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Effect of low-dose cyclophosphamide on endoplasmic reticulum stress and inflammatory reaction of acute renal ischemia reperfusion injury

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Abstract. – OBJECTIVE: To explore the effect of low-dose cyclophosphamide (CP) on endoplasmic reticulum stress (ERS) and inflammatory reaction of acute renal ischemia reperfusion injury (IRI), providing new ideas for clinical treatment.

MATERIALS AND METHODS: Sixty healthy adult male mice were selected and divided randomly into sham operation group (n = 20), IRI group (n = 20 cases), and the experimental group (n = 20 cases). Mice in the experimental group were pretreated with low-dose CP and acute IRI model construction. Intercellular adhesion molecule-1 (ICAM-1) immunohistochemical staining was conducted after the 24h and the detection of CHOP protein by Western blot method. Postoperative 3h and 7 day survival rate, the results of ICAM-1 immunohistochemistry staining and the gray values of C/EBP homologous protein (CHOP) of mice were compared between the three groups.

RESULTS: The survival rates of mice in the IRI group and the experimental group after operation were decreased significantly ($p < 0.05$). However, the survival rates of mice in the experimental group were higher than that in the IRI group. The differences were significant ($p < 0.05$). The expression of ICAM-1 was significantly reduced ($p < 0.05$). The difference was statistically significant. The expression of ICAM-1 was significantly reduced ($p < 0.05$). The gray values of mice in the IRI group and the experimental group were significantly increased. The difference was statistically significant ($p < 0.05$). And the gray value in the IRI group increased more than that of the experimental group. The difference was statistically significant ($p < 0.05$).

CONCLUSIONS: The pretreatment with low-dose CP for mice with acute renal IRI can reduce effectively the ERS and inflammation, which reduces kidney damage for mice and have good effect on prolonging its life. It provides a good way for the guidance of clinical research.

Key Words:

Cyclophosphamide, Ischemia reperfusion, Endoplasmic reticulum stress- inflammation reaction, ICAM-1, CHOP protein.

Abbreviations

CP = cyclophosphamide; ERS = endoplasmic reticulum stress; IRI = ischemia reperfusion injury; ICAM-1 = intercellular adhesion molecule-1; CHOP = C/EBP homologous protein; SP = streptovidin-peroxidase; NC = nitrocellulose.

Introduction

The kidney is one of the most sensitive organs of the body to ischemia and hypoxia. And the ischemia reperfusion injury (IRI) is one of the characteristics of acute renal dysfunction, which is also one of the major causes of failure of renal transplantation. It is a hot and difficult problem for now[1]. A large number of animal models revealed from different aspects that stress and inflammatory reaction may be one of the most important mechanisms involved[2-4]. In addition to the mitochondrial pathway and death receptor pathway, various evidences prove that endoplasmic reticulum (ER) is also the main point for perceiving damage or apoptosis signal integration. Endoplasmic reticulum stress (ERS) and inflammatory reaction theory means that after the ERS activation of the unfolded protein response (UPR), it can induce the expression of inflammatory transcription factor and a variety of inflammatory factor which induce inflammatory reaction starting, thereby, increasing the cell damage

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process[5,6]. The glucose regulated protein GRP78 of the heat shock protein family is one of the markers of ERS. Signaling molecules NF- B and JNK mediate the ERS signal inflammation transfer process[7]. Cyclophosphamide (CP) is a double alkylating agent which has different effect with different doses of it. Low-dose CP can act on DNA of cells, inhibiting the immune cell proliferation, which has strong anti-inflammatory inhibitory effect. It was speculated that anti-inflammatory effect of low-dose CP may reduce injury degree of acute renal ischemia reperfusion through the inhibition of ERS and inflammation action[8-10].

Materials and Methods

Materials

Sixty healthy adult male mice in the experiment were provided by Shanghai Experimental Animal Center of Chinese Academy of Sciences. The mice were 4~5 weeks with body weight of (24.8 + 4.3) g and clean grade. The paraffin and different gradient ethanol for section making were bought from Shanghai Experiment Reagent Co. ICAM- 1 immunohistochemistry kit, pentobarbital sodium, and CP injection were products of Jiangsu Hengrui Medicine Co. Ltd. CHOP-SiRNA was provided by Life, Shanghai. Microscope and centrifuge are products of Germany Leica Company (Weitzhar, Germany). Operation equipment and ALC-HTP animal constant temperature system were provided by Shanghai Alcott Biological Technology Co. Ltd (Shanghai, China). Refrigerator was a product of Haier Electric Company (Quingdao, China).

Methods

The mice were divided randomly into sham operation group (n = 20), ischemia reperfusion injury (IRI) (n = 20), and small dose of CP-ischemia reperfusion experimental group (n = 20 cases). Age and body weight of mice in three groups had no statistically significant difference ($p > 0.05$). The mice in experimental group were injected with CP preoperative (0.5 mL/g). Mice in IRI group and sham operation group were injected with saline of the same volume.

Construction of Acute IRI Model

According to reports in the literature[11,12], mice in the experimental group and the IRI group were anesthetized with an intraperitoneal injection of 1% pentobarbital sodium (60 mg/kg). Abdominal skin was prepared after anesthesia and mice were fixed. Routine disinfection of skin with dehydrated alcohol and prepare the sterile towels. The abdominal median incision was with the upper edge of xiphoid and lower edge of the superior margin of the pubic symphysis. The skin was separated layer by layer until it reached the abdominal cavity. Mice were only subjected to blunt separation of bilateral ureteral with ligation. Then, free the right renal pedicle. The right kidney was resected after doubly ligated with 5-0 suture. Thirty milliliters of heparin was injected into the intraperitoneal for the systemic heparinization. The left renal pedicle aorta was repeatedly clamped 5 minutes for ischemia and reperfused for 20 minutes until the renal blood flow recovered. Observe the change of organizational color. Then, close the abdomen to complete the operation. The successful modeling standard is that the left renal pedicle renal color turns from red into black purple by clamping. While it goes back to bright red after loosening the clamp. And the mice recovered and resumed eating, drinking water and other normal activities 1-3 hours after the surgery. Mice in the sham operation group were treated with the same operation methods but with no occlusion of the left renal pelvis. The body temperature of mice maintain in the 36 ± 0.4°C during the whole operation, then, they were placed in 24-28°C environment to keep warm after the mice awake. Sufficient feed and water were provided.

ICAM-1 Immunohistochemical Staining

The renal tissues were taken 24 hours after Ischemia reperfusion and fixed by saline and paraformaldehyde. The tissues were taken at the juxtamedullary regions and made of wax block made with fixed-dehydration-embedding method. Serial sections were cut with 5 μm. The tissues were subjected to immunohistochemical staining of ICAM-1 using SP method. The dyeing process was conducted in strict accordance with the kit protocol. The brown staining appeared in the corticomedullary junction under the microscope represents ICAM-1 positive. Five different areas in the corticomedullary junction were taken at high magnification (400 times). Positive blood vessels were counted. The numbers of positive vessels results at 5 different views were added to show the expression of ICAM-1

Table I. The difference between the postoperative mortality.

Groups	Death caused by operation failure	Death after 3 hours	Death after 7 days
Sham operation group (n = 20)	1	0	2
IRI group (n = 20)	0	2	11
Experimental group (n = 20)	1	1	7
χ^2	0.784	0.044	2.453
p	0.912	0.122	0.028

Detection of the Expression of CHOP Protein by Western Blot

The kidney tissue was taken 24 hours after IR for total protein extraction. Protein content was measured by bicinconic acid (BCA) assay and underwent SDS-PAGE electrophoresis and transferred to nitrocellulose (NC) membrane. Block with blocking buffer for 2 hours. The ratio of 1:200 mouse anti-rat CHOP antibody was added and sealed in 4°C overnight. Wash the liquid membrane with washing buffer. The ratio of 1:2000 dilution of alkaline phosphate (AP) labeled Goat anti-rabbit IgG was added and incubated at room temperature. Wash the liquid membrane with washing buffer for exposure and development. Gray scale analysis was analyzed after scanning. The gray scale values of CHOP protein of each group were compared after scanning.

Observation Index

Compare the 3 hour and 7 days survival rate after operation between the three groups, the results of immunohistochemistry staining for ICAM-1 and gray value of CHOP protein.

Statistical Analysis

All data are analyzed using SPSS19.0 statistical software (IBM Company, New York, NY, USA). Measurement data was listed with (± s). *t*-test was conducted for comparison between groups. The enumeration data was expressed by the number of cases or percentage and compared with the c^2-test. $p < 0.05$ means that the difference was statistically significant.

Results

The Difference of 3 Hour and 7 Day Survival Rate After Operation

The mice were put in the same environment postoperatively and given with sufficient water and feed. The survival rate observation results proved that the survival rate of mice in both IRI group and the experimental group were significantly decreased ($p = 0.032$). However, the survival rate of mice in the experimental group was higher compared with that in the IRI group. The difference was statistically significant (Table I).

Effect of CP on the Expression of ICAM-1

The mice of the three groups died in 24hours were excluded (1 rat in the sham operation group and 1 in the experimental group). No ICAM-1 positive blood vessel ($n = 0$) was seen in the mice of the sham operation group ($n = 19$). ICAM-1 positive vessels can be seen at the corticomedullary junction in the mice of the IRI group ($n = 20$, ICAM-1 vessels positive in 43 ± 5). The number of mice with ICAM-1 ICAM-1 vessels positive in the experimental group ($n = 19$, ICAM-1 positive blood vessel with 29 ± 5) was less than that of the IRI group ($t = 2.983$, $p = 0.019$). The difference was statically significant.

Appraisal Result of the Expression of CHOP Protein

CHOP western blot results of three groups were shown in Figure 1. The gray values of the IRI group and the experimental group were significantly increased than that of the sham opera-

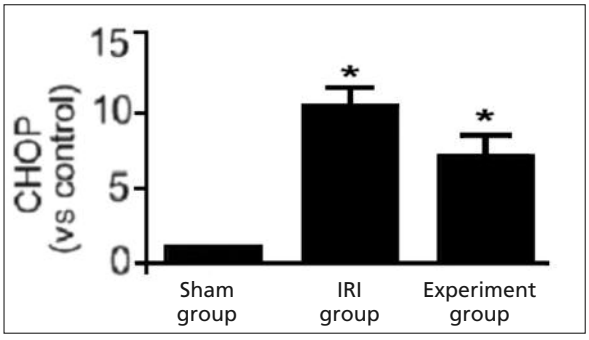

Figure 1. Western blot results of three groups.

tion group. The difference was statistically significant ($p < 0.05$). The gray value of the experimental group is significantly lower compared with the IRI group. The difference was statistically significant ($p < 0.05$, Figure 2).

Discussion

CP is a clinical common immune inhibitor which can interfere with cell gene sequence. It is an alkylating agent playing a dual role according to different dosage and different methods of administration. The anti-inflammatory effect currently has been widely accepted and commonly used in clinical as chemotherapy drugs and treatment of autoimmune diseases such as glomerulonephritis[13-16]. The inhibitory effect on the immune function was realized by its product of phosphoramide mustard under the catalysis of hepatic enzymes *in vivo*.

It shows nonspecific cytotoxicity to immune cells such as lymphocyte. CP, as a nonspecific cell cycle drugs, can identify immune cells such as lymphocyte in the mitosis state and change normal structure of chromosome inhibiting DNA synthesis, so as to have an inhibition effects on the immune cell proliferation. The strong effect of CP on immune system is making it become one of the positive markers of immune suppression cell model[17-20].

Renal IRI is one of the most popular researches in recent years. Renal anatomical features with huge blood flow and double blood supply are one of the reasons for the high rate of renal IRI. How to reduce to the degree of kidney damage caused by IRI is a key to ensure the success rate of renal transplantation and prevent renal failure[21]. There are many factors for IRI such as oxygen free radical theory, mitochondrial damage, etc. One of the important factors to promote the development of IRI process is inflammatory reaction. Studies on renal cells injury in IRI in recent years show that Endoplasmic reticulum stress (ERS) and inflammation play key roles in the regulation[22,23].

Figure 2. Contrast analysis of CHOP in three groups.

The ER as organelles with the largest area within the cell is an important factor for the regulation of protein function and stable within the cellular microenvironment. When the cells are stimulated with hypoxia ischemia and energy shortage, the ER function disorders occur with the error modification and processing of protein, resulting in savings of ineffective protein with no physiological function and activity. This state is called the ERS and inflammatory reaction. Cells can be activated by protection mechanism of accelerating degradation of the protein and other signal transductions in the compensated phase. However, when calcium overload exceeded the limit of compensation in the decompensated stage, cell will be apoptosis[24-27].

CP can be used to alleviate renal cell injury caused by reperfusion through its strong anti-inflammatory effect in the model of renal IRI[28]. The main mechanism is that low-dose CP administration can significantly reduce the number of ischemic lesions of neutrophils. It is speculated that CP can reduce the expression of adhesion molecule ICAM-1. ICAM-1 plays a key role in invasion and migration process of inflammatory cells. The reduction of it can reduce the adhesion between neutrophils and endothelial cells, thereby, reducing the infiltration of inflammatory cells and inhibiting the reaction of inflammation[29-31]. Secondly, low-dose CP may reduce the damage degree of ERS state by reducing the level of CHOP for protecting. CHOP is an ER-derived transcription factor. The expression of it is very low under normal circumstances. However, it can be activated and integrated into the nucleus to initiate apoptosis program in a variety of inflammatory stress state to promote apoptosis. The decreased level of CHOP can significantly reduce the number of apoptotic cells in the ERS state, which has protective effects for the kidney cells, vascular endothelial cells, and renal tubular epithelial cells[31,32]. Through this study, the expression of ICAM-1 in the experimental group was significantly decreased than that in the IRI group, while the gray value of CHOP protein was significantly increased, and the survival rate was significantly increased.

Conclusions

Although the detailed pathway for CP reducing the inflammatory reaction is not fully understood, the anti-inflammatory effect of it and its

visible protective effects on renal IRI have been confirmed. In view of the double effect of CP caused by different doses on administration, how to use small dose drug for reducing reperfusion injury is the key of the study. And the anti-inflammatory effects of CP on cell level reducing ERS inflammation are the new direction of future researches.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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