Ozone-oxidative-preconditioning alleviates hepatic ischemia-reperfusion injury in rats via PKC-ERK1/2 pathway

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Research

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ABSTRACT

BACKGROUND:
To explore the protective effect of ozone-oxidative-preconditioning (OzoneOP) through the protein kinase C (PKC), ERK1/2, and heat shock protein70 (HSP70) signal transduction in rats during hepatic ischemic-reperfusion (IR) injury.

METHODS:
We constructed an IR model by occluding all vessels of the rats’ left and median liver lobes for 45 min, followed by reperfusion for 3 h. Afterwards, we constructed the OzoneOP model via intraperitoneal injection of 1 mg · kg⁻¹ · d⁻¹ of 50 mg · L⁻¹ ozone for 5 days to investigate the significance of PKC, ERK1/2 and HSP70 signal
transduction in OzoneOP. The PKC inhibitor chelerythrine chloride (CHE), activator phorbol12-myristate13-acetate (PMA) and MEK inhibitor PD98059 were utilized to analyze the phosphorylation of PKC and the expression levels of ERK1/2 and HSP70. After ischemia and reperfusion, alanine aminotransferase (ALT) and aspartase aminotransferase (AST) were detected in the abdominal aorta blood. Meanwhile, the expression of HSP70 protein and the activities of PKC and ERK1/2 in the left hepatic lobe were analyzed, and the ultrastructure of the hepatic was observed.

RESULTS:

Compared with the control group, the phosphorylation of PKC and ERK1/2 and the expression of HSP70 were higher in the OzoneOP-treated model ($P<0.05$). Conversely, inhibiting PKC and ERK1/2 abolished the protection conferred by OzoneOP ($P<0.05$).

CONCLUSION:

OzoneOP significantly increased the expression of HSP70 by activating PKC and ERK1/2 signaling pathways, thus significantly alleviating hepatic IR injury in rats.

KEYWORDS: ERK1/2 MAPKs; HSP70; Liver ischemia-reperfusion; Ozone-oxidative-preconditioning; PKC
BACKGROUND

Hepatic ischemic reperfusion (IR) injury is one of the most common complications associated with liver transplantation, hepatic lobectomy, massive trauma, and hemorrhagic shock. It is unavoidable and usually leads to postoperative liver failure and mortality. However, the protective measures for hepatic IR injury are limited.

Heat shock protein (HSP) 70 is involved in the protection and regeneration of liver tissue following liver resection in rats, and high HSP70 expression can improve the survival[1, 2]. Furthermore, ischemic preconditioning can activate the protein kinase C (PKC)-mediated p44/42 mitogen-activated protein kinase (MAPKs) signaling pathway and increase the expression of HSP70[3]. Moreover, ozone-oxidative-preconditioning (OzoneOP) could alleviate hepatic IR injury in rats through HSP70[4]. It is still unclear whether OzoneOP can alleviate hepatic IR injury in rats by activating the PKC-mediated ERK1/2 signaling pathway. Thus, we designed the following experiments to confirm this hypothesis.

MATERIAL AND METHODS

Grouping and experimental protocol

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Fujian Medical University.

Specific-pathogen-free (SPF) male Sprague-Dawley (SD) (8 weeks old, weighing 250–300g) were obtained from Shanghai Slack Laboratory Animal Co., Ltd. (Animal Certificate: SCXK (Shanghai) 2017-0005) were kept in the animal room of the Fujian Medical University. Animals were housed in wire mesh floor cages under 12h light/ dark cycles, a controlled temperature of 22-24 °C and a relative humidity of 60 ± 10%.

Animals were fasted for 12 hours before surgery, free to drink water, anesthesia by 3% sodium pentobarbital (35mg· kg⁻¹) through intraperitoneal injection (i.p.), and
then fixed on a small animal operating table, continuous monitoring of rat anal
temperature and pulse oximetry. The heated bulb and blankets were used to maintain
the rat’s body temperature at 36 ± 1 °C. After disinfection, 20G venous indwelling
needle for tracheal intubation and connect to a small animal ventilator (respiratory
frequency 60-70 beats / min, suction ratio 1:1.5).

The rats were randomly divided into six groups (10 rats in each group) and
subjected to the following experimental protocols.

(1) S group (sham group: neither IR nor OzoneOP): 5 mL of normal saline was
slowly injected through dorsal veins of subjects’ penis for 10 min, after that the liver
hilus was exposed, but not occluded.

(2) IR group (ischemic reperfusion): The vessels leading to the left and median
liver lobes were occluded for 45 min with a vascular clamp after the same as S group.
Then the clamp was removed and blood flow was reperfused for 3 h.

(3) O₃ group (both OzoneOP and IR): To induce OzoneOP, animals were given
50 mg·L⁻¹ ozone which create by the zoned Basic Ozone Therapy Apparatus, 1
mg·kg⁻¹·d⁻¹ for 5 days by i.p. Then the same as IR group.

(4) O₃+PD98059 group: 5 mL PD98059 (SIGMA P215) (5 mg·kg⁻¹)[3] was
repeat normal saline, then the same as O₃ group.

(5) O₃+CHE group: 5 mL chelerythrine chloride (CHE: MERCK 220285) (5
mg·kg⁻¹)[3] was repeat normal saline, then the same as O₃ group.

(6) IR+PMA group: 5 mL phorbol 12-myristate 13-acetate (PMA: SIGMA
P1585) (4 ug · kg⁻¹)[3] was repeat normal saline, then the same as IR group.

Measurement of serum ALT and AST

Three hours after the reperfusion, the abdomen of each group was re-opened. Blood
samples of abdominal aorta were obtained. After standing at room temperature for
30-60 minutes, 4°C, 3000 rpm, centrifugation for 15 minutes, serum was taken and
Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) were
detected by an automatic biochemical analyzer.

**Western blotting analysis of HSP70, ERK1/2 MAPKs, PKC**

The subjects were euthanized 3 hours after the reperfusion. Tissue samples (0.3cm × 0.3cm × 0.3cm) were rapidly taken from left liver lobe. After extracted from hepatocytes, protein samples were separated on SDS-polyacrylamide gel (100 g·L⁻¹) and transferred to nitrocellulose membranes. After membranes were transferred, they were blocked for 1 hour with 50mL·L⁻¹ of nonfat milk in Tris-buffered saline. Membranes were randomly divided into three groups, each group was separately incubated with murine monoclonal antibody to HSP70 (ABCAM America), phosphorylated ERK1/ERK2 MAPK Rabbit monoclonal antibody (CST America) and antibody to PKC (ABCAM America). Membranes were kept overnight at 4 °C, and washed 3 times for 5 minutes in TBST before addition of goat anti-mouse-HRP conjugated secondary antibody for 1 hour at room temperature. Membranes were washed 3 times for 5 minutes with TBS, and peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by the ECL Western blotting detection system.

**Statistical analysis**

SPSS 22.0 statistical software was used for all statistical analyses of this study. The measurement data were expressed as mean ± standard deviation (mean±SD). Comparison between groups was performed using analysis of variance, and two pairs of comparison were using SNK-q test; when the variance was not uniform, comparisons were performed using rank sum test. $P<0.05$ for the difference was considered statistically significant.

**RESULTS**

1. **ALT and AST**: Compared with the S group, the serum concentrations of ALT and AST were significantly higher than those in the other groups ($P<0.05$). Compared with the IR group, the ALT and AST levels were markedly higher than those in the other groups ($P<0.001$). Moreover, the ALT and AST levels of $O_3$+CHE and
O₃+PD98059 groups were significantly higher than those in the O₃ group (P<0.05). (Table. 1)

Table 1: Changes in serum concentration of ALT and AST (n=10, mean±SD)

<table>
<thead>
<tr>
<th>GROUP</th>
<th>ALT(U/L)</th>
<th>ALT(U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>60.67±6.50</td>
<td>313.50±36.07</td>
</tr>
<tr>
<td>IR</td>
<td>1445.68±154.41*</td>
<td>2523.02±204.24*</td>
</tr>
<tr>
<td>O₃</td>
<td>251.33±36.87##</td>
<td>646.50±95.92##</td>
</tr>
<tr>
<td>O₃+PD98059</td>
<td>481±147.12*##&amp;</td>
<td>965.00±116.29*##&amp;</td>
</tr>
<tr>
<td>O₃+CHE</td>
<td>770.00±50.17*##&amp;</td>
<td>2118.99±122.86*##&amp;</td>
</tr>
<tr>
<td>IR+PMA</td>
<td>897.17±83.47##</td>
<td>1442.83±105.90##</td>
</tr>
</tbody>
</table>

*P<0.05 versus S group, #P<0.001 versus IR, &P<0.05 versus O₃

2. **HSP70**: The expression of HSP70 in the O₃, O₃+PD98059, O₃+CHE and IR+PMA groups was significantly higher than those in the IR group (P<0.001) and the S group (P<0.01). The expression of HSP70 in the O₃ group was significantly higher than those in the O₃+PD98059 (P<0.05) and O₃+CHE groups (P<0.001). (Figure. 1)

Figure 1: Expression of HSP70 in rat liver (n=10, mean±SD)

*P<0.01 versus S group, #P<0.001 versus IR group, &P<0.05 versus O₃ group

3. **PKC77/40**: PKC activity levels in the IR group was significantly higher than those
in the S group \((P<0.001)\). PKC activity levels in the \(O_3\), \(O_3+PD98059\), \(O_3+CHE\) and IR+PMA groups was obviously higher than those in the IR group \((P<0.001)\). Moreover, PKC activity levels in the \(O_3+PD98059\) and \(O_3+CHE\) groups was significantly lower than those in the \(O_3\) group \((P<0.01)\). (Figure. 2)

Figure 2: Expression of PKC in rat liver \((n=10,\text{mean±SD})\)

*\(P<0.001\) versus S group, \#\(P<0.001\) versus IR group, \&\(P<0.01\) versus \(O_3\) group

4. **p-MAPK44/42**: The p-MAPK44/42 activity levels in the IR group was markedly higher than those in the S group \((P<0.001)\). Moreover, the p-MAPK44/42 activity levels in the \(O_3\), \(O_3+PD98059\), \(O_3+CHE\), IR+PMA groups was significantly higher than those in the IR group \((P<0.01)\). The p-MAPK44/42 activity levels in the \(O_3+PD98059\) and \(O_3+CHE\) groups was significantly lower than those in the \(O_3\) group \((P<0.001)\). (Figure. 3)

Figure 3: Expression of ERK1/2 MAPKs in rat liver \((n=10,\text{mean±SD})\)

*\(P<0.001\) versus S group, \#\(P<0.01\) versus IR group, \&\(P<0.001\) versus \(O_3\) group
DISCUSSION

Our study showed that OzoneOP can markedly reduce the levels of serum ALT and AST following hepatic IR in the rats, and significantly alleviate pathological damage. The expression levels of HSP70, PKC and ERK1/2 MAPKs in hepatic IR model in the rats pretreated with OzoneOP were significantly upregulated. Moreover, when PKC inhibitor CHE and ERK1/2 blocker PD98059 were used, the effect of OzoneOP was obviously weakened.

We established the model of hepatic IR with reference to literature[5]. The subjective index to judge the success of our hepatic ischemia model was that the color of liver changed from bright red to yellowish within 0.5 seconds after clamping the non-invasive vascular clamp, and the color of liver changed from yellowish to bright red within 0.5 seconds after loosening the vascular clamp. The ALT and AST levels were used as objective indicators to judge the our success of liver ischemia model because they are reliable indicators of early acute liver injury.

In this study, the ALT and AST levels in the IR group was significantly higher than those in the S group ($P<0.001$), thus indicating that IR caused acute liver damage in rats. Further, ALT and AST levels in the O$_3$ group were significantly lower than those in the IR group ($P<0.001$), thus indicating that OzoneOP can significantly alleviate acute liver damage in rats. According to our previous studies, OzoneOP has an important role in limiting or downregulating liver IR injury[3, 6].

In this study, we reduce the influence of body temperature changes on the experimental results by placed the rats on blankets during ischemia and reperfusion periods and maintain an anal temperature of 36 °C±1 °C because of the HSP70 concentration increased significantly during liver damage or stress[7]. Our results indicate that OzoneOP can significantly induce HSP70 expression which is consistent with previous studies that hyperbaric oxygen pretreatment and ozone can induce biosynthesis of HSP70[8, 9]. Moreover, our study demonstrate that the higher the expression level of HSP70, the lighter the liver ischemia-reperfusion injury in rats.
The addition of this result corroborates previous studies which had shown that HSP70-expressing cells can survive even under lethal conditions[10] because of HSP70 is a non-specific cytoprotective protein with anti-inflammatory, anti-oxidative and anti-apoptotic effects[11, 12].

In this study, the phosphorylation of ERK1/2 in the liver tissues of rats were pretreated with OzoneOP were significantly increased, serum ALT and AST levels were significantly decreased, and HSP70 expression was also significantly increased. When PD98059 was used the phosphorylation of ERK1/2 was reduced correspondingly, and serum ALT and AST levels were also increased. Moreover, HSP70 expression was decreased accordingly. These suggest that the protective effect of OzoneOP was weakened and HSP70 expression was negatively correlated with liver injury. These suggest that OzoneOP can activate ERK1/2, and then enhance HSP70 transcription and expression. However, the effects were markedly decreased when PD98059 was used because it counteracted some effects of OzoneOP.

In this study, upon administration of the PKC activator PMA (PMA simulates DAG to activate conventional and new PKCs[13]), the PKC and ERK1/2 signaling pathways were activated and HSP70 expression was significantly increased; whereas upon administration of the PKC inhibitor CHE (CHE can reduce cell proliferation and upregulate cell apoptosis[14]), the activation of PKC was blocked, and the ERK1/2 MAPKs pathway was also blocked. Furthermore, the decreased HSP70 expression suggested that PKC mediates ERK1/2 signaling pathway and participates in the protection of hepatic IR, and this findings is consistent with those of Tang et al.[15] and Peng P et al.[16]. That is, after PKC activation, via Ras/Raf/MEK, the cascade reaction of MAPK increases and activates ERK1/2 step by step, thereby regulating the increase of protective protein expression and producing cytoprotective effects[17]. Previous studies have shown that PKC signaling pathways are involved in regulation of cell growth, metabolism, proliferation, differentiation, and apoptosis[18]. Furthermore, ERK1/2 is related to cell survival and death, proliferation and differentiation[19]. And it is an anti-apoptotic kinase of the MAPK family and has
anti-apoptotic effects[20, 21]. Thus, inhibiting ERK can increase cell apoptosis and activating ERK can promote the phosphorylation of cytoplasmic target proteins or regulate other protein kinase activities, then promote phosphorylation of various transcription factors, and then enhance transcriptional activity[22, 23]. Studies have showed that HSP70 is directly or indirectly involved in the protection and regeneration of rat liver tissue, and the increased HSP70 expression can significantly improve the survival rate of rats [1, 2]. Therefore, OzoneOP regulates the expression of HSP70 through PKC mediated ERK1/2 signaling pathway by protecting the rat liver from IR injury, and OzoneOP may become a future therapeutic target for liver IR injury.

This study is limited in that it only examined changes in the liver after 45 minutes of ischemia and 3 hours of reperfusion, and no dynamic monitoring of multiple time points was performed. Further, we only concentrated on the PKC and ERK1/2 pathways. Thus, whether other signaling pathways are involved in OzoneOP hepatic IR injury is unclear. Additionally, the mechanism of OzoneOP is complicated, and the protective effect of HSP70 synthesis induced by OzoneOP on hepatic IR injury may be only one aspect of its protective mechanism. Therefore, the exact mechanism and clinical application require further research. However, this is an animal study, and therefore clinical evaluation is required to evaluate responses in humans. Furthermore, the hepatic protective effect of OzoneOP provides new insights for research and clinical prevention of liver IR injury.

In summary, we found that OzoneOP can alleviate the hepatic IR injury in rats. Moreover, we also found that OzoneOP regulates the expression of HSP70 through PKC mediated ERK1/2 signaling pathway by protecting the rat liver from IR injury. Furthermore, Our study may provides evidence that OzoneOP may be a potent candidate for the treatment of hepatic IR.

CONCLUSION

OzoneOP can significantly reduce hepatic IR in rat through activating PKC and
ERK1 / 2 signaling pathways to cause high expression of HSP70.

Declarations

Ethics approval and consent to participate

Our studies was approved by the Animal Protection and Use Committee of Fujian Medical University.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Zhenyun Tao, Zutai Jiang, Weixin Zhong, Lianghui Li performed the ozone pretreatment and the liver ischemia reperfusion on the rats. Zhenyun Tao and Zutai Jiang performed histological examination of the liver. Zhenyun Tao, Zutai Jiang, Wanglan Lan, Junle Liu and Wenhua Chen analyzed and explained relevant data on ozone pretreatment and liver ischemia-reperfusion injury, and were the main contributors in writing the manuscript.

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Not applicable.
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Figures

**Figure 1**
Expression of HSP70 in rat liver (n=10, mean±SD)

**Figure 2**
Expression of PKC in rat liver (n=10, mean±SD)
Figure 3

Expression of ERK1/2 MAPKs in rat liver (n=10, mean±SD)