Inhibitory effect of aminoguanidine on bleomycin-induced pulmonary toxicity in rat

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KEY WORDS bleomycin; toxicity; pulmonary fibrosis; guanidines

ABSTRACT

AIM: To observe the inhibitory effect of aminoguanidin (AG) on the pulmonary toxicity induced by bleomycin A_5($BLM-A_5$) in rat. **METHODS**: The contents of hydroxyproline, nitrite/nitrate (NO_2^-/NO_3^-), and malondialdehyde (MDA), which reflect fibrosis, nitric oxide (NO) production, and hyperoxidative injury of lung, were investigated by colorimetry. Histologic and morphometric examination of lung was also carried out on histological sections stained with hematoxylin and eosin (HE). **RESULTS**: (1) The content of hydroxyproline in lung and MDA in out-going pulmonary blood (OPB) increased from 14 d to 30 d after intratracheal administration of $BLM-A_5$. Collapsed alveoli and fibrotic areas were seen and a lot of fibroblasts appeared in the lung interstitum of the rats 30 d after $BLM-A_5$. (2) The NO_2^-/NO_3^- content increased in supernatant of alveolar macrophage culture and in OPB. (3) The increment of hydroxyproline in lung and MDA in OPB induced by $BLM-A_5$ was alleviated by aminoguanidine (AG, 20 $mg \cdot kg^{-1} \cdot d^{-1}$, ip). AG also reduced the histologic and morphometric changes in lung interstitum. (4) The augment of NO_2^-/NO_3^- in OPB was blocked by AG. **CONCLUSION**: AG had inhibitory effect on pulmonary injury and fibrosis induced by $BLM-A_5$, and the inhibitory effect was related to the decrease of NO production in lung.

INTRODUCTION

Bleomycin-induced pulmonary toxicity involved pulmonary inflammatory injury and fibrosis. Despite the high efficiency of bleomycin-A_5 ($BLM-A_5$) as a chemotherapeutic agent against various carcinomas, the pulmonary toxicity, especially the potentially lethal and chronic fibrotic response of the lung, is a major dose-limiting side effect[1,2]. So far, there was not effective therapy for the pulmonary fibrosis.

It has been reported that nitric oxide (NO) was involved in inflammatory response[3] and fibrosis of lung[4,5]. Quinlan *et al*[6] reported that NO generation might be important in cell injury and inflammation by asbestos. NO is produced by a five-electron oxidation of *L*-arginine to *L*-citrulline by NO synthase (NOS). Two general classes of NOS have been identified: the calcium-dependent, constitutively expressed NOS (cNOS) and the calcium-independent, transcriptionally inducible NOS (iNOS)[7]. It is well-accepted that cNOS was involved in physiological regulations. On the other hand, inflammatory stimuli, such as cytokines and endotoxin, upregulated iNOS. Produced in excess by iNOS, NO may cause tissue injury.

Aminoguanidine (AG), containing the guanido-group of *L*-arginine linked to hydrazine, *in vitro*, displays 10 to 100 folds higher potency as inhibitor of iNOS than cNOS[8]. In endotoxemic rodents and dogs, AG suppresses activation of iNOS and peroxynitrite production in the lungs and decreases plasma levels of the stable NO metabolites, nitrites, and nitrates. AG also reduces lung edema, improves gas exchange, and increases survival by counteracting circulatory failure[9-12]. Cruz *et al*[13,14] found that AG significantly attenuated the *N*-nitroso-*N*-methylurethane (NNMU)-induced alveolar injury. According above, We hypothesized that AG might suppress the progression of pulmonary injury and fibrosis induced by $BLM-A_5$. Unfortunately, these have rarely been reported.

The current study was to prove enhancement of NO during pulmonary injury and fibrosis induced by $BLM-A_5$, and to investigate the effect of AG in the above processes.

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MATERIALS AND METHODS

Experimental animals and groups Sprague-Dawley rats (♂, $n = 70$, weighing 180 – 200 g, Grade Ⅱ, Certificate № 06057) were provided by the Experimental Animal Center of Hebei Province, China. For the duration of the experiment, the animals were fed with commercial rat food and water *ad libitum*. The rats were randomly allocated to two treatment parts: (1) Rats received instillation of BLM-A_5(5 mg/kg, in 0.5 mL of sterile saline) via tracheotomy under anesthesia (ip 2 % pentobarbital sodium 2 mL/kg) and sacrificed on d 7, d 14, d 21, and d 30, respectively (BLM-A_5 7 d, BLM-A_5 14 d, BLM-A_5 21 d, BLM-A_5 30 d group); or received intratracheal injection of the same amount of 0.9 % sterile saline as control group (Control); (2) Rats were given the same amount of BLM-A_5 as part (1), followed by daily intraperitoneal (ip) injection of AG (20 $mg \cdot kg^{-1} \cdot d^{-1}$) or saline for 14 d or 21 d. The rats were sacrificed on d 14 or d 30 after BLM-A_5(BLM-A_5 + AG 14 d, BLM-A_5 + AG 30 d, BLM-A_5 + saline 14 d, BLM-A_5 + saline 14 d group).

Collection of samples Alveolar macrophages (from bronchoalveolar fluid) were placed in 96-well plates (in RPMI 1640 medium, 20 % fetal bovine serum, 5×10^8 cells/L, 0.1 mL/well) and incubated for 24 h at 37 ℃ with 5 % CO_2. After incubation, the culture supernatants of alveolar macrophage were used for detecting NO_2^-/NO_3^-.

Out-going pulmonary blood (OPB) was collected under anesthesia (ip 2 % pentobarbital sodium 2 mL/kg) for detecting the nitrite/nitrate (NO_2^-/NO_3^-) and malondialdehyde (MDA). Lung was taken for evaluating collagen by determining hydroxyproline and for morphometric examination.

Measurements of nitrite/nitrate and MDA The content of nitrite/nitrate (NO_2^-/NO_3^-) in culture supernatants of alveolar macrophage and in OPB were measured by using detecting kit (Nanjing Jiancheng Biochemical Institute, China). The content of MDA in OPB was measured by using MDA detecting kit (Nanjing Jiancheng Biochemical Institute, China).

Assay of hydroxyproline in lung Hydroxyproline was released from lung tissue homogenates by acid hydrolysis (105 ℃, 18 h). The free hydroxyproline was then oxidized by chloramine T to produce a pyrrole-type compound. The addition of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde dissolved in perchloric acid) resulted in the formation of a chromophore with a wavelength maximum at 550 nm. The absorbances were read using water as reference and corrected for the reagent blank. Absorbance values were plotted against nanomoles of added hydroxyproline and the negative intercept on the x axis was found by linear regression analysis. The absolute value of this intercept was taken to represent the hydroxyproline content of the tissue[15].

Histological and morphometric examination of lungs Lungs from 6 rats of BLM-A_5 14 d and BLM-A_5 + AG 14 d groups were fixed by 4 % formaldehyde. Sections stained with hematoxylin and eosin (HE) were examined by light microscopy for evidence of pulmonary injury and fibrosis.

Drugs Bleomycin-A_5(BLM-A_5) was produced by Tianjin Taihe Pharmaceutical Co, China. Aminoguanidine hemisulfate salt (AG) was produced in Germany, *L*-4-hydroxyproline was from Sigma Chemical Co, USA. RPMI 1640 was from GIBCO, USA. All other chemicals were of analytical reagent.

Statistics Data were expressed as $\bar{x} \pm s$ and compared by t-test.

RESULTS

The toxicity of BLM-A_5 on lung The degree of pulmonary fibrosis and injury was estimated by testing hydroxyproline in lung, MDA in OPB and histologic and morphometric changes of lungs. The contents of hydroxyproline in lung and MDA in OPB in BLM-A_5 14 d, 21 d, and 30 d groups were higher than those in the control group (Tab 1). Collapsed alveoli and fibrotic areas were seen and a lot of fibroblasts appeared in the lung interstitum of the rats in BLM-A_5 30 d group (Fig 1).

Tab 1. Hydroxyproline in lung and MDA and NO_2^-/NO_3^- in out-going pulmonary blood after intratracheal administration of BLM-A_5 in rats. $n = 6$ rats. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Groups	Hydroxyproline /$mg \cdot g^{-1}$	MDA /$\mu mol \cdot L^{-1}$	NO_2^-/NO_3^- /$\mu mol \cdot L^{-1}$
Control	19±6	8.5±1.5	60±20
BLM-A_5 7 d	24±10	9.1±1.1	103±19^c
BLM-A_5 14 d	33±8^b	11.7±0.8^b	171±32^c
BLM-A_5 21 d	52±6^c	11.9±1.0^b	77±22
BLM-A_5 30 d	56±10^c	12.8±0.5^c	77±31

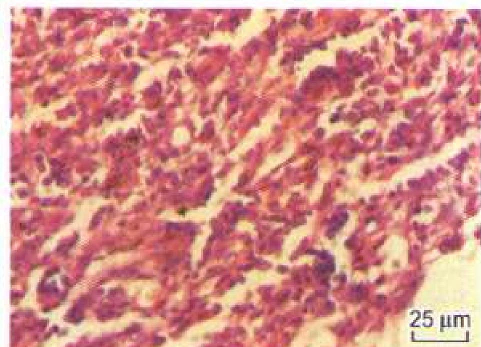

Fig 1. Typical morphology of lung tissue was observed in SD rat d 30 after intratracheal injection of BLM-A_5. HE stain. ×400.

Change of NO metabolites in lung induced by BLM-A_5 The NO_2^-/NO_3^- in OPB were significantly increased in BLM-A_5 7 d and BLM-A_5 14 d group, and returned in BLM-A_5 21 d and 30 d groups (Tab 1). Compared with control, the contents of NO_2^-/NO_3^- in culture supernatants of alveolar macrophages were high in BLM-A_5 14 d and BLM-A_5 30 d groups. But the NO_2^-/NO_3^- in supernatants in BLM-A_5 30 d group was lower than that in BLM-A_5 14 d group (Tab 2).

Tab 2. The content of NO_2^-/NO_3^- in culture supernatants of alveolar macrophages after administration of BLM-A_5 in rats. $n=4$ rats. $\bar{x} \pm s$. ${}^bP<0.05$, ${}^cP<0.01$ vs control at the same day. ${}^eP<0.05$ vs BLM-A_5 d 14.

Groups	NO_2^-/NO_3^- ($\mu mol \cdot L^{-1}$) d 14	d 30
Control	242 ± 32	241 ± 29
BLM-A_5	442 ± 38^c	333 ± 38^{bc}

Effects of AG on the pulmonary injury and fibrosis induced by BLM-A_5 In BLM-A_5 + AG 30 d group, both hydroxyproline in lung and MDA in OPB were lower than those in BLM-A_5 + saline 30 d group. (Tab 3). In lung interstitum of rats in BLM-A_5 + AG 30 d group, the morphometric changes of BLM-A_5 30 d group reduced (Fig 2).

Effects of AG on NO in OPB of rats after administration of BLM-A_5 In BLM-A_5 + AG 14 d group, NO_2^-/NO_3^- in OPB was lower than that in BLM-A_5 + saline 14 d group (Tab 4). There was no significant difference of the content of NO_2^-/NO_3^- in OPB between BLM-A_5 + AG 30 d group and BLM-A_5 + saline 30 d group (Tab 4).

Tab 3. Effects of AG on contents of hydroxyproline in lung and MDA in out-going pulmonary blood of rats on d 30 after intratracheal administration of BLM-A_5. $n=6$ rats. $\bar{x} \pm s$. ${}^cP<0.01$ vs BLM-A_5 + saline.

Groups	Hydroxyproline/ $mg \cdot g^{-1}$	MDA/$\mu mol \cdot L^{-1}$
BLM-A_5 + Saline	57 ± 4	12.5 ± 1.9
BLM-A_5 + AG	44 ± 4^c	8.0 ± 1.2^c

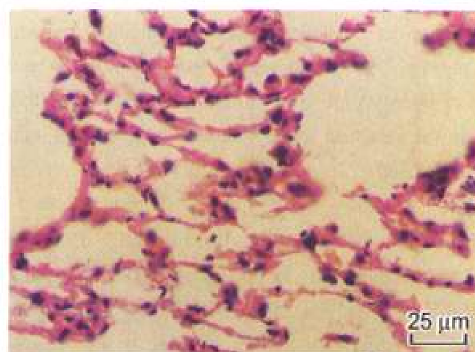

Fig 2. Typical morphology of lung tissue was observed in SD rat d 30 after intratracheal injection of BLM-A_5, followed by ip AG 20 $mg \cdot kg^{-1} \cdot d^{-1}$ for 21 d. HE stain. ×400.

Tab 4. Effects of AG on contents of NO_2^-/NO_3^- in out-going pulmonary blood of rats on d 14 and d 30 after intratracheal administration of BLM-A_5. $n=6$ rats. $\bar{x} \pm s$. ${}^cP<0.01$ vs BLM-A_5 + saline.

Groups	NO_2^-/NO_3^- ($\mu mol \cdot L^{-1}$) d 14	d 30
BLM-A_5 + Saline	176 ± 17	96 ± 20
BLM-A_5 + AG	93 ± 19^c	80 ± 8

DISCUSSION

In the present study, a large amount of NO was generated in lung during the period of hyperoxidative injury induced by BLM-A_5. AG could block the above process and pulmonary fibrosis, as shown by the decreases of MDA level in OPB, hydroxyproline level in lung and reduction of the morphometric changes in lung interstitum. It was well known that NO was an important inflammatory mediator. High concentrations of NO may result in

greater accessibility of inflammatory cells to sites of injury[16]. Moreover, peroxynitrite formed by the reaction of NO˙ with superoxide ($O_2^{\cdot}$) may cause oxidant injury *per se* or after formation of a hydroxyl-like radical[17]. Saleh *et al*[18] had already found the increased production of peroxynitrite in the lungs of patients with idiopathic pulmonary fibrosis. Paredi *et al*[5] found that exhaled NO was increased in active fibrosing alveolitis. These results suggested that NO might be one of the important factors to cause pulmonary injury. The inhibitory effect of AG might be related to block NO formation in lung.

Rezende *et al*[19] reported that after intratracheal administration of bleomycin, aminoguanidine bicarbonate (50 $mg \cdot kg^{-1} \cdot d^{-1}$ for 4 weeks) led to significant reductions of hydroxyproline in murine lung, and the collagen area in the axial and septal zones of lung sections stained with Sirius red. In our experiment, the apparently high level of NO_2^-/NO_3^- in OPB appeared from d 7 to d 21 after injection of BLM-A_5, and 20 $mg \cdot kg^{-1} \cdot d^{-1}$ AG for 14 d could abolished above process. In addition, 20 $mg \cdot kg^{-1} \cdot d^{-1}$ AG for 21 d could partly block the progression of pulmonary fibrosis. These results suggested that 20 $mg \cdot kg^{-1} \cdot d^{-1}$ AG was enough to eliminate the influence of NO on pulmonary toxicity induced by BLM-A_5.

Animal experiments listed some methods to modify the incidence and severity of pulmonary toxicity induced by BLM-A_5. Anti-transforming growth factor-β antibodies[20-22] and human recombinant soluble tumor necrosis factor receptor[23] provided potentialities for intervening in the fibrotic process in the lung. *In vivo*, angiotensin-converting enzyme inhibitor captopril or the caspase inhibitor Z-Val-Ala-Asp-fluoromethylketone was used to block apoptosis of alveolar epithelial cell and lung fibrosis in response to BLM[24]. Keratinocyte growth factor, a potent proliferation and differentiation factor for rat alveolar type Ⅱ cells, could prevent BLM-induced lung fibrosis in rats[25]. Our study was another way to prevent pulmonary toxicity induced by BLM. The level of NO in lung of patients could be estimated by NO_2^-/NO_3^- in out-going pulmonary blood. So, it's easy to determine the time when AG should be administered. This work demonstrated AG produced an inhibitory effect on pulmonary toxicity induced by BLM-A_5. The observation suggests that AG may be a hopeful chemical reagent to prevent and cure the pulmonary fibrosis induced by BLM-A_5.

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氨基胍对博莱霉素诱发大鼠肺毒性的抑制作用

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关键词 博莱霉素；毒性；肺纤维化；胍类

目的：观察氨基胍(AG)对博莱霉素 A_5($BLMA_5$)诱发大鼠肺毒性作用的影响．**方法**：BLM-A_5(5 mg·kg^{-1})气管滴注诱发大鼠肺损伤和肺纤维化形成；在气管滴注 BLM-A_5 的同时及之后 21 d 腹腔注射 AG (20 mg·kg^{-1}·d^{-1}, ip)．化学比色法测定肺组织羟脯氨酸含量、肺泡巨噬细胞培养上清液中 NO_2^-/NO_3^- 含量、出肺血中 NO_2^-/NO_3^- 含量；HE 染色组织切片上观察组织形态学变化．**结果**：(1) 滴注 BLM-A_5 后 d 14 至 30，肺组织羟脯氨酸含量和出肺血中的 MDA 含量逐渐升高；注 $BLMA_5$ 后 d 30，肺间质可见萎陷的和纤维化的肺泡，并出现大量的成纤维细胞．(2) 滴注 BLM-A_5 后 d 7 至 14，出肺血 NO_2^-/NO_3^- 含量升高；肺泡灌流液中巨噬细胞培养上清液中 NO_2^-/NO_3^- 含量升高．(3) AG 明显抑制出肺血 MDA 含量和肺羟脯氨酸含量的升高；AG 还减轻肺间质组织形态学变化．(4) AG 明显抑制出肺血 NO_2^-/NO_3^- 含量的升高．**结论**：AG 对 BLM-A_5 诱发的大鼠肺损伤和纤维化有抑制作用，此作用与其抑制肺内 NO 大量生成有关．

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