Diazoxide on preserved kidney

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

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Abstract

Background. Successful clinical organ preservation 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

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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.

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).

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
The kidneys were weighed, minced with scissors and homogenized into 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% diaminobenzidine (DAB) plus 0.3% H2O2 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.

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

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of positive cells</th>
<th>Optical density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celsior 0 h</td>
<td>3.53 ± 1.03/mm²</td>
<td>179.24 ± 8.21</td>
</tr>
<tr>
<td>DE 0 h</td>
<td>3.43 ± 1.02/mm²</td>
<td>182.33 ± 4.64</td>
</tr>
<tr>
<td>Celsior 24 h</td>
<td>7.79 ± 1.20/mm²</td>
<td>207.71 ± 3.88</td>
</tr>
<tr>
<td>DE 24 h</td>
<td>8.70 ± 1.14/mm²</td>
<td>216.12 ± 3.31</td>
</tr>
<tr>
<td>Celsior 48 h</td>
<td>5.90 ± 1.50/mm²*</td>
<td>198.83 ± 3.95*</td>
</tr>
<tr>
<td>DE 48 h</td>
<td>6.80 ± 1.05/mm²*</td>
<td>208.42 ± 4.24*</td>
</tr>
</tbody>
</table>

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 (\( P < 0.05 \)).

The kidneys 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, MgCl2 13, CaCl2 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 malondialdehyde (MDA) in the kidneys. The kidneys were then removed and stored in different preservation solutions for 0, 24 or 48 h at 4°C Celsior solution (mM: NaOH 100, KCl 15, MgCl2 13, CaCl2 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.

For the MDA assay, 10 μL of supernatant (10% tissue homogenate) was transferred to the cuvette for assay. The absorbance of the solutions for 0, 24 or 48 h at 4°C was measured at 550 nm using a UV spectrometer. Twenty microliters of 5 μL 5′ RT buffer, 2.5 μL olig d(T), 5 μmol/L deoxynucleotide triphosphates (dNTPs) and 20 U RNAasin (RNase inhibitor). The hexamers were annealed by 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-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/L 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-PCR to amplify GAPDH (forward: 5′-GGTGACCTCATGGGCTTACAT-3′, reverse: 5′-GCCCTTCTGTGCTACATTCA-3′), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in rT-PCR to amplify GAPDH (forward: 5′-GGTGACCTCATGGGCTTACAT-3′, reverse: 5′-GCCCTTCTGTGCTACATTCA-3′) as an internal control of CHOP (forward: 5′-CGAGGGTACCGACGACATCA-3′, reverse: 5′-CTGGTCTTCACATGGGCTTGTG-3′) and caspase-12 (forward: 5′-AGGAGGCGGCGGTCGTA TACAGA-3′, reverse: 5′-CTGGTCTTCACATGGGCTTGTG-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 of 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. The results were statistically analyzed using a t-test.
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, \(\bar{x} \pm s\))

<table>
<thead>
<tr>
<th>Groups</th>
<th>Number of positive cells (\text{mm}^2)</th>
<th>Optical density (\text{mm}^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celsior 0 h</td>
<td>1.92 ± 0.74</td>
<td>170.56 ± 3.46</td>
</tr>
<tr>
<td>DE 0 h</td>
<td>2.15 ± 0.54</td>
<td>169.78 ± 6.94</td>
</tr>
<tr>
<td>Celsior 24 h</td>
<td>6.06 ± 0.83</td>
<td>194.1 ± 4.36</td>
</tr>
<tr>
<td>DE 24 h</td>
<td>6.58 ± 1.12</td>
<td>197.09 ± 6.30</td>
</tr>
<tr>
<td>Celsior 48 h</td>
<td>4.50 ± 1.29</td>
<td>185.97 ± 6.23</td>
</tr>
<tr>
<td>DE 48 h</td>
<td>5.15 ± 0.89</td>
<td>189.45 ± 3.30</td>
</tr>
</tbody>
</table>

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 \((P < 0.05)\).

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 \((P < 0.05, \text{Figure 1})\) and the quantity of MDA was significantly lower in the DE groups compared with the Celsior groups at 24 and 48 h \((P < 0.05, \text{Figure 2})\).

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 \((P < 0.05, \text{Table 1, Figure 4})\). 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 \((P < 0.05, \text{Table 2, Figure 5})\).

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 48 h \((P < 0.01, \text{Tables 3 and 4})\), 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 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 pre-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 executor 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
...and Celsior 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 3. Comparison of CHOP mRNA levels using the comparative CT method (n = 8, X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>CHOP, average CT</th>
<th>GAPDH, average CT</th>
<th>(△△CT)</th>
<th>Fold difference CHOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celsior 0 h</td>
<td>34.06 ± 0.02</td>
<td>20.81 ± 0.06</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DE 0 h</td>
<td>34.07 ± 0.02</td>
<td>20.76 ± 0.04</td>
<td>0.02 ± 0.04</td>
<td>0.98 ± 0.03</td>
</tr>
<tr>
<td>Celsior 24 h</td>
<td>31.02 ± 0.9</td>
<td>21.37 ± 0.89</td>
<td>−3.63 ± 0.03</td>
<td>12.4 ± 10.28</td>
</tr>
<tr>
<td>Celsior 48 h</td>
<td>30.72 ± 0.57</td>
<td>21.21 ± 0.56</td>
<td>−3.77 ± 0.03</td>
<td>13.65 ± 0.3</td>
</tr>
<tr>
<td>DE 24 h</td>
<td>31.7 ± 0.47</td>
<td>21.64 ± 0.53</td>
<td>−3.22 ± 0.04</td>
<td>9.35 ± 0.25 **</td>
</tr>
<tr>
<td>DE 48 h</td>
<td>30.58 ± 0.03</td>
<td>20.78 ± 0.04</td>
<td>−3.48 ± 0.05</td>
<td>11.2 ± 0.04 ***</td>
</tr>
</tbody>
</table>

△CT, CHOP−GAPDH; △△CT, △CT−△CTCelsior 0 h; fold difference, 2△△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 CT method (n = 8, X ± s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Caspase-12, average CT</th>
<th>GAPDH, average CT</th>
<th>(△△CT)</th>
<th>Fold difference caspase-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Celsior 0 h</td>
<td>34.3 ± 0.2</td>
<td>20.82 ± 0.06</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>DE 0 h</td>
<td>34.3 ± 0.21</td>
<td>20.77 ± 0.05</td>
<td>0.06 ± 0.25</td>
<td>0.97 ± 0.17</td>
</tr>
<tr>
<td>Celsior 24 h</td>
<td>30.74 ± 0.6</td>
<td>20.74 ± 0.63</td>
<td>−3.47 ± 0.19</td>
<td>11.2 ± 1.58</td>
</tr>
<tr>
<td>Celsior 48 h</td>
<td>30.49 ± 0.41</td>
<td>20.81 ± 0.4</td>
<td>−3.79 ± 0.19</td>
<td>14.02 ± 1.98</td>
</tr>
<tr>
<td>DE 24 h</td>
<td>32.17 ± 0.47</td>
<td>21.78 ± 0.41</td>
<td>−3.08 ± 0.2</td>
<td>8.54 ± 1.23 **</td>
</tr>
<tr>
<td>DE 48 h</td>
<td>30.72 ± 0.03</td>
<td>20.79 ± 0.03</td>
<td>−3.54 ± 0.2</td>
<td>11.72 ± 1.68 **</td>
</tr>
</tbody>
</table>

△CT, caspase-12−GAPDH; △△CT, △CT−△CTCelsior 0 h; fold difference, 2△△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).

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 it controls peroxide generation. Because DE acts as an antioxidant by decreasing in its enzymatic activity has 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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Bone marrow mononuclear cells shift bioactive lipid pattern in injured kidney towards tissue repair in rats with unilateral ureteral obstruction

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Abstract

Background. Bioactive lipids are important in tissue injury and regeneration. Ceramide (Cer) is known for its pro-apoptotic action and sphingosine-1-phosphate (S1P) for inducing proliferation and cell survival; diacylglycerol (DAG) and lysophosphatidic acid (LPA) are involved in...