African Journal of Biotechnology Vol. 11(9), pp. 2359-2364, 31 january, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.3270
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

The adverse reaction of chitooligosaccharides in rats

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Accepted 7 December, 2011

In this study, hair removal effect after subcutaneous injection of chitooligosaccharides in mice was investigated. Different methods of observation of hair removal, tissue slices and detection of hematological parameters, like aminacridone (AMAC) labeled and antagonism treat were employed in this research. It was shown that Chitooligosaccharides exhibited renal toxicity, which was similar to aminoglycoside antibiotics (AmAn) toxicity.

Key words: Chitooligosaccharides, adverse reaction, nephrotoxicity, *in vivo* metabolism, antagonism.

INTRODUCTION

Chitooligosaccharide is the degraded product of chitosan, which has good biocompatibility and a variety of physiological functions, such as anti-tumor, enhancing body's immunity, reducing blood lipids and antibacterial activity (Muzzarelli et al., 1990; Kim et al., 1997). As to the safety of chitosan, there have been reported cases worldwide, for example Wu and Yuan (1995) and Shi et al. (2000) used chitin and chitosan for studying objects and gavage the largest amount of drugs in mice, respectively. While Li et al. (2004) used the largest concentration of chitosan for intraperitoneal injection in mice. Chitosan was also used abroad as absorbed sutures and drug carriers. All results obtained showed that the large molecular weight of chitosan was almost non-toxic. However, as for carbohydrates, we know that molecular weight and its spatial structure would affect other properties. We don't know whether this is also true for chitooligosaccharides. Referring to related experiments conducted previously, we found that intraperitoneal injection of chitooligosaccharides showed strong toxicity like appetite declined, response lags, weight decrease, hair rarefaction or fallen, which were similar to aminoglycoside antibiotics (AmAn). With the application of chitooligosaccharides in medicine, food and other aspects, it is necessary to seriously evaluate the safety of chitooligosaccharides, rather than rather than following the previous viewpoints entirely.

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MATERIALS AND METHODS

Drugs and reagents

Medium molecular weight chitosan, 2-aminacridone (2-AMAC), sodium cyanoborohydride were purchased from Sigma-Aldrich Co. (USA). Chitooligosaccharides (molecular weight<2000) were purchased from Jinan Haidebei Marine Bioenineeting Co. (Shandong, China). Ligustrazine phosphare and sodium chloride injection were obtained commercially from Tianjin Jinyao Group Co. (Tianjin, China), Gentamicin sulfate injection were purchased from Henan Tianfang Group Co. (Henan, China). Melatonin were purchased from New Zealand Life-power Pharmaceutical industry Limited Co. (New Zealand), Fermentation Cordyceps Powder was purchased from Institute of Fungus Resources, Guizhou University (Guiyang, China). Silica gel plate was provided by Branch of Qingdao Haiyang Chemical Co. (Qingdao, China).

Experimental animals

Adult male Sprague-Dawley rats (180-200 g) and Kunming mice (18-22 g) were provided by Laboratory Animal Center of Academy of Military Medical Sciences, Chinese People's Liberation Army (Grade II).

Detection of the characteristics of raw materials

Dual-channel atomic fluorescence spectrometer, by GB7917.3-1987 method, was used to detect the materials' arsenic and

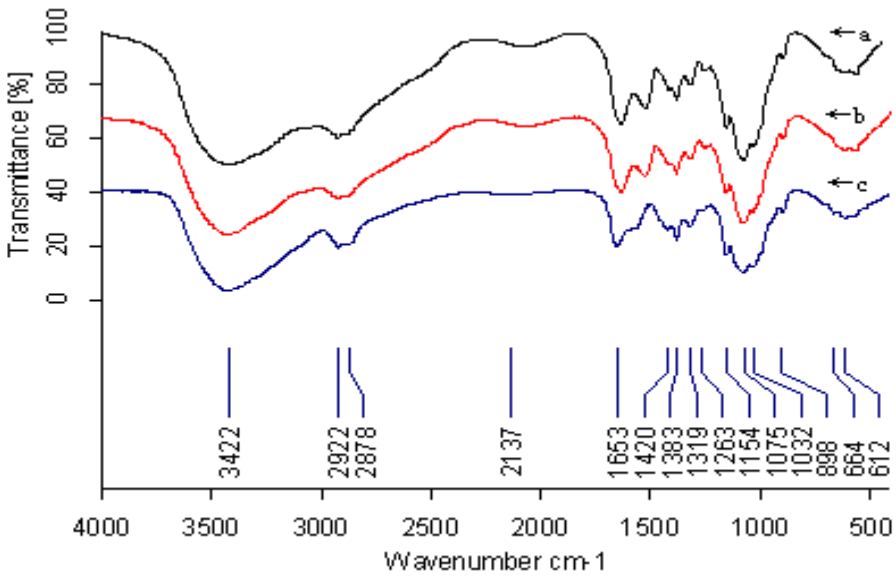

Figure 1. The Infrared spectra of K5000, K2000, KSIG...a: K5000, b: K2000, c: KSIG

mercury concentrations. Fourier transform infrared spectrometer was carried out for infrared detection.

Chitooligosaccharides hydrolysis and compared monosaccharides

Exacts called K_{5000}, K_{2000} and K_{SIG} were prepared for 25 mg each, respectively. Add 1.0 ml trifluoroacetic acid soluble (2 mol/L), nitrogen-sealed at 100°C in a water bath, hydrolyzed for 10 h at 40°C blown to dryness with nitrogen. It was then dissolved in methanol before it was blown to dryness with nitrogen. The process was repeated three times. Thin-layer silica gel plates were used for detection (Developing agent: butanol/acetic acid/water (60/25/25, v/v/v), chromogenic agent：aniline/diphenylamine/phosphate (Yang et al., 2003).

Chitooligosaccharides and AmAn comparative experiment

40 mice were randomly divided into four groups: (1) The blank group (injecting physiological saline); (2) K_{SIG} (injecting middle molecular weight chitosan); (3) K_{2000} (injecting Chitooligosaccharides); (4) Gentamicin (injecting gentamicin). Each group was injected a dose of 150 mg/kg, weighed and recorded the observed phenomena.

Antagonism treatment

40 mice were randomly divided into five groups: (1) The blank group (injected with physiological saline); (2) K_{2000} group (injected with 150 mg/kg of Chitooligosaccharides); (3) Ligustrazine phosphare group (injected with 5000 U/d Ligustrazine phosphare and sodium chloride; and then150 mg/kg chitooligosaccharides); (4) Melatonin group (infused in the paunch with 150 mg/kg of melatonin and then injected with150 mg/kg chitooligosaccharides); (5) Fermentation cordyceps powder group (infused in the paunch with 150 mg/kg fermentation cordyceps powder and then injected with 150 mg/kg chitooligosaccharides). The mice were weighed daily and any observed phenomenon was recorded. Then experimental mice were killed and their fresh kidneys were taken out for assays on N-acetyl-beta- D-glucosaminidase (NAG), Superoxide dismutase (SOD) and malondialdehyde (MDA) according to specification. Also 10% formalin was used to fix the samples. The samples were section after embedding the in paraffin wax before they were stained with haematoxylene and eosin.

Label chitooligosaccharides observed in *in vivo* metabolism

According to Okafor (1997), 10 mg chitooligosaccharides was weighed and 100 µl of 1M $NaCNBH_4$ and 10 µl of 0.1 M 2-AMAC/acetic acid solution (12/55, V/V) were added for blending. This was heated at 90°C in a water bath for 40 min. It was then suspended in an ice water. Ethanol extraction of free 2-AMAC (put in darkness) was carried out and then, it was dried at room temperature and was dissolved in physiological saline again. The resulting solution was injected into the peritoneal cavity of SD rats. The treated rats were sacrificed after 1, 2, 4, 8, 16 and 24 h of injection, and their livers as well as their kidneys, spleens, lungs were collected. Their recovery ratio was measured by fluorescence spectrometer.

$$\text{Recovery, } (\%) = \frac{F_1 - F_0}{F_0} \bullet \frac{C_0}{C_1} \bullet 100\ \%$$

Where, F_1 is the fluorescence value of injecting labeled chitooligosaccharides; F_0, is the fluorescence value of blank; C_1, is the concentration of labeled tissue homogenate and C_0, is the concentration of blank tissue homogenate.

RESULTS

Analysis of infrared spectrogram

The infrared spectra of K_{5000}, K_{2000} and K_{SIG} were similar and all of them showed a characteristic peak of chitosan, for example, -NH, -OH, etc (Figure 1). All of them exhibited

Table 1. Comparison of the Concentration of arsenic and mercury.

Parameter	K_{2000} (mg/kg)	K_{5000} (mg/kg)	Standard (mg/kg)
Arsenic	0.0357	0.0588	2
Mercury	0.0036	0.0054	1

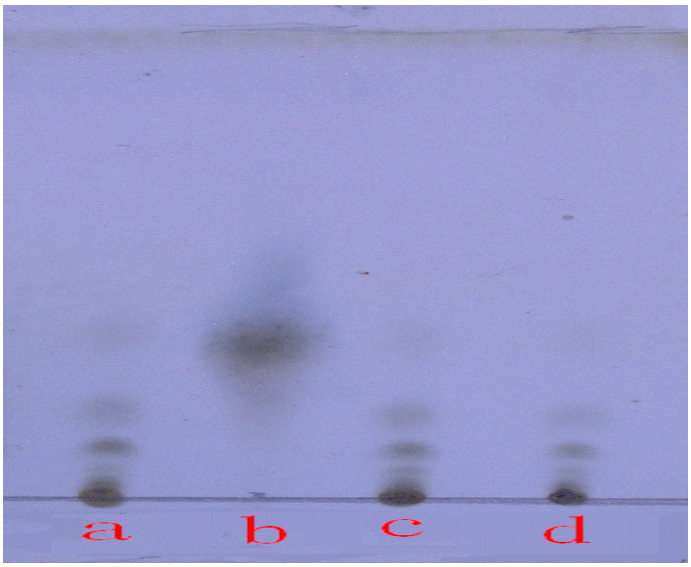

Figure 2. The TLC of hydrolysate (a: KSIG, b: glucosamine, c: K2000, d: K5000)

major absorption peaks, for example, amino stretching at 3422 cm^{-1} and methyl stretch vibration at 2878 cm^{-1}, carbonyl stretching at 1653 cm^{-1} and C-N stretching vibration at 1319 cm^{-1}. But the -N-H shear vibration and -N-H stretching vibration peaks of chitooligosaccharides were more obvious than chitosan. This showed that they were not apparent distinction in basic skeleton structure. However, some absorption peaks changed, by the role of molecular weight decrease, glycosidic bond fractured, and the hydrogen bonds weakened (Figure 1).

Arsenic and mercury concentration

According to the results obtained, the concentrations of arsenic and mercury in chitooligosaccharides were very low (Table 1). The current arsenic standard concentration in drinking water is 0.01 mg/m^3 (USA) while its concentration in food is 2 mg/kg (Thailand). The national standards concentration of mercury is 1 mg/kg (Table 1).

Monosaccharides compared

The hydrolysis products of three chitosans also had similar results in silica gel thin-layer plate test (Figure 2). There was no substantive distinction except concentration. Experimental results showed the chitooligosaccharides, (from Jinan Haidebei Biological Engineering Company Limited) and chitosan (from Sigma) were assuredly the same type of material. The only differences were molecular weight, the degree of polymerization and deacetylation (Figure 2).

Contrast of chitooligosaccharides and AmAn

From this investigation, we found that K_{SIG} group did not have specific response and showed no obvious toxic action. This group also almost corresponded with the blank group. These results agree with that reported by Li et al. (2004). While there were certain similarities between the K_{2000} group and the gentamicin group, such as decrease in activities, appetite declined, response lags, decrease in weight, hair rarefaction and fallen (Figure 3), abnormal urine, liver leaf number, kidney; and some physiological indicators were abnormal.

Label chitooligosaccharides and observation of *in vivo* metabolism

According to the recovery rate results in different times, almost no chitooligosaccharides distributions were observed in the spleen and lung appeared after injecting the subjects with label chitooligosaccharides. Their recovery rates were nearly zero or negative. Chitooligosaccharides were mainly distributed in kidney and they reached their peak after injection for 3 to 7 h (Figure 4). This was because most of its prototype were excreted from urine, with the drug increasing in the renal cortex, thereby leading to toxic damage. The recovery rate of renal tissue was still as high as 21% because of its low rate of excretion. Such a high concentration seriously affected the functions of the renal tissue repair. A maximum of 27% could be achieved in liver (Figure 5).

Antagonism treatment

After 12 days, K_{2000} group still had adverse reactions, while the treatment group, such as fermentation cordyceps powder group, melatonin group and ligustrazine phosphate group had more or less antagonistic function. All of them had no hair shedding phenomenon. But their weights still decreased and their liver and abdominal membranes were linked together in ligustrazine phosphate

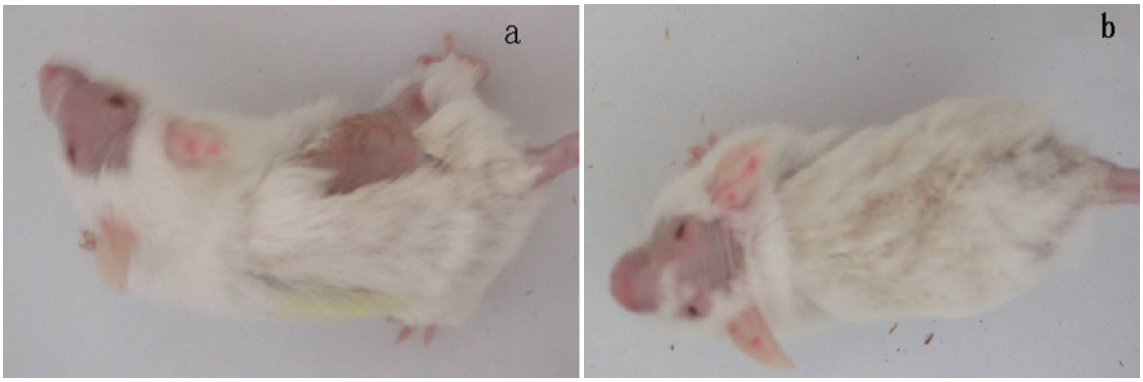

Figure 3. The photo of paunch injecting oligochitosan result in hair removing in mouse model (a: K2000 group, b: gentamicin group).

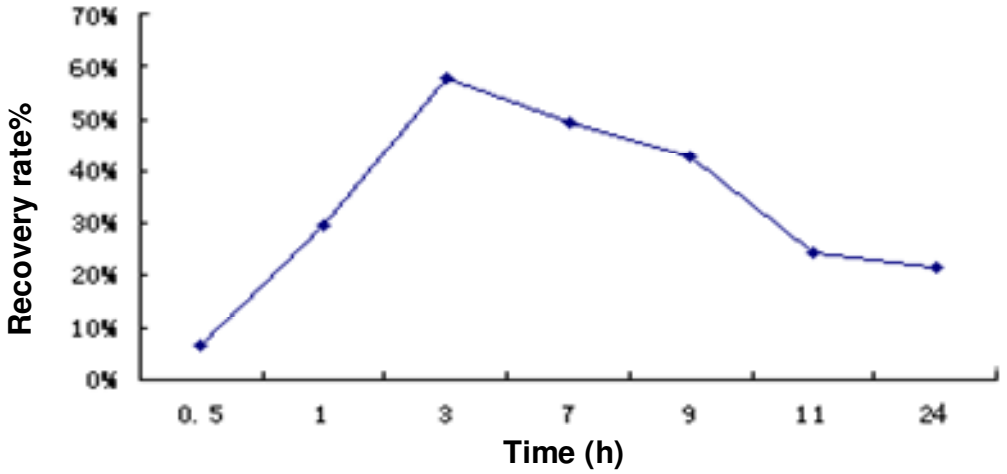

Figure 4. The recovery of AMAC-oligochitosan in kidney.

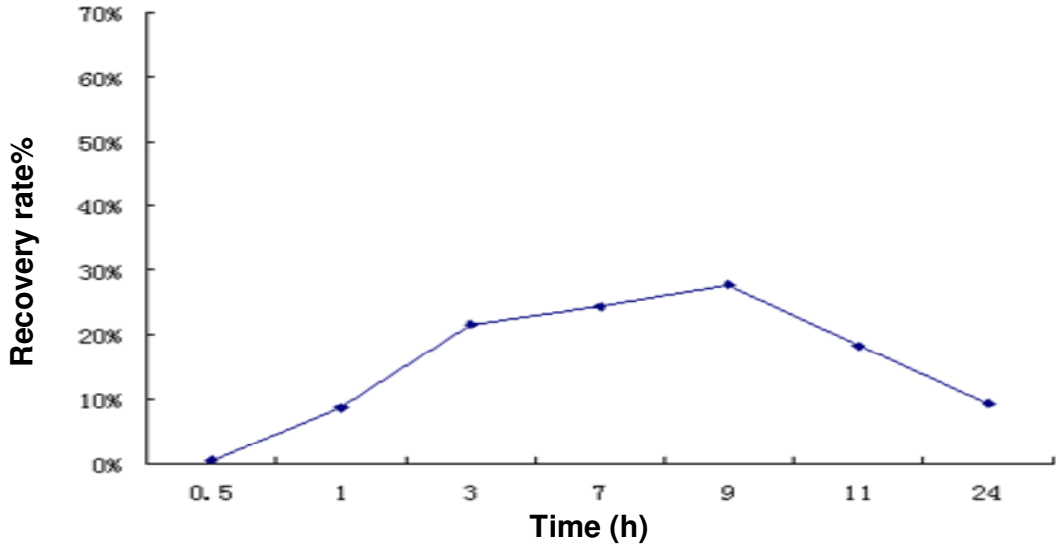

Figure 5. The recovery of AMAC-oligochitosan in the liver.

Table 2. Enzymatic activities of NAG, SOD and MDA in kidney.

Group	NAG	SOD	MDA
blank group	17.6 ± 4.2	47.1 ± 8.2	1.99 ± 0.11
K_{2000} group	52.2 ± 7.8$^{\Delta\Delta}$	29.7 ± 4.9$^{\Delta\Delta}$	6．38 ± 0.24$^{\Delta\Delta}$
ligustrazine phosphare group	35.4 ± 9.7**	31.1 ± 2.7	5.30 ± 0.30
melatonin group	32.6 ± 6.7**	36.6 ± 2.2*	3.15 ± 0.21**
fermentation cordyceps powder group	28.9 ± 7.6**	42.8 ± 8.7**	2.99 ± 0.14**

Compared to the blank group, ΔΔP<0.01; Compare to the K_{2000} group, *P<0.05, ** P<0.01. NAG, N-acetyl- β-D-glucosaminidase; SOD, souperoxide dismutase; MDA, malondialdehyde

group, melatonin group's hair had some loses; fermentation cordyceps powder had the best performance. The results of the measurement of NAG, SOD and MDA are summarized in Table 2. The quantity of the MDA could be used to represent renal activities of the substances. It was significantly higher in gentamicin and the K_{2000} groups. On the other hand, oxygen free radical scavenging enzymes SOD were significantly decreased. The results obtained indicate that K_{2000} and gentamicin enhanced oxidative stress in the kidney. While melatonin, fermentation cordyceps powder and ligustrazine phosphate treatments as well as the MDA had dropped significantly, while SOD increased.

NAG is a lysosomal enzyme, which cannot get through glomerular filtration membrane. And the increase of NAG reflects the injury of renal tubule. In the measurement, we found that the treated group could decrease the value of NAG.

DISCUSSION

According to the pertinent reports and the preliminary research results in our laboratories, we found that chitin and chitosan and either gavage or intraperitoneal injection showed no obvious toxicity in animal experiments while chitooligosaccharides for gavage showed no obvious toxicity. But intraperitoneal injection exhibited obvious toxic action in the kidney, which was similar to AmAn.

When compared theoretically, the experiments on detecting the characteristics of raw materials and monosaccharides could remove the toxic phenomenon that has been introduced by the toxic substances of the materials. Chitosan easily chelated many kinds of metal ions. Previous reports suggested that it was chelated arsenic that showed some toxicity. But this was denied by the results of the experiments on arsenic and mercury concentrations' measurement.

The contrast experiment of gentamicin and chitooligosaccharides also indicated that there was a certain similarity. 2-AMAC labeled chitooligosaccharides metabolized *in vivo* were mainly distributed in the kidney of the rats. This result was similar with Hiraku's reports (Hiraku and Yoshiharu, 1999). Isothiocyanate (FITC) labeled chitosan was also distributed in the kidney and urine but was distributed less, in other organs. In other words, it was toxic to kidney. And this strong nephrotoxicity caused by chitooligosaccharides was similar to AmAn's toxic effects on human body. Current research showed that gentamicin, in the normal physiological pH, contained free positively charged amino groups and combined with renal tubular epithelial cells or bristle cells surface specific receptor; and then enters the cells to form vesicles, which combined with junior lysosomal, forming secondary lysosomal. Because the permeability of lysosomal membrane increased, it released a lot of lysosomal enzymes. It acted on mitochondria, specifically inhibiting mitochondrial respiratory enzyme synthesis, cellular energy metabolism and then damaged the cell structure. In addition, after gentamicin might have entered the body, almost all of them are filtered by the glomerulus in the final prototype. If the concentration increased to an intoxicating concentration in renal tubular, gentamicin could directly damage vascular patterns, causing lymphatic circulatory disturbance, which would increase the hair cell toxicity (Trichlya et al., 1997).

Its similarity was mainly manifested in the following aspects:

(1) A certain similarity in the main structure. Both of them are amino sugars linking the chain structure with glycosidic bond;
(2) A certain similarity in the molecular weight range, for example, sulfate tobramycin's molecular weight is 1425.45, netilmicin's molecular weight is 1441.54;
(3) Both of them have a certain antimicrobial function, in which amino structure of the antimicrobial activity plays an important role. And the more content, the more antibiotics activity, accompanied by more toxicity;
(4) Both of them were absorbed a little at the oral region. But they were quickly absorbed and appeared to exhibit toxic effects after *in vivo* injection.

Their toxicity was demonstrated to be renal toxicity, which had the same symptoms, such as abnormal urine, yellowing and variable stiffness. Their toxicity was shown to be reversible, in other words, it was mitigated or disappeared after resumption. Gentamicin can cause acute kidney damage, although, the toxicological role is not yet fully clear. However, the study also found that it was related

to the increase of oxygen free radical activity. So ligustrazine phosphate, melatonin and fermentation cordyceps powder were used to alleviate the side effects of AmAn (Tian et al., 2002; Cai and Wang, 2005).

In this paper, they were also used to treat the side effects of intraperitoneal injection of chitooligosaccharides. And in the experiment of antagonism treatment, the results showed that the treated group exhibited more or less antagonistic function.

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