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Toxicity of halloysite nanotubes in liver of mice after oral administration

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Abstract: Halloysite is widely used in many fields due to its natural hollow nanotubular structure. To assess the liver toxicity of the purified halloysite nanotubes (HNTs) in mice *via* oral route, purified HNTs were orally administered to mice at 4, 20 and 100 mg/kg BW body weight (BW) every day for 30 d. Oral administration of HNTs stimulates the growth of the mice at low dose (4 mg/kg BW) with no liver toxicity, but inhibits the growth of the mice and induces oxidative stress in the liver at high dose (≥20 mg/kg BW). In addition, oral administration of HNTs at high dose causes Al accumulation in liver, which induces hepatic dysfunction and histopathologic changes.

Key words: halloysite nanotube; in vivo toxicity; liver

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埃洛石纳米管的口服毒性研究

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摘要:埃洛石是一种广泛应用的天然纳米管状材料。研究了纯化后的埃洛石经口服后对小鼠的肝脏的毒性.实验按口服纯化埃洛石的剂量不同分为4组:对照组,4,20和100 mg/kg BW组.实验结果表明,低剂量(4 mg/kg BW)的埃洛石对小鼠的肝脏没有毒性,且可促进小鼠体重增长;而当剂量高于20 mg/kg BW时小鼠的肝脏产生氧化应激反应,体重增长也受到抑制.而且,高剂量的埃洛石还会导致Al在肝脏聚集,并导致肝脏功能障碍和病理变化.

关键词:埃洛石纳米管;体内毒性;肝脏

0 Introduction

Halloysite is a type of aluminosilicate clay with a hollow nano-tubular structure [1]. The internal lumen diameter and outside lumen are in the range 10～20 nm and 50～100 nm, respectively[2].

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Halloysite nanotubes (HNTs) have been applied in various fields (dye degradation, catalysis, antibacterial, optics, hydrogen storage, drug and gene delivery) because of their unique properties, including abundant deposits, nanoscale lumens[3]. The broad applications of HNTs increase the chances of human beings and animals exposed to them, during the process of purification and application. The toxicity associated with HNTs exposure has been widely documented [4-5]. Zhang et al.[6] show that halloysite has no antibacterial activity against Gram-negative bacteria and Gram-positive bacteria. Fakhrullina et al. [7] reported that HNTs were found safe for Caenorhabditis elegans even at a high concentration of 1 mg/mL, However the toxicity of HNTs in mammals is still unclear.

The commercially available HNTs are usually inhomogeneous and contain various kinds of impurities, such as quartz and kaolinite [8]. Recently, we developed a method for preparing homogeneous and length controllable HNTs by the combination of ultrasonic treatment and uniform viscosity centrifugation [8]. This research sought to investigate the *in vivo* toxicity of high-purity HNTs in mice. Liver is commonly believed to be the first accumulating organ for xenobiotics and nanoparticles en-route to systemic circulation after oral administration [9]. Therefore HNTs-induced hepatic toxicity in mice was assessed by measuring organ coefficient, oxidative stress parameters, and serum biochemical parameters, biodistribution of Al and Si and pathologic changes.

1 Experimental

1.1 Chemicals and preparation

The raw HNTs powders were obtained from YanBo Minerals Processing Company (Hebei, China) and purified by the combination of ultrasonic treatment and uniform viscosity centrifugation as previously described[8]. The purified HNTs were characterized by TEM (JEM-2010, JEOL Ltd, Japan). The hydrodynamic diameter of the sample was determined by a dynamic light scattering (DLS) instrument (Zetasizer μV, Malvern Instruments Ltd., UK).

1.2 Animals and treatment

Kunming mice (male, 22 g±2 g, 6～8 weeks old) were purchased from the Animal Center of Anhui Medical University (China). The mice were housed in stainless steel cages with filter tops under standard conditions (22 ℃±2 ℃; relative humidity at 55%±5%; 12 h light/dark cycle). Distilled water and sterilized food for mice were available *ad libitum*. After 7 d acclimation, mice were randomized into 4 groups with 15 mice in each group: control group (treated with physiological saline) and three experimental groups (4, 20, 100 mg/(kg BW·d) HNTs). The mice were orally exposed to HNTs every day for 30 d. The symptom and mortality of mice were observed every day and their weight was recorded. After an overnight fast, the mice were anesthetized, and blood samples were collected, and then the mice were sacrificed by cervical dislocation. The liver was excised for further analysis. The coefficient of liver to body weight was calculated as the ratio of organ (wet weight, mg) to body weight (g). All experiments were conducted in accordance with the guidelines of University of Science and Technology of China for the care and use of laboratory animals.

1.3 Oxidative stress assay

The liver tissues were assayed for the oxidative biomarkers by the conventional methods described previously [8]. The activities of glutathione peroxidase (GSH-Px), superoxide dismutase(SOD), and lipid peroxidation product malondialdehyde (MDA) were determined using commercial kits (Nanjing Jiancheng Bioeng Inst., Nanjing, China) following the manufacturer's protocol.

1.4 Serum biochemical parameters assay

Serum was harvested by centrifuging blood at 3000 r/min for 10 min at 4 ℃. We estimate liver function by alanine aminotransferase (ALT),

aspartate aminotransferase (AST), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), total protein (TP), albumin (ALB), globulin (GLO), direct bilirubin (DBIL), indirect bilirubin (IBIL) and total bilirubin (TBIL). All serum biochemical parameters were determined by commercial kits (Roche Ltd., Switzerland) using biochemical auto analyzer (Roche Modular DPP, Roche Ltd., Switzerland).

1.5 Histopathological examination

The livers were removed from mice and immediately fixed in 10% (volume fraction) formalin, and then the tissues were embedded in paraffin blocks, sliced into 5 μm thick sections, which were then stained with hematoxylin and eosin (H&E). The optical microscope (IX-81, Olympus, Japan) was used to observe the sections and take photos.

1.6 Analysis of Al and Si biodistribution

The liver tissues (0.1～0.3 g) were weighed and digested with guarantee reagent nitric acid overnight, and then the solutions were heated at 180 ℃ to remove the remaining nitric acid, followed by additional 0.5 mL of 70% perchloric acid. Then, they were heated again until they were colorless and clear. Finally, the samples were cooled to room temperature and diluted to 3 mL with deionized water, and filtered through a 0.22 μm cellulose membrane filter for analysis. The Al and Si contents in the sample solutions were detected by inductively coupled plasma-atomic emission spectrometry (ICP-AES) (Perkin Elmer Corporation, USA).

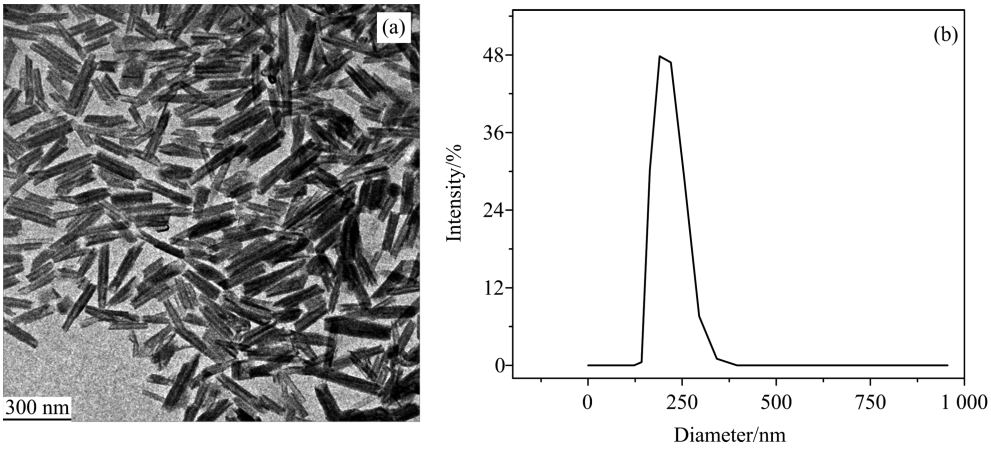
1.7 Statistical analysis

Results were expressed as means ± SD. Data was analyzed by one-way analysis of variance (ANOVA) test using SPSS 19.0 (SPSS Inc., Chicago, IL). The differences between the control group and the HNT-treated groups were tested by Dennett's test. The statistical significance for all tests was set at $p<0.05$.

2 Results

2.1 Characterization of HNTs

The purified HNTs were obtained by the combination of ultrasonic treatment and uniform viscosity centrifugation as described previously [8]. Fig.1(a) displays TEM images of purified HNTs. As shown in the images, halloysite had a tubular structure with an average length of 180 nm ± 8 nm and good dispersity. DLS measurement showed that the average hydrodynamic diameter of purified HNTs was 200 nm ± 6 nm, which was slightly larger than that determined by TEM because of the thickness of the electrical double layer on the surface of the HNTs. The result indicated that HNTs did not aggregate in the physiological solution.

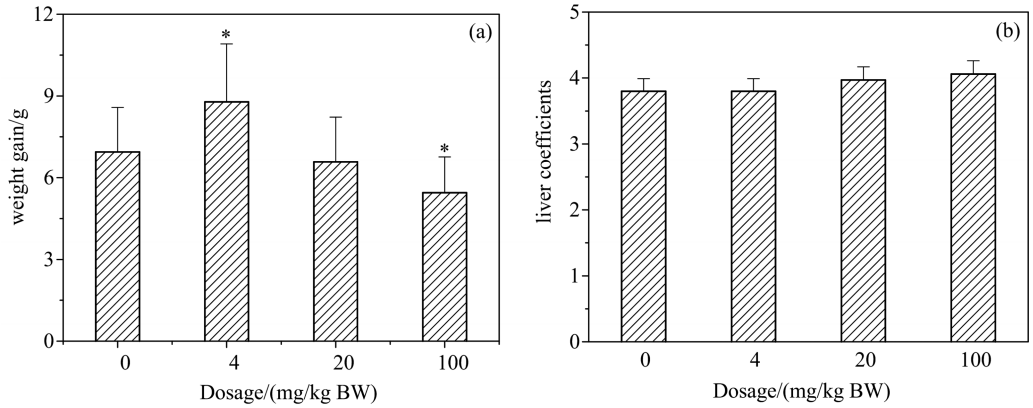


(a) TEM image of purified HNTs. (b) Size distributions of purified HNTs in physiological solution measured by DLS

Fig.1 Characterization of purified HNTs

2.2 Effects on weight and coefficients of liver

During the 30 d study period, no animals showed anyabnormal daily activity and symptoms in the control and low dose (4 mg/kg BW) groups, while the mice exhibited changes such as loss of appetite, and passive behavior in the middle (20 mg/kg BW) and high (100 mg/kg BW) dose groups. The average food consumption of mice in the control group, the low dose (4 mg/kg BW) group, the middle (20 mg/kg BW) and high (100 mg/kg BW) dose groups were 9.15g±0.46g, 9.53 g±0.58 g, 8.93 g±0.51 g and 7.80 g±0.32 g, respectively. Fig.2(a) reveals that the weight gain in the low dose group was higher than the control group with a significant difference ($p<0.05$), and the mice in the high dose group grew slower than the control ($p<0.05$), and no significant changes were observed between the middle dose group and control group. This suggests that HNTs had a positive effect on the growth of mice in the low dose group but a negative effect in the high dose group. As shown in Fig.2(b), the liver coefficient did not significantly change between each halloysite-treated group and the control group.



(a) The weight gain of mice after 30 d oral administration of HNTs (means±SD, $n=10$).

(b) The liver coefficients of mice in different dose groups (means±SD, $n=10$). * $p<0.05$ *versus* the control

Fig.2 The weight gain and the liver coefficients of mice after the experiment

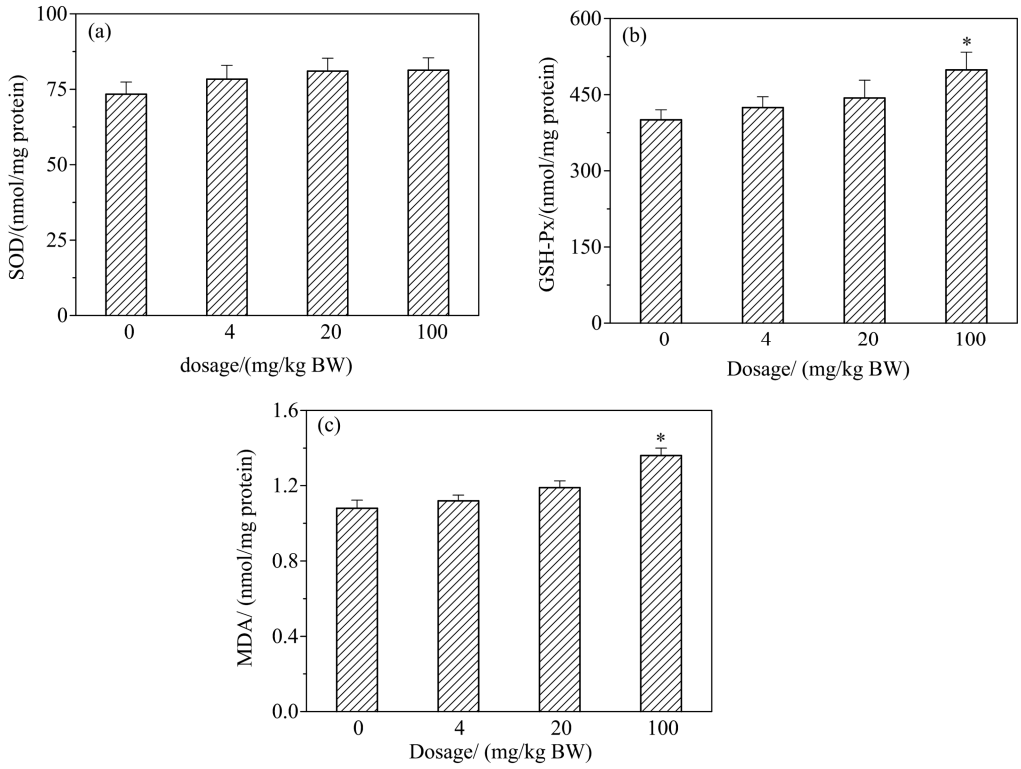
2.3 Oxidative damage in liver tissues

The effect of oral HNTs on the oxidative stress in the liver was investigated by assaying the endogenous antioxidative enzymes, GSH-Px and in the organs. It was reported that decreased levels of antioxidant enzymes are associated with increased oxygen free radical production [10]. As shown in Fig. 3, administration of HNTs did not cause significant change ($p > 0.05$) in the SOD activity in the liver. However, the GSH-Px activity significantly increased in liver in the middle and high dose groups ($p<0.05$) in a dose-dependent manner, compared to the control. MDA is the product of lipid peroxidation, the level of which reflects the level of oxidative damage in tissues. The MDA levels were not significantly changed in liver except in the high dose group (100 mg/kg BW) ($p<0.05$). The significant increase in MDA level shows descended antioxidant capacity. These results together indicate that administration with HNTs does not cause oxidative stress in the liver at the low dose, but induces significant oxidative stress in the liver at the middle or high dose.

2.4 Serum biochemical parameters

The serum biochemical parameters are shown in Tab. 1. In the HNT-treated groups, there were no significant changes in ALT levels compared with the control group ($p > 0.05$). The LDH and AST activities became significantly higher in the middle and high dose groups than the control ($p<0.05$). The TBIL, IBIL and ALP levels were significantly higher in the high dose group than the control. All the results demonstrate that the liver is impaired when given the middle or high dose of HNTs.

Tab.1 Changes of biochemical parameters in serum of mice after 30 d oral administration of HNTs



The mice were oral administrated with purified HNTs for 30 consecutive days. Results were presented as mean±SD (n = 5). * $p<0.05$ *versus* the control

Fig.3 The activities of SOD, GSH-Px and MDA in the liver tissues

Index	control	4 mg/kg BW	20 mg/kg BW	100 mg/kg BW
LDH (U/L)	876±54	1135±82	1211±98*	1344±119*
TBIL (μmol/L)	1.85±0.3	1.73±0.52	1.88±0.16	2.26±0.21*
IBIL (μmol/L)	1.20±0.15	1.08±0.37	1.24±0.11	1.60±0.23*
ALT (U/L)	21±1.73	28±5.00	25±1.53	26±3.32
AST (U/L)	79±2.10	81±7.00	100±1.40*	110±4.90*
ALP (U/L)	69±7.00	72±5.50	78±4.11	98±8.28*

【Note】 Results were expressed as mean±SD(n=5). * $p<0.05$ versus the control.

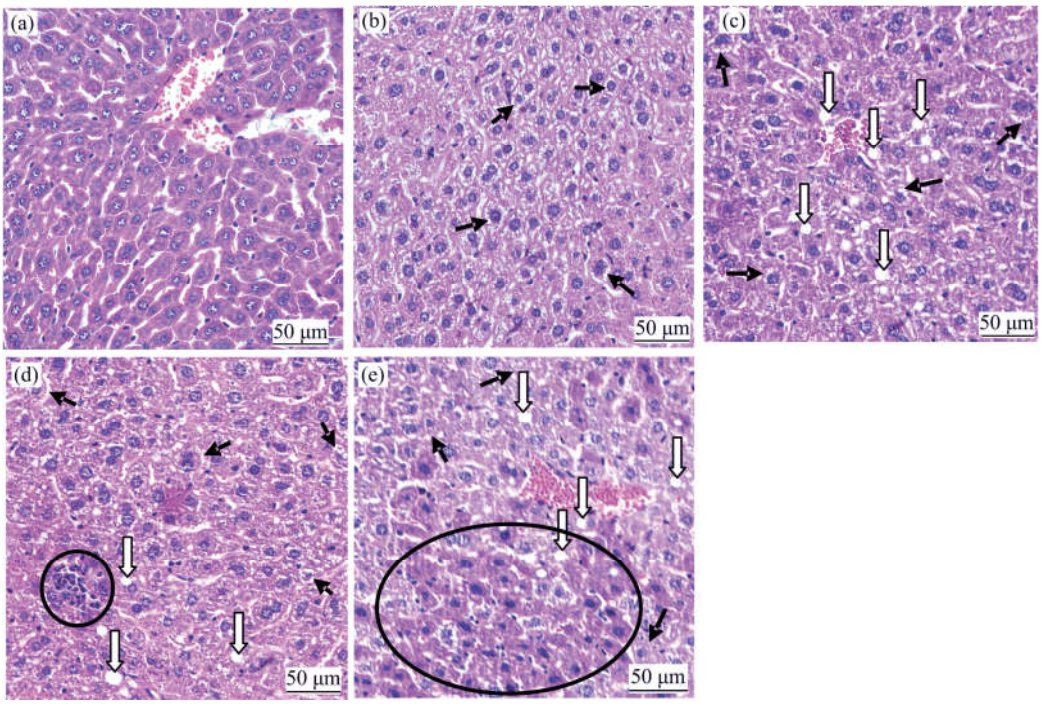
2.5 Biological effects by HNTs in liver tissue

The liver tissues in the control group showed a normal liver architecture (Fig.4(a)). Administration of 4 mg/kg BW HNTs did not cause observable histological changes except slight hydropic degeneration, revealing that HNTs at the low dose had no remarkable adverse effects on the liver. However, in the middle and high dose groups, serious hydropic degeneration, spotted and focal hepatocyte necrosis and fatty degeneration were observed in the liver tissue. Severe acidophilic changes were discovered in the liver tissue in the high dose group. The severity of fatty changes and hydropic degeneration were dose-dependent. All these changes in the liver tissue indicate that HNTs may be hepatotoxic at the middle and high dose.

2.6 Biodistribution of Al and Si

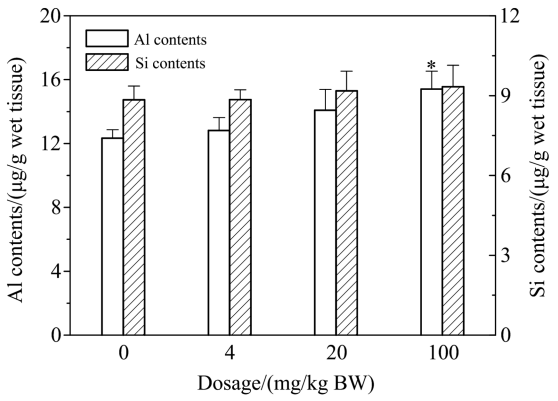
The Al and Si contents in liver tissues after 30 d repeated administration of HNTs are shown in Fig.5. The Si content in HNTs-treated groups in liver was similar to that of the control group. Administration of HNTs at the low and middle doses did not cause significant change in Al content

in the liver. However, the Al content increased significantly in the liver in the high dose group ($p<0.05$). Previous work showed that Si is more rapidly excreted from the body than Al [11]. The different biodistributions of Al and Si in the liver should be caused by the different excretion rates of Al and Si.



(a) In control group, (b) 4 mg/kg BW group, (c) 20 mg/kg BW group, (d) 100 mg/kg BW group, (e) 100 mg/kg BW group. Hydropic degeneration (solid arrow) appeared in all HNTs treated groups. Fatty degeneration (hollow arrow) was discovered in middle and high dose groups. Spotted and focal necrosis (circle) and acidophilic changes (ellipses) appeared only in high dose group

Fig.4　Histopathological sections of the liver tissue in mice following daily oral administration of purified HNTs for 30 d



The mice were oral administrated with purified HNTs for 30 consecutive days. Values represent means ± SD ($n=5$). * $p<0.05$ versus the control.

Fig.5　Biodistributions of Al and Si in liver tissues of mice determined by ICP-AES

3　Discussion

Because HNTs have been widely used in many fields, estimation of their toxicity is extremely important. In the present study, we used a series of dosages from 4 mg/kg BW to 100 mg/kg BW to evaluate the *hepatic* toxicity of oral HNTs in mice. The results showed that oral HNTs at the high dose decreased body weight gain. Similarly, Zhang et al.[12] reported that supplementation of HNTs in the diets could decrease the average daily weight gain of rats. The slight increase in liver coefficient indicated that the pathological changes might be induced in mice after they were treated with HNTs, which was confirmed by the further morphological examinations.

The liver, as a main detoxification organ, is activated to eliminate the negative effects induced by the ingested nanoparticles [13]. It will be damaged first when it takes up excessive nanoparticles beyond its detoxification capability. In our study, biodistribution experiment showed

that the Al accumulated in the liver in the high dosage groups (100 mg/kg BW), and the 4, 20 mg/kg BW groups showed no significant difference from the control. This suggests that the dosage of 100 mg/kg BW may be too high for the liver and will result in liver damage. But there was no significant difference in Si level between each HNTs-treated group and the control. Jani et al.[14] found that only 3.8 % particles were detected in the liver which had been absorbed from the intestine when the particle size reached 100 nm. HNTs must be dissolved in body fluids and then transported to the liver after uptake by the gastrointestinal tract. Earlier studies reported that nanoparticles may elicit oxidative stress [9, 15]. In our study, the changes in GSH-PX and MDA levels shows that the balance of the oxidative/antioxidative system was broken down, which indicates that HNTs can induce certain oxidative impairment in the liver. We also find changes in several serum biochemical indexes. The elevated serum LDH in all HNTs-treated groups indicate cell membrane destruction in liver tissues. The dose dependence of the elevation of LDH suggested that the changes of serum LDH was probably induced by the administration of HNTs. The serum AST and ALP levels were also raised in the experiment groups, which may result from the serious damage and necrosis of the liver tissue. The rise of serum TBIL and IBIL levels in the 100 mg/kg BW group indicate the occurence of hepatic injury, as confirmed by the observation of histopathology. Severe fatty degeneration, acidophilic changes, hydropic degeneration, spotted and focal necrosis were observed on the liver tissue slices. Park et al.[16] reported that aluminum predominantly accumulates in the liver, increasing the serum LDH level, and inducing apathological lesion in the liver. Berthon[17] confirmed that aluminum salt was dissolved and then absorbed by the gastrointestinal tract. Based on the above results, the liver dysfunction and injury probably might be induced by the Al dissolved from HNTs.

The effect of HNTs on mice shows that appropriate low dose of HNTs is benefitial to mice (gain more body weight), and liver dysfunction will occur if the dose is too high to the mice. The result reveals that it is crucial to ensure the correct dose in the application of HNTs, especially as drug delivery and a feed additive in breeding industry.

4 Conclusion

Oral HNTs can cause Al accumulation in the liver at the middle or high dose. Low dosage of HNTs increases the weight gain, while high dosage of HNTs induces serious liver damage, including necrosis, fatty degeneration, acidophilic changes, hydropic degeneration. The liver dysfunction and the oxidative stress are induced by HNTs in the liver at the middle or high dose. More extensive studies are required to investigate the *in vivo* toxicity of HNTs.

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