPP is superior to CS in achieving optimal early graft functioning, when kidneys are harvested after warm ischemia as with non-heart-beating donors. HPP significantly reduces the level of acute tubular necrosis. Furthermore, during conservation, perfusion data can predict and discriminate kidney functioning after transplantation [8, 21]. Tesi et al. proposed the acceptance of less than 60 mmHg stable perfusion pressure kidneys for a flow >0.7 ml/min per gram [30] and Inman et al. to measure perfusion flow and the glomerular filtration rate [15, 16].

In this study, the initial perfusion pressure was set at 60 mmHg with a flow rate of 0.7 ml/min/g. When the kidneys were directly perfused on the HPP machine after harvesting (HPP and HPP-CS), the pressure initially decreased for 10–25 min before increasing to reach 90 mmHg, even if the kidneys were not subjected to warm ischemia. These results are not in agreement with other studies [4, 18, 21]. This could be explained by our use of low Na^+-high K^+ UW CSS for kidney perfusion. We chose UW CSS because Barber et al. showed that HPP with UW CSS improved the prompt onset of graft functioning compared to Belzer's gluconate HPP [2]. Moreover, it is commonly used for harvesting in France. Conversely, when HPP preservation is used, several authors reported the advantage of low K^+-high Na^+ Belzer's gluconate solution [14]. UW CSS contains starch, with a high viscosity, which can increase perfusion pressures [24]. Inman et al. used a 3.75 hydroxyethyl starch concentration to produce a ureteral effluent [15]. Furthermore, a low K^+ high Na^+ solution may provide better results during perfusion [14]. Moreover, this increasing pressure occurred on the same times scale as the "no reflow phenomenon," observed during reperfusion after declamping [3, 26].

When the kidneys were perfused for 12 h by CS before being perfused by HPP (CS-HPP), the perfusion pressures were significantly higher, showing successive spikes. These spikes were most likely the consequence of vasoconstriction episodes, showing that the kidneys were metabolically active. The pressure normalization observed by adding verapamil to UW CSS demonstrates that Ca^{++} plays a critical role. Lack of ATP and hypothermia lead to inactivation of the ATP dependent Ca^{++} uptake system.

The significant differences in perfusion kinetic pressure between HPP and CS-HPP preserved kidneys shows that HPP and CS may have different consequences for kidney quality.

Perfusion pressure kinetics and histological data are not significantly influenced by the duration of warm ischemia. The mode of preservation induces a massive modification of the kidney structure. All CS preserved kidneys exhibited congestion but little or no signs of tubular damage were seen. In HPP preserved kidneys (alone or associated with CS), a high level of acute tubular necrosis was associated with low blood congestion. The use of HPP seems to have induced cell damage. These results can be compared with the in vivo ischemic reperfusion model presented by Mason et al. [19, 20]. They showed that immediately after ischemic reperfusion, the kidneys were massively congested but that the cellular structures were well preserved. After being subjected to 18 h of blood reperfusion, the glomeruli were still congested, and necrosis of the proximal convoluted tubules was intense. The histological results observed in the HPP preserved kidneys are comparable to those obtained after blood reperfusion. Davies et al. obtained the same results after 3 days of reperfusion, and Ozden et al. after 24 h [10, 25].

Moreover, there were no significant histological differences between HPP, HPP-CS and CS-HPP preserved kidneys, and the biochemical investigation of the tissue oxidative status was done to evaluate the specific consequences of each mode of preservation on the cell metabolism and particularly on peroxidation.

It is widely accepted that warm ischemia results in free radical production. An increasing intracellular concentration of free radicals induces lipid, protein, and nucleic injury, leading to membrane damage, enzymatic and mitotic deregulation, and finally to cell death. Antioxidant enzyme expression is reported to be controlled by specific regulatory genes in response to oxidative stress. Cu/Zn SOD, Mn SOD, GPx, and Cat transcription levels increase with moderate oxidative stress [27, 28, 29]. Inversely, free radicals have been shown to be toxic to enzymes, leading to their inhibition, inactivation, and proteolysis [13]. When free radical formation increases, the detoxifying system is overwhelmed, resulting in a significant decrease in the activity of these enzymes [29]. This phenomenon is amplified by the increase in intracellular Ca^{++}. The oxidant status leads to spontaneous aggravation, leading to membrane injury, and the appearance of lipid breakdown products such as MDA [9]. Contrary to the perfusion and the histological results, we found a correlation between the duration of warm ischemia and a decrease in cytosolic enzyme activity (Cu/Zn SOD, Cat). The decrease in Cu/Zn SOD, Cat activity indicates that these kidneys were subjected to oxidative stress during ischemia, in agreement with Eschwege et al. [12].

The increase in MDA level after CS indicates that the kidneys were subjected to oxidative stress, resulting in membrane damage. This peroxidation is balanced by an increase in the activity of Cat, GPx, and SOD Cu/Zn resulting from their transcription stimulation or activation allowed by the length of CS. Their efficiency provides sufficient protection to avoid cell death. The well-preserved histological aspect of the tissues supports this explanation. Only SOD Mn activity is decreased,

which may reflect mitochondrial alteration by free radicals.

Antioxidant enzyme activity depends on the mode of kidney preservation. During HPP preservation Mn SOD and GPx activity decreases, Cu/Zn SOD is not modified, and Cat increases less than after CS. After HPP, the kidney tissues are less protected against free radicals, as confirmed by the high levels of MDA.

The G V group (HPP-CS) provides similar results to G IV (HPP alone). The only difference is a higher level of MDA in the HPP-CS preserved kidneys.

On the contrary, the use of CS prior to HPP leads to kidneys with a worse oxidative status, as shown by a decrease in Mn SOD, Cu/Zn SOD, and GPx activity. The tissues are subjected to a higher oxidative stress, as reflected by the increased MDA level.

All of these results indicate that kidneys are subjected to oxidative stress during both warm ischemia and surgical procedures, in agreement with other studies [12]. Decrease in Mn SOD and GPx Se induce the accumulation of superoxide anions, leading to mitochondrial dysfunction which amplifies the oxidative stress. The enzyme kinetics observed during HPP preservation are similar to those observed during in vivo reperfusion. In vivo reperfusion induces oxygen free radical formation providing O_2 [29]. With a high rate of radical input, the enzyme inactivation prevails and the enzyme activity is reduced. The worst results obtained after CS-HPP preservation could have been successively induced by previous oxidative stress induced by the surgical technique, warm ischemia, and CS. When kidneys are subjected to HPP, the O_2 supply creates a major oxidative stress.

It was surprising to observe that the higher MDA level was obtained after CS preservation. We would expect a higher MDA level after HPP preservation, which should lead to lower enzyme activity compared to CS. This phenomenon could be due to the reperfusion injury itself, leading to more significant peroxidation lesions than with CS. These lesions can induce cell impairment and finally cell death. MDA is a very reactive species that quickly binds to the nearest protein structure. These proteins are eliminated during cell destruction and MDA levels seem to be less significant than after CS, in spite of higher production. During CS, cells were less broken-down and MDA was not eliminated. This finding is in agreement with the low perfusion pressure obtained after the addition of verapamil to the solute. Verapamil could be active in preventing the cell penetration by Ca^{++}. UW CSS does not contain Ca^{++}, which could be released during cell death, as well as MDA. This hypothesis of reperfusion injury occurring during HPP preservation is supported by both the histological and perfusion pressure results.

In kidneys subjected to ischemia/reperfusion, the level of injury increases with the length of reperfusion, leading to acute tubular necrosis as observed during HPP preservation [10, 25, 30]. This observation confirms that under our experimental conditions, HPP with UW-CSS can simulate in vitro reperfusion lesions. Moreover, the perfusion pressure increase occurs in the same period as the "no reflow" phenomenon observed after kidney revascularisation. This phenomenon is characterised by capillary level obstruction, which results in increased pressure. Many factors might be responsible, including cellular swelling during ischemia, compression of the capillary bed and accumulation of interstitial fluid in the post-ischemic tissues, which can result in the compression of the microvasculature, or imbalance between PGI_2 and TxB_2. Collectively, these phenomena are responsible for pressure increases after reperfusion, which is exacerbated by a previous period of CS, as we saw in the CS-HPP group.

Perfusion pressure, histological and biochemical data combine to show that HPP induced, in our model, reperfusion injuries, particularly in the CS-HPP group. The major point, which probably leads to such damage, is perfusion with oxygenated UW CSS. After oxidative cell disruption, toxic molecules are eliminated in the perfusate which create additional damage. This is the reason why the association of CS and HPP is more deleterious when kidneys have previously undergone warm ischemia. We think that it would be better to keep only one mode of preservation, either CS or HPP, throughout the duration of cold ischemia, and particularly after a period of warm ischemia. Because there is no simple cold storage before HPP, machine perfusion technology is clinically efficient, especially for the NHB donor kidney. However, the preservation efficacy of the CS + HPP association would probably be better if an extracellular perfusion solution was used.

A perfusion machine could be used to create the conditions leading to reperfusion injuries, in order to explore ischemia–reperfusion lesions. Using such a machine, it would be very easy to control all of the perfusion conditions: temperature and oxygenation of the perfusate. It would also be possible to prepare the graft for transplantation. HPP can allow a progressive oxygenation rather than rapid oxygenation of the perfusate, leading to better results. It would also help to eliminate toxic waste incurred with cell damage. The use of HPP could facilitate the prevention of peroxidation, vasospasm and intracellular Ca^{++} increase.

We consider machine reperfusion after a period of cold ischemia as an undesirable preservation method. If we had to choose the mode of preservation, it is best to use only one method throughout the period of cold ischemia (HPP or CS). The association of CS and HPP is more deleterious when a kidney has undergone warm ischemia. The reason may be due to the recirculation of numerous deleterious molecules or cell components during the reperfusion step which were produced during CS. Because there is no CS before HPP, machine perfusion technology is clinically efficient, especially for the NHB donor kidney.

Moreover, if we consider the CS + HPP association to be relevant, the preservation efficacy would probably be better using an extracellular perfusion solution.

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