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Research Article

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Effects of L-arginine on the pulmonary heme oxygenase-1 during lung ischemia-reperfusion injury in rabbits

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ABSTRACT

Single lung ischemia-reperfusion injury animal model was used in vivo. Twenty rabbits were randomly divided into two groups (n=10, in each), pulmonary ischemia-reperfusion (IR) group and L-Arg group. The tissue slides were stained by in situ hybridization(ISH) for HO-1 to detect the expression of HO-1 in lung and to analyze the absorbance. Wet to dry ratio of lung tissue weight (W/D) and the injured alveoli rate (IAR) were measured. The lung tissue slide was prepared for electron microscopic observation after 180 min reperfusion. HO-1 expression was upregulated in the pulmonary endothelial cells in two groups. In some pulmonary vascular smooth muscle cells, extima of vessels and epithelial cells of airway, the value of absorbance was 0.148±0.013, 0.158±0.012 , respectively, and the activity of HO-1 was 526±65, 784±84, respec tively. The L-Arg group showed higher absorbance and activity of HO-1 than the IR group (P<0.05 and P<0.01), lower W/D and IAR values than the IR group (P<0.01) significantly and slighter abnormal changes of the lung tissue in morphologically than the IR group. L-Arg possesses notable protective effects on reperfusion lung in rabbits by increasing the expression and activity of HO-1 in lung tissue.

Key words: L-arginine, Lung, Reperfusion injury, Heme oxygenase-1, CO

INTRODUCTION

In these years, pulmonary ischemia reperfusion injury (PIRI) is still an important factor that influences prognosis, although new cure methods of pulmonary disease such as lung artery sleeves-resection, lung transplantation, combined heart and lung transplantation, lung thrombolysis have developed[1,2]. Ischemia - reperfusion (IR) lung injury is a multifactorial common complex role in the pathological and physiological processes, but the exact Pathological, physiological mechanism is still not entirely clear. Therefore, it is extremely urgent to discover a way to prevent PIRI[3,4]. Our previous research [5,6] have found that L-arginine(L-Arg) can protect lung from PIRI. Xuejun Xiao et al[7] reported, in their research, heart and lung transplantation canine model had be finished successfully by using standard method , they had used grafts preserved up to 4-6 hours successfully, administration of L-arginine to cardioplegic and lung perfusion solution increased the release of nitric oxide, can improve protection of heart and lung in heart and lung transplantation. However, there is no report precisely saying whether that function of L-Arg is worked by increasing the expression and activity of heme oxygenase -1(HO-1). This study observed the effects of L-Arg on wet to dry ratio of lung tissue weight (W/D), injured alveoli rate (IAR) and lung histomorphology changes, in vivo model. We investigated the mechanism of L-Arg protection in order to provide a theory base for clinical research.

EXPERIMENTAL SECTION

Animals; Main Equipments and Main Reagents:

Twenty healthy Japanese big ear rabbits, either gender, body weight of 1.7-2.6Kg (offered by Experimental Animal Center of Wenzhou Medical College). All animals received humane care in compliance with European Convention on Animal Care. The following experimental protocol was approved by the Wenzhou Medical College Animal Care

and Use Committee.

DW2000-animal breathing machine(Shanghai Pengjiakeji Co., Ltd.), UNIVERSAL 32R-low temperature high speed centrifuge (Germany HETTICH Company), electric airblast drier (Shanghai Laboratory Instrument Factory),752-spectrophotometer(Shanghai Precise Scientific Instrument Co.,Ltd.), BS210S-automatic electronic Balance(Beijing Sartorius Group),electric thermostatic waterbath (Shanghai Medical Thermostatic Equipment Factory), JY92- II ultrasonic wave cell crusher (Ningbo Xinzhi Instrument Institute) , FSH-II-highspeed electric homogenizer(Jiangsu Jintan Jincheng Guosheng Laboratory Instrument Factory),common pathological section machine, light microscope, LKB-V2088- Ultramicrotomy, H-600- Transmission Electron Microscope (Japan Hitachi) , CMIA-color medical image analysis system.

The primary antibody against HO-1; diethylpyrocaronate(DEPC); 20% ethyl urethane; L-Arg solution(Sigma); ethanol; hybridization in situ kit for HO-1 (Wuhan Boster Biological Technology Co.,Ltd.).

Animal Model And Grouping:

Rabbit PIRI model was duplicated referred to Sekido's method[8].After some conventional operations: vein anesthesia by 20% ethyl urethane (1.0g/kg), normal saline I.V. (0.5-1.5ml/min) through external jugular vein, cannula of carotid artery ready to draw blood, the rabbit were ventilated with pure O_2 (8-10 ml/kg) at breaths 30-40/min, expiration aspiration ratio 1:1.25. NO.3,4,5 ribs were cut off along the left breast bone, the thoracic cavity was opened, and hilum which a ligature was passed around was dissociated. Ligation establish PIRI model.

The rabbits were divided randomly into two groups, 10 rabbits each group: (1) ischemia- reperfusion group (IR group): after ligatures passing hilum 20min, tie it to perform ischemia for 1h, then immediately unclamp to proceed reperfusion for 3 h. （2）L-Arg group: L-Arg solution (Sigma) was treated by100mg/kg in i.v pre-ischemia 20min.

Lung HO-1 activity examination:

Based on the theory that HO-1 can degrade heme into bilirubin and CO, bilirubin level was detected to reflect the activity of HO-1. The result was expressed by $pmol \cdot mg^{-1}\ prot \cdot h^{-1}$.

Lung HO-1 hybridization in situ analysis:

(1) lung tissue species disposal: fixed lung species was dehydrated by ethanol series, embedded in paraffin, sectioned.

(2) HO-1 oligonucleotide probe sequence: ①5'—AGAAT GCTGA GTTCA TGAGG AACTT TCAGA —3'; ②5'—GCTGC TGGTG GCCCA CGCCT ACACC CGCTA —3'; ③5'— TTCCT GCTCA ACATC CAGCT CTTTG AGGAG—3'.

(3) operation steps: 1) paraffin section of lung tissue baked at 60℃ for an hour, then conventionally deparaffinized to water. And sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase, washed 3 times by water. The sections were digested by the fresh pepsinum diluted 3% citric acid at 37℃ for 12min to expose mRNA nucleic acid fragment then were washed by PBS buffer 3 times (5min per time), distilled water one time. 20% glycerinehe 20ml was added to the bottom of the dry box to keep wet. After prehybridization solution 20μl for each section at 42℃ 3 h , discard redundancy, do not wash, dip hybridization solution (containing probe) 20μl every section, cover the slides and then incubate at 42℃ overnight in thermostatic waterbath box(about 17 h).2) Negative control sections were dropped hybridization solution without probe on. Uncover the coverslip, wash by 2×SSC at 37℃ 5min 2 times, then 0.5×SSC 15min one time , 0.2×SSC 15min one time at the same temperature sequentially. Apply Blocking Solution and incubate at 37℃ for 30 min. Remove the blocking reagent and do not wash. Incubate the slides in biotinylated digolan rat antibody at 37℃ for 60 min, wash 5 min for 4 times with PBS which is used specially for hybridization, add biotinylated peroxydase to the sections and incubate at 37℃ for 20min, repeat washing 5min 4 times with PBS special for hybridization, employ DAB to finish the color reaction, wash sufficiently with water. Afterstain the sections with Hematoxylin , then wash sufficiently with water. Finally, sections were washed, mounted, dried, dehydrated, cleared, and sealed. 4 test under microscope Buffy is the staining of positive express. 3) apply the software (developed by East China University of Science and Technology) to read the absorbance.

Lung W/D determination:

Harvest specimen of lung near the side of hilum and rinse with normal sodium, remove the superfluous water and weigh as wet weight. Dry the specimen in electric airblast drier at 70℃ for 24 hours, and get dry weight. So we can

get the wet to dry weight ratio of lung tissue.

The injured alveoli rate (IAR):
Lung tissue was fixed in 4% paraformaldehyde treated by DEPC, dehydrated by ethanolic series, embedded in paraffin, sectioned and stained by HE conventionally. We observed the changes of histological structure under microscope, as Murata[9] reported. At 200 magnification field of vision we continuously observed 200 pulmonary alveoli, counted the quantity of those injury alveoli which there were more than 2 erythrocyte or/and leucocyte inside of, and calculated the percentage of the injury alveoli as a quantitative assessment index of pulmonary alveoli impair.

Observation of lung tissue at electron microscope:
Harvest 2 or 3 pieces of lung tissue near the left hilum ; the size of each piece is about 0.1×0.1×0.1cm. Transfer the specimen to 2.5% glutaric dialdehyde, then post-fix with 1% osmic acid, dehydrate through ethanol and acetone series, make embedment, ultrathin sections and double staining with acetic acid and lead nitrate, finally observe at transmission electron microscope.

Statistical analysis:
We employed statistical software SPSS 11.5 to analyze data. All data were processed by the test of normality and were expressed as means ±SD (standard deviation). Differences between groups were analyzed by using *t* test. $P < 0.05$ was considered statistically significant. Correlation analysis among HO-1 activity, HO-1 ISH, W/D and IAR was finished by Pearson interclass correlation in the Bivariate, we got the coefficient by utilizing linear regression.

RESULTS AND DISCUSSION

Lung HO-1 activity and ISH absorbance:
HO-1 activity in L-Arg group was higher than that in IR group ($P<0.01$). HO-1 expression in lung blood vessel endothelium (including arteriola and vein), parts of vessel smooth muscle, extima of vessels and epithelial cells of airway was positive and ISH absorbance obviously increased (vs IR group, $P<0.05$)（Tab 1,Fig 1, Fig 2）.

Tab.1 changes of HO-1 activity and ISH absorbance in two groups (*n=10*，*x*±*s*)

groups	HO-1 (pmol·mg^{-1} prot·h^{-1})	OD of ISH
IR	526±65	0.148±0.013
L-Arg	784±84$^{\#\#}$	0.159±0.009*

Vs IR group， P<0.05, # # P<0.01*

Fig.1 HO-1 expression in IR group

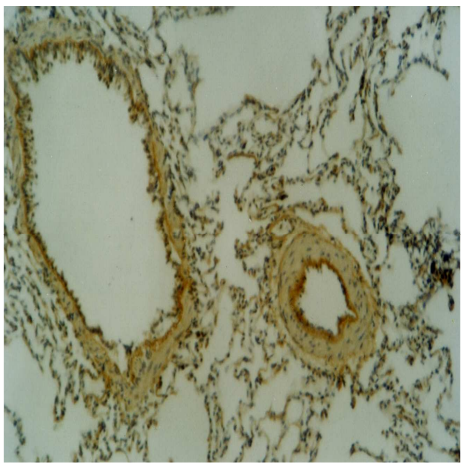
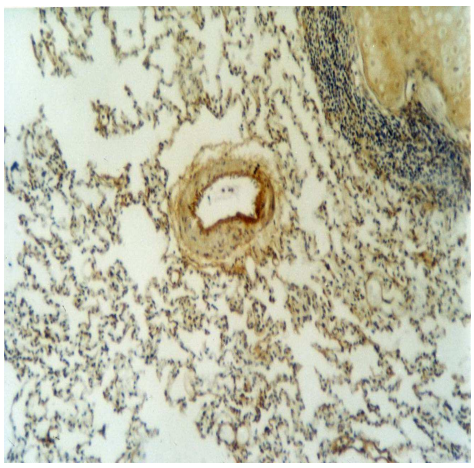


Fig.2 HO-1 expression in L-Arg group



Change of W/D and IAR values in lung tissue:
W/D and IAR values of L-Arg group were significantly lower than those of IR group ($P<0.01$)（Tab 2）

Tab.2 Change of W/D and IAR values in lung tissue in two group(*n=10*，*x*±*s*)

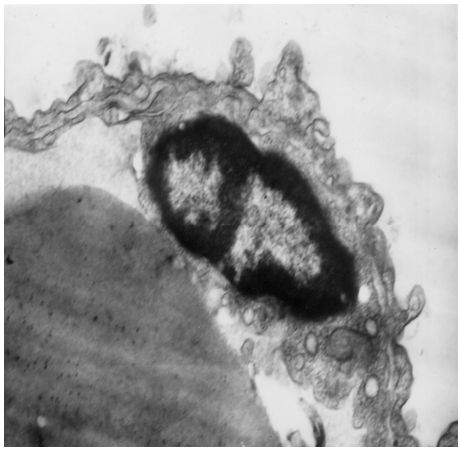
Groups	W/D	IAR (%)
IR	6.33±0.71	49.85±5.85
L-Arg	5.44±0.80*	21.80±4.12$^{\#\#}$

Vs IR group， $P<0.05$, ## $P<0.01$*

Ultramicrostructure change of lung tissue:

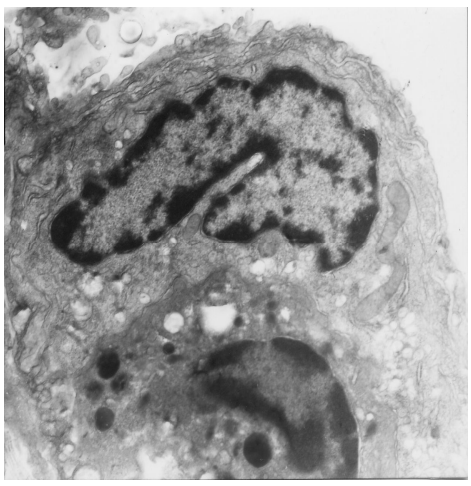
IR group: capillary endothelial cells swelled, nuclear chromatin was seen to concentrate at the side of nuclear membrane, nucleus intended to condense, and the gaps enlarged. There were a few of pinocytosis vesicles in type Ⅰ alveolar epithelial cells. Microvilli reduced on the surface of type Ⅱ alveolar epithelial cells, mitochondrium swelled, lamellar body was rare, some vacuoles appeared. interalveolar septum got oedematous. Inflammatory cells were affiliated to interalveolar septum and capillary lumen, neutrophil played the main role in this process (Fig 3).

Fig.3 ultramicrostructure change in IR group(x 20k)



L-Arg group: inflammatory cells in the capillary reduced, chromatin scattered normally. There were many pinocytosis vesicles in Ⅰtype alveolar epithelial cells. Microvilli is normal on the surface of type Ⅱ alveolar epithelial cells and lamellar body increased. Interalveolar septum lightly got oedematous (Fig 4).

Fig.4 ultramicrostructure change in L-Arg group(x 20k)



Dependability analysis:

W/D and IAR of lung tissue became positive correlation(r=0.811, P<0.01); the activity of HO-1 was negative correlation with W/D, IAR, coefficient was -0.767 and -0.812 respectively, each P <0.01; HO-1 ISH appeared negative correlation with IAR, coefficient r was -0.817, P <0.01.

In this experiment, we found L-Arg decreased W/D and IAR greatly, and improved ultramicrostructure injury increased by IR. It suggested that L-Arg can protect lung from IR in some extent. Treatment of L-Arg solution can obviously increase the activity and expression of HO-1. There was a negative correlation between the activity and

expression of HO-1 and IAR. It showed the use of L-Arg pre-ischemia 20min can induce the expression of HO-1 and increase its activity to improve CO level[10] for anti-PIRI. And then, our previous research[11] have found that the upregulation of HO-1 can be one of the results to reduce the damage of lung ischemia-reperfusion.

Previous studies[12] showed that L-Arg can up-regulate HO-1 gene to prevent lung for PIRI through increasing CO content and activating PKG signal pathway. But its mechanism was not elucidated totally, maybe involved some factors below: ① anti-oxydation: HO-1 degradated biliverdin and bilirubin that are strong antioxidant. HO-1 can degradate iron to induce synthesis of ferritin which can reduce reactive oxygen. Elbirt et al[13] have discover that low content CO conduce cells to anti- oxidative stress. Low dose of CO (1/20 fatal dose) can improve the tolerance of animals in hyperxia lung injury. Jialiang Zhu et al[14] reported that their study results supported this view that L-arginine can reduce lung ischemia-reperfusion injury caused by the response to oxidative stress play a main role, but whether there are other mechanisms to participate in and whether there is a dose-dependent relationship to be studied. ② improvement microcirculation: endogenous CO can suppress platelet aggregation and reduce blood glutinousness to reduce thromb in microcirculation by activating guanine nucleotide cyclase to increase cyclic guanosine monophosphate content. CO can relax vessels by activating K^+ channel to make smooth muscle hyperpolarization. CO also can reduce the production of vaso-excitor material to maintain activating prostaglandin cycloxygenase and suppressing cytochrome P450 dependent monoamine oxidase[15]. ③ regulation of cell cycle: Zhang et al[16] evidenced that CO can suppress the expression of Fas/FasL to inhibit the activation of caspase 3, 8, 9 and the release of cytochrome C, increase the expression of anti-apoptosis gene Bcl-XL, Bcl-2. ④anti-inflammatory: Otterbein et al[17] reported that low content CO (250ppm) can selectively downregulate the expression of proinflammatory cytokine TNF-α, IL-1β, MIP-1 , and upregulate anti-inflammatory cytokine IL-10.And Yin Kai Chao et al.[18]reported that Pulmonary perfusion with L-arginine could further attenuate Post-cardiopulmonary bypass(CPB)-induced lung injury by restoring endothelial dysfunction and decreasing inflammatory response.

CONCLUSION

Summarily, L-Arg can upregulate HO-1 expression in lung, strengthen HO-1 activity to increase CO level and protect lung from PIRI effectively.

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