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Effect of levocarnitine on cerebral ischemia-reperfusion rats *via* activating Nrf2/ARE signaling pathway

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Abstract. – OBJECTIVE: Levocarnitine plays a crucial role in the metabolism of organisms. The aim of this study was to explore the impact of Levocarnitine on cerebral ischemia-reperfusion (I/R) rats and the underlying mechanism.

MATERIALS AND METHODS: Cerebral I/R model was first successfully established. Two groups were set up, including drug group (I/R + Levocarnitine group) and control group (I/R group). The influences of Levocarnitine on brain injury and oxidative stress in cerebral I/R rats were evaluated. Furthermore, the impacts of Levocarnitine on the nuclear factor E2-related factor 2 (Nrf2)/antioxidant responsive element (ARE) signaling pathway and neuronal apoptosis in rats were detected.

RESULTS: Compared with I/R group, I/R + Levocarnitine group exhibited markedly lowered neurological deficit score and cerebral infarct volume. However, superoxide dismutase (SOD) and notably decreased malondialdehyde (MDA) were significantly up-regulated in I/R + Levocarnitine group. This suggested that Levocarnitine could relieve cerebral nerve injury and oxidative stress in cerebral I/R rats. Additionally, in I/R + Levocarnitine group, the protein expressions of Nrf2, heme oxygenase-1 (HO-1), and B-cell lymphoma 2 (Bcl-2) were significantly up-regulated, whereas cleaved Caspase-3 (c-Caspase-3) was notably down-regulated. Furthermore, neuronal apoptosis in cerebral I/R rats was remarkably inhibited.

CONCLUSIONS: Levocarnitine alleviates brain injury and neuronal apoptosis in cerebral I/R rats by activating the Nrf2/ARE signaling pathway.

Key Words:
Levocarnitine, Nrf2/ARE signaling pathway, Ischemia-reperfusion (I/R), Injury, Cell apoptosis.

Introduction

Cerebral ischemia is usually caused by cerebral arterial occlusion and lack of blood supply to the brain. Currently, the restoration of blood supply into brain tissues is an effective therapy. However, ischemia-reperfusion (I/R) can cause pathophysiological cascades, resulting in further injuries[1]. It has been proved that cerebral I/R is able to induce several injuries, including neuronal death and white matter injury[2,3]. Previous studies[4,5] have demonstrated that the mechanism of I/R is a complex mechanism, which may be related to oxidative stress, inflammatory infiltration, and cell apoptosis. The oxidative stress mechanism in I/R injury has gradually been uncovered in recent years. In this process, activated reactive oxygen species (ROS) induces oxidative stress and is regarded as a key factor[6]. In the case of I/R, ROS will activate various signaling pathways and amplify inflammations. However, other proteins can be activated, such as nuclear factor E2-related factor 2 (Nrf2). With the function of regulating environmental and oxidative stress[7,8], Nrf2 together with Kelch-like ECH-associated protein 1 (Keap1) are stored in the cytoplasm of normal resting cells *via* cytoskeleton-related specific inhibitory proteins[7]. After being activated by oxidative stress, cysteine residues in the Keap1 hinge region are modified and conformation changed. Therefore, the binding of Nrf2 is ineffective. Nrf2 can only bind to antioxidant responsive elements (AREs) in the nucleus, including heme oxygenase-1 (HO-1), superoxide dismutase (SOD), glutathione peroxidase GSH-Px, and NADPH. Eventually, all of the above molecules can antagonize ROS-induced oxidative stress[8].

As the main component of Levocarnitine, L-carnitine was first found in muscular tissues. Skeletal muscle tissue contains over 95% of the systemic carnitines, which are also enriched in other organs[9]. Thal et al[10] have found that L-carnitine plays a vital role in metabolism in organisms. According to the systematic evaluation and analysis of 13 control experiments in a clinical study, Levocarnitine can lower the mortality and morbidity rates of ventricular arrhythmias and angina. Trimethylamine oxide (TMAO) has been identified as a metabolite of L-carnitine, choline

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or betaine. Meanwhile, the correlation between plasma TMAO and the development of atherosclerosis has been elucidated in the latest clinical study[11] on humans. When not being absorbed by intestines, these precursors can be metabolized into trimethylamines (TMAs). They can be further absorbed by intestines into the circulation and oxidized by the FMO family[12]. The metabolite TMAO generated is subsequently discharged along with urea[13]. Tang et al[13] have demonstrated the relationship between the level of plasma TMAO and the progression of atherosclerosis. However, the possible underlying mechanism remains unclear.

In recent years, it has been proved that the Nrf2/ARE signaling pathway is important in visceral I/R injury. Therefore, the aim of this work was to investigate whether Levocarnitine could prevent cerebral I/R injury by activating the Nrf2/ARE signaling pathway.

Materials and Methods

Experimental Materials

The SOD detection kit was purchased from Sigma-Aldrich (St. Louis, MO, USA); cleaved Caspase-3 (c-Caspase-3), Caspase-3, and B-cell lymphoma 2 (Bcl-2) antibodies from Abcam (Cambridge, MA, USA); malondialdehyde (MDA) content determination kit from Solarbio (Beijing, China); reverse transcription (RT) kit from TaKaRa (TaKaRa Biotechnology Co., Ltd., Dalian, China) and annexin V-FITC/PI cell apoptosis detection kit from BioLegend (San Diego, CA, USA).

Establishment of Cerebral I/R Rat Model

The rats were anesthetized to separate the right common carotid artery. Subsequently, a fishing line with 0.26 mm in diameter was inserted into the internal carotid artery along the right common carotid artery until the origin of the middle cerebral artery was blocked. The penetration depth was about 18 mm. Next, the incision was sutured. The rats were subjected to a focal ischemia for 2 h. The fishing lines were pulled out 3, 6, 12, 24, or 48 h later, respectively. In sham operation group, the right carotid artery was separated without blocking the common carotid artery. The operations were performed at (22 ± 2)°C. The success of modeling was evaluated *via* neurological scoring. This research was approved by the Animal Ethics Committee of Linyi Central Hospital Animal Center.

Evaluation of Neurological Function

72 h after modeling, neurological function was evaluated based on the revised six-point scale. 0 point: no neurological deficit; 1 point: not fully extended contralateral forelimbs; 2 points: not extended contralateral forelimbs, 3 points: slight circular motion in the contralateral forelimbs, 4 points: evident circular motion in the contralateral forelimbs; 5 points: complete contralateral forelimb immobility. A higher score indicated a more severe nerve function impairment.

Measurement of Infarct Volume

After anesthesia, the rats were sacrificed and brain tissues were isolated on ice. Subsequently, brain tissues were cut into 5 parts, followed by staining with 2% trithienocyclotriyne (TTC) containing foil cover and incubation at 37°C for 15-30 min. This was to ensure that brain tissues evenly contacted dyeing solution. Finally, the images were acquired and analyzed using a digital camera.

Determination of SOD Content

Brain tissues were added with pre-cooled saline and homogenized in the icebox until no suspended solid was visible. Subsequently, the resulting tissues were centrifuged at 4°C and 5,000 rpm for 10 min. The supernatant was then taken for determining the content of SOD. All the operations were conducted according to the instructions of the kits.

Determination of MDA Level

At 24 h after reperfusion, the rats were executed to rapidly remove the brain tissues. The right cortical samples were weighed. Finally, the content of lipid peroxides was determined by the thiobarbituric acid method, representing MDA level.

Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA in tissues was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, extracted RNA was reverse transcribed into complementary DNA (cDNA) according to instructions of the M-MLV reverse transcriptase kit. The mRNA expressions of Nrf2 and HO-1 were detected using the SYBR PrimeScript™ RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) on the Roche LightCycler480 fluorescence quantitative PCR system. 3 replicates were set for each sample. The relative expression of miRNA was calculated by the $2^{-\Delta\Delta CT}$ method. Primers used in this study were as follows: Nrf2 primer: (sense)

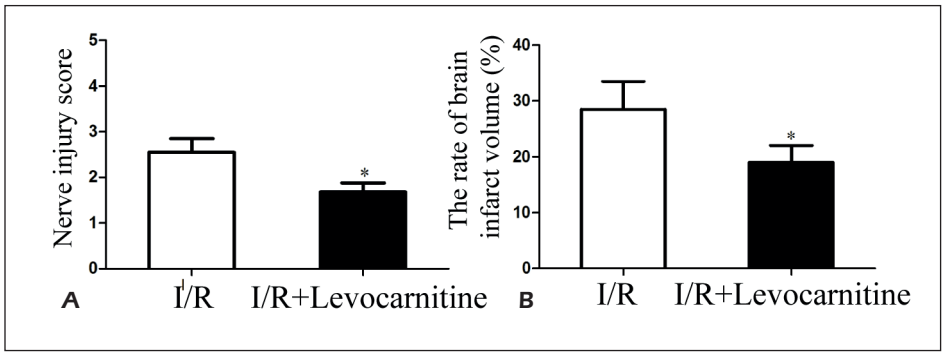

Figure 1. Influences of Levocarnitine on neurological deficit score and cerebral infarct volume in cerebral I/R rats. **A**, Impact of Levocarnitine on neurological deficit score in cerebral I/R rats (*p<0.05). **B**, Influence of Levocarnitine on cerebral infarct volume in cerebral I/R rats (*p<0.05).

ACACGGTCCACAGCTCATC and (antisense) TGCCTCCAAGTATGTCAATA-3, HO-1 primer: (sense) TCAGTCCCAAACGTCGCGGT and (antisense) GCTGTGCAGGTGTTGAGCC-3, and β-actin: (sense) GAAGTGTGACGTTGACATCCG and (antisense) TGCTGATCCACATCTGCTGGA.

Western Blotting

After brain tissues were taken, lysed and homogenized, the total proteins were extracted *via* bicinchoninic acid (BCA) assay (Abcam, Cambridge, MA, USA). Subsequently, protein samples were separated by dodecyl sulfate, sodium salt-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membranes were incubated with primary antibodies at 4°C overnight. After washing with Trisphosphate-Buffered Saline (TPBS) for three times, the membranes were incubated with the diluted horseradish peroxidase (HRP)-labeled secondary antibodies at room temperature for 2 h. Next, the membranes were washed again with Tris-Buffered Saline and Tween-20 (TPBS) solution for 3 times. Immuno-reactive bands were developed using super-sensitive enhanced chemiluminescence (ECL) reagent.

Cell Apoptosis

The influence of Levocarnitine treatment on neuronal apoptosis was analyzed using flow cytometry. Cells cultured under different conditions were first harvested. After rinsing using phosphate-buffered saline (PBS) and centrifugation, the cells were re-suspended in 0.5 μL of solution containing propidium iodide (PI) and RNases. Finally, cell apoptosis was detected *via* flow cytometry.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 12.0 software (SPSS, Chicago, IL, USA) was used to analyze the experimental data of nerve function impairment, infarct volume, as well as SOD and MDA activities. The results of RT-PCR and Western blotting were subjected to analysis of variance. p<0.05 was considered statistically significant.

Results

Levocarnitine Alleviated Brain Injury in Cerebral I/R Rats

The influence of Levocarnitine on brain injury in cerebral I/R rats was first explored. Two indicators, including neurological deficit score and cerebral infarct volume, were used to evaluate the injury. As shown in Figure 1A, I/R + Levocarnitine group showed substantially lowered neurological deficit score than I/R group [(1.88±0.22) points *vs.* (2.55±0.32) points, p<0.05]. This implied that Levocarnitine could alleviate brain injury in cerebral I/R rats.

Cerebral infarct volume is an important indicator indirectly reflecting nerve injury. According to the results of this study (Figure 1B), cerebral infarct volume was significantly reduced in I/R Levocarnitine group when compared with I/R group [(19.21±3.34)% *vs.* (28.84±5.10)%, p<0.05]. This suggested that Levocarnitine treatment decreased cerebral infarct volume in I/R rats.

Levocarnitine Reduced Oxidative Stress in Cerebral I/R Reperfusion

To further verify whether Levocarnitine relieved injury in cerebral I/R rats, the influence of Levocarnitine on the oxidative stress in rats was

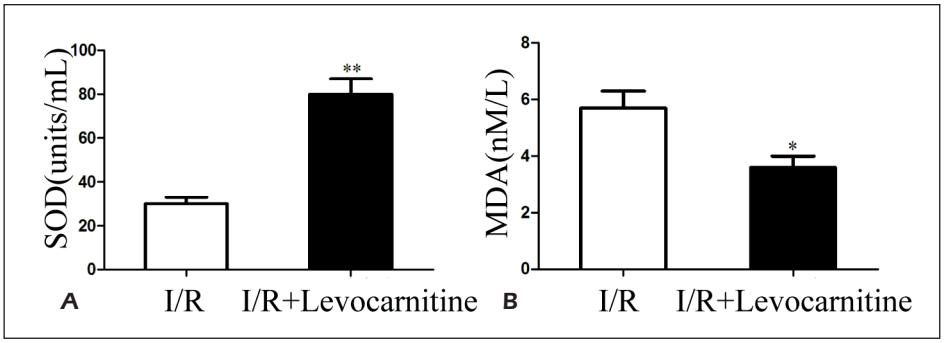

Figure 2. Influence of Levocarnitine on oxidative stress in cerebral I/R reperfusion. **A**, Influence of Levocarnitine on SOD content in the brains of cerebral I/R rats detected via enzyme-linked immunosorbent assay (ELISA) (**p<0.01). **B**, Impact of Levocarnitine on MDA content in the brains of cerebral I/R rats detected via ELISA (*p<0.05).

investigated. As shown in Figure 2A, the level of SOD in I/R + Levocarnitine group was significantly higher than that of I/R group [(80.8±7.12) U/mL *vs*. (30.5±3.2) U/mL, p<0.01], indicating that Levocarnitine up-regulated the level of SOD in cerebral I/R rats.

However, the level of MDA was markedly lower in I/R + Levocarnitine group than that of I/R group [(3.6±0.4) nM/L *vs*. (5.7±0.6) nM/L, p<0.05] (Figure 2B). This indicated that Levocarnitine could down-regulate the level of SOD in cerebral I/R rats.

Impact of Levocarnitine on the Nrf2/ARE Signaling Pathway

Previous reports have suggested that the Nrf2/ARE signaling pathway is remarkably changed in cerebral I/R. Therefore, the impact of Levocarnitine on the Nrf2/ARE signaling pathway was explored in our study. The influences of Levocarnitine on the expressions of Nrf2 and HO-1 were detected *via* RT-PCR and Western blotting, respectively. As shown in Figure 3A-C, compared with I/R group, the messenger ribonucleic acid (mRNA) and protein expressions of Nrf2 and

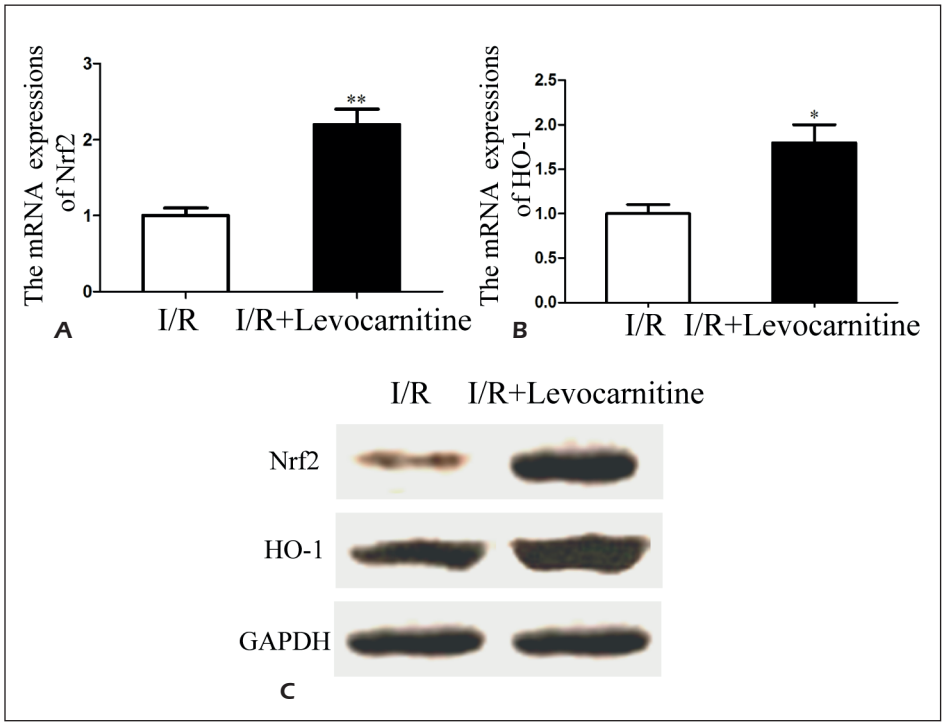

Figure 3. Influences of Levocarnitine on the expressions of Nrf2 and HO-1 detected via RT-PCR and Western blotting. **A-B**, MRNA expressions of Nrf2 and HO-1 detected via RT-PCR (*p<0.05, **p<0.01). **C**, Influences of Levocarnitine on the protein expressions of Nrf2 and HO-1 detected via Western blotting.

HO-1 were markedly up-regulated in I/R + Levocarnitine group ($p<0.05$).

Influence of Levocarnitine on Brain Injury in Cerebral I/R Rats

The impacts of Levocarnitine on the expressions of Bcl-2, Caspase-3, and c-Caspase-3 in neurons of cerebral I/R rats were finally detected *via* Western blotting. Meanwhile, the influence of Levocarnitine on neuronal apoptosis in rats were detected *via* flow cytometry. According to the results (Figure 4A-B), compared with I/R group, I/R + Levocarnitine group exhibited substantially up-regulated protein expression level of Bcl-2 ($p<0.01$), while the down-regulated protein expression level of c-Caspase-3 ($p<0.05$). However, no significant changes were observed in the protein level of Caspase-3. Flow cytometry results indicated that (Figure 4C) I/R + Levocarnitine group exhibited a significantly lower proportion of apoptotic cells than I/R group ($p<0.05$).

Discussion

We evaluated the protective effect of Levocarnitine on the cerebral I/R rat model. Our findings revealed the mechanism of Levocarnitine in alleviating cell apoptosis *via* the Nrf2/ARE signaling pathway.

According to our results compared with I/R group, Levocarnitine substantially relieved nerve function impairment and cerebral infarct volume, enhanced SOD activity, and lowered the MDA level. These results suggested that Levocarnitine reduced oxidative stress in I/R injury. Meanwhile, we found that Levocarnitine could up-regulate Bcl-2 expression and down-regulate c-Caspase-3 expression by activating the Nrf2/ARE signaling pathway. Ultimately, this might help to reduce neuronal apoptosis. A previous study[14] has demonstrated that the interaction of Nrf2 with PKC is more prominent in all the biochemical reactions involving Nrf2. The Nrf2/HO-1 signaling pathway is an important intracellular anti-oxidative stress pathway[15]. An excessive activation of this pathway exerts protective effects on ischemic tissue damage. The results of our study manifested that Nrf2 could catalyze hypoxic-ischemic brain damage in the model of cerebral I/R injury. Therefore, properly activating Nrf2 could improve the prognosis of the disease with certain clinical significance.

L-carnitine, a main component of Levocarnitine, was first found in muscular tissues. Skeletal muscle tissues contain over 95% of the systemic carnitines, which are also enriched in other organs[16]. L-carnitine serves as a vital player in the metabolism in organisms. Since free L-carnitine mainly forms acetyl-L-carnitine (ALC) and propionyl-L-carnitine (PLC) with its acyl group, carnitines have been applied in different clinical practices[17,18]. ALC passes through the thrombosis barrier and exerts beneficial effects on brain tissues and nerve cells in several diseases, such as neuropathy, depression, fatigue, and cerebropathy[18]. PLC shows high specificity for heart and skeletal muscles, which is widely applied in the treatment of heart diseases[18]. At the end of the 20th century, large numbers of researchers focused on the effect of L-carnitine on the prevention of I/R injury[19,20]. Currently, numerous studies have explored the protective role of L-carnitine in I/R-induced cardiac injury.

Oxidative stress has been found important in ischemic stroke, which is characterized by a significant increase in ROS[21]. In normal cells, the antioxidant system protects them from various oxidative stresses.

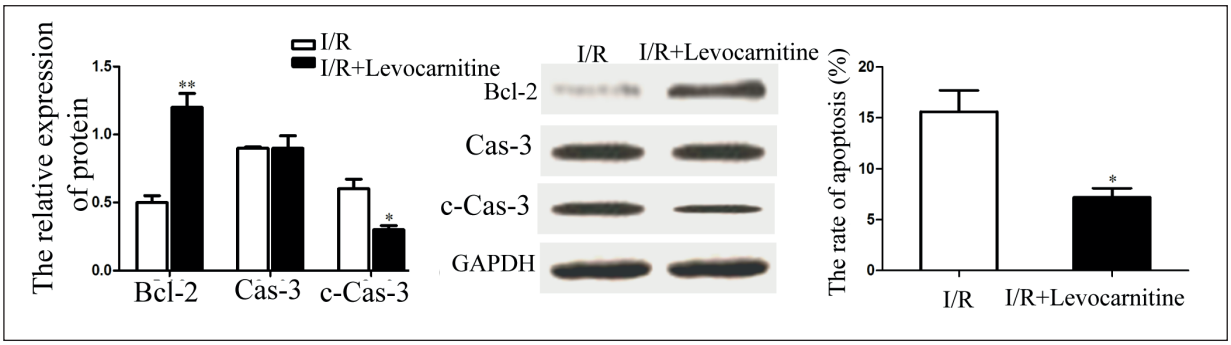

Figure 4. Impact of Levocarnitine on neuronal apoptosis in cerebral I/R rats. **A-B**, Influences of Levocarnitine on the expressions of Bcl-2, Caspase-3 and c-Caspase-3 in the neurons of cerebral I/R rats detected via Western blotting (*$p<0.05$, **$p<0.01$). **C**, Impact of Levocarnitine on neuronal apoptosis in cerebral I/R rats detected via flow cytometry.

Neuronal apoptosis is a major pathological process in ischemic stroke[22,23]. Toldo et al[24] have proved that inhibiting cell apoptosis can alleviate ischemic injury. Although two apoptotic pathways, namely extrinsic and intrinsic ones have been recognized, the final stage of cell apoptosis involves the activation of Caspases such as Caspase 3[25]. In the present work, Levocarnitine could reduce cerebral I/R-induced oxidative stress. Therefore, after I/R injury, the expression levels of both Nrf2 and HO-1 were significantly up-regulated. In addition, L-carnitine pretreatment remarkably up-regulated their expression levels.

Conclusions

We revealed for the first time the impact of Levocarnitine on I/R rats *via* the Nrf2/ARE signaling pathway. Levocarnitine could relieve brain injury and neuronal apoptosis in I/R rats by activating the Nrf2/ARE signaling pathway.

Conflict of Interests

The authors declared that they have no conflict of interests.

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