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Oral halloysite nanotubes-induced subacute toxicity in the large intestine of mice and recovery

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Abstract: Natural halloysite nanotubes (HNTs) with a hollow lumen have been widely applied in many fields, such as traditional Chinese medicines, drug carriers, cosmetics, feed additives, antibacterials and water purification. However their toxicity in the gastrointestinal tract is still unclear. The aim of this study is to evaluate subacute oral toxicity of HNTs in the large intestines of mice and their recovery from it. Oral HNTs at low dose (5 mg/kg) for 30 days had no obvious adverse effect on the large intestine. Oral HNTs at high dose (50 mg/kg) for 30 days induced Al and Si accumulation and oxidative stress in the large intestine as indicated by the significant decreases in GSH-Px and SOD activities and the significant increase in MDA level in the large intestine, which caused significant increases in COX-2 and iNOS levels and inflammatory response and iNOS-mediated damages in the large intestine. Oral HNTs-induced changes at high dose described above were not observed after a 30 days recovery period, suggesting that oral HNTs-induced subacute toxicity in the large intestine was reversible.

Key words: halloysite nanotubes; toxicity; oxidative stress; large intestine

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口服埃洛石纳米管对小鼠大肠的亚急性毒性及其恢复情况

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摘要: 天然埃洛石纳米管(HNTs)**具有空心腔结构,它作为传统中药、药物载体、化妆品、饲料添加剂、抗菌材料、污水处理剂等广泛地应用于许多领域.然而目前人们对埃洛石纳米管诱导的肠道毒性仍不清楚.本文研究口服** HNTs **对小鼠大肠亚急性毒性及其恢复情况.结果表明,小鼠连续** 30 **天口服低剂量**(5 mg/kg) HNTs,**未对其大肠造成明显的** Al **和** Si **聚集和损伤.小鼠连续** 30 **天口服高剂量**(50 mg/kg)HNTs,**导致其大肠中** Al **和** Si **聚集和氧化应激反应,表现为大肠中谷胱甘肽过氧化物酶活性和超氧化物歧化酶活性显著**

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降低，以及脂质氧化产物丙二醛含量显著升高.该氧化应激导致大肠细胞炎症反应，表现为大肠组织中一氧化氮合酶和环氧化酶-2 的表达水平升高以及炎细胞浸润病变.这说明口服高剂量 HNTs 能引起由一氧化氮合酶参与的大肠损伤.高剂量组小鼠口服给药 30 天后再经过 30 天恢复期，未发现其大肠组织有明显损伤，说明口服高剂量 HNTs 引起的小鼠大肠的急性毒性是可逆的和可以恢复的.

关键词：埃洛石纳米管；毒性；氧化应激；大肠

0 Introduction

Halloysite ($Al_2Si_2O_5(OH)_4 \cdot nH_2O$) nanotubes (HNTs) are natural aluminosilicate mineral with a tubular nanostructure[1]. HNTs have a length of 0.2～15μm, an inner lumen of 10～20nm, and an outer diameter of 50～100nm[2]. Due to their low toxicity, large surface area and tubular nanostructure, HNTs have wide applications in numerous fields, such as water purification[3], antibacterials[4], catalyst carriers[5], drug carriers[6], cosmetics[7], and feed additives[8-9] and traditional Chinese medicines[10]. The wide applications of HNTs increase the potential risk for their release to the surroundings[11-12], which are commonly found in natural soils and water[13]. Thus, the clarification of the toxicity of HNTs towards humans and animals is crucially important. An increasing number of investigations have been conducted to examine in vitro toxicities of HNTs. HNTs showed low cytotoxicity to many cell lines, such as HepG2, HCT116, Caco-2/HT29-MTX, MCF-7, HeLa and yeast cell[14-17]. In contrast to studies on the in vitro toxicity of halloysite, there have been few articles published on the in vivo toxicity of halloysite. Fakhrullina et al. studied the in vivo toxicity of HNTs to Caenorhabditiselegans nematode and found that HNTs reduced the size of nematodes, stimulated the gastrointestinal tract and affected nutrient absorption of nematodes [18]. Marina Kryuchkova et al. studied the in vivo toxicity of halloysite on large paramecium, and found that halloysite had no effect on cell viability at low concentrations, but the cell survival rate decreased with increasing concentration of halloysite[19]. However, so far, the toxicity of HNTs in gastrointestinal tract has remained unclear.

In this study, thesubacute toxicity of oral HNTs in the large intestine of mice was investigated after intragastric administration of HNTs at different doses for 30 days. In addition, the recovery from oral HNTs-induced subacute toxicity in the large intestine was also assessed after 30 day of recovery.

1 Experimental

1.1 Materials

Rawhalloysite powders were purchased from Yan-Bo Minerals Processing Company (Hebei, China) and purified as described previously[20]. The purified HNTs were characterized by transmission electron microscopy (TEM) (JEM-2010, JEOL Ltd, Japan). The elemental contents of the purified HNTs were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (OPTIMA 7300DV, PerkinElmer, USA). All chemicals were purchased from Shanghai Chemical Reagent Co. Ltd. (Shanghai, China).

1.2 Animals and treatment

The male Kunming mice (6～8 weeks old, Animal Center of Anhui Medical University) were housed in stainless-steel cages at a relative humidity of 55%～65% and 25℃ using a 12h light/dark cycle, with free access to food and water. 72 male mice were divided randomly into three groups (24 mice in each group): control group (saline solution), low dose group (5mg HNTs per kilogram of body weight (BW) per day) and high dose group (50 mg HNTs per kilogram of body weight per day). The doses of HNTs were used in this study according to OECD Test Guideline 420[21]. The mice were intragastrically injected with chemicals for 30 consecutive days. After the last administration, one half of the mice in each group were fasted 12 h and another half of

the mice in each group were fed with mouse food for another 30 days and then fasted 12 h. After a 12-h fasting, all mice were sacrificed. The large intestine was dissected out. All experiments were performed with the approval of the Animal Ethical Committee of University of Science and Technology of China.

1.3 Detection of Al and Si contents in the large intestine

According to the previous methods[22], Al and Si contents in the large intestine were detected by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (OPTIMA 7300DV, PerkinElmer, USA).

1.4 Histopathological examination

The large intestine tissues were removed from the mice and immediately stored in 10% formalin at 4 ℃, and then the tissues were embedded in paraffin blocks, sliced into 5 μm thick sections. After being stained with hematoxylin and eosin, the sections were observed by an optical microscope (IX-81, Olympus, Japan).

1.5 Oxidative stress analysis

Oxidative stress in the large intestine tissues was determined using the previous methods[22]. The level of malondialdehyde (MDA), and the activities of glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) were measured using commercial kits (Nanjing Jiancheng Bioeng Inst., China), following the manufacturer's instructions.

1.6 Cytokine expressionanalysis

The cyclooxygenase-2 (COX-2) andnitric oxide synthase (iNOS) levels in the large intestine tissues were determined by ELISA using commercial kits (Shanghai Jianglai Biotechnology Co., Ltd., Shanghai, China), according to the instructions of the manufacturer. The absorbance of all samples (iNOS and COX-2) was detected at 450nm on a microplate reader (Bio-Tek, ELx-800, Vermont, USA).

1.7 Statistical analysis

One-way analysis of variance (ANOVA) was performed to analyze the data using SPSS 19.0 software (SPSS Inc., Chicago, IL). Differences between the control group and the HNTs-treated groups were compared by Dennett's test. A p-value of less than 0.05 was considered statistically significant.

2 Results

2.1 Characterization of the purified HNTs

The size and homogeneity of the purified HNTs were examined by TEM. As shown in Fig.1 (a), (b), the purified HNTs were well-dispersed and homogeneous with an average length of 1 88 nm$\pm$7 nm. The average outer diameter and the average inner diameter were 42.6 nm$\pm$3.5 nm and 14.3 nm$\pm$1.3 nm, respectively, and their specific surface area was 33.3 m^2/g. ICP-AES measurements indicated that the purified HNTs consisted of 60.57% SiO_2, 35.54% Al_2O_3, 2.71% Fe_2O_3, 0.61% CaO and 0.53% MgO. No toxic heavy metals were detected in the purified HNTs.

2.2 Coefficients of the large intestine

The coefficient of the large intestine to body weight was calculated as the ratio of large intestine (wet weight, mg) to body weight (g). As shown in Fig.2, oral administration of HNTs for 30 days caused a slight increase in the coefficient of the large intestine at low dose ($p > 0.05$) and a significant increase in the coefficient of the large intestine at high dose ($p < 0.05$), suggesting that HNTs might have caused damage in the large intestine at high dose. No significant changes were found in large intestine coefficients between the control group and the low or high dose group ($p > 0.05$) after 30 days of recovery, indicating that the mice in the high dose group might have recovered from oral HNTs-induced damage in the large intestine.

2.3 Contents of Al and Si in the large intestine

As shown in Fig.3(a), (b), the contents of Al and Si in the large intestine slightly increased at low dose ($p > 0.05$) and significantly increased at high dose ($p < 0.05$) after administration of HNTs for 30 days, suggesting that oral HNTs at high dose caused accumulation of Al and Si in the large intestine. The contents of Al and Si in the large intestine at high dose significantly decreased ($p < 0.05$) after 30 days of recovery, which were very close to the control, indicating that all accumulated Al and Si were cleared out of the large intestine after 30 days of recovery.

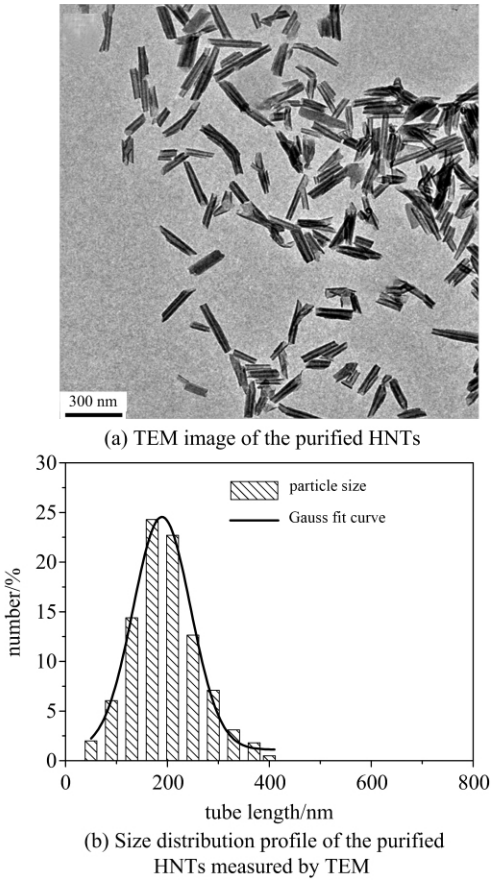
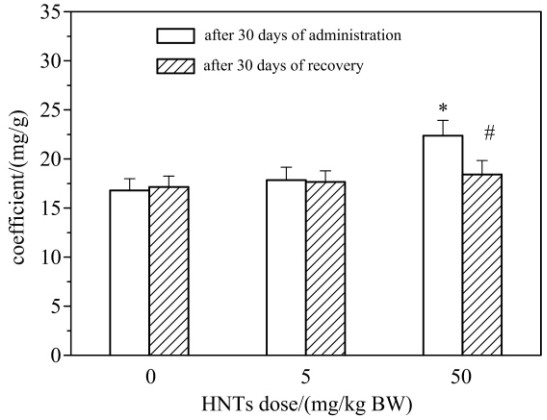


Fig.1　Characterization of purified HNTs by TEM

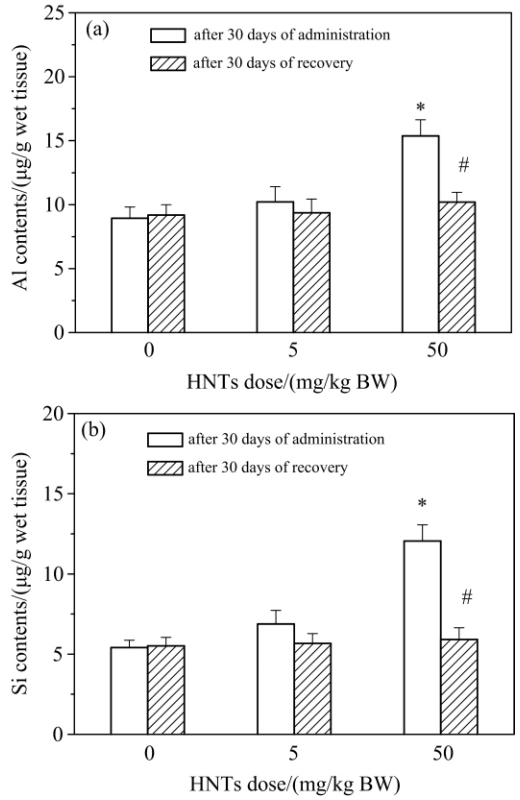


Mice were orally administrated with HNTs for 30 days following a 30-d recovery period. * $p<0.05$ versus the control, # $p<0.05$ versus the value measured after 30 days of administration

Fig.2　Coefficient of the large intestine of mice (means±SD, $n=6$)

2.4　Histopathological evaluation

In order to analyze the effect of accumulated Al and Si in the large intestine on its tissue architecture, histopathological examination was performed for the large intestine. As shown in Fig. 4, the large intestine tissues in the control and low dose groups showed the normal architectures, revealing that HNTs at low dose had no observable adverse effects on the large intestine. However, histopathological changes were observed in the large intestine tissues in the high dose group. Local bleeding in mucosal and submucosal layers and inflammatory cell infiltration in mucosal layers were observed in large intestine tissue in the high dose group. Compared to the control, no histopathological changes were observed in the tissues of the large intestine in the high dose group after 30 days of recovery, indicating the recovery of the organ from HNTs-induced injury.

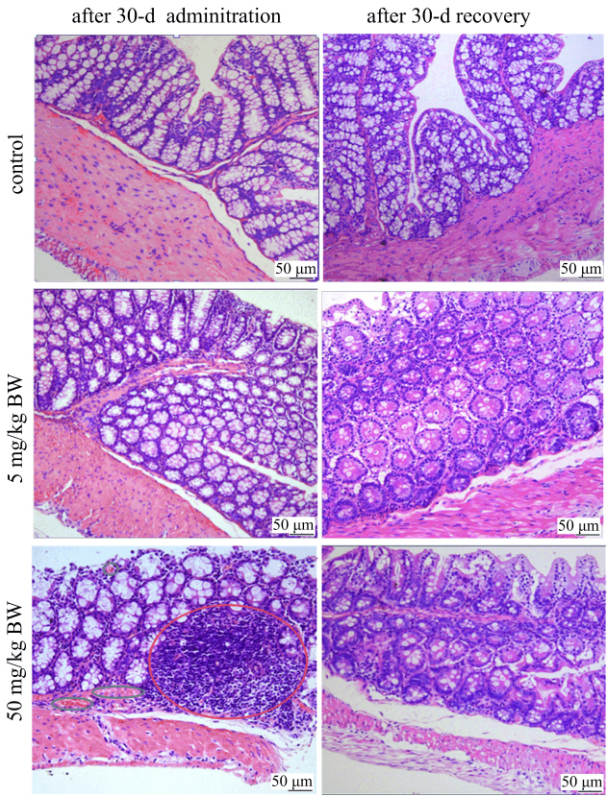


Mice were orally administrated with HNTs for 30 days following a 30-d recovery period. * $p<0.05$ versus the control, # $p<0.05$ versus the value measured after 30 days of administration

Fig.3　The contents of Al (a) and Si (b) in the large intestine measured with ICP-AES (means±SD, $n=6$)

2.5　HNTs-induced oxidative stress

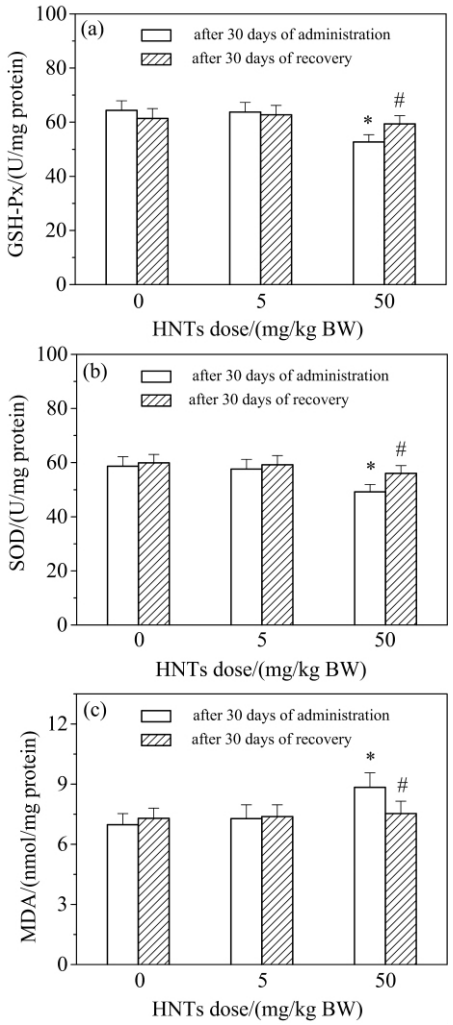
In order to investigate the mechanism of the toxicity of oral HNTs in the large intestine, HNTs-induced oxidative stress in the tissue of the large intestine was analyzed by measuring the endogenous anti-oxidative enzymes, SOD and



Mice were orally administrated with HNTs for 30 days following a 30-d recovery period. Red ellipses indicates inflammatory cell infiltration in mucosal layers. Green circle indicates local bleeding in mucosal layers. Green ellipses indicates local bleeding in submucosal layers

Fig.4 Histopathological changes in the large intestine of mice

GSH-Px, and the product of lipid peroxidation, MDA in the two organs. As shown in Fig.5(a), (b), oral administration of HNTs for 30 days did not cause significant changes in SOD and GSH-Px activities in the large intestine at low dose ($p>0.05$) but induced significant increases in SOD and GSH-Px activities in the large intestine at high dose ($p<0.05$), suggesting that oral HNTs might cause oxidative stress in the large intestine at high dose. As shown in Fig.5(c), oral administration of HNTs for 30 days did not induce a marked change ($p>0.05$) in MDA level in the large intestine at low dose, but caused a significant increase ($p<0.05$) in MDA level in the large intestine at high dose. These results further indicated that oral HNTs caused oxidative stress in the large intestine at high dose. The SOD and GSH-Px activities and the MDA level in the large intestine at high dose were very close to the control after 30 days of recovery, suggesting the recovery of the large intestine from HNTs-induced oxidative stress after 30 days of recovery.



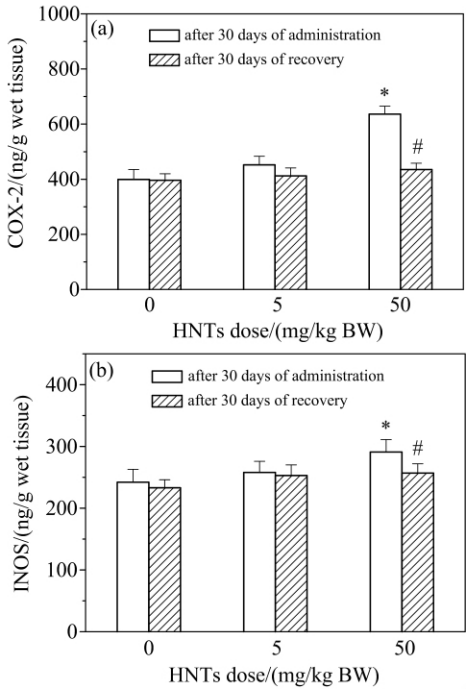
Mice were orally administrated with HNTs for 30 days following a 30-d recovery period. * $p<0.05$ versus the control, # $p<0.05$ versus the value measured after 30 days of administration

Fig.5 Changes in GSH-Px (a) and SOD (b) activities, and MDA (c) level in the mouse large intestine (means±SD, $n=6$)

2.6 HNTs-induced expression of iNOS and COX-2

To analyze the HNTs-induced inflammatory response in the large intestine, the levels of two inflammation indicators, iNOS and COX-2, were examined by ELISA. As shown in Fig. 6, oral administration of HNTs for 30 days did not induce significant changes ($p>0.05$) in the iNOS and COX-2 levels in the large intestine at low dose, but led to significant increases ($p<0.05$) in the iNOS and COX-2 levels in the large intestine at high dose, which confirmed that oral HNTs induced inflammatory response in the large intestine at high dose. The iNOS and COX-2 levels in the large

intestine at high dose were very close to the control after 30 days of recovery, suggesting that HNTs-induced inflammatory response in the large intestine disappeared after 30 days of recovery.



Mice were orally administrated with HNTs for 30 days following a 30-d recovery period. * $p<0.05$ versus the control, # $p<0.05$ versus the value measured after 30 days of administration

Fig.6　Changes of COX-2 (a) and iNOS (b) levels in the mouse large intestine (means±SD, $n=6$)

3　Discussion

The present study was designed to examine the subacute oral toxicity of HNTs in the large intestines of the mice and their recovery from it. Although oral HNTs at low dose for 30 days caused slight accumulation of Al and Si in the large intestine, no observable damages were found in the large intestine after oral administration with HNTs for 30 days at low dose, indicating that slight accumulation of Al and Si in the large intestine was not enough to cause damage in the organ. Previous work showed that oral HNTs at low dose for 30 days have no toxicity in the liver and lung of mice[22-23]. These results together with the present data demonstrate the safety of the application of HNTs in mice at low dose. In a routine biomedical application in humans, the oral dosages of HNTs are usually lower than 5 mg/kg BW[21].

Oral HNTs at high dose for 30 days led to significant increases in the contents of Al and Si in the large intestine owing to the accumulation of HNTs and their degradation products in the organ. The Al accumulation in the large intestine was slightly higher than that of Si in the high dose group, which might be attributed to the different dissolution rates of Al and Si ions in HNTs in the large intestine. Recently, we reported that HNTs were very stable in artificial intestinal fluid (pH 6.8), in which only 0.31% of Si ions and 0.032% of Al ions in HNTs were released into the artificial intestinal fluid after 4 h[23]. The dissolved Si ions and Al ions re-entered into the systemic circulation and excreted from the body. The dissolution rate of Si ions in HNTs in the large intestine was higher that of Al ions. Therefore, the excretion rate of Si was also higher than than that of Al. As a result, the Al accumulation in the large intestine was slightly higher than that of Si in the high dose group.

The accumulation of HNTs and their degradation products in the large intestine induced the oxidative stress in the large intestine as indicated by the significant decreases in GSH-Px and SOD activities and the significant increase in MDA level in the large intestine (Fig. 5). The MDA level indirectly reflects the severity of free radical attack and the degree of oxidative damage in the tissue.

The exposed surfaces of HNTs are silica, thus the toxicity of HNTs may be compared with that of SiO_2 nanoparticle[15]. Both HNTs and silica nanoparticles can induce reactive oxygen species[24]. The surface interactions of the nanoparticles with media can directly produce reactive oxygen species. On the other hand, Al ions have been shown to increase peroxidation in liposomes[25]. This prooxidant action occurs through interactions of the Al^{3+} ion with membranes. The binding of Al^{3+} to the membrane results in subtle changes in the rearrangement of lipids which promotes liposome aggregation and permeability, increases the possibility of fatty acids to be attacked by free radicals and facilitates the propagation of lipid peroxidation[26].

Therefore, oral HNT-induced oxidative stress in the large intestinal tract should be caused not only by deposited HNTs but also by accumulated Al in the organ. Oral HNTs-induced oxidative stress in the large intestinal caused the large intestinal dysfunction and histopathological abnormalities.

The HNTs-induced oxidative stress in the large intestine at high dose caused significant increases in the COX-2 and iNOS levels in the tissues of the large intestine. COX-2 is an important enzyme in the process of inflammation, and its overexpression is a marker of inflammation[27]. COX-2 serves as one of the bridging molecules in linking oxidative stress and inflammation. ROS induces the expression of COX-2, which causes tissue inflammation. The COX-2 overexpression in the large intestine indicated the oral HNTs-induced inflammatory response in the large intestine, which was confirmed by histopathological examination (Fig. 4). INOS is the rate-limiting enzyme for NO synthesis. It is reported that increased iNOS expression leads to the production of high concentrations of NO, which can stimulate ROS-induced lipid oxidation[28]. In the presence of oxygen, the high level of NO in the tissue can produce ROS, thus reducing the activity of the antioxidant enzyme[29]. MDA is the product of lipid peroxidation. The significant increases in the MDA and iNOS levels in the large intestine indicated that iNOS might participate in oral HNTs-induced inflammation and damage in the large intestine. INOS-mediated damage in the large intestine at high dose caused significant increase in the coefficient of the large intestine.

A previous work showed that the lung is the major host organ in mice for oral HNTs and the liver, kidney and spleen are also the target organs in mice[23]. The present result indicates that the large intestine is also the target organ for oral HNTs (Fig.3).

This study was also designed to understand whether oral HNTs-induced subacute toxicity in the large intestine was reversible. HNTs accumulated in the tissue of the large intestine underwent slight dissolution. The dissolved Si and Al ions re-entered into the systemic circulation and were excreted from the body. We found that almost all of accumulated Al and Si were cleared out of the large intestine after 30 days of recovery. Oral HNTs-induced oxidative stress, inflammation and damage in the large intestine at high dose essentially disappeared after 30 days of recovery. The large intestine coefficient in the high dose group returned to normal values at the end of the recovery period. These results supported that oral HNTs-induced subacute toxicity in the large intestine was reversible. The degradation of HNTs and the excretion of their degradation products resulted in the recovery of the large intestine from oral HNTs-induced injury.

4 Conclusion

In summary, oral HNTs for 30 days had no obvious adverse effect on the large intestine at low dose, but induced Al and Si accumulation and oxidative stress in the large intestine at high dose, which in turn caused inflammatory response and iNOS-mediated damages in the large intestine. Oral HNTs-induced oxidative stress, inflammation and damage in the large intestine at high dose were not observed after 30 days of recovery, suggesting reversibility. The present results can be used to evaluate the health risks from exposure to HNTs in environments contaminated by HNTs.

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