Protective Effects of Flavonoid and Polyphenol from Lotus Leaf on Lung Damage Induced by Inhalation of N₂O₄ in mice

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Abstract. To study the protective effects of flavonoid and polyphenol (FP) from lotus leaf on the damage induced by N₂O₄ in mice. Constructing an animal damage model through exposing mice to 45μl N₂O₄ in a 120 L sealed cabinet for 30 minutes. ICR male mice were randomly divided into normal group, physiological saline+N₂O₄ group, 1.25g/kgFP +N₂O₄ group, 2.50g/kgFP + N₂O₄ group, 3.75g/kg FP +N₂O₄ group. 1.25, 2.50, 3.75 g/kg FP were orally administered to mice respectively for 5 days, equal volume physiological saline for normal group and physiological saline+N₂O₄ group. Then, the three FP groups and the physiological saline+N₂O₄ group were exposed to N₂O₄ in the cabinet on 5th day. In an hour after N₂O₄ exposure, killing the mice by dislocation to measure the SOD, GSH-Px activity and MDA content in lung tissue, checking pathology change in lung tissue slice. Results were shown: the 1.25, 2.50g/kg FP increased SOD (U/mg pro) by 30.3% and 24.4%, GSH-Px (U/mg pro) by 77.4% and 60.9%, respectively. Pathological observation of lung tissue showed that three FP groups had lighter damage than the physiological saline+N₂O₄ group. So, FP has significant protective effects on damage caused by N₂O₄ in mice.

1 INTRODUCTION

Lotus (*Nelumbonucifera Gaertn*) is an important member of medicinal aquatic plants. Its leaf is widely applied in Indian or Chinese traditional medicine for treatment on various diseases and alleviation of corresponding symptoms. Because of its good anti-tumor, anti-oxidation, inhibition of liver fibrosis, hypoglycemia^[1], anti-inflammatory, antiarrhythmic, antipyretic, immunomodulatory, antibacterial, anti-inflammatory, cardiovascular protection, and other activities^[2], deep development for lotus leaf is paid attention to, especially, main chemical components polyphenol and flavonoid, as well as polysaccharides, volatile oils and trace elements. N₂O₄ is a kind of liquid propellant of large launch rocket used in the United States and other countries^[3]. It is a strong oxidant, through inhalation which can cause bronchial epithelial damage, reduce the phagocytosis activity of alveolus phagocytes, increase the incidence of chronic infection of respiratory tract significantly, and then cause the chronic inflammation of the respiratory tract ^[4, 5]. Flavonoid and polyphenol are important active substances in plants, such as scavenging free radical, detoxification, maintaining DNA biosynthesis, maintaining normal growth and immunity of cell, widely used in biomedical and food processing fields ^[6]. In this

paper, the protective effects of flavonoid and polyphenol on lung damage induced by the inhalation of N₂O₄ in mice was studied.

2 MATERIALS AND METHODS

Preparation of flavonoid and polyphenol from lotus leaf. Fresh lotus leaves were gathered from Hou Guan lake of Wuhan city, and its flavonoid and polyphenol were extracted with ethanol at our laboratory at following conditions: ethanol concentration 40%, ratio of leaf and liquid 1:30, extraction temperature 30°C, extraction time 120s. The extraction liquid was concentrated and vacuum freeze dried to collect lotus leaf extraction powder. Flavonoid and polyphenol were determined by spectrophotometry method (rutin and gallic acids standard ample, Sigma Co.). The flavonoid content was 93.7mg/g and polyphenol 11.2mg/g.

2.1 Experimental animals

Healthy male ICR mice, of clean grade, with the body weight of 18-22g, provided by the Experimental Animal Science Department of Medical College, Beijing University. Experimental animal license number: SYXK (Beijing) 2016-0025. The temperature of animal

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breeding room was maintained at $22 \pm 1^\circ\text{C}$, humidity 50%, free feeding and drinking.

2.2 Instruments and Equipments

120 L toxicant exposure counter, Special Medical Center of 306 Hospital.

2.3 Reagents

Liquid N_2O_4 was provided by 101 Institute of Aerospace Industrial Research Academy. Coomassie brilliant blue protein and the assay kits of SOD, GSH-Px, MDA were provided by Nanjing Jiancheng Bioengineering Institute.

2.4 Construction of animal damage model

Referring to the method of Mingliang Ye^[7], by the way of inhaling N_2O_4 in the toxicant exposure cabinet. Volume of the toxicant exposure cabinet was 120 L. Temperature in the cabinet was maintained at $25 \pm 0.5^\circ\text{C}$. Liquid N_2O_4 (45 μl) was injected by the micro-syringe and vaporized. Start the fan to make the N_2O_4 steam distributed uniformly in the cabinet. Expect the mice of normal control group, all mice were exposed to N_2O_4 in the toxicant exposure cabinet. Mice breathed freely in the cabinet. Inhalation exposure time was 30 minutes, adding liquid N_2O_4 (1 μl) to the cabinet every 5min. After exposure, the mice were removed from the cabinet, feeding and drinking normally.

2.5 Animals grouping and treatment

Totally 70 healthy male ICR mice, of clean grade, were randomly divided into 5 groups: normal control group, saline+ N_2O_4 group, 1.25g/kgFP+ N_2O_4 group, 2.50g/kgFP+ N_2O_4 group and 3.75g/kg FP+ N_2O_4 group, 15 mice in each group except the normal control group (n=10). Adding FP powder into the physiological saline to prepare the solution for oral administration, then the mice in three FP intervention groups were administered with FP by oral administration at the dosage of 1.25, 2.50, 3.75 g/kg respectively, 0.4ml for each mouse, once a day for 5 days. The mice in physiological saline+ N_2O_4 group were administered with physiological saline by oral gavage, 0.4ml for each mouse, once a day for 5 days. The exposure was conducted at 1st after intragastric administration on the 5th day, mice were killed by dislocation at 12th hour after the inhalation intoxication of N_2O_4 , then taking 10 mice from each group to make lung tissue slice and determine the SOD activity, GSH-Px activity and MDA content of lung tissue, the other 5 mice were killed to make lung tissue slice on the 7th day after exposure.

2.6 Indexes Determination

Place the lung tissue into the glass tube, add a certain amount of ice saline accurately into the glass tube with micropipette, then homogenize the tissue. Centrifugate

the prepared homogenate at 14000rpm, at 4°C , for 10min. Take appropriate supernatant to determine SOD activity, GSH-Px activity, MDA content.

2.7 Pathological examination

After the mice were killed, their thoracic cavity were opened and general lung pathological changes were observed. The whole lung tissue was soaked in 10% formalin solution and fixed for a week. Pathological changes were observed under light microscope according to conventional pathology methods of progressively dehydrating, transparency, embedding, sectioning and HE staining.

2.8 Statistical analysis

The data of measurement result were expressed with $\bar{x} \pm s$ and carried out t-test statistical analysis with SPSS16.0 software.

3 RESULTS AND ANALYSIS

3.1 Effect of FP on activities of SOD, GSH-Px and MDA content in lung tissue of mice exposed to N_2O_4 .

Compared with the normal control group, SOD activity (42.95 U/mg pro) in saline+ N_2O_4 group, showing significant decrease ($P < 0.01$). Compared with the saline+ N_2O_4 group, SOD activity (55.97 U/mg pro in 1.25g/kgFP+ N_2O_4 group, 53.44 U/mg pro in 2.50g/kgFP+ N_2O_4 group) increased by 30.3% and 24.4% ($P < 0.01$) respectively, while SOD activity (45.26 U/mg pro) in 3.75g/kgFP+ N_2O_4 group showed no difference (Table 1).

Compared with the normal control group, GSH-Px activity (4.17 U/mg pro) in saline+ N_2O_4 group, exhibiting significant decrease, too ($P < 0.01$). Compared with the saline+ N_2O_4 group, GSH-Px activity (7.40 U/mg pro in 1.25g/kgFP+ N_2O_4 group, 6.71 U/mg pro in 2.50g/kgFP+ N_2O_4 group, 6.52U/mg pro in 3.75g/kgFP+ N_2O_4 group) increased by 77.4%, 60.9%, and 56.3% ($p < 0.01$) respectively.

MDA content of lung tissue in all N_2O_4 exposure groups decreased, Compared with the normal control group. Especially, MDA content (5.99 mmol/L) in saline+ N_2O_4 group, showing significant decrease ($P < 0.01$). Compared with saline+ N_2O_4 group, MDA content (11.66 mmol/L) in 1.25g/kg FP+ N_2O_4 group increased by 94.6% ($P < 0.01$).

Table 1 Effect of flavonoid and polyphenol on activities of SOD, GSH-Px and MDA content in lung tissue of mice after 12h of exposed to N₂O₄ ($\bar{x} \pm s$, n=10)

Groups	SOD activity (U/mg pro)	GSH-Px activity (U/mg pro)	MDA content (mmol/L)
Normal	56.84±3.90	9.74±1.39	16.47±2.97
saline+N ₂ O ₄	42.95±2.10**	4.17±0.26**	5.99±0.54**
1.25g/kg FP+N ₂ O ₄	55.97±2.72 $\Delta\Delta$	7.40±0.95 $\Delta\Delta$	11.66±1.75 $\Delta\Delta$
2.50g/kg FP+N ₂ O ₄	53.44±1.80 $\Delta\Delta$	6.71±0.63 $\Delta\Delta$	9.80±1.57 Δ
3.75g/kg FP+N ₂ O ₄	45.26±2.02	6.52±0.56 $\Delta\Delta$	10.37±1.53 Δ

**: $P < 0.01$ vs normal group; Δ : $P < 0.05$, $\Delta\Delta$: $P < 0.01$ vs N₂O₄ group

3.2 Effects of FP on the lung tissue pathological changes in mice exposed to N₂O₄.

Taking the 1.25g/kg FP+N₂O₄ group to observe the lung tissue pathological changes. Compared with the normal control group, the mice in saline+N₂O₄ group showed significant pathological changes in the lung tissue, pulmonary alveolus structure was damaged, the number of pulmonary alveolus was reduced, pulmonary alveolus size was unequal, alveolus interstitium was widened, matrix was increased significantly, mononuclear cell infiltration occurred, some tissue was fiberized and showed cord-like distribution. Dilatation and congestion of small veins within the lung tissue and capillary within pulmonary alveolus wall were observed, pink serous fluid was exuded, part of the pulmonary alveolus wall was destructed into small cysts. 12h after inhalation of N₂O₄ lung tissue was swelled and the volume was increased. Lung tissue swelling and turning white, mixed with some substantial lesion areas were observed in saline+N₂O₄ group on 7th day after exposed to N₂O₄. Compared with the saline+N₂O₄ group, the severity of pathological lesions in FP+N₂O₄ group was obviously relieved, pathological examination showed that the severity of pulmonary alveolus structure damage, the decline of the number of pulmonary alveolus, the severity of mononuclear cell infiltration and lung tissue fibrosis were eased significantly (Figure 1). 1.25g/kg FP has significantly protective effects on lung injury induced by the inhalation of N₂O₄ in mice.

a. Lung tissue in normal control mice (HE, $\times 100$) b. Pulmonary hemorrhage, edema and inflammatory cell infiltration in lung tissue in physiological saline+N₂O₄ group at 12th hour after injury (HE, $\times 100$) c. Pulmonary hemorrhage, edema and inflammatory cell infiltration in lung tissue in physiological saline+N₂O₄ group at 7th day after injury (HE, $\times 100$) d. Pulmonary hemorrhage, edema and inflammatory cell infiltration in lung tissue in FP+N₂O₄ group at 12th h after injury (HE, $\times 100$) e. Pulmonary hemorrhage, edema and inflammatory cell infiltration in lung tissue in FP+N₂O₄ group on 7th day after injury (HE, $\times 100$)**Figure 1.** Lung tissue of mice was sectioned and enlarged by HE

4 DISCUSSION

4.1 N₂O₄ injury mechanism analysis.

N₂O₄ has strong oxidizing capacity, unstable, and easily decomposed into NO₂ at room temperature. The main target organ of NO₂ damage is lung. NO₂ can cause a variety of injury of bronchial epithelial cells and alveolus epithelial cells, its mechanism of action is related to free radicals and peroxidation damage [4]. Oxidative and peroxidation damage and function change of antioxidant system can lead to the changes of lipid peroxidation products MDA content, such as increase of MDA content, the decrease of SOD and FP-Px, catalase (CAT) activities and so on.

FP is widely present in plant tissues. This study shows that flavonoid and polyphenol at low dosage can significantly increase activities of SOD, GSH-Px in the lung tissue of mice. Mechanism of the protective effects of flavonoid and polyphenol on acute lung damage induced by the inhalation of N₂O₄ in mice is attributed to their reduction capacity: FP is small molecule antioxidants, which can directly react with N₂O₄ inhaled into lung and reduce its harm. In this study, the mice with lung damage induced by inhalation of N₂O₄ were given high doses of FP (3.75g/kg), the lung tissue SOD activity was not significantly higher than the normal control group, its detailed mechanism needed further study.

The study showed that after exposed to N₂O₄, lung tissue MDA content in the mice of all N₂O₄ poisoning group significantly declined. This is obviously different from general oxide. Under normal circumstances, oxides can decrease activities of SOD and GSH-Px in blood or tissue, at the same time, increase MDA content.

However, our study showed that N_2O_4 poisoning could decrease the activities of SOD, GSH-Px and MDA content in tissue of mice simultaneously. Its mechanism may be correlated with N_2O_4 (NO_2) promoting conversion and elimination of MDA, through changing metabolic pathways of lipid peroxidation and forming other products [8], resulting in lung tissue MDA content showing a certain downward trend.

FP widely exists in plants, for example, lotus leaf, grapes, sweet potatoes. Personnel engaged in production and storage of special rocket fuel N_2O_4 , taking more foods such as yeast, lotus leaf, grapes, and sweet potatoes for their daily recipe, is wise. Lotus leaf, as basic materials, utilized to develop special functional food against N_2O_4 damage will exhibit good prospect.

5 CONCLUSION

N_2O_4 resulted in SOD, GSH-Px and MDA levels decreasing in mouse lung tissue. This study presented that FP has significant protective effects on damage caused by N_2O_4 in mice. FP were a promising approach to developing functional food against N_2O_4 poisoning.

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DISCLOSURE

The authors report no conflicts of interest with this work.

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