Original Article
Prolonged warm ischemia aggravates hepatic mitochondria damage and apoptosis in DCD liver by regulating Ca^{2+}/CaM/CaMKII signaling pathway

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Abstract: This study was conducted to investigate the effect of warm ischemia duration on hepatocyte mitochondrial damage after liver transplantation, and confirm the role of CaMKIIγ in this process. Rat donation after cardiac death (DCD) liver transplantation model was established by exposing donor liver to 0 (W$_0$ group), 15 (W$_{15}$ group), and 30 (W$_{30}$ group) min warm ischemia. Some rats in W$_{15}$ group were transfected with CaMKIIγ and CaMKIIγ-shRNA lentivirus. On day 1, 3, and 7 post-transplantation, a series of experiments, including HE staining, TEM observation, ALT and AST measurement, flow cytometry analysis, qRT-PCR, and Western blotting were performed to evaluate the extent of hepatic and mitochondria damage. Within 7 days post-transplantation, prolonged ischemia led to an obvious deterioration of hepatic and mitochondria damage, presenting with a marked increase of apoptotic hepatocytes, ALT and AST levels, cells with low MMP, and AIF and Cyt C expression. CaMKIIγ overexpression caused the significant ultrastructural damage of hepatic cells, increase of cells with low MMP, enhancement of AIF and Cyt C expression, and augmented Ca^{2+}/CaM/CaMKIIγ, while blocking CaMKIIγ showed an opposite result. In conclusion, ischemia duration is proportional to the extent of hepatic mitochondria damage, and CaMKIIγ plays a negative regulatory role in this process by regulating the Ca^{2+}/CaM/CaMKII signaling pathway.

Keywords: Warm ischemia, mitochondria damage, DCD liver, Ca^{2+}/CaM/CaMKII

Introduction

Liver transplantation is an effective form of therapy for patients with end-stage liver diseases. However, the shortage of organ donors has become a bottleneck impeding liver transplantation development. Thus, liver donation after cardiac death (DCD) and donation after brain death (DBD) have become the important ways to acquire human organs. However, some problems resulting from the widespread use of DCD donor present gradually, that is, DCD donors inevitably undergo warm ischemia, cold preservation, and reperfusion injury, causing detrimental effect of prolonged warm-ischemia on the graft function. Ischemia-reperfusion (I/R) injury, the usual clinical practice for organ and tissue injury in liver transplantation surgery, is

an important indicator of liver function recovery after hepatectomy and transplantation [1]. Severe hepatic I/R injury could contribute to liver failure that is negatively associated with the prognosis of disease, success rate of operation, and survival rate of patients [2]. In early post transplantation, about 10% graft failure is caused by I/R injury [3]; meanwhile, the incidence of acute and chronic graft rejection after transplantation may also increase markedly [4]. Therefore, how to protect ischemic liver and reduce reperfusion-induced injury is an urgent problem to be solved in the medical field.

As the main productive organelle of eukaryotic cells, mitochondria are one of the target organelles in I/R injury. Upon the action of various injury factors, mitochondria undergo osmotic

displacement, then leads the related proteins between the mitochondrial bilayer membranes to go into the cytoplasm, and eventually initiates apoptosis [5, 6]. Previous studies observed severe mitochondrial damage in the liver model of I/R injury, manifesting as swelling mitochondria and mitochondrial cavity, a disorganized cristal body, and the loose matrix accompanied by varying degrees of vacuolation and mitochondrial membrane rupture, which has a high positive correlation with the increase of serum ALT and AST levels [7, 8]. Thus, the mitochondrial apoptosis pathway plays a key role in liver warm ischemia-reperfusion injury because mitochondrial dysfunction will significantly reduce the activity of transplanted organs [9].

Ca^{2+} is one of the first discovered factors involved in the progress of ischemic injury. The corresponding Ca^{2+} channels affect the severity of I/R injury by regulating the intracellular Ca^{2+} level [10]. Calmodulin (CaM), a major Ca^{2+} carrier protein in non-muscle cells, and its downstream signaling pathways were revealed to be implicated in the regeneration and proliferation of liver cells [11]. CaMKII (CaM kinase II) is an important member of the calmodulin-dependent kinases cascade family that is a class of proteins activated by the presence of Ca^{2+} and calmodulin. Kang *et al.* [12] reported that CaMKII plays a negative role in cardiomyocyte apoptosis in heart failure. *In vitro* in human macrophages, over expression of CaMKII serves as an independent factor to trigger macrophage death [13]. Similarly, CaMKII can also induce the apoptosis of mitochondria and cardiomyocytes by direct action on mitochondria in myocardial cells undergoing irreversible I/R injury, while the blocking of CaMKII causes the opposite result [14]. Considering this evidence, we speculate that the Ca^{2+}/CaM/CaMKII signaling pathway may play an important role in mitochondrial apoptosis.

At present, most of the studies involving hepatocyte damage post-liver transplantation concentrate more on oxygen radicals [15], antibody-dependent cell-mediated cytotoxicity (ADCC), and T cell-mediated apoptosis [16]. Whereas, the relationship between warm ischemia time (WIT) of donor liver and hepatocytes mitochondria damage, as well as Ca^{2+}/CaM/CaMKII signaling pathway has not been reported. In this study, to explore the mechanism of hepatic

I/R injury, we successfully established a rat model of DCD liver transplantation, and assessed the effect of the ischemia duration on liver function mitochondrial damage, and the related protein expression. Further, we also evaluated the potential role of CaMKIIγ in ischemia caused-hepatocyte mitochondria apoptosis after DCD orthotopic liver transplantation. Our study may provide a valuable theoretical basis for clinical application of DCD and a novel research point for improving the quality of liver donation.

Materials and methods

Animals

One hundred thirty-eight healthy Sprague-Dawley (SD) rats, aged 10-12 weeks, weight 250-280 g were obtained from Experimental Animal Center of Fudan University (Shanghai, China). Before formal experimentation, all animals were housed under standard laboratory conditions for 7 days. Animals were randomly divided into three groups: 0 min-ischemic group, namely sham-operated group (n = 36), 15 min-ischemic group (n = 66), and 30 min-ischemic group (n = 36). In 0 min-ischemic and 30 min-ischemic groups, half of the group was used as the donor and the other half as the recipient for liver transplantation. In 15 min-ischemic group, thirty rats were selected to as the recipient of lentiviral transfection. All experimental procedures were approved by the animal ethics committee of Kunming Medical University (Approval No.: KMMU2018015) and in accordance with the institutional guidelines for the Care and Use of Laboratory Animals (National Institutes of Health 2003).

Animal model of warm ischemia

After ether aspiration anesthesia, a midline laparotomy was performed in the supine position to expose the heart. All animals were intravenously injected with 1 ml of heparin sodium solution (25 U heparin sodium) by the dorsum of penis vein to heparinize the donor liver. Next, the basilar part of the heart was clamped to block the thoracic aorta for 15 and 30 min. Thus, a donor liver warm ischemia model was established. Sham-operated rats underwent the same protocol without clamping of the heart vessels. According to the duration of warm ischemia, the rats were randomly divided

into three groups: W0 (WIT: 0 min), W15 (WIT: 15 min), and W30 (WIT: 30 min).

Construction of lentiviral vector targeting CaMKIIγ

The recombinant lentivirus CaMKIIγ protein (LV-CaMKIIγ), lentivirus-encoding short hairpin RNA targeting CaMKIIγ (LV-CaMKIIγ-RNAi), and their corresponding empty vectors including LV-NC and RNAi-NC were constructed by Shanghai GenePharma Co. Ltd (Shanghai, China). The following sequences were used: 5'-CCAACTT-TGTGCCAACCGGTCGCCACCATGGCCACCACCG CCACCTGCAC-3' and 3'-AATGCCAACTCTGAGC-TTCTGCAGCGGTGCAGCAGGGGCTC-5' for LV-Ca-MKIIγ; 5'-GGAGAGCGTCAACCACATC-3' and 3'-GATGTGGTTGACGCTCT CC-5' for LV-CaMKIIγ-RNAi.

Lentiviral transfection

Prior to orthotopic liver transplantation, half of the rats in W_{15} group were randomly divided into five groups (6 rats in each group): 1) Control group: the rats were injected with 500 μl normal saline; 2) LV-NC group: the rats were injected with 500 μl saline containing 10^8 TU/ml LV-CaMKIIγ empty vector; 3) LV-CaMKIIγ group: the rats were injected with 500 μl saline containing 10^8 TU/ml LV-CaMKIIγ protein; 4) RNAi-NC group: the rats were injected with 500 μl saline containing 10^8 TU/ml RNAi empty vector; 5) CaMKIIγ-RNAi group: the rats were injected with 500 μl saline containing 10^8 TU/ml LV-CaMKIIγ-RNAi. All rats were injected with saline by the penile dorsal vein. After 6 h of administration, we detected the transfection efficiency by fluorescence analysis. Real-time PCR and western blotting were used to identify overexpression or knockdown efficiency of CaMKIIγ.

Animal model of liver transplantation

After warm ischemia duration, 20 mL lactic acid Ringer's solution (0-4°C, containing 50 u/mL heparin sodium) was perfused into the abdominal aorta by an infusion pump. Then, we dissected the liver ligaments and ligated the pyloric vein adjacent to the portal vein when the portal vein and hepatic proper artery were freed. After isolating the infra-hepatic inferior vena cava, the right suprarenal vein and right renal vein, the supra-hepatic inferior vena cava,

the hepatic artery, the portal vein, and the infra-hepatic inferior vena cava were all resected. The collected donor liver specimens were stored in 4°C Ringer's solution. Rat DCD liver transplantation surgery was performed according to the method reported by Kamada and Calne [17] with minor modification. In this study, the cold ischemia time was 50 min, and no animals died during the study. On days 1, 3, and 7 after liver transplantation, the rats in three groups were randomly executed and the hepatic tissues were collected for subsequent analysis.

Determination of liver function

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed by a 7170-A automatic analyzer (Hitachi, Tokyo, Japan) using commercially available kits (Boehringer Mannheim, Lewes, East Sussex, UK). Both values were showed as units/liter (U/L).

Liver morphological examination

Liver tissues were fixed in 10% neutral buffered formalin for 24 h, embedded in paraffin, cut into 3 μm thick sections, and then stained with hematoxylin-eosin (HE). For transmission electron microscopy, liver samples were fixed in 2.5% glutaraldehyde, embedded, sectioned, and then observed using an electron microscope (JEOL JEM-1400, Japan).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential was determined using JC-10 probe (Invitrogen, Eugene, OR, USA) that emits red-orange fluorescent from the cells with high mitochondrial potential (HMP) or green fluorescent light from the cells with low (LMM) and medium (MMM) mitochondrial potential. Samples were analyzed by flow cytometry excited at 488 nm, and measured at 590 nm. Data analysis was conducted using FlowJo software.

Measurement of Ca^{2+}

The total Ca^{2+} of hepatic tissues was determined using an atomic absorption spectrophotometer (FAAS; TAS-986, Persee; China) according to the manufacturer's instructions.

Cell apoptosis analysis

Briefly, the hepatic cells were collected and then transferred into a flow tube. Annexin V-FITC and Propidium Iodide (PI) were added to each sample and incubated in the dark for 15 min. Samples were analyzed using a flow cytometer (FACStar, BD Biosciences).

RT-qPCR

RNA was extracted from liver tissues using Trizol reagent (Life Technologies, Carlsbad, CA, USA). Quantitative real time-PCR was carried out as previously described [18]. The primer sequences were as follows: CaM, forward 5'-GACGGTAATGGCACAATC-3', reverse 5'-ACG-GAACGCTTCTCTAAT-3'; CaMKII, forward 5'-AC-ACAGGAATATGCTGCAAAAAT-3', reverse 5'-CAG-TAACAAGGTCAAACACGAGG-3'; AIF, forward TA-GAGAACAGCGAGTCCCGA, reverse GACCCGC-TTGTCTACGACTT; Cyt C (Cytochrome C), forward TGGACCCAATCTGCATGGTC, reverse TCC-AGGTACTCGAACAGGGT; β-actin, forward 5'-TG-GGTATGGAATCCTGTGGCA-3', reverse 5'-TGTTG-GCATAGAGGTCTTTACGG-3'. Gene expression levels were calculated as $2^{-\Delta\Delta Ct}$ values normalized to β-actin levels.

Western blot analysis

Protein extraction and western blotting were performed as previously described with minor modification [19]. Briefly, equal amounts of protein were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, Hercules, USA) and then transferred to a PVDF membrane (Millipore, Bedford, USA). After incubation with 5% non-fat milk in Tris-buffered saline (TBS), the membranes were probed with primary antibody (all antibodies were purchased from Abcam, Cambridge, UK) overnight at 4°C, and then with the corresponding secondary antibody conjugated with HRP (1:1000, Cell Signaling Technology, Danvers, MA, USA) at room temperature for 1 h. Finally, the membranes were detected using a multispectral imaging system (UVP, California, USA).

Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. Values are shown as mean ± standard deviation (SD). Two-tailed unpaired Student's *t*-test or one-way analysis of variance (ANOVA) is used to evaluate the significance between groups. P < 0.05 was considered significant.

Results

The duration of ischemia is proportional to the extent of hepatic damage

To evaluate the link between the duration of liver ischemia and hepatocellular injury in rats, H&E staining was first performed to observe the manifestation of hepatocellular damage and the results are shown in **Figure 1A**. At 1 d post transplantation, the non-ischemic group showed approximately normal morphology of liver tissues with intact liver tissues structure. In group W_{15}, hepatic damage was represented by slight hepatocyte swelling, necrosis, vacuoles, and neutrophil infiltration. These pathologic changes were dramatically aggravated in W_{30} group, with severe vacuolar degeneration necrosis in liver cells and infiltration of massive neutrophils. With prolongation of postoperative time, hepatic damage in all groups gradually deteriorated, represented by the hepatic sinusoid narrowing and edema, and derangement of extensive hepatocytes with turgescence. At 7 d post transplantation, livers in the 30-min ischemia group were entirely pale and had disorganized liver morphology.

In the results of the apoptosis assay, we observed that both W_{15} and W_{30} groups showed a significant increase of the proportion of apoptotic liver cells compared with W_0 group within 7 days post-transplantation (P < 0.05, P < 0.01), and the same trend was also found in the comparison of W_{15} and W_{30} groups (P < 0.05) (**Figure 1B**, **1C**). Also, warm ischemia also caused hepatotoxicity in liver tissues, as indicated by the elevation of the serum ALT and AST levels in the rats tolerating 15 and 30 min of ischemia at 1 d after surgery (P < 0.05) (**Figure 1D**, **1E**). The serum ALT and AST levels almost returned to normal levels in three groups at 3 d after transplantation and no significant difference was found, but they significantly increased again in livers in the 30-min ischemia group at 7 d post transplantation (P < 0.001) (**Figure 1D**, **1E**). This may be associated with the hepatic damage induced by reperfusion. The above results strongly confirmed that the extent of hepatocel-
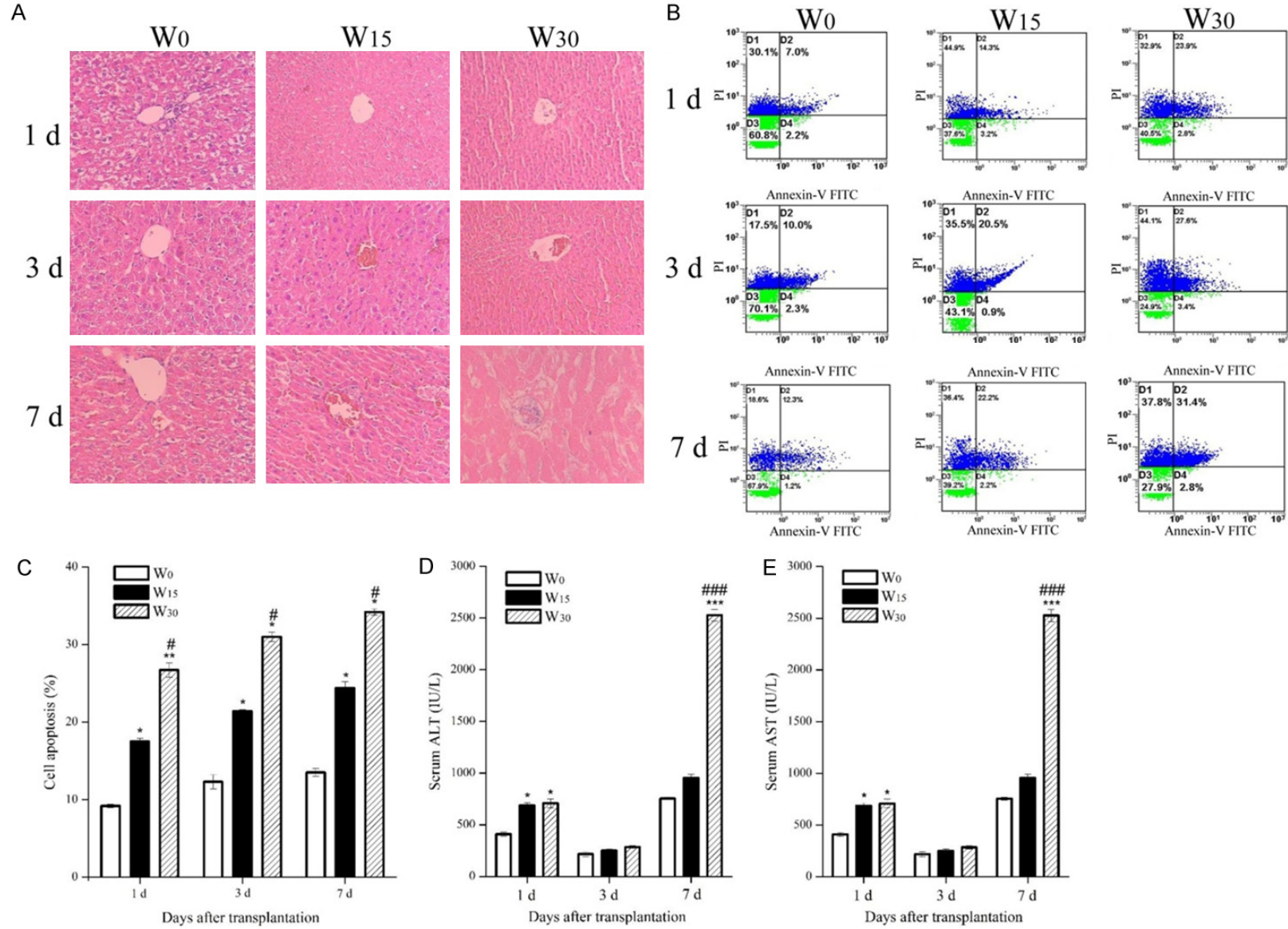
Figure 1. The duration of ischemia is proportional to the extent of hepatic damage. The rats were randomly divided into W_0, W_{15}, and W_{30} groups, and then respectively exposed to 0, 15, and 30 min-warm ischemia. At 1, 3, and 7 d post transplantation, the rats were sacrificed and the liver tissues were collected for analysis. A. Representative hematoxylin and eosin-stained liver sections (400 × magnification). B. Representative results of the cell apoptotic rate detected by flow cytometry. C. Statistical data of the apoptosis rate. D. Serum aspartate aminotransferase (AST) level. E. Serum alanine aminotransferase (ALT) level. *P < 0.05, **P < 0.01, ***P < 0.001 versus W_0 group; #P < 0.05, ##P < 0.01, ###P < 0.001 versus W_{15} group.
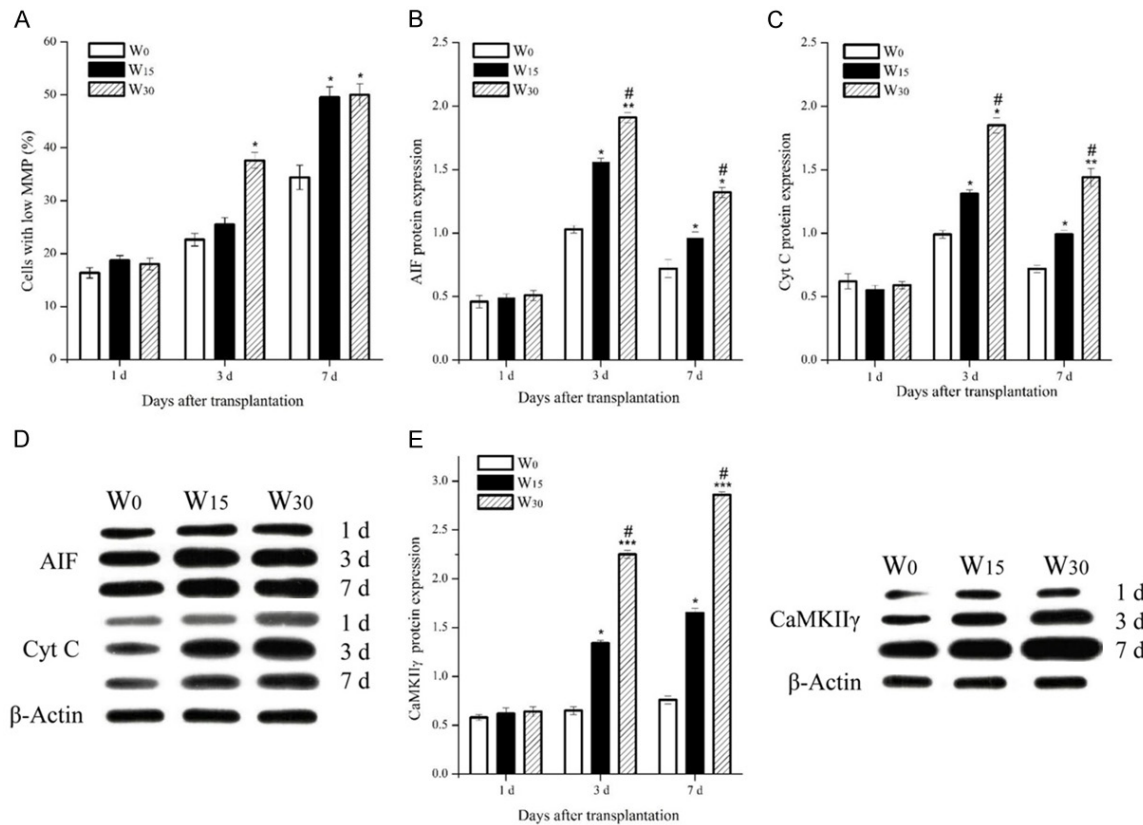

Figure 2. The duration of ischemia is proportional to the extent of mitochondrial apoptosis. The rats were randomly divided into W_0, W_{15}, and W_{30} groups, and then respectively exposed to 0, 15, and 30 min-warm ischemia. At 1, 3, and 7 d post transplantation, the rats were sacrificed and the liver tissues were collected for analysis. (A) The statistic data of the cells with low MMP in liver tissues detected by JC-10 staining. (B, C) Statistical data of the relative protein expression of AIF (B) and Cyt C (C). (D) The relative protein expression of AIF and Cyt C measured by western blotting. (E) The relative protein expression of CaMKIIγ measured by western blotting and the corresponding statistical data. *P < 0.05, **P < 0.01, ***P < 0.001 versus W_0 group; #P < 0.05, ##P < 0.01, ###P < 0.001 versus W_{15} group.

lular damage is proportional to the duration of ischemia.

The duration of ischemia is proportional to the extent of mitochondrial apoptosis

Next, we evaluated the effect of WIT on hepatic mitochondrial damage and apoptosis. As shown in **Figure 2A**, ischemia treatment contributed to a significantly increased number of the cells with lower MMP in the livers in W_{15} and W_{30} groups compared with the control non-ischemic

group at 3 or 7 d post transplantation (P < 0.05); meanwhile, the relative protein expression of AIF and Cyt C was also increased with the prolongation of ischemia implemented within 7 days after DCD orthotopic liver transplantation (P < 0.05, P < 0.01) (**Figure 2B**, **2C**). The above manifestations were attributed to mitochondrial apoptosis under the stimulation of ischemia. Previous evidence reported that CaMKIIγ was abnormally expressed in several injured tissues or cells induced by various risk factors [20, 21]. Thus, we assumed that warm
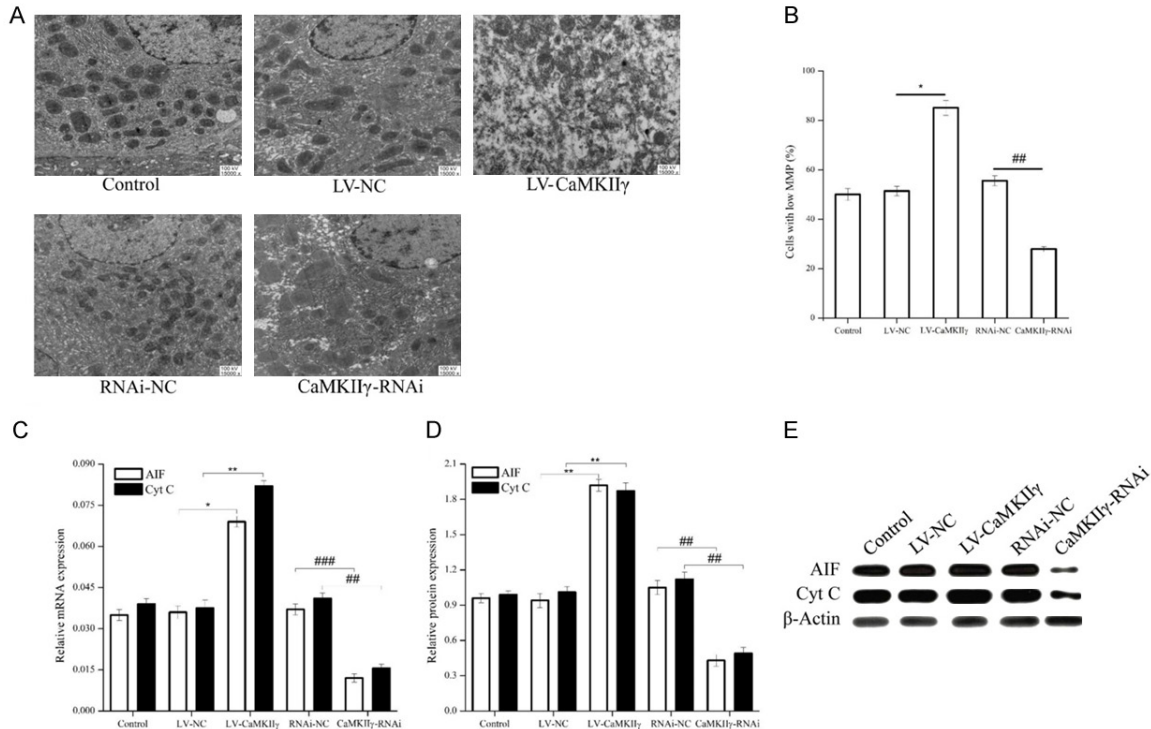
Figure 3. CaMKIIγ aggravates I/R-induced hepatic mitochondria apoptosis. Rats in W_{15} group were randomly divided into five groups and then respectively injected with saline, LV-CaMKIIγ empty vector, LV-CaMKIIγ protein, LV-CaMKIIγ-RNAi, and RNAi empty vector. On day 7 after liver transplantation, the rats in five groups were randomly executed and the hepatic tissues were collected for subsequent analysis. A. Ultrastructural changes of liver tissues determined by transmission electron microscopy (15000 × magnification). B. Statistical data of the cells with low MMP in liver tissues detected by JC-10 staining. C. Statistical data of the relative mRNA expression of AIF and Cyt C measured by RT-qPCR. D. Statistical data of the relative protein expression of AIF and Cyt C. E. Results of the relative protein expression of AIF and Cyt C tested by western blotting. D. E. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus LV-NC group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus RNAi-NC group.

ischemia may result in an aberrant expression of CaMKIIγ in the liver tissues. As expected, the protein expression of CaMKIIγ in rats exposed to 15 or 30 min ischemia was much higher than that in the W_0 group at 3 and 7 d after transplantation ($P < 0.05$, $P < 0.01$) (**Figure 2E**), confirming the positive association between WIT and CaMKIIγ level. Thus, it can be concluded that the duration of ischemia is proportional to the degree of mitochondrial apoptosis and the expression of CaMKIIγ.

CaMKIIγ aggravates I/R-induced hepatic mito-chondria apoptosis

In consideration of the significant increase in CaMKIIγ expression in the livers subjected to with warm ischemia, we assumed that CaMKII may be implicated in the process of I/R-induced mitochondrial injury. To identify this, we transferred CaMKIIγ-RNAi and CaMKIIγ overexpres-sion lentivirus into the rats exposed to 15 min-ischemia, and then evaluated mitochondrial damage at 7 d post transplantation. As shown in **Figure 3A**, ischemia only caused insignificant ultrastructural damage of liver tissues in control group, including slight hepatocyte edema and mitochondrial condensation, but the mito-chondria exhibited regular cristae and intact membrane without swelling. Similar changes were also observed in the LV-NC and RNAi-NC groups. However, the ultrastructural damage was much worse in the rats after transferred with CaMKIIγ, as shown by obvious mitochon-drial swelling and hepatocellular necrosis, me-mbrane rupture and atrophy, and the loss of mitochondrial cristae. When CaMKIIγ expres-sion was blocked, damaged morphology was improved markedly, in which the hepatic cells were normal and no swollen mitochondrial or hepatocytes were found.
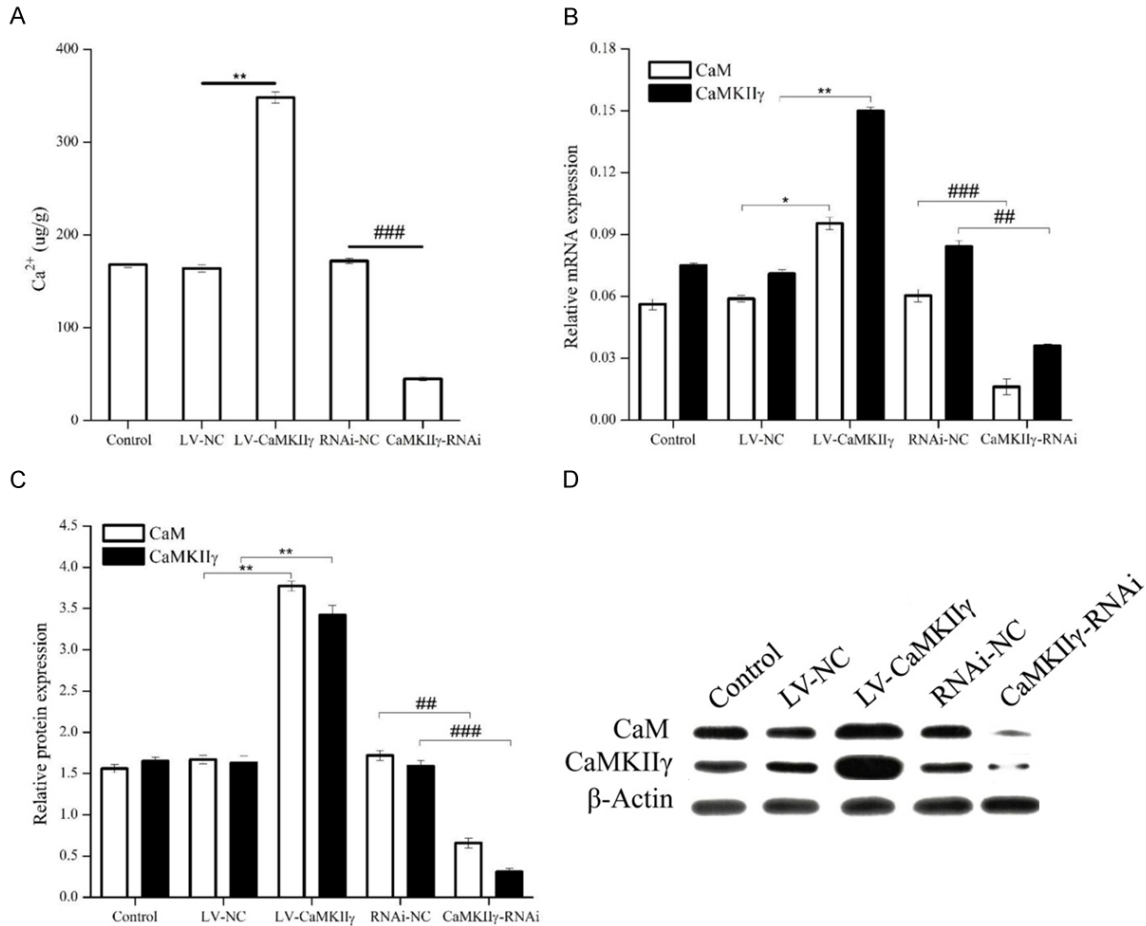
Figure 4. CaMKIIγ upregulates Ca²⁺/CaM/CaMKIIγ level in rat model of I/R injury. The rats in W_{15} group were randomly divided into five groups and then respectively injected with saline, LV-CaMKIIγ empty vector, LV-CaMKIIγ protein, LV-CaMKIIγ-RNAi, and RNAi empty vector. On day 7 after liver transplantation, the rats in five groups were randomly executed and the hepatic tissues were collected for subsequent analysis. A. The results of Ca²⁺ level detected by an atomic absorption spectrophotometer. B. Statistical data of the relative mRNA expression of CaM and CaMKIIγ measured by RT-qPCR. C. Statistical data of the relative protein expression of CaM and CaMKIIγ. D. Relative protein expression of CaM and CaMKIIγ measured by western blotting. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus LV-NC group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ versus RNAi-NC group.

Next, JC-10 staining was performed *in vitro*, for assessing mitochondrial damage. Control group exhibited relatively few cells with low MMP at 7 d after surgery, and there were no differences between control and empty vector groups. CaMKIIγ overexpression resulted in a significant increase of the percentage of the cells with low MMP relative to the empty vector group ($P < 0.05$), suggesting apoptosis in the mitochondria; whereas, CaMKIIγ knockdown caused an opposite result ($P < 0.01$) (**Figure 3B**). Similarly, AIF and Cyt C release was significantly enhanced by CaMKIIγ overexpression or reduced by CaMKIIγ-RNAi when compared with the corresponding negative control ($P <$

0.05, $P < 0.01$, $P < 0.001$), as confirmed by both RT-qPCR (**Figure 3C**) and western blotting (**Figure 3D**, **3E**) assays. Collectively, these findings revealed a destructive effect of CaMKIIγ on I/R-induced hepatic mitochondrial apoptosis post-orthotopic liver transplantation.

CaMKIIγ upregulates Ca²⁺/CaM/CaMKIIγ levels in rat model of I/R injury

To further explore the potential mechanism of CaMKIIγ involving I/R-induced mitochondrial injury, we then detected the changes of Ca²⁺/CaM/CaMKIIγ expression in different treatment groups. Clearly, there was an obvious increase of Ca²⁺ level in rats administered with CaMKIIγ

overexpression compared with empty vector group (P < 0.01); whereas, the Ca^{2+} level in CaMKIIγ-RNAi group was much lower than that in RNAi-NC group because of the downregulation of CaMKIIγ (P < 0.001). We also found that the relative expression of CaM and CaMKIIγ was upregulated markedly in the CaMKIIγ group and downregulated significantly in the CaMKIIγ-RNAi group when compared with their negative controls at both the mRNA (P < 0.05, P < 0.01, P < 0.001) (**Figure 4B**) and protein levels (P < 0.01, P < 0.001) (**Figure 4C**, **4D**). Combined with above and prior results, we can conclude that the overexpression of CaMKIIγ that aggravates I/R-induced mitochondrial injury in liver tissues may be achieved by upregulating of Ca^{2+}/CaM/CaMKIIγ.

Discussion

Orthotopic liver transplantation has been widely recognized as the only effective method for treating end-stage liver disease [22]. Currently, donor liver is one of the main sources of liver transplantation, and its global demand has been growing steadily over the past few years [23]. However, ischemia-reperfusion caused-liver injury has been identified as an important risk factor that determined the success rate of liver transplantation by affecting the prognosis of disease, success rate of operation, and survival rate of patients [2]. Therefore, to maintain the functional activity of donor liver and improve the success rate of liver transplantation, it is necessary to explore the effects of warm ischemia duration on transplanted liver, as well as identify the potential mechanism and related target molecules.

To evaluate the effect of warm ischemic time on hepatocellular damage, we first established a rat model of liver I/R injury by exposing the donor liver to 0, 15, and 30 min-ischemia respectively, and then assessed the extent of liver damage in rats on 1, 3, and 7 d post transplantation. The representative images of H & E staining revealed that the livers that tolerated 30-min ischemia showed the worst histomorphology, exhibited by a large amount of neutrophil infiltration and severe vacuolar degeneration necrosis. The morphology of liver tissues in W_{15} group was much better than the W_{30} group but worse than the W_0 group. With prolongation of postoperative time, hepatic damage in all

groups gradually deteriorated. It is well known that apoptosis is the primary pathogenesis of I/R injury [24], thus the Annexin V-FITC/PI double staining was subsequently performed to explore the apoptotic rate of hepatocytes. As expected, the percentage of apoptotic liver cells was also proportional to the duration of ischemia within 7 days after transplantation surgery, which further confirmed the observation of H&E staining. ALT and AST are the most useful indicators of liver function. Enhanced levels of ALT and AST indirectly reflect the degree of hepatocyte damage [25]. In this study, the serum ALT and AST improved rapidly at 1 d after operation and then almost returned to the normal level at 3 d, which was consistent with the previous finding [26] that showing the activities of ALT and AST increased markedly within a few hours after transplantation, then reached a peak within 24 h, and finally dropped rapidly. However, we also found that the levels of ALT and AST significantly increased again on 7 days, which may be caused by reperfusion induced-hepatic injury post-operation of liver transplantation.

Mitochondrial dysfunction, a key pathological process of I/R injury, can directly affect cell energy metabolism and induce necrosis or apoptosis [27, 28]. The decline in mitochondrial membrane potential is the most significant manifestation of damaged mitochondria. In our research, the proportion of cells with low MMP rose gradually with the increase of ischemic time within 7 days after orthotopic liver transplantation, and the difference was statistically significant, indicating a positive relationship between the extent of mitochondria apoptosis and the duration of warm ischemia. As for the mechanism of I/R induced mitochondria injury, a previous study revealed that ischemic treatment generally caused mitochondrial osmotic translocation through the action of risk factors, then caused the related proteins (including cytochrome C and apoptosis inducing factor, AIF) between the mitochondrial bilayer membrane to move into the cytoplasm [5], and finally initiated apoptosis [6]. Not surprisingly, the protein expression levels of AIF and Cyt C were found to be proportional to the duration of warm ischemia in this study. Similarly, we also observed a significant upregulation of CaMKIIγ expression when warm ischemia time was increased, which revealed that CaMKIIγ may be

one potential target molecule involved in I/R induced-mitochondria damage. Actually, CaM-KIIγ was found to be overexpressed in several injured cells caused by various wounding factors, such as in the brain tissues in rats with acute severe carbon monoxide poisoning [20] and in PC12 cells exposed to fluoride [21]; these studies partly agreed with our results.

Considering the abnormal upregulation of CaMKIIγ, we tested a hypothesis that CaMKII plays an important role in the process of mitochondrial apoptosis induced by I/R. To verify this, we overexpressed or down CaMKIIγ level in the rats exposed to 15 min-ischemia by transfecting CaMKIIγ overexpression and CaMKIIγ-RNAi lentiviral vectors. On 7 d post transplantation, CaMKIIγ overexpression caused a significant deterioration of ultrastructural morphology in liver tissues compared with the negative control, while blocking CaMKIIγ exhibited opposite effect. These findings preliminarily revealed the negative regulatory role of CaMKIIγ in I/R induced mitochondria injury, which is supported by several previous studies that revealing CaMKII regulates ischemia mediated-cardiomyocyte apoptosis and necrosis by directly inducing mitochondrial apoptosis [29, 30]. Membrane potential is involved in maintaining mitochondrial function and plays a crucial part in inhibiting hepatocyte apoptosis [31]. Previous research observed that the apoptotic rate of renal tubular epithelial cells induced by clindamycin in CaMKIIγ knockout rats was significantly lower than the control group. Similar results were also found in the loss of mitochondrial membrane potential and mitochondrial apoptosis [32, 33]. In our study, an obvious increased percentage of cells with low MMP was observed under the administration of CaMKIIγ, which directly suggested that CaMKIIγ can induce the permeability transition and the disruption of the mitochondrial membrane potential. In addition, the protein level of Cyt C and AIF was also up-regulated when CaMKIIγ was up-regulated, which reflected that CaMKIIγ upregulates the level of apoptosis-related proteins, such as Cyt C and AIF, by acting on the mitochondrial membrane, and then initiate apoptosis.

According to much evidence, we learned that Ca^{2+}/CaM/CaMKII signaling pathway was associated with cell damage or apoptosis triggered by many factors. For example, activation of the Ca^{2+}/CaM/CAMKII pathway could positively regulate high glucose-induced apoptosis in human umbilical vein endothelial cells [34], and also was confirmed to be implicated in myocardial ischemia induced-heart injury [35]. In this study, CaMKIIγ overexpression caused a significant increase of Ca^{2+} level, as well as the expression of CaM and CaMKIIγ at both the mRNA and protein levels, and an opposite result was found when CaMKIIγ was downregulated. The above observation indicated that the induction of mitochondrial damage by I/R may be achieved by activating Ca^{2+}/CaM/CaMKII signaling pathway, which is supported by the prior article that confirming the involvement of the Ca^{2+}/CaM/CaMKII pathway in oxidative stress-induced mitochondrial permeability transition and apoptosis in isolated rat hepatocytes [36].

Based on our results, the potential mechanism of hepatocyte apoptosis induced by warm ischemia can be tentatively summarized as follows: during DCD orthotopic liver transplantation, hepatocellular I/R injury contributes to the abnormal expression of CaMKIIγ. Upregulation of CaMKIIγ induces a decline and permeability shift of mitochondrial membrane potential, then leads to mitochondrial swelling, rupture, and apoptosis. Next, AIF and Cyt C proteins enter into the cytoplasm and initiate apoptosis, and eventually promote hepatocyte apoptosis. Considering the auxo-action of CaMKIIγ overexpression in ischemia induced-mitochondrial apoptosis, it can be inferred that specific blockage of the CaMKII signaling pathway post DCD liver transplantation may effectively relieve hepatocyte damage and improve prognosis.

Acknowledgements

Disclosure of conflict of interest

None.

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