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Original Article

Diazoxide attenuates hypothermic preservation-induced renal injury via down-regulation of CHOP and caspase-12

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Abstract

Background. Successful clinical organ preservations are a prerequisite for organ transplantation. Diazoxide (DE), which shows a concentration-dependent selectivity for mitoK$^+$-ATP over plasma membrane K$^+$-ATP, displays protective effects during organ preservation. The current study investigated possible protective effects of DE on rat kidneys injured by hypothermic preservation.

Methods. Forty-eight Sprague–Dawley rats were randomly divided into six groups ($n = 8$): Celsior groups with kidneys preserved in Celsior solution for 0, 24 and 48 h and DE groups with kidneys preserved in DE (30 μM) plus Celsior solution for 0, 24 and 48 h. Superoxide dismutase (SOD) activity and the quantity of malonaldehyde (MDA) in the kidneys from each group were measured, and the levels of C/EBP homologous protein (CHOP) and caspase-12 were determined by immunohistochemistry staining and real-time reverse transcription quantitative polymerase chain reaction analysis.

Results. SOD activity was significantly higher and the quantity of MDA was significantly lower in the DE groups compared with the Celsior groups at both 24 and 48 h ($P < 0.05$). The expressions of CHOP and caspase-12 were also lower in DE groups at 24 and 48 h ($P < 0.05$).

Conclusions. The present results demonstrate that DE exerts protective effects by attenuating oxidative stress injury through up-regulation of SOD activity and down-regulation of MDA quantity and by decreasing the cell apoptosis in kidneys by reducing the levels of CHOP and caspase-12 during hypothermic preservation.

Keywords: caspase-12; CHOP; diazoxide; hypothermic preservation; kidney

Introduction

Although the success of clinical organ preservation is a prerequisite for organ transplantation, many questions remain despite decades of clinical experience. At present, the largest problem for organ preservation is to minimize the negative effects of ischaemia and hypothermia [1]. Hypothermic preservation solutions are designed to allow an effective organ flush that removes blood and cools the organ. The Celsior solution, one of the standard solutions for perfusion and organ preservation purposes, was originally developed and used as a preservation solution for the lung and heart and has also been proposed for the liver and kidney [2]. However, during the preservation period, oxygen deficiency in cells leads to irreversible changes in the transplanted organ [3]. Although antioxidants are often added to reduce damage resulting from anoxia, reperfusion and oxidative stress, the current hypothermic preservation solutions do not achieve the desired result, and reducing injury to the organ during hypothermic preservation remains a key issue.

Previous studies indicate that endoplasmic reticulum (ER) responses play a pivotal role in cellular apoptosis after exposure to various stresses, such as hypoxia, anoxia and oxidative stress [4,5]. At least three pathways contribute to ER stress-mediated cell death: transcription activation of the C/EBP homologous transcription factor (CHOP) [6], activation of the IRE1-tumour necrosis factor receptor-associated factor (TRAF2) pathway [7] and activation of ER-associated caspase-12 [8,9]. Sargsyan [10] has shown that diazoxide (DE) can improve β-cell function by reducing ER stress caused by exposure to elevated levels of glucose and fatty acids. DE shows a concentration-dependent selectivity for mitoK+-ATP over plasma membrane K+-ATP and also displays a protective effect during *in vitro* heart preservation [11,12]. Additional evidence shows that DE may significantly enhance myocardial protection during long-term hypothermic preservation and may decrease the number of apoptotic cells following hypothermic preservation [13].

The aim of the present study was to investigate a possible protective influence of DE during hypothermic preservation and to explore possible mechanisms, such as an attenuation of oxidative stress injury.

Materials and methods

Animals

Forty-eight Sprague–Dawley (SD) male rats were purchased from the Experimental Animal Center of Zhejiang University. All procedures were

conducted with the approval of the local animal care committee (under National Institutes of Health (NIH) policies).

Experimental groups

Forty-eight male SD rats weighing 220–250 g were randomly divided into two groups: Celsior groups, in which the kidneys were stored in Celsior solution after perfusion, and DE groups, in which the kidneys were stored in Celsior solution containing DE (30 μmol/L; St Luis, MO, USA) after perfusion. Each group was further subdivided according to preservation times at 0, 24 and 48 h.

Kidney hypothermic preservation

All rats in each of the six groups were anaesthetized by intraperitoneal injection with a lethal dose of nembutal. The kidneys from each rat were fully exposed and the renal vessels were ligated to block blood supply to the kidneys. The renal artery was cannulated using a Tibbs arterial cannula connected to a 50 mL syringe and was perfused with a 4°C Celsior solution (mM: NaOH 100, KCl 15, $MgCl_2$ 13, $CaCl_2$ 0.25, mannitol 60, lactobionate 80, histidine 30, glutamate 20; pH = 7.4) or with a 4°C Celsior solution containing 30 μM DE. Kidneys were perfused until the solution effusing from the renal vein appeared clear. The kidneys were then removed and stored in different preservation solutions for 0, 24 or 48 h at 4°C.

SOD activity and MDA level assay

The right kidneys from each rat were preserved in liquid nitrogen to measure the activity of superoxide dismutase (SOD) and the quantity of malonaldehyde (MDA) using a UV spectrometer (Bio-Tek Instruments, USA). The kidneys were weighed, minced with scissors and homogenized into 10% tissue homogenate (homogenized for 3 × 10 s intervals on ice). From 5 to 10 mL of 10% tissue homogenate was centrifuged at 1000 r.p.m. for 10 min (4°C) and the supernatant was transferred to a new tube; some supernatant was diluted to a concentration of 0.1 g/mL for SOD assay. A Coomassie brilliant blue kit (NanJin JinCheng ShenWu YanJiuSuo, NanJin, China) was used to measure protein concentration.

The SOD assay kit (NanJin JinCheng ShenWu YanJiuSuo, NanJin, China) used the xanthine oxidase method, and the xanthine oxidase assay used a compilation of methods from various sources. Xanthine oxidase catalyses the oxidation of xanthine to uric acid and in the process generates O_2^-. The O_2^- production oxidizes hydroxylamine to nitrite, then nitrite would generate a prunosus colour production under the function of specific chromogenic agent, and the prunosus colour production was followed spectrophotometrically, allowing for quantitative measurement. For the SOD assay, 20 μL of supernatant (0.1 g/mL) was added to the reaction system; this was incubated at 37°C for 40 min, 2 mL of chromogenic agent was added and the mixture was incubated to room temperature for 10 min. The relative absorbance of the supernatants was immediately measured at 550 nm using a UV spectrometer. Twenty microliters of ddH_2O was used instead of supernatants as a negative control. A 1 mg protein of a sample solution that established 50% inhibition was used to determine the SOD unit in an assay solution as 1 U.

The MDA kit (NanJin JinCheng ShenWu YanJiuSuo, NanJin, China) used thio-malonylurea methods. MDA was condensed with thiobarbituric acid to generate a red colour production (a colorimetric reaction), which was followed spectrophotometrically, allowing for quantitative measurement. For the MDA assay, 10 μL of supernatant (10% tissue homogenate) was added into the reagent of the MDA kit; this was incubated at 95°C for 40 min, centrifuged at 4000 r.p.m. for 10 min (4°C) and cooled, and the supernatant was transferred to the cuvette for assay. The absorbance of supernatants was measured at 532 nm using a UV spectrometer. Ten microliters of alcohol was used instead of supernatants as a negative control, and the standard substance (10 nmol/mL) from the MDA kit was used as a positive control.

Histologic staining and immunohistochemistry

Half of the left kidneys from each rat were preserved in 4% paraformaldehyde for 24 h, and the other half were put into liquid nitrogen for assay of real-time RT–PCR. The tissues were embedded in paraffin, and transverse paraffin sections (5 mm thick) were mounted on silane-coated slides (10 slide series with 10 sections per slide). Sections were deparaffinaged and rehydrated. Some sections were then stained with cresyl violet (0.3%; VWR International, Buffalo Grove, IL, USA) for histological analysis, while other sections were treated for antigen retrieval with 10.2 mmol/L sodium citrate buffer, pH 6.1, for 20 min at 95°C for immunohistochemistry.

The remaining sections were washed in 0.01 M PBS containing 0.3% Triton X-100 (pH 7.4, PBS-T), then immersed in 2% normal goat serum in PBS for 2 h at 37°C, incubated overnight at 4°C with polyclone CHOP antibody (1:50, Santa Cruz Biotechnology, USA) and polyclone caspase-12 antibody (1:50, Santa Cruz Biotechnology, USA) in PBS containing 1% bovine serum albumin, washed in PBS (3 × 5 min), incubated in biotinylated goat-anti-rabbit IgG (1:200, Boster) in PBS for 2 h at room temperature, washed in PBS-T (3 × 5 min), incubated in avidin–biotin–peroxidase complex solution (ABC, 1:100, Boster) for 2 h at room temperature, then rinsed again in PBS-T (3 × 5 min). Immunolabelling was visualized with 0.05% diaminobenzdine (DAB) plus 0.3% H_2O_2 in PBS. After staining, the sections were counterstained by haematoxylin, and the sections were then dehydrated with ethanol and xylene before coversliping with Permount. Rat immunoglobulin IgG (1:200, Biomeda Corporation, USA) was used instead of primary antibody as a negative control.

Real-time reverse transcription quantitative PCR analysis of CHOP and caspase-12

For total RNA isolation in RT–PCR analysis, RNA was isolated from the specimens using the Trizol reagent kit (Invitrogen, USA) as per

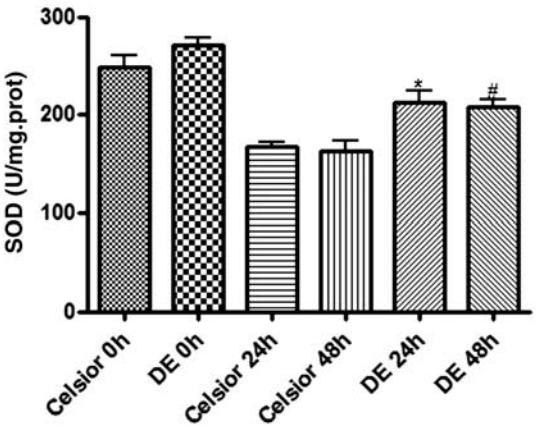

Fig. 1. The activity of superoxide dismutase (SOD) in rat kidneys at different time periods of hypothermic preservation. The Celsior 0 h and DE 0 h groups were not different. However, compared with Celsior 24 and 48 h groups, SOD activity was increased in the DE 24 h group (*$P < 0.05$) and in the DE 48 h group ($^{\#}P < 0.05$).

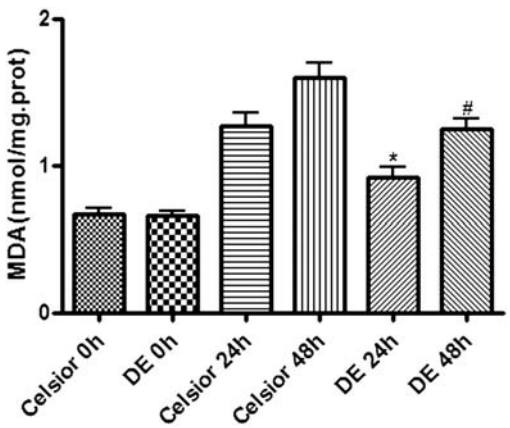

Fig. 2. The quantity of malonaldehyde (MDA) in rat kidneys at different time periods of hypothermic preservation. The Celsior 0 h and DE 0 h groups were not different. However, compared with the Celsior 24 and 48 h groups, the quantity of MDA was significantly decreased in both the DE 24 h (*$P < 0.05$) and DE 48 h group ($^{\#}P < 0.05$).

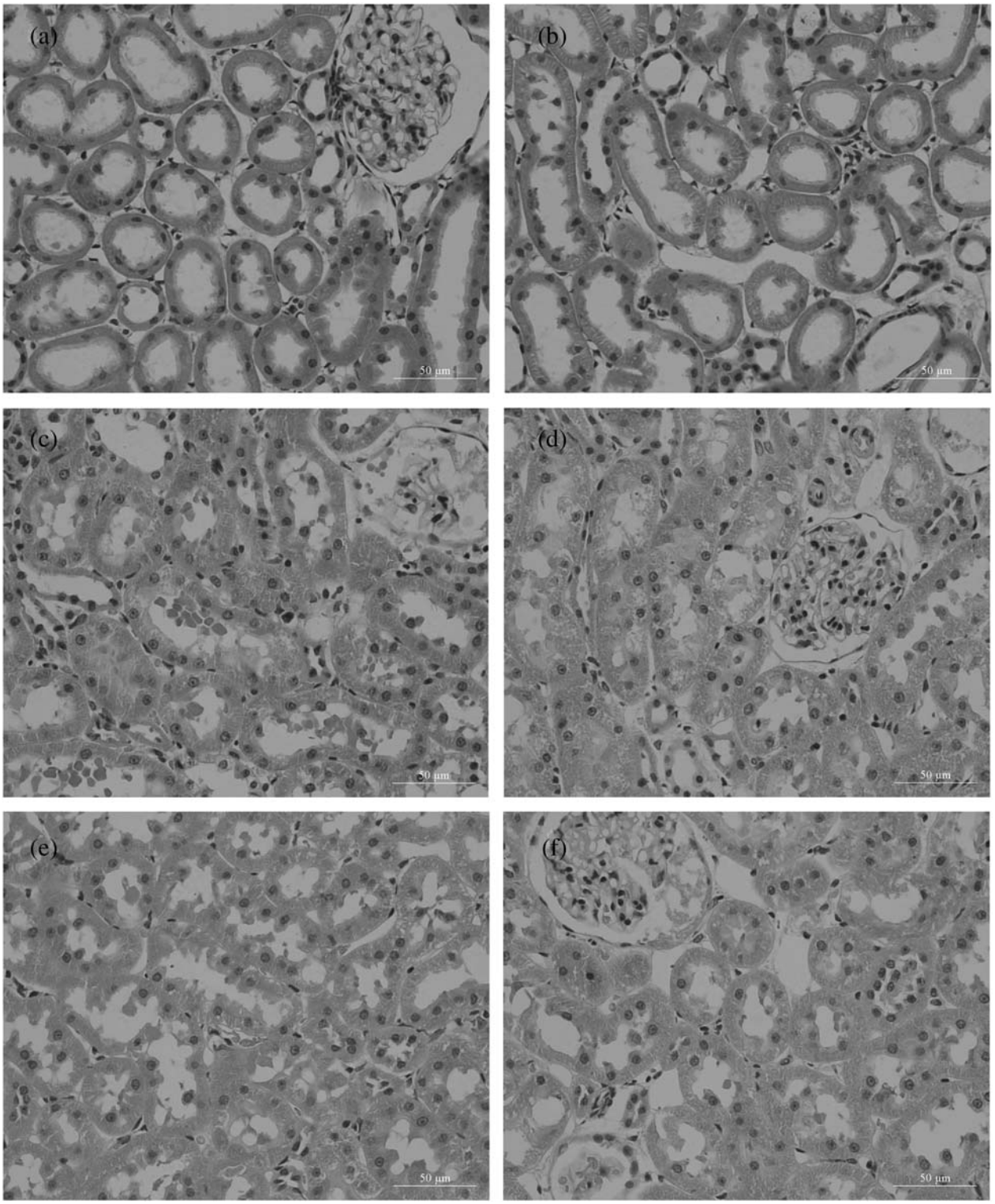

Fig. 3. The morphology of rat kidney tissues in each group by H&E staining, ×400. (**a**) Celsior 0 h group; (**b**) DE 0 h group; (**c**) Celsior 24 h group; (**d**) Celsior 48 h group; (**e**) DE 24 h group; (**f**) DE 48 h group. The walls of proximal renal tubules in the groups with 0-h hypothermic preservation were composed of monolayer cuboidal epithelial cells without clear cell demarcation (a, b). Compared with the Celsior 24 h group after hypothermic preservation (c), the injury after hypothermic preservation was clearly reduced in DE group with 24 h (e). Furthermore, the injury after hypothermic preservation in the DE group with 48 h (f) was also reduced compared with the Celsior 48 h group (d).

the manufacturer's protocol. For reverse transcription, RNA concentration was measured spectrophotometrically and 2 μg total RNA was added to the cDNA synthesis reaction system (20 μL) on a FTC2000 (Funglyn, Canada). The reaction mixture consisted of 4 μL 5× RT buffer, 2.5 μmol/L oligd (T), 5 mmol/L deoxynucleotide triphosphates (dNTPs) and 20 U RNAasin (RNase inhibitor). The hexamers were annealed by

Table 1. Comparison of CHOP-positive cells and optical density in CHOP-positive cells from rat kidneys in the different study groups ($n = 8$, $\bar{\chi} \pm s$)

Groups	Number of positive cells	Optical density
Celsior 0 h	$3.53 \pm 1.03/mm^2$	179.24 ± 8.21
DE 0 h	$3.43 \pm 1.02/mm^2$	182.33 ± 4.64
Celsior 24 h	$7.79 \pm 1.20/mm^2$	207.71 ± 3.88
Celsior 48 h	$8.70 \pm 1.14/mm^2$	216.12 ± 3.31
DE 24 h	$5.90 \pm 1.50/mm^{2}$*	199.83 ± 3.95*
DE 48 h	$6.80 \pm 1.05/mm^{2\#}$	$208.42 \pm 4.24^{\#}$

CHOP expression was not different between the Celsior 0 h and DE 0 h groups. However, when compared with the Celsior 24 h group, the number of positive cells and the optical density were significantly decreased in the DE 24 h group (*$P < 0.05$). Similarly, the number of positive cells and the optical density were reduced in the DE 48 h group compared with the Celsior 48 h group ($^{\#}P < 0.05$).

incubating the samples to 70°C for 5 min. Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (200 U; Promega, USA) was added, then incubated at 42°C for 60 min. The reaction was stopped by heating to 72°C for 10 min. For rt-RT–PCR, the reaction mixture (40 μL) consisted of 4 μL cDNA, 35.2 μL SYBR® Premix Ex Taq™ (TaKaRa, China), 0.5 μL of 5 U Taq DNA polymerase and 0.3 μL of 20 pmol/μL CHOP or caspase-12 primer (Invitrogen, USA). The cDNA was denatured by heating to 94°C for 3 min. The template was amplified by 40 rounds of PCR (denaturation at 94°C for 10 s, annealing at 60°C for 30 s, extension at 72°C for 30 s) before measuring fluorescence at 72°C. Meanwhile, the primers were used for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in rt-RT–PCR to amplify GAPDH (forward: 5′-GGTGGACCTCATGGCCTACAT-3′, reverse: 5′-GCCTCTCTCTTGCTCTCAGTATCCT-3′) as an internal control of CHOP (forward: 5′-CGGAGTGTACCCAGCACCATCA-3′, reverse: 5′-CCCTCTCCT TTGGTCTACCCTCA-3′) and caspase-12 (forward: 5′-AGGGATAGCCACTGCTGA TACAGA-3′, reverse: 5′-CTGTCTCCACATGGGCCTTTGTT-3′).

Statistical analysis

The sections were examined at ×400 magnification with UTHSCSA Image Tools 3.0 (University of Texas Medical School at San Antonio, TX, USA), and the number and optical density of the CHOP and caspase-12-positive cells were determined. A probability of 95% was taken to indicate a significant difference. Data are presented as means ± SD.

Results

Effect of DE on SOD activity and MDA level

Although there was no significant difference between the Celsior 0 h and DE 0 h groups, the activity of SOD was significantly higher ($P < 0.05$, Figure 1) and the quantity of MDA was significantly lower in the DE groups compared with the Celsior groups at 24 and 48 h ($P < 0.05$, Figure 2).

Morphologic changes of proximal tubular epithelial cells from rat donor kidney under light microscope

The walls of the proximal renal tubules in the 0-h hypothermic preservation groups were composed of monolayer cuboidal epithelial cells without clear cell demarcation. The intensely eosinophilic cytoplasm and the deep-blue stained nuclei were seen and the brush border could be observed on the luminal surface of the renal tubules (Figure 3a, b). The renal tubular epithelial cells of the Celsior group with 24-h hypothermic preservation were slightly swelled, and tubular cellular debris was present in the renal tubules (Figure 3c). The renal tubular epithelial cells of the Celsior group with 48-h hypothermic preservation had prominent swelling, visible vacuolar areas within the cytoplasm and debris from the tubular epithelial cells in the lumen of the renal tubules (Figure 3d). In contrast with the Celsior group, the DE group with 24-h hypothermic preservation showed slight swelling of renal tubular epithelial cells without clear demarcation (Figure 3e). In the DE group with 48-h hypothermic preservation, the vacuolar areas were smaller with a decrease in amount of debris from the tubular epithelial cells (Figure 3f). Hence, it is clear that injury after hypothermic preservation was reduced in the DE groups at 24 and 48 h.

Immunoreactivity assay

CHOP immunoreactivity was visualized as a granular immunostain pattern in the nuclei of the cells. In the DE groups at 24 and 48 h, quantitative analysis of the number and optical density of CHOP-positive cells with DAB immunostaining showed significant decreases in glomerular mesangial cells, glomerular endothelial cells and epithelial cells from the renal and collecting tubules ($P < 0.05$, Table 1, Figure 4). Caspase-12 immunohistochemistry staining positive cells with DAB staining showed buffy granules in the cytoplasm. Also in the DE groups at 24 and 48 h, the glomerular mesangial cells, glomerular endothelial cells and epithelial cells from the renal and collecting tubules had decreased caspase-12 expression compared with the Celsior groups ($P < 0.05$, Table 2, Figure 5).

rt-RT–qPCR assay

According to rt-RT–qPCR results, CHOP and caspase-12 mRNA levels were up-regulated in the Celsior groups compared to the DE groups at 24 and 48h ($P < 0.01$, Tables 3 and 4), indicating that the ER stress responses were stronger in the Celsior groups.

Discussion

In the last half century, hypothermic preservation has been recognized as an effective measure for organ preservation because hypothermia can reduce the metabolic rate of organs. However, improvements are needed in hypothermic preservative solutions to prevent cellular oedema and acidosis in order to extend the preservation time of organs. Because of recent interest in chronic allograft nephropathy, there has been an increasing awareness that ischaemia/reperfusion injury can significantly affect outcomes after transplantation. This has stimulated research on preservation damage and on the development of new preservation solutions [14,15].

A number of insults can lead to protein misfolding in the ER, and these include nutrient deprivation, alterations

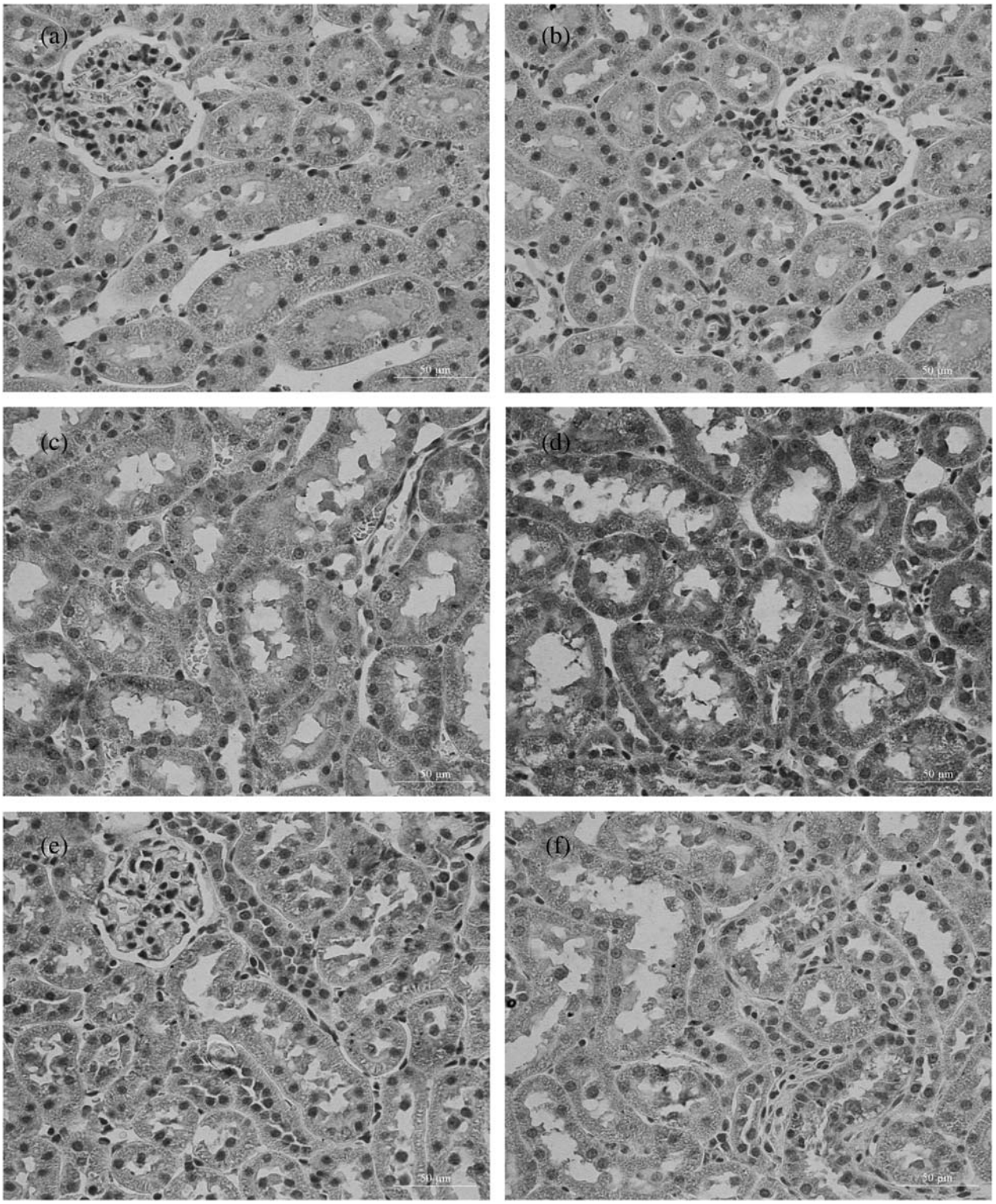

Fig. 4. The expression of CHOP detected by immunohistochemistry in rat kidneys after different periods of hypothermic preservation, ×400. (**a**) Celsior 0 h group; (**b**) DE 0 h group; (**c**) Celsior 24 h group; (**d**) Celsior 48 h group; (**e**) DE 24 h group; (**f**) DE 48 h group. Yellow brown granules within the nucleus and cytoplasm were identified as positive CHOP protein staining. There was no significant difference between Celsior 0 h and DE 0 h groups (a, b). Compared with Celsior 24 h group (c), the expression of CHOP was significantly decreased in the DE 24 h group (e). The expression of CHOP was also reduced in the DE 48 h group (f) compared with the Celsior 48 h group (d).

Table 2. Number of caspase-12-positive cells and optical density in caspase-12-positive cells in rat kidneys in the different study groups (n = 8, $\bar{\chi} \pm s$)

Groups	Number of positive cells	Optical density
Celsior 0 h	1.92 ± 0.74/mm^2	170.56 ± 3.46
DE 0 h	2.15 ± 0.54/mm^2	169.78 ± 4.94
Celsior 24 h	6.06 ± 0.83/mm^2	194.1 ± 4.36
Celsior 48 h	6.58 ± 1.12/mm^2	197.09 ± 6.30
DE 24 h	4.50 ± 1.29/mm^2*	185.97 ± 6.23*
DE 48 h	5.15 ± 0.89/mm$^{2\#}$	189.45 ± 3.30$^{\#}$

Caspase-12 expression was not between the Celsior 0 h group and DE 0 h groups. When compared with Celsior 24 h group, the number of positive cells and the optical density were significantly decreased in the DE 24 h group (*$P < 0.05$). Similarly, the number of positive cells and the optical density were reduced in the DE 48 h group compared with the Celsior 48 h group ($^{\#}P < 0.05$).

in the oxidation reduction balance, changes in calcium concentration, failure of post-translational modifications or simply increases in secretory protein synthesis [16]. In response to accumulation of unfolded proteins in the ER, ER resident protein chaperones and protein foldases are expressed, the ER compartment proliferates and ER-associated degradation factor is activated to eliminate the irreparably misfolded proteins. When these pro-survival efforts are exhausted, ER stress-related apoptosis commences.

CHOP-mediated ER stress-induced cell death appears to be involved in several neurodegenerative diseases and in brain ischaemia [17–20]. In addition, deletion of the CHOP gene is known to delay the onset of β-cell destruction and hyperglycaemia in heterozygous Akita mice [6]. CHOP activation occurs concomitantly with the activation of caspase-12, and activated caspase-12 in turn produces activation of the caspase cascade [21].

Caspase-12 activation is mediated mainly by calpain, which is released from the ER membrane by tumour necrosis factor receptor-associated factor. Subsequently, caspase-12 interacts with caspase-9, which forms part of the 'intrinsic' apoptotic pathway, leading to activation of the executer caspase-3. The present work confirmed that CHOP and caspase-12 levels are increased after hypothermic preservation. As preservation times progressed from 24 to 48 h, deteriorations in renal morphologic integrity were accompanied by elevations in CHOP and caspase-12 levels. Therefore, CHOP and caspase-12-mediated ER stress-induced cell death appears to be one of the major mediators of apoptotic cellular death after hypothermic preservation.

Opening the mitoK$^+$-ATP channel with DE has been shown to improve the recovery of the rate–pressure product after reperfusion and to attenuate oxidant generation during both ischaemic and reperfusion periods [22]. In response to oxidant generation, animals have developed a natural defence system to cope with these toxic species. Such defence mechanisms include SOD, glutathione peroxidase and other systems [23]. SOD is important in that increases in its enzymatic activity have been shown to increase cellular capabilities for scavenging/quenching of free radicals. MDA is a degradation product of oxygen-derived free radicals and lipid oxidation, and its levels reflect damage caused by reactive oxygen species. In agreement with previous work [24,25], we found that DE can down-regulate the quantity of MDA and up-regulate the activity of SOD after hypothermic preservation. Moreover, our histological staining experiments revealed a more dramatic pathological destruction of renal tissue in the Celsior groups compared with the DE groups. At the same time, the expression of CHOP and caspase-12 was lower in the DE groups after 24 and 48 h of hypothermic preservation. In addition to the ability of DE and related compounds to improve glucose tolerance in non-diabetic individuals and obese subjects with T2DM [26], DE can also be used to prolong the time of residual β-cell mass in individuals with T1DM. Since both T1DM and T2DM can easily induce ER stress, the possibility that DE may attenuate ER stress may explain an important aspect of its beneficial effects.

Conclusion

In conclusion, the present studies provided evidence that DE can attenuate the activation of ER stress, which is triggered by alterations in oxidation–reduction balance or by the accumulation of oxygen free radicals during the period of hypothermic preservation. DE acts by decreasing the expression of CHOP and caspase-12 by down-regulating the quantity of MDA and up-regulating the activity of SOD. Because DE acts as an antioxidant that controls peroxide generation, it may be added to Celsior solutions to provide an effective and promising agent for renal hypothermic preservation.

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Conflict of interest statement. None declared.

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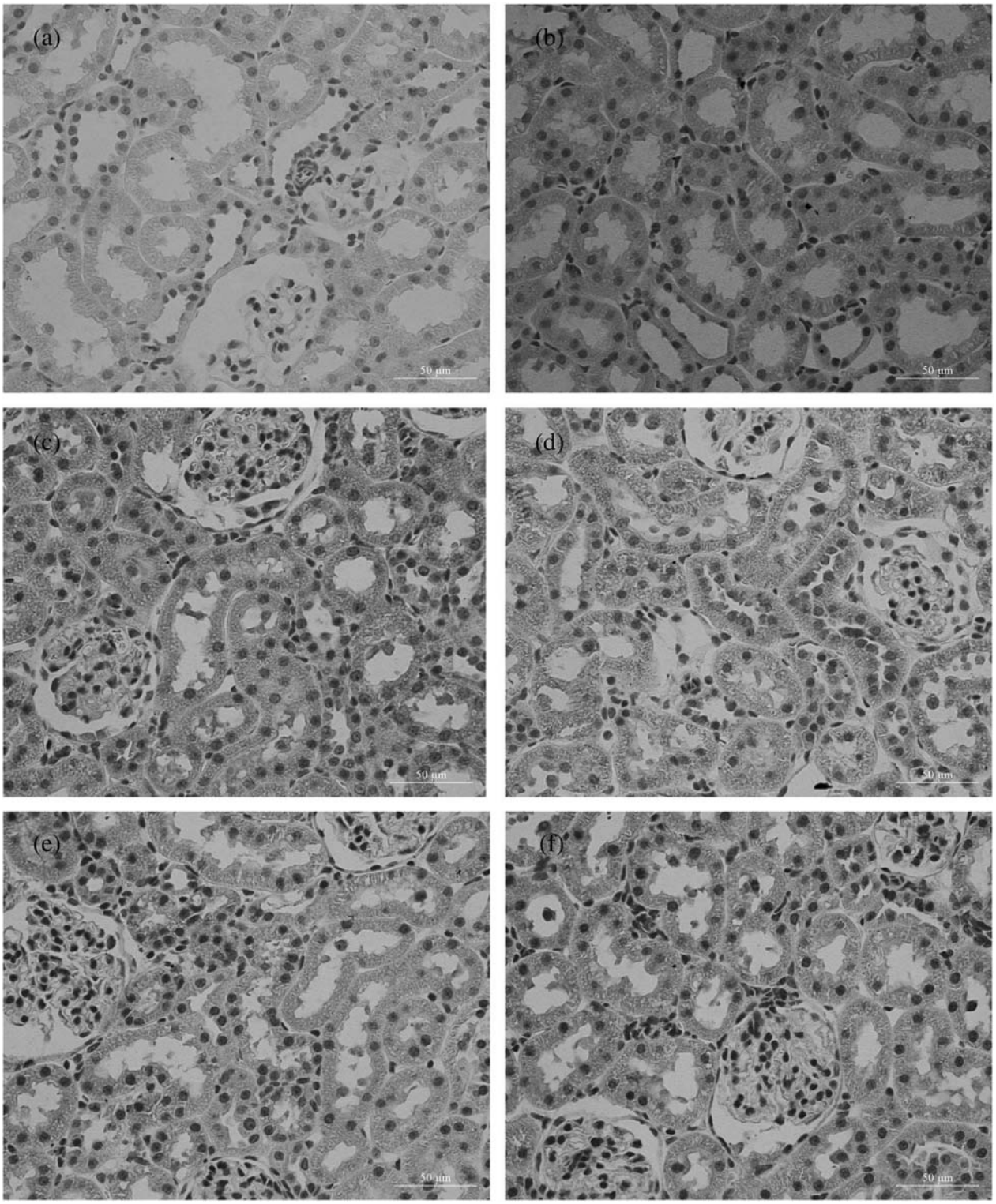

Fig. 5. The expression of caspase-12 detected by immunohistochemistry in rat kidneys after different periods of hypothermic preservation, ×400. (**a**) Celsior 0 h group; (**b**) DE 0 h group; (**c**) Celsior 24 h group; (**d**) Celsior 48 h group; (**e**) DE 24 h group; (**f**) DE 48 h group. Yellow brown granules within the cytoplasm were identified as positive caspase-12 protein staining. There was no significant difference between the Celsior 0 h and DE 0 h groups (a, b). Compared with the Celsior 24 h group (c), the expression of caspase-12 was significantly decreased in the DE 24 h group (e). The expression of caspase-12 was also reduced in the DE 48 h group (f) compared with the Celsior 48 h group (d).

Table 3. Comparison of CHOP mRNA levels using the comparative C_T method ($n = 8$, $\bar{\chi} \pm s$)

Group	CHOP, average C_T	GAPDH, average C_T	($\triangle\triangle C_T$) CHOP	Fold difference CHOP
Celsior 0 h	34.06 ± 0.02	20.81 ± 0.06	0	1
DE 0 h	34.07 ± 0.02	20.76 ± 0.04	0.02 ± 0.04	0.98 ± 0.03
Celsior 24 h	31.02 ± 0.9	21.37 ± 0.89	−3.63 ± 0.03	12.4 ± 10.28
Celsior 48 h	30.72 ± 0.57	21.21 ± 0.56	−3.77 ± 0.03	13.65 ± 0.3
DE 24 h	31.7 ± 0.478	21.64 ± 0.53	−3.22 ± 0.04	9.35 ± 0.25**
DE 48 h	30.58 ± 0.03	20.78 ± 0.04	−3.48 ± 0.05	11.2 ± 0.04$^{\#\#}$

$\triangle C_T$, CHOP−GAPDH; $\triangle\triangle C_T$, $\triangle C_T - \triangle C_T^{\text{Celsior 0h}}$; fold difference, $2^{-\triangle\triangle CT}$. The expression of CHOP mRNA was not different between the Celsior 0 h and DE 0 h groups. Compared with the Celsior 24 h group, the expression of CHOP mRNA was significantly decreased in the DE 24 h group (**$P < 0.01$). Similarly, the expression of CHOP mRNA was reduced in the DE 48 h group compared with the Celsior 48 h group ($^{\#\#}P < 0.01$).

Table 4. Comparison of caspase-12 mRNA levels using the comparative C_T method ($n = 8$, $\bar{\chi} \pm s$)

Group	Caspase-12, average C_T	GAPDH, average C_T	($\triangle\triangle C_T$) caspase-12	Fold difference caspase-12
Celsior 0 h	34.3 ± 0.2	20.82 ± 0.06	0	1
DE 0 h	34.3 ± 0.21	20.77 ± 0.05	0.06 ± 0.25	0.97 ± 0.17
Celsior 24 h	30.74 ± 0.6	20.74 ± 0.63	−3.47 ± 0.19	11.2 ± 1.58
Celsior 48 h	30.49 ± 0.41	20.81 ± 0.4	−3.79 ± 0.19	14.02 ± 1.98
DE 24 h	32.17 ± 0.47	21.78 ± 0.41	−3.08 ± 0.2	8.54 ± 1.23**
DE 48 h	30.72 ± 0.08	20.79 ± 0.03	−3.54 ± 0.2	11.72 ± 1.68$^{\#}$

$\triangle C_T$, caspase-12−GAPDH; $\triangle\triangle C_T$, $\triangle C_T - \triangle C_T^{\text{Celsior 0h}}$; fold difference, $2^{-\triangle\triangle CT}$. Caspase-12 mRNA expression was not different between the Celsior 0 h and DE 0 h groups. Compared with the Celsior 24 h group, the expression of caspase-12 mRNA was significantly decreased in the DE 24 h group (**$P < 0.01$). Similarly, the expression of caspase-12 mRNA was reduced in the DE 48 h group compared with the Celsior 48 h group ($^{\#}P < 0.05$).

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