

# Protective effect of tanshinone IIA on rat kidneys during hypothermic preservation

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Received July 28, 2011; Accepted October 13, 2011

DOI: 10.3892/mmr.2011.639

**Abstract.** Renal preservation is a universal problem since ischemia/reperfusion (I/R) injury remains an unresolved issue during the procedure of renal transplantation. Tanshinone IIA, one of the effective components of the traditional Chinese medicine Danshen, was reported to exhibit a variety of biochemical activities, including protection against I/R injury. Therefore, identifying the specific molecular pathway mediating tanshinone IIA protection of renal preservation would be of great value to the patients concerned. In this study, rats were divided into two groups and the kidneys were isolated and preserved in two solutions separately, one with Celsior solution and the other with tanshinone IIA additionally added to the Celsior solution. The superoxide dismutase (SOD) activity and the quantity of malonaldehyde (MDA) were measured, the expression of CHOP and caspase-12 were assessed by immunohistochemistry staining, and real-time quantitative reverse transcription-polymerase chain reaction analysis was performed after 0, 24 and 48 h of preservation. A significant increase in the activities of SOD and a decrease in the quantity of MDA were observed in the kidneys preserved with tanshinone IIA at 24 and 48 h ( $P < 0.01$ ). The expression of CHOP and caspase-12 was lower in the kidneys preserved with tanshinone IIA at 24 and 48 h than that in the kidneys preserved with Celsior solution alone ( $P < 0.05$ ). The results suggest that the supplementation of tanshinone IIA in standard Celsior solution may significantly improve long-term kidney preservation. Attenuating oxidative stress injury and decreasing endoplasmic reticulum (ER) stress-mediated apoptosis may play a role in the protection of kidney hypothermic preservation.

## Introduction

Due to tremendous progress in immunosuppressive therapy and surgical techniques, renal transplantation is the gold

standard therapy for patients with end-stage renal disease (1). However, problems involving ischemia/reperfusion (I/R) damage remain unresolved since, to date, the procedure of renal transplantation requires the grafted organ to go through phases of cold preservation and warm reperfusion (2). It has been reported that the storage conditions of the donor kidney may affect the consequences of I/R injury (3), and the development of better organ preservation methods is a major target which has attracted scientific consideration.

Recently, increasing evidence suggests that endoplasmic reticulum (ER) stress may play a significant role in mediating allograft injury. Transplanted organs are challenged by various insults, including I/R injury, acute rejection episodes or inflammation, which could cause biological disturbances of ER homeostasis, thus leading to ER stress (4). Emadali *et al* reported that the expression of BiP, CHOP/GADD153 and GADD34, which are known to be induced specifically upon ER stress, was differentially affected upon I/R, thus suggesting that distinct ER stress responses were triggered during each phase of human liver transplantation (5). Xiang *et al* suggested that ER stress-induced apoptosis is involved in allogeneic T-cell apoptosis, which affects the cardiac allograft survival outcome (6). CHOP and caspase-12 as the index factors of the ER stress-mediated apoptosis pathway may be novel targets in the prevention and therapy of allograft injury. However, it has yet to be fully studied in the procedures of organ preservation.

Danshen (DS), a dried root of *Salvia miltiorrhiza*, is a traditional Chinese drug, which has proved to be an effective treatment for diseases such as stroke, myocardial infarction, occlusive vasculitis and atherosclerosis, and has been available as a standardized substance for decades in eastern Asia (7). Extensive studies have proven that tanshinone IIA is one of the most abundant components of DS, exhibiting a variety of biochemical activities, including vasorelaxation, elimination of oxygen-derived free radicals, stabilization of vascular endothelial function and protection against I/R injury (8). Investigators have reported that tanshinone IIA improves the protective effects of University of Wisconsin (UW) solution for skeletal muscle preservation by inhibiting ICAM-1 expression and apoptosis (9). In this study, tanshinone IIA was added to the standard hypothermia preservation solution in order to investigate a possible protective effect of tanshinone IIA during hypothermic preservation and explore the possible mechanisms.

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**Key words:** tanshinone IIA, kidney, hypothermic preservation, CHOP, caspase-12

## Materials and methods

**Animals.** A total of 48 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 NIH policies).

**Experimental groups.** A total of 48 male SD rats weighing 220–250 g were randomly divided into two groups: the Celsior group, in which the kidneys were stored in Celsior solution following perfusion, and the tanshinone IIA group, in which the kidneys were stored in Celsior solution containing tanshinone IIA (100  $\mu\text{mol/l}$ ; Zhejiang Institute for Drug Control) following 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 (NaOH 100 mM, KCl 15 mM,  $\text{MgCl}_2$  13 mM,  $\text{CaCl}_2$  0.25 mM, mannitol 60 mM, lactobionate 80 mM, histidine 30 mM, glutamate 20 mM; pH 7.4), or with a 4°C Celsior solution containing 30  $\mu\text{M}$  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.

**Superoxide dismutase (SOD) activity and malonaldehyde (MDA) level assay.** Following 0, 24 or 48 h at 4°C in different preservation solutions, the right kidneys from each rat were preserved in liquid nitrogen to measure the activity of SOD and the quantity of MDA using a UV spectrometer (Bio-Tek Instruments, Winooski, VT, USA). The kidneys were weighed, minced with scissors and homogenized in 10% tissue homogenate (homogenized for 3x10 sec intervals on ice). Tissue homogenate (5–10 ml of 10%) was centrifuged at 1,000 rpm 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 the SOD assay. A Coomassie Brilliant Blue kit (NanJin JinCheng ShenWu YanJiuSuo, NanJin, China) was used to measure the protein concentration.

The SOD assay kit (NanJin JinCheng ShenWu YanJiuSuo) used the xanthine oxidase method, and the xanthine oxidase assay used a compilation of methods from various sources. Xanthine oxidase catalyzes the oxidation of xanthine to uric acid and in the process generates  $\text{O}_2^-$ . The  $\text{O}_2^-$  production oxidizes hydroxylamine to nitrite, then nitrite generates a prunosus color production under the function of a specific chromogenic agent; the prunosus color production was followed spectrophotometrically, allowing for quantitative measurement. For the SOD assay, 20  $\mu\text{l}$  of supernatant (0.1 g/ml) was added to the reaction system, and this was incubated at 37°C for 40 min. Chromogenic agent (2 ml) was added and the mixture was incubated at room temperature for 10 min. The relative absorbance of the supernatants was immediately measured at 550 nm using a UV spectrometer.  $\text{ddH}_2\text{O}$  (20  $\mu\text{l}$ ) was used instead of supernatants as a negative control. A 1 mg prot of

the sample solution that established 50% inhibition was used to determine the SOD unit in an assay solution as 1 unit.

The MDA kit (NanJin JinCheng ShenWu YanJiuSuo) used thio-malonylurea methods. MDA was condensed to thibarbituric acid (TBA) to generate a red color production (a colorimetric reaction), which was followed spectrophotometrically, allowing for quantitative measurement. For the MDA assay, 10  $\mu\text{l}$  of supernatant (10% tissue homogenate) was added to the reagent of the MDA kit. This was incubated at 95°C for 40 min, centrifuged at 4,000 rpm for 10 min (4°C), cooled and the supernatant was transferred to the cuvette for assay. The absorbance of the supernatants was measured at 532 nm using a UV spectrometer. Alcohol (10  $\mu\text{l}$ ) was used instead of the supernatants as a negative control, and the standard substance (10 nmol/ml) from the MDA kit was used as a positive control.

**Immunohistochemistry assay.** Following 0, 24 or 48 h at 4°C in different preservation solutions, one-half of the left kidneys from each rat was preserved in 4% paraformaldehyde for 24 h, and the other half was put into liquid nitrogen for assay of real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). The tissues were embedded in paraffin, and transverse paraffin sections (5-mm) were mounted on silane-coated slides (ten slide series with ten sections per slide). Sections were deparaffinized and rehydrated. The 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 sections were washed in 0.01 M phosphate-buffered saline (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, Santa Cruz, CA, USA) and polyclone caspase-12 antibody (1:50; Santa Cruz Biotechnology) in PBS containing 1% bovine serum albumin, and washed in PBS (3x5 min), incubated in biotinylated goat-anti-rabbit IgG (1:200; Boster) in PBS for 2 h at room temperature, washed in PBS-T (3x5 min), incubated in avidin-biotin-peroxidase complex solution (ABC; 1:100; Boster) for 2 h at room temperature, then rinsed again in PBS-T (3x5 min). Immunolabeling was visualized with 0.05% DAB plus 0.3%  $\text{H}_2\text{O}_2$  in PBS. Following staining, the sections were counterstained by hematoxylin and then dehydrated with ethanol and xylene before coverslipping with Permount. Rat immunoglobulin IgG (1:200; Biomeda Corporation, USA) was used instead of the primary antibody as a negative control.

**Real-time quantitative RT-PCR analysis of CHOP and caspase-12.** Following 0, 24 or 48 h at 4°C in different preservation solutions, the remaining one-half of the left kidney was used for total RNA isolation in RT-PCR analysis, and RNA was isolated from the specimens using the TRIzol reagent kit (Invitrogen, USA) according to the manufacturer's instructions. For reverse transcription, RNA concentration was measured spectrophotometrically and 2  $\mu\text{g}$  total RNA was added to the cDNA synthesis reaction system (20  $\mu\text{l}$ ) on a FTC2000 (Funglynn, Canada). The reaction mixture consisted of 4  $\mu\text{l}$  5X RT-Buffer, 2.5  $\mu\text{mol/l}$  oligo d(T), 5 mmol/l dNTPs and 20 units RNAasin (RNase inhibitor). The hexamers were annealed by incubating the samples at 70°C for 5 min. M-MLV

reverse transcriptase 200 units (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 real-time RT-PCR, the reaction mixture (40  $\mu$ l) consisted of 4  $\mu$ l cDNA, 35.2  $\mu$ l SYBR<sup>®</sup> Premix Ex Taq<sup>™</sup> (Takara, China), 0.5  $\mu$ l 5 units Taq DNA polymerase and 0.3  $\mu$ l of 20 pmol/ $\mu$ l CHOP or caspase-12 primer (Invitrogen). The cDNA was denatured by heating at 94°C for 3 min. The template was amplified by 40 rounds of PCR (denaturation at 94°C for 10 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec) prior to measuring fluorescence at 72°C. Meanwhile, the primers used were: for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), in real-time RT-PCR to amplify GAPDH (forward: 5'-GGTGGACCTCATGGCCTACAT-3', reverse: 5'-GCCTCTCTTGTCTCTCAGTATCCT-3') as an internal control of CHOP (forward: 5'-CGGAGTGTA CCCAGCACCATCA-3', reverse: 5'-CCCTCTCCTTTGGTC TACCCTCA-3') and caspase-12 (forward: 5'-AGGGAT AGCCACTGCTGATACAGA-3', reverse: 5'-CTGTCTCCA CATGGGCCTTTTGT-3').

**Statistical analysis.** The sections were examined at x400 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 statistically significant difference. Data are presented as the means  $\pm$  SD. We made relative quantitation using the comparative C<sub>T</sub> method. The  $\Delta$ C<sub>T</sub> value was determined by subtracting the average GAPDH C<sub>T</sub> value from the average CHOP or caspase-12 C<sub>T</sub> value. The calculation of  $\Delta\Delta$ C<sub>T</sub> involved subtraction of all group's  $\Delta$ C<sub>T</sub> by the  $\Delta$ C<sub>T</sub><sup>control</sup> calibrator value. CHOP and caspase-12 mRNA level in all groups relative to the control was determined by calculating the formula: 2<sup>-( $\Delta\Delta$ C<sub>T</sub>)</sup>.

## Results

The activity of SOD and the quantity of MDA were measured. There was no significant difference between the Celsior group and tanshinone IIA group at the beginning. Following 24 and 48 h of preservation, the activity of SOD significantly increased and the quantity of MDA decreased in the tanshinone IIA group compared to the Celsior group (P<0.01, Table I).

The expression of CHOP and caspase-12 was assayed by immunohistochemistry (Figs. 1 and 2). Fig. 1 shows the expression of CHOP in the Celsior and tanshinone IIA groups. CHOP expression was not different between the Celsior and tanshinone IIA groups at 0 h. However, the number of CHOP-positive cells and the optical density were significantly decreased in the tanshinone IIA group following hypothermic preservation for 24 and 48 h (Table II). Fig. 2 shows the expression of caspase-12 in the Celsior and tanshinone IIA groups. Similarly to CHOP, the number of caspase-12-positive cells and the optical density were decreased in the tanshinone IIA group at 24 and 48 h of preservation compared to the Celsior group (Table II).

Table III shows the mRNA level of CHOP and caspase-12 assayed by real-time quantitative RT-PCR. The expression of CHOP and caspase-12 mRNA was not different between the

Table I. Activity of SOD and MDA in the rat kidneys at different time periods following hypothermic preservation.

Groups	MDA (nmol/mg.prot)	SOD (U/mg.prot)
Celsior 0 h	0.65 $\pm$ 0.13	250.21 $\pm$ 35.60
Tanshinone IIA 0 h	0.65 $\pm$ 0.12	255.41 $\pm$ 32.62
Celsior 24 h	1.32 $\pm$ 0.27	201.52 $\pm$ 28.11
Tanshinone IIA 24 h	1.09 $\pm$ 0.22 <sup>b</sup>	208.23 $\pm$ 29.25 <sup>a</sup>
Celsior 48 h	1.70 $\pm$ 0.25	185.36 $\pm$ 18.31
Tanshinone IIA 48 h	1.14 $\pm$ 0.26 <sup>c</sup>	204.57 $\pm$ 21.15 <sup>c</sup>

vs. the Celsior 24 h group, <sup>a</sup>P<0.01 and <sup>b</sup>P<0.01; vs. the Celsior 48 h group, <sup>c</sup>P<0.01.

Table II. Comparison of CHOP- and caspase-12-positive cells from the rat kidneys in all of the groups.

Groups	CHOP-positive cells (/mm <sup>2</sup> )	Caspase-12-positive cells (/mm <sup>2</sup> )
Celsior 0 h	3.58 $\pm$ 0.34	2.11 $\pm$ 0.23
Tanshinone IIA 0 h	3.56 $\pm$ 0.42	2.06 $\pm$ 0.25
Celsior 24 h	7.86 $\pm$ 1.43	5.86 $\pm$ 0.64
Tanshinone IIA 24 h	5.68 $\pm$ 1.42 <sup>a</sup>	4.26 $\pm$ 0.64 <sup>a</sup>
Celsior 48 h	8.96 $\pm$ 1.65	6.75 $\pm$ 1.20
Tanshinone IIA 48 h	6.95 $\pm$ 1.57 <sup>b</sup>	5.10 $\pm$ 0.95 <sup>b</sup>

vs. the Celsior 24 h group, <sup>a</sup>P<0.05; vs. the Celsior 48 h group, <sup>b</sup>P<0.05.

Celsior 0 h and the tanshinone IIA 0 h groups. CHOP and caspase-12 mRNA levels were up-regulated in the Celsior groups compared to the tanshinone IIA groups at 24 and 48 h, which was consistent with the results of the immunohistochemical staining.

## Discussion

Renal transplantation has been accepted as a successful therapeutic option for patients with end-stage renal failure. However, the persistent shortage of donor kidneys remains a universal problem due to the inclusion of older, marginal and non-heart-beating donor kidney sources in the donor pool at present (10). These kidneys are more susceptible to ischemic damage, leading to delayed graft function or primary non-function, as well as worse graft function and survival. Two storage approaches have been developed: cold static storage and machine pulsatile perfusion. Although hypothermic perfusion was reported to improve outcome following renal transplantations, static cold storage remains the most practiced preservation method in kidney transplantation (11).

The traditional preservation solution is a type of static cold crystalloid solution that contains impermeants to maintain osmoregulation and control edema, reactive oxygen species scavengers to prevent free radical damage, substrates to maintain energy and buffers to prevent acidosis, such as UW solution,

Table III. Relative quantification of CHOP and caspase-12 mRNA levels using the comparative  $C_T$  method followed by real-time quantitative RT-PCR.

Groups	$\Delta C_T$		Fold difference	
	CHOP	Caspase-12	CHOP	Caspase-12
Celsior 0 h	13.25	13.48	1.00	1.00
Tanshinone IIA 0 h	13.21	13.60	0.97	1.09
Celsior 24 h	9.65	9.92	12.12	11.79
Tanshinone IIA 24 h	10.04	10.30	9.25	9.06
Celsior 48 h	9.51	9.67	13.36	14.03
Tanshinone IIA 48 h	9.74	9.95	11.39	11.55

$(\Delta\Delta C_T) = \Delta C_T - \Delta C_{T(\text{control})}$ . Fold difference =  $2^{-(\Delta\Delta C_T)}$ .

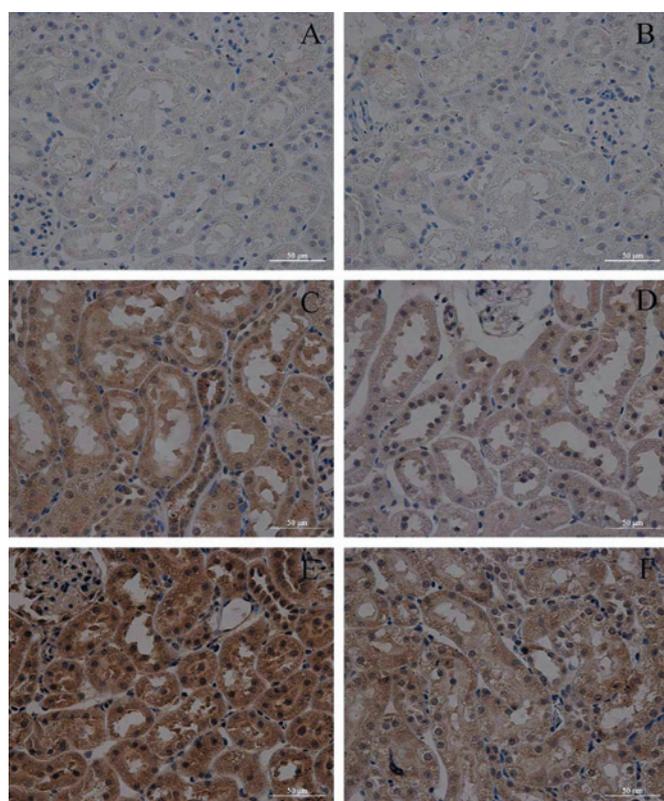


Figure 1. Expression of CHOP detected by immunohistochemistry in rat kidneys following different periods of hypothermic preservation (magnification,  $\times 400$ ). (A) Celsior 0 h group; (B) tanshinone IIA 0 h group; (C) Celsior 24 h group; (D) tanshinone IIA 24 h group; (E) Celsior 48 h group; (F) tanshinone IIA 48 h group. Yellow brown granules within the nucleus and cytoplasm were identified as positive CHOP protein staining. Bar, 50  $\mu\text{m}$ .

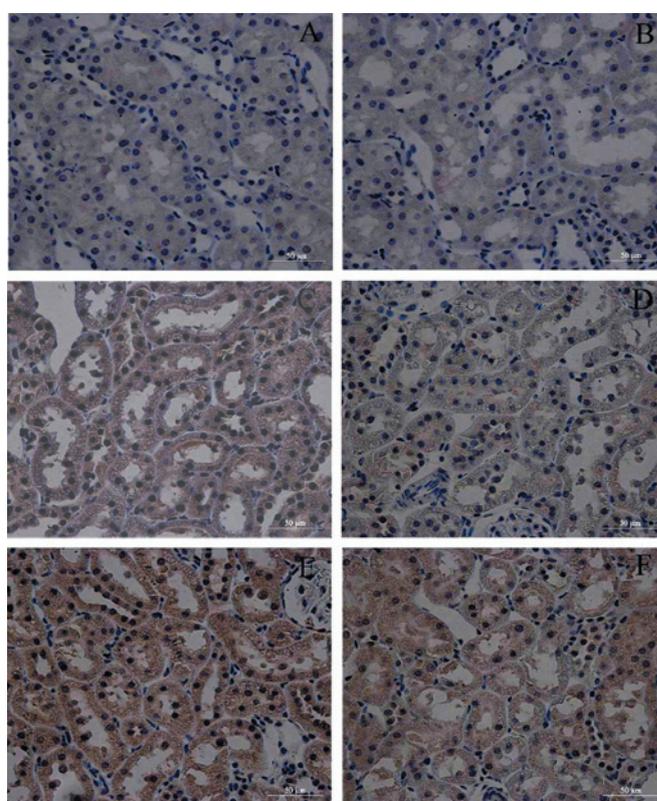


Figure 2. Expression of caspase-12 detected by immunohistochemistry in rat kidneys following different periods of hypothermic preservation (magnification,  $\times 400$ ). (A) Celsior 0 h group; (B) tanshinone IIA 0 h group; (C) Celsior 24 h group; (D) tanshinone IIA 24 h group; (E) Celsior 48 h group; (F) tanshinone IIA 48 h group. Yellow brown granules within the cytoplasm were identified as positive caspase-12 protein staining. Bar, 50  $\mu\text{m}$ .

which has become a gold standard (12). It has been reported that cold storage is a simple and effective method, which keeps the donor kidney stored for up to 24 h when the kidney is from a standard deceased donor (13). However, a long-time cold ischemia would be directly related to the severity of I/R injury following transplantation, particularly for allograft retrieved from extended criteria donors (14). I/R has been an inevitable event accompanying kidney transplantation, which may lead to a variety of cellular lesions, including disruption of cell polarization, disassembly of actin cytoskeleton organization,

disruption of tight junctions, necrosis and apoptosis (3). Recent evidence markedly suggests that I/R injury may initiate ER stress leading to cellular phenotypic alterations and death (15). ATP deficiency caused by hypoxia and glucose deprivation during ischemia initiates protein misfolding, thereby inducing the unfolded protein response (16). Reperfusion of the affected organ triggers oxidative stress with subsequent alterations of redox-dependent reactions, protein disulfide bond formation and protein folding. CHOP and caspase-12 are two distinct pro-apoptotic transcription factors induced

uniquely during ER stress. It has been reported that induction of CHOP could be detected between 4 and 8 h of exposure to ischemia, and pro-caspase-12 was also detectable in the same time frame (17). Minor *et al* showed significant ultrastructural alterations of ER and marked up-regulation of caspase-12 mRNA following cold storage or oxygenated machine perfusion of rat liver for 18 h, while mitochondrial appearance was unaffected, suggesting the ER to be an early subcellular target of preservation injury (18). Our previous study reported that ER stress was triggered during the hypothermic preservation, and reducing the levels of CHOP and caspase-12 may preserve the donor organ viability (19). Therefore, attempts to improve and prolong preservation conditions with the development of preservation solutions to minimize cold ischemic damage may be an alternative choice (20).

DS has been extensively used in traditional Chinese medicine for decades as an effective remedy for cerebrovascular disorders, angina pectoris and hypertension with only minor side effects (21). Recent medical research has found more than 15 types of chemical structures extracted from DS, and tanshinone IIA was the main effective components of anti-oxidative activity (22), adjustment of the metabolism of blood lipids (23), improvement of rheology, body microcirculation and activity of fibrinolysis (24). Investigators have reported that tanshinone IIA displayed significant protective effects on I/R injury in skeletal muscle preservation (9). In this study, we found that tanshinone IIA down-regulated the quantity of MDA and up-regulated the activity of SOD in preserved renal tissues, which revealed the anti-oxidant activity of tanshinone IIA. Moreover, our histological staining experiments revealed minor pathological destruction of renal tissue in the tanshinone IIA groups compared to the Celsior groups. It clearly showed that tanshinone IIA improved graft survival and reduced renal injury following hypothermic preservation. The mechanism by which tanshinone IIA prolonged kidney allograft survival remains to be determined.

In conclusion, the present study demonstrated that the supplement of tanshinone IIA in standard Celsior solution may significantly improve the long-term kidney preservation consequences by attenuating oxidative stress injury through up-regulation of SOD activity and down-regulation of MDA quantity, and may decrease cell apoptosis by reducing the expression levels of CHOP and caspase-12. The ER stress-mediated apoptosis pathway may be a novel target for long-time preservation of donor organs. Further efforts are required to explore the specific mechanism involved.

### Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 30971124) and the Zhejiang Provincial Natural Science Foundation of China (no. Y2090120).

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