Effects of niacin on nitric oxide synthase expression in rat lungs exposed to silica*

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Abstract The aim of this study was to evaluate the effects of niacin in diet on the expression of nitric oxide synthase (NOS) in rat lungs of the animal model of silicosis established by direct tracheal instillation of silica particles into rat lungs surgically. The niacin concentration in serum was analyzed by high performance liquid chromatography (HPLC). The expression of inducible nitric oxide synthase (iNOS) protein in paraffin-embedded lung sections was determined by streptavidin/peroxidase (SP) staining. Quantitative analysis by Image-Pro Plus was also performed on the expression of iNOS. The results showed that niacin concentration in serum of the niacin-treated rats was significantly higher than that in the control and silica-treated rats. After 7 days of silica instillation, iNOS integrated optical density (IOD) in rat lungs and total NOS and iNOS activities in bronchoalveolar lavage fluid (BALF) in silica-treated rats rose by 273420.75, 2.61 units/mL and 1.89 units/mL respectively, when compared with those in the control rats. Niacin treatment significantly reduced silica-induced iNOS IOD in rat lung tissues and total NOS and iNOS activities in BALF supernatant by 248292.35, 1.50 units/mL and 0.91 units/mL, respectively, as compared with those in silica-treated rats. Therefore, niacin can effectively attenuate the pathological expression of NOS in rat lung tissues induced by silica particles.

Keywords: niacin, silica, rat lung, nitric oxide synthase.

Nowadays, major medicines for the prevention and treatment of silicosis in China include polyvinylpyridine-N-oxide, piperaquine phosphate, quincolyl piperazine hydroxy phosphate, hanfangchin A, aluminium citrate, Xinin and some traditional Chinese medicines with the therapeutic effects such as blocking and delaying of the pathological process, but the improvement on X-ray images is not satisfactory. Most patients receiving clinical treatment are at the stages of Ⅱ and Ⅲ, but it is urgent for the 70% patients at stage Ⅰ to receive early treatment, so it is essential to develop and manufacture highly effective medicines with low toxicity against silicosis[1]. Niacin, including nicotinic acid and nicotinamide, participates in the composition of electron transport chain and a great many metabolic responses in the form of nicotinamide adenine dinucleotide (NAD, enzopride) and nicotinamide adenine dinucleotide phosphate $NADP^+$ (nadide phosphate) *in vivo*[2,3]. Previous studies suggested that supplementation of niacin in diet can effectively prevent lung damage and pulmonary fibrosis induced by bleomycin[4] and its mechanism may be the inhibitory effect of niacin on the bleomycin-induced nitric oxide (NO) production[5]. Nitric oxide synthase (NOS), catalyzing L-arginine and oxygen molecules to produce NO, includes neuronal NOS(nNOS), endothelial NOS(eNOS) and inducible NOS(iNOS)[6]. A large number of reports have demonstrated that reactive nitrogen free radical NO is involved in the early process of inflammatory reaction of silicosis and is closely associated with the formation of pulmonary fibrosis[7]. In this study, we investigated the effects of niacin on NOS expression in the early process of inflammatory lung damage in rats exposed to silica using tissue microarray technology.

1 Materials and methods

1.1 Materials

A total of 144 Wistar rats (both genders), weighing 200~240 g each, were provided by Experimental Animal Center, Academy of Military Medical Science.

Silicon dioxide was purchased from Sigma Chemical Co. with 99% purity and size of 1~5 μm for 80% particles; Niacin (nicotinic acid, niacin, Vita-

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min B_5) with purity > 99.5% was purchased from Sanland International Inc.; 0.1% polylysine and rabbit anti-rat iNOS antibody was purchased from Santa Cruz Biotechnology, Inc.; SP Test Kit was purchased from Beijing Zhongshan Biotechnolgy Co. Ltd.; and the total NOS and iNOS Test Kit was purchased from Nanjing Jiancheng Bioengineering Institute.

1.2 Methods

1.2.1 Establishment of the animal model Rats were anesthetized by intraperitoneal injection of 0.3% pentobarbital, and then tracheas of the rats were exposed under sterile conditions. Silicon suspension of 1 mL at the concentration of 50 mg/mL (prepared using 0.9% NaCl solution and SiO_2, 8000 U/mL benzylpenicillin added before use for the purpose of prevention from pulmonary infection), was slowly injected into the tracheas and then the skin was sewn up and sterilized. The control group was treated in the same way but injection of 1 mL sterilized saline instead. After the operation, rats in both groups were fed with common forage. The rats in the niacin-treated group were fed with forage containing 2.5% niacin (wt/wt) 3 days before the operation day.

1.2.2 Collection of samples and detection of iNOS and NOS After establishment of the animal model, 8 rats were selected from each group and sacrificed on day 1, 3, 7, 14, 21 and 28, respectively. Rat blood was collected from the abdominal aorta for the preparation of plasma by high performance liquid chromatography. Bronchoalveolar lavage liquid was collected by injecting 2 mL sterilized saline at 37 ℃ into the lungs and drawing out the liquid after an interval of 30 s until 5 mL bronchoalveolar lavage liquid was harvested. The bronchoalveolar lavage liquid was centrifuged at 1500 r/min and at 4 ℃ for 10 min and the supernatant was collected. The concentrations of iNOS and NOS in the bronchoalveolar lavage liquid were determined using iNOS and NOS test kits following the manufacturer's instructions.

1.2.3 Histological observation After the bronchoalveolar lavage operation, the left lobe of the lungs was collected and fixed in 10% neutral buffered formaldehyde solution. The tissue sections were made according to the methods of Zhang et al.[8]. A total of 50 samples with 1.6 mm in diameter were placed on each biochip of 5 μm in thickness. The iNOS expression was determined using immunohistochemical SP method according to the directions of the test kit. The first antibody was rabbit anti-rat iNOS (diluted to 1∶200), and phosphate-buffered saline (PBS) in place of the first antibody served as the negative control. The iNOS expression in pulmonary tissues was observed by a Nikon Eclipse E800 microscope. Five vision fields on each slice were selected for images taken by a WV-CP240/G Panasonic colour CCTV camera. Quantitative analysis of iNOS expression was performed using an Image-Pro Plus Image Analysis System. The integral optical density of the positive cells was used to represent iNOS expression[9].

1.2.4 Statistical analysis All values were expressed by $\bar{x} \pm s$. Comparison of the 3 groups at different time points was conducted using LSD method of one-factor analysis of variance. The minimum standard of significant difference was represented as $p < 0.05$. Statistical processing of all data was performed by SPSS11.5 software.

2 Results

2.1 Effects of niacin on iNOS expression in rat pulmonary tissues exposed to silica

Plate I shows the immunochemical staining of the lung tissues of the rats with different treatments.

The figures demonstrated that on the same day of the operation, no expression of iNOS in the lungs of the control rats was observed (a), while in silica-treated rats, iNOS started to express in alveolar macrophages (b), and in niacin-treated group, iNOS expression could be observed in a few phagocytes (c). One week after the operation, a strong iNOS expression in a large number of aggregated macrophages and neutrophils in pulmonary tissues of silica-treated rats was observed (e) with a lower level of iNOS expression in the aggregated macrophages in lungs of niacin-treated rats (f). Two weeks after the operation, silica-treated group showed that the number of phgocytes expressing iNOS became smaller in the pulmonary tissues (h) and niacin-treated group remained at a low level of iNOS expression as compared with that in silica-treated group (i). Four weeks after the operation, we could see that weak iNOS expression in a few cells in pulmonary tissues of silica-treated rats remained (k) and niacin-treated group had almost no iNOS expression in their pulmonary tissues (l). These results demonstrated that niacin could regulate the expression of iNOS in the lung tissues of silica-treated

rats, and reduce the damage caused by silica.

2.2 Plasma niacin content in rats exposed to silica

Table 1 gives the plasma niacin content in rats of different groups. According to the table, we can see that concentration of plasma niacin in niacin-treated group is significantly higher than that in the other two groups.

Table 1. Changes of plasma niacin in rats exposed to silica at different time points (μg/mL, $\bar{x}\pm s$)

Group	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
Control	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Silica	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Niacin	5.9464 $\pm$6.5815 $^{*\#}$	17.422 $\pm$6.9989 $^{**\#\#}$	21.398 $\pm$5.0306 $^{**\#\#}$	16.0910 $\pm$6.5388 $^{**\#\#}$	4.4143 $\pm$2.8593 $^{**\#\#}$	7.1305 $\pm$5.0931 $^{**\#\#}$

* $p<0.05$ *vs* control; ** $p<0.01$ *vs* control; $^{\#}$ $p<0.01$ *vs* silica-treated group; $^{\#\#}$ $p<0.01$ *vs* silica-treated group.

2.3 Effects of niacin on the integral optical density of iNOS positive cells in rat pulmonary tissues exposed to silica

Table 2 gives the measured integral density of iNOS in rat lungs of different groups. Compared with the control group, the integral density of iNOS in silica-treated group increased from day 1 and peaked on day 7 (increased by nearly 1 fold), then recovered to the normal level on day 14. Significant difference was found on day 3 and day 7. However, from day 21 to day 28, the integral density of iNOS in silica-treated group was lower than that in the control group and significant significance was found on day 28. The integral density of iNOS in niacin-treated group increased to a lesser extent than that in silica-treated group, and no significant difference was found between the two groups. On day 28, the integral density of iNOS in niacin-treated group was significantly lower than that in the control group. The results suggest that niacin has a protective effect on the prevention of damage from silica.

Table 2. Effects of niacin on the integral density of iNOS in rat pulmonary tissues exposed to silica at different time points ($\bar{x}\pm s$)

Group	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
Control	226510.13 $\pm$116633.59	159508.87 $\pm$92644.36	234437.04 $\pm$49054.53	217118.50 $\pm$59052.01	286096.47 $\pm$46527.45	287268.65 $\pm$51114.47
Silicon	241300.70 $\pm$81884.38	306878.39 $\pm$131103.68 *	507857.79 $\pm$292943.94 *	237763.88 $\pm$104300.47	214361.98 $\pm$61830.54	176429.17 $\pm$46965.45 **
Niacin	237358.26 $\pm$66564.99	234504.08 $\pm$35595.24	259565.44 $\pm$44674.88 $^{\#}$	275391.51 $\pm$97110.89	211910.08 $\pm$68454.70	144772.82 $\pm$35869.28 **

* $p<0.05$ *vs* control group; ** $p<0.01$ *vs* control group; $^{\#}$ $p<0.05$ *vs* silica-treated group.

2.4 Effects of niacin on the BALF iNOS activity in rats exposed to silica at different time points

In the BALF of silica-treated group, iNOS activity increased from day 1 and peaked on day 7. iNOS activity in BALF increased by 2- and 5- fold compared with that in the control group. From day 14, iNOS activity in BALF in silica-treated group reduced or even was lower than that of the control group, but no significant difference was found, while iNOS activity in BALF of niacin-treated group was higher than that of the control group with statistical significance (Table 3).

Table 3. Effects of niacin on the BALF iNOS activity in rats exposed to silica (U/mL, $\bar{x}\pm s$)

Group	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
Control	0.45$\pm$0.38	0.49$\pm$0.62	0.36$\pm$0.25	0.40$\pm$0.34	0.51$\pm$0.57	0.39$\pm$0.29
Silicon	0.62$\pm$0.54	1.35$\pm$0.23 *	2.25$\pm$1.23 **	1.32$\pm$0.59 **	0.60$\pm$0.46	0.33$\pm$0.34
Niacin	0.51$\pm$0.22	1.14$\pm$0.75	1.34$\pm$0.27 $^{*\#}$	1.55$\pm$0.67 **	1.20$\pm$0.79 *	0.34$\pm$0.55

* $p<0.05$ *vs* the control group; ** $p<0.01$ *vs* the control group; $^{\#}$ $p<0.05$ *vs* the silica-treated group.

2.5 Effects of niacin on total NOS activity in BALF of rats exposed to silica

Table 4 demonstrates the total NOS activity in BALF of rats in different groups. Compared with the control group, the total NOS activity in BALF in silica-treated group began to increase on day 1 and kept increasing until day 7. From day 21, the total NOS

activity in BALF in silica-treated group became lower than that of the control group, but no significant difference was found. For niacin-treated group, it shows a higher NOS activity than that of the control group for the first 21 days, and a lower NOS activity than that of the silica-treated group for 28 days.

Table 4. Effects of niacin on total NOS activity in BALF of rats exposed to silica at different time points (U/mL, $\bar{x} \pm s$)

Group	Day 1	Day 3	Day 7	Day 14	Day 21	Day 28
Control	1.85±0.24	1.52±0.67	1.20±0.35	1.42±0.40	1.90±0.57	1.54±0.86
Silicon	3.28±1.53 *	3.57±0.89 * *	3.81±1.37 * *	3.61±0.94 * *	1.86±0.74	1.52±0.89
Niacin	2.52±0.80	2.55±0.59 * #	2.31±0.61 * # #	2.83±1.05 # #	2.14±1.06	1.10±0.52

* $p < 0.05$ *vs* control group; * * $p < 0.01$ *vs* control group; # $p < 0.05$ *vs* silica-treated group; # # $p < 0.01$ *vs* control group.

3 Discussion

This study proved that niacin has a dual effect on the expressions of iNOS and NOS induced by silica. Niacin has an inhibitory effect on the excessive increase of NOS at the early stage of exposure to silica (14 days), and has a promoting effect of increasing NOS at the later stage of exposure to silica (14 ~ 28 days). This protective effect is beneficial to the organisms susceptible to the damage caused by silica.

The mechanism of the inhibitory effect of niacin on the production of iNOS may be that niacin inhibits the activation of nuclear factor-kappa B (NF-κB)[5]. NF-κB activity is closely associated with NO production of pneumocytes induced by silica. NO production from NOS induced by silica dust is regulated at multiple levels, including transcription, post-transcription and post-translation levels[10]. At transcription level, NF-κB participates in the expression of iNOS gene. There is a binding site at 5′ end of iNOS gene. In cells at resting state, NF-κB is located in the cytoplasm and binds to inhibitory proteins IκBα, IκBβ, p^{105} and p^{100}, so NF-κB is in an inactivated state. When cells are stimulated, the inhibitory proteins are degraded by the protease, and then activated NF-κB enters the nucleus and binds to the specific site of iNOS gene and hence the gene transcription is controlled[11, 12].

Niacin can inhibit NOS expression, and more importantly, it can inhibit the damage effect of NO. Ogata et al.[13] studied the activity of niacin-related compounds to clear free radicals by electron spin resonance and found that nicotinic acid hydrazide and isonicotinic acid hydrazide could clear NO free radicals. Excessive release of NO can result in DNA break and the fragment of DNA chain can further activate ribozyme-poly-ADP-ribose polymerase (PARP) that participates in the repair of DNA damage. NAD is the substrate of PARP, but excessive production of PART can evacuate NAD stored in tissues, which results in evacuation of ATP and cell death[14, 15]. Niacin, the precursor of NAD, can increase or keep the intracellular NAD and ATP levels in lung tissues and can inhibit the evacuation of NAD[16, 17], but its participation in poly-ADP-ribose reaction in the form of NAD^+ cofactor is helpful to the repair of broken fragment of DNA chain[18]. Effect of high dose of nicotinamide on the ADP-ribose reaction of pancreatic β-cells, immunocytes and endothelial cells can change the cell death pathway and patterns of gene expression, which is helpful to the survival of β-cells and maintenance of immunologic balance[19]. The above-mentioned results prove that niacin inhibits tissue damage caused by NO through increasing NAD level in tissues and affecting intracellular ADP-ribose reaction.

Our study found that supplementation of niacin in foods can attenuate lung tissue damages caused by silica through increasing niacin concentration in plasma and regulating the appropriate silica-induced NOS expression in lung tissues.

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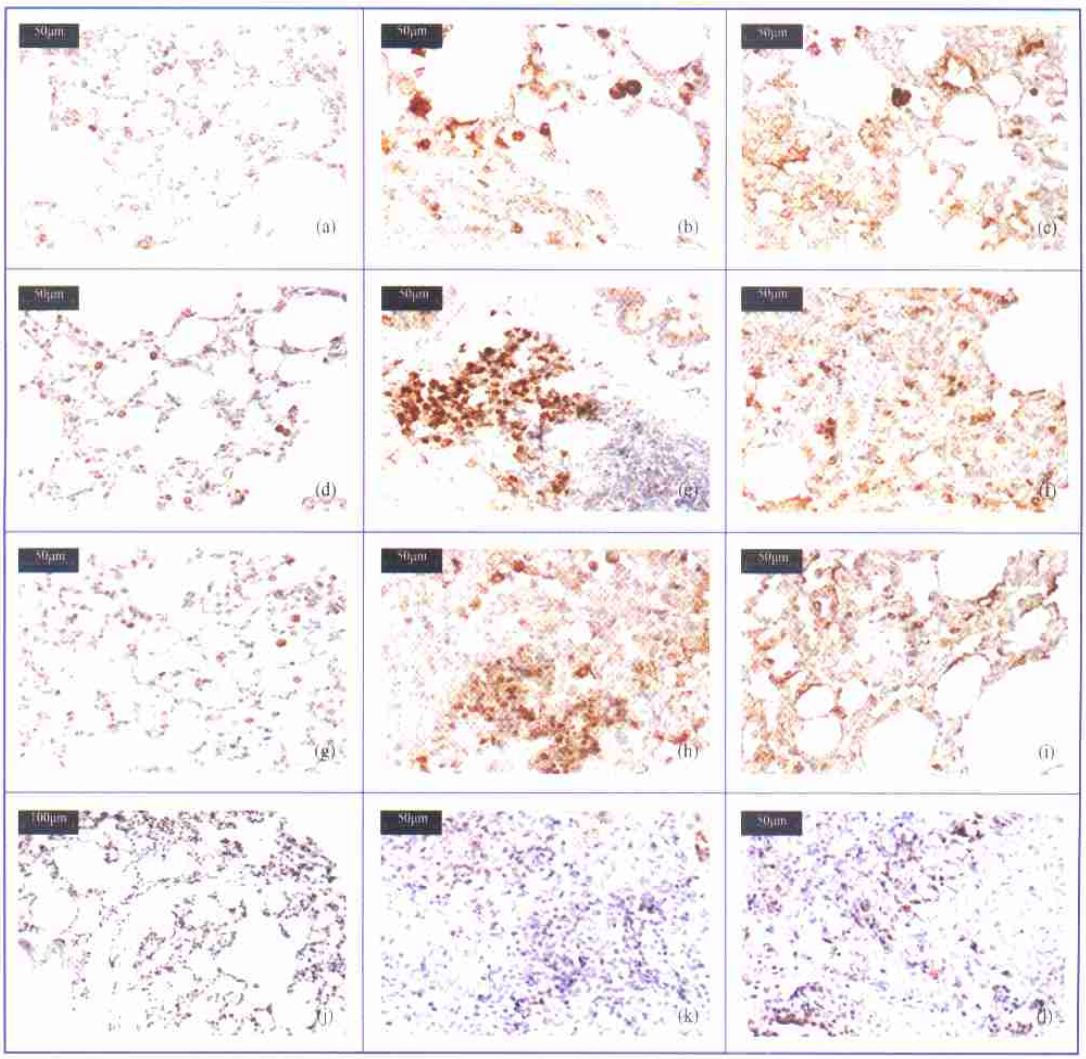
Plate I



Effects of niacin on iNOS expression in rat pulmonary tissues exposed to silica (a)~(k), see text.