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Subchronic Toxicity and Genotoxicity Assessment of Low Molecular Mass Konjac Mannan Oligosaccharide *in vitro* and *in vivo**

JIANG Min[1], LI Heng[1], WANG Miao[1], LUO Chun-Qin[2],
XU Zheng-Hong[1], SHI Jin-Song[1]**, MA Yan-He[3]
([1] *School of Pharmaceutical Sciences, Jiangnan University, Wuxi* 214000, China;
[2] *Yongan Pharmaceutical Co. Ltd., Chengdu* 610000, China; [3] *Institute of Microbiology, Chinese Academy of Sciences, Beijing* 100101, China)

Abstract Konjac mannan oligosaccharide (KMOS) is a new food additive with prebiotic function in China. In this study, KMOS with degree of polymerization (DP) of 2～7 was prepared by enzymatic method followed by organic solvent precipitation. Then its subchronic toxicity and genotoxicity was investigated. In the subchronic toxicity test, KMOS was administered to rat orally for 90 days at dose of 0, 2.25, 5.25, 7.50 g/kg body weight (BW) daily, respectively. No significant toxicological manifestation in clinical examination as well as clinical pathology was noted. At terminal necropsy, histopathology changes in the liver and kidney were observed, which was considered to be spontaneous and incidental in nature and unrelated to KMOS-treatment. In addition, a battery of tests including the Ames test, micronucleus test and sperm abnormality test suggested no mutagenicity potential. In conclusion, the results of this study supported that ingestion of KMOS appeared to be safe as a food additive for oral consumption.

Key words KMOS, subchronic toxicity, Ames test, micronucleus test, sperm abnormality test
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In decades, a lot of attention has been paid to functional oligosaccharide, because of its important physicochemical and physiological properties associated with health benefit[1]. Among them, mannan oligosaccharide (MOS) became more popular in recent years because of its unique biological characteristic, especially the most well-known prebiotic function[2-6]. Amongst all the sources available, MOS obtained from microbe (MMOS) is predominant so far. For example, the commercial MMOS products (Bio-MOS ®, Active MOS® and so on) are extracted from yeast, and mainly used as dietary supplement in animal feeding industry. Owing to the gut health benefit and immune modulate function[2,7-9], these products are considered as promising alternative for antibiotic in EU and US.

Recently, MOS derived from plants, especially konjac, has also been widely studied. As compared with MMOS, the prebiotics effect of KMOS was found to be more significant in certain species[10]. Numerous researches have already demonstrated that KMOS, especially the low molecular mass fragment [10-12], modulates the intestinal environment *via* a number of mechanisms[4, 12-17]: (1) it could selectively stimulate the proliferation of probiotic bacteria (especially *Bifidobacterium* and *Lactobacillus*); (2) it could promote the production of short-chain fatty acid (SCFA) , thus lower gut pH; (3) by binding to the pathogenic bacteria (especially *Escherichia coli* and *Salmonella*), it could block the adhesion between pathogenic bacteria and mucosa surface of animals' intestinal wall, and further results in the excretion of harmful pathogens into feces. As a result of these, the researches on the relation between KMOS and dysbacteriosis related diseases became more and more

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**Corresponding author.
Tel: 86-510-85327353, E-mail: shijs@163.com
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popular. Suwannaporn [18] demonstrated that KMOS was effective in easing the symptoms associated with inflammatory bowel disease (IBD) in human clinical test. Furthermore, the physiological effects of KMOS in the gut could reflect in/on other ecological systems of the body, such as skin [19-20] and vagina [21]. Bateni [19] found that a spray formulation containing 5% KMOS could significantly improve the skin health, implying that it could be used as a prophylactic or novel topical therapeutic product for acne vulgaris and to improve skin health more generally. Tester[21] found that KMOS could help to recover the healthy microflora of vagina due to its prebiotics effect.

It is important to note that konjac is a local perennial plant in China, containing abundant mannan, and right now is mainly used for low-added value product manufacturing. Thus, economically, it is more meaningful to use KMOS from konjac source for diet supplement.

Although the intrinsic safety of KMOS is supported by its natural occurrence in konjac, the use of enzymatic prepared KMOS as a new food additive requires a thorough safety assessment. In this study, we firstly prepared low molecular mass KMOS(DP=2～7) using enzymatic method and organic solvent precipitant. Then, the safety test of KMOS was performed, including a 90-day repeated oral dosing in rats and genotoxicity characterization both *in vitro* and *in vivo*. These results could provide accurate information about the potential hazards of KMOS to be used orally.

1 Materials and methods

1.1 LMW KMOS preparation

According to the previously reported method[22-23], KMOS was prepared by enzymatic method with some modification. Briefly, β-mannanase (WelPont Biotechnology Company, Guangxi, China) was added to KMOS (500U/g KMOS). Hydrolysis was carried out in phosphate buffer (pH 7.0) for 8 h at 55℃. Then the reaction solution was centrifuged at 5 000 r/min followed by desalination with ion exchange resins. Pigments and macromolecular were removed by extraction with 90% (*w*/*w*) ethanol. The supernatant was concentrated by rotary evaporators to one-tenth of its original volume followed by extraction with 90% (*w*/*w*) acetone, and the precipitate was lyophilized. The final product is white power containing mixed monosaccharide(glucose and mannose) and oligomers.

1.2 KMOS constituent analysis

The constituent of KMOS was analyzed by Ultrahigh Performance Liquid Chromatography-Mass Spectrum(UPLC-MS) system (Waters, USA) equipped with BEH Amide column (2.1 mm×100 mm, 1.7 μm) thermo stated at 45℃. Analysis was completed with a gradient elution of 80% (*w*/*w*) acetonitrile in 0.1% (*w*/*w*) ammonia water (A) -30% (*w*/*w*) acetonitrile in 0.1% (*w*/*w*) ammonia water (B) within 15 min at a flow rate of 0.3 ml/min. The gradient elution was: 0～15 min, 100% A; 15～17 min, 100% A→40% A; 17～30 min, 40% A →100% A. For the MS detection (Waters MaldiSynapt Q-TOF), the optimal parameters were as follows: capillary voltage 3.0 kV; cone voltage 30 V; Detector voltage 1.8 kV; source temperature 100℃; desolvation temperature 400℃; desolvation gas flow 500 L/h; cone gas flow 50 L/h. Mass detection was performed in full scan mode for *m*/*z* in the range 100～1500. The UPLC-MS analysis showed that the constituent of KMOS was mainly disaccharide, tri-saccharide, tetra-saccharide, penta-saccharide, hexa-saccharide and hepta-saccharide (data not shown).

The content of monosaccharide was determined by high performance liquid chromatography (HPLC) (Dionex U3000, USA) equipped with HILIC amide column (4.6 mm×250 mm, 35 μm) thermostated at 30℃ and refractive index detector (Shodex RI-101, Shodex, Japan) thermostated at 35℃. The flow rate was 0.4 ml/min with acetonitrile ∶ water =75 ∶ 25 (*w*/*w*) in 0.1% (*w*/*w*) ammonia water. The moisture content was measured by drying samples at 105℃ to constant weight in an oven, and ash content was determined by an incineration in a muffle furnace at (550 ± 25)℃ [24]. The compositional analysis result of KMOS was shown in Table 1.

Table 1 Composition analysis result of KMOS

Macronutrient	Value	Unit
Total sugar (dry wt. basis)	> 98.6	%
Monosaccharide (dry wt. basis)	< 9.2	%
Ash	< 0.4	%
Moisture	< 6.2	%

1.3 Animals

The weaning Sprague-Dawley (SD) rats and mice

were supplied by the Laboratory Animal Center of Sichuan Academy of Chinese Medicine Science (Sichuan, China). The animals were quarantined for a week prior to test. Animals were housed individually in solid bottom polycarbonate cages under standard environmental conditions (20 ℃ ～26℃ , 50% ～60% relative humidity and 12 h light/12 h dark cycle). Soft wood shaving underneath the cage was changed at least three times per week. Standardized feed and tap water were provided *ad libitum*. All animal experiments were conducted in compliance with the Guide of The Care and Use of Laboratory Animals[25].

1.4 Subchronic toxicity study

Rats were randomly divided into four groups (10/sex/group). After 7-day acclimation period, KMOS was dissolved in distilled water and administered orally for 90 days at dose of 0, 2.25, 5.25, 7.50 g/kg BW daily. During the study, the viability, signs of gross toxicity and behavioral symptoms such as diarrhea, immobility, neuromuscular problems of animals were observed once daily. On the 91st day, animals were anesthetized and sacrificed after fasting overnight. Blood were collected and stored at –80℃ for further assay.

1.4.1 Body weight and food consumption

Individual animal body weight and food consumption were recorded weekly. Final body weight was recorded prior to the scheduled necropsy. Mean body weight, mean change of body weight as well as mean food consumption was calculated for the corresponding intervals. The weekly food utilization rate of each group was calculated as follows:

$$\text{Food consumptions}/\% = \frac{\text{Mean body weight change}}{\text{Mean food consumption}} \times 100$$

1.4.2 Hematology and clinical biochemistry analysis

Hematological parameters were determined with an automatic blood analyzer (MEK-7222, Tokyo, Japan), and included red blood cell count [RBC], hemoglobin concentration [HB], total white blood cells count [WBC], granular leukocyte count [GLC] and lymph leukocyte count [LLC].

Clinical biochemistry parameters were detected using an automatic chemical analyzer (Hitachi 7080, Japan), and included alanine aminotransferase [ALT], aspartate aminotransferase [AST], total protein [TP], albumin [ALB], blood urea nitrogen [BUN], creatinine [CR], blood glucose [GLU], cholesterol [CHO], triglyceride [TG], potassium and sodium.

1.4.3 Organ weight and histopathological analysis

A complete necropsy was conducted in all animals. The necropsies included examination of the external surface, all orifices, and the cranial, thoracic, abdominal and pelvic cavities, including viscera. At necropsy, the weight of brain, heart, liver, kidney, spleen, lung, adrenals, thymus, testis (males), epididymis (males), ovaries (females) and uterus (females) was measured in addition to the terminal body weight. Then the individual organ weight/body weight were calculated.

Representative samples from each animal of all the organs and tissues in the necropsy were fixed in 10% neutral buffered formalin and embedded in paraffin, sectioned, stained with hematoxylin and eosin (H&E) for light microscopically examination.

1.5 Genetic toxicity study

1.5.1 Ames assay

We adopted five standard types of histidine-requiring *Salmonella typhimurium* (*S. typhimurium*) strains TA 1535, TA 97, TA 98, TA 100 and TA 102. KMOS was assayed at dose of 8, 40, 200, 1 000 and 5 000 μg/plate, with and without an exogenous metabolic activation (S9 mix). Deionized water was used as negative control. The positive controls without S9 were 2, 4, 7-trinitro-9-fluorenonesodium azide, mitomycin C and sodium azide with while the positive ones with S9 were 2-aminofluorene, 1, 8-dihydroxyanthraquione and 2-aminoanthracene. For an adequate estimate of variation, triplicate plating should be used at each dose level.

1.5.2 Erythrocyte micronucleus assay

For erythrocyte micronucleus assay, 25 male and 25 female mice (25～30 g BW, 4 weeks old) were randomly separated into five groups (5/sex/group). KMOS was dissolved in distilled water and administered by oral gavage at dose levels of 2.5, 5.0, and 10.0 g/kg BW, respectively. Deionized water and endoxan served as the negative and positive control substances, respectively. Bone marrow cells were collected and analyzed after methanol fixing and Giemsa staining. The polychromatic erythrocyte(PCE)/ norm chromatic erythrocyte (NCE) was estimated based on a total of 200 erythrocytes for each mouse.

1.5.3 Sperm malformation test

25 male mice (25～30 g BW, 4 weeks old) were assigned to five groups randomly (5/sex/group). KMOS-treated group was administered at dose levels of 2.5, 5.0, and 10.0 g/kg BW, respectively, twice daily by oral gavage for 5 days. The negative control and positive control were deionized water and endoxan (40 mg/kg), respectively. Immediately after sacrifice

on 35th day, the epididymis was excised and shredded in 2 ml saline. The mass was filtered and diluted to an appropriate concentration. Then the liquid was smeared, dried, fixed with methyl hydrate, dyed with H&E and observed under optical microscope. 1000 no overlap sperms per mouse were analyzed for the morphological abnormalities (amorphism, banana shape, no hook, fat head, two-head, two-trailed and so on) and the sperm malformation rate (%) was calculated.

1.6 Statistical analysis

The data was performed with the statistical software Package for Encyclopaedia Medical Statistics 3.0 (PEMS3.0), which was provided by Sichuan University. Values are expressed as mean ± SD. The homogeneity of variance was analyzed using Levenes's test firstly. When variance was homogeneous, the comparisons were performed by one-way analysis of variance followed with Duncan's test. Where variances were considered significantly different, groups were compared using Kruskal-Wallis non-parametric analysis of variance followed by the Dunnett's *t*-test. $P < 0.05$ was considered statistically significant.

2 Results

2.1 Clinical observations, body weight and food utilization

No occurrence of death was observed in the KMOS-treated rats throughout the duration of the experiment at any dose used. No abnormal clinical symptoms but softer and wetter feces were found in KMOS treated group as compared with the untreated group. In addition, there is no treatment-related effects on body weights (Figure 1) and food utilization rate (Figure 2) in KMOS-treated groups as compared with the control group.

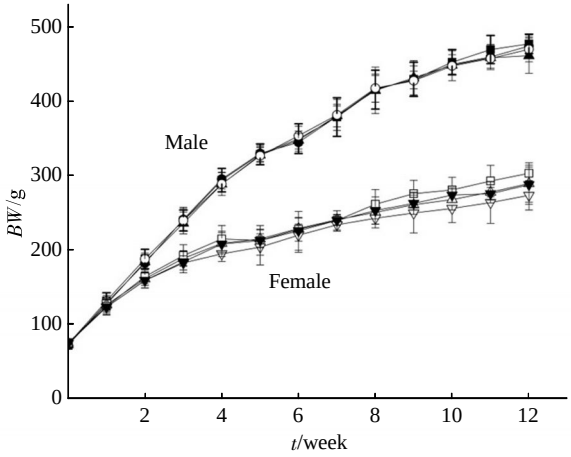

Fig. 1 Effect of KMOS on mean body weight in male and female rats for 90 days

Male. ●—●: 0 g/kg; ▲—▲: 2.25 g/kg; ■—■: 5.25 g/kg; ○—○: 7.5 g/kg;
Female. △—△: 0 g/kg; □—□: 2.25 g/kg; ▽—▽: 5.25 g/kg; ▼—▼: 7.5 g/kg.

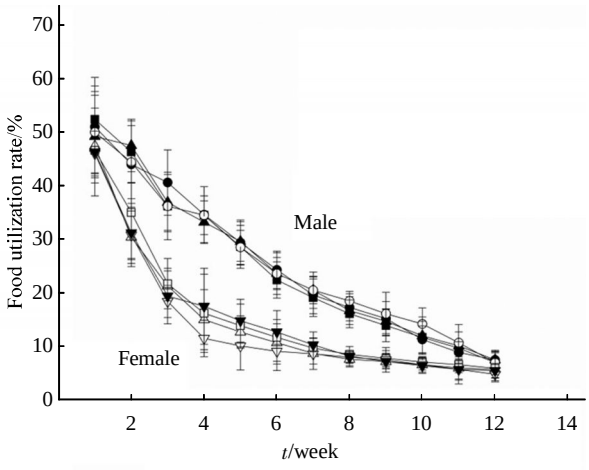

Fig. 2 Effect of KMOS on food utilization rate in male and female rats for 90 days

Male. ●—●: 0 g/kg; ▲—▲: 2.25 g/kg; ■—■: 5.25 g/kg; ○—○: 7.5 g/kg;
Female. △—△: 0 g/kg; □—□: 2.25 g/kg; ▽—▽: 5.25 g/kg; ▼—▼: 7.5 g/kg.

2.2 Hematology and clinical biochemistry analysis

The hematology and clinical biochemical parameters of rats were analyzed(Table 2 and Table 3).

Table 2 Effect of sub-chronic oral administration of KMOS on hematological parameters of male and female rats

Sex	Parameters					
	Doses/(g•kg^{-1}•d^{-1})	RBC/(10^{12}•L^{-1})	HB/(g•L^{-1})	WBC/(10^{9}•L^{-1})	GLC/%	LLC/%
Male	0	7.55 ± 0.29	162.0 ± 8.6	7.8 ± 2.4	17.8 ± 4.6	82.2 ± 4.6
	2.25	7.66 ± 0.14	164.4 ± 3.9	7.7 ± 2.5	18.1 ± 4.9	81.9 ± 4.9
	5.25	7.60 ± 0.10	161.5 ± 5.0	8.4 ± 2.8	17.0 ± 4.2	83.0 ± 4.4
	7.50	7.48 ± 0.22	159.5 ± 5.0	9.5 ± 1.8	18.2 ± 5.1	81.8 ± 5.1
Female	0	7.08 ± 0.14	153.1 ± 4.6	4.0 ± 1.4	18.2 ± 2.4	81.8 ± 2.4
	2.25	6.98 ± 0.27	151.6 ± 5.2	4.0 ± 1.6	18.5 ± 3.9	81.5 ± 3.9
	5.25	6.98 ± 0.24	153.8 ± 7.8	4.5 ± 1.6	18.1 ± 4.3	81.9 ± 4.3
	7.50	7.09 ± 0.25	155.1 ± 5.5	5.1 ± 1.7	17.2 ± 3.7	82.8 ± 3.7

0: The negative control. Abbreviation of hematological parameters: red blood cell count [RBC], hemoglobin concentration [HB], total white blood cells count [WBC], granular leukocyte count [GLC], lymph leukocyte count [LLC].

Table 3 Effect of sub-chronic oral administration of KMOS on blood biochemistry parameters of male and female rats

	Parameters	Doses/(g•kg^{-1}•d^{-1})			
		0	2.25	5.25	7.50
Male	ALT/(U•L^{-1})	51.62±6.50	57.30±9.20	53.00±9.10	52.60±5.50
	AST/(U•L^{-1})	168.71±15.73	158.40±14.0	165.03±9.81	160.70±11.60
	TP/(g•L^{-1})	64.72±3.34	66.10±2.91	65.10±3.72	65.29±2.80
	ALB/(g•L^{-1})	34.82±1.09	34.60±1.00	34.50±0.86	34.52±0.88
	BUN/(mmol•L^{-1})	6.12±0.56	6.04±0.60	6.18±0.45	5.93±0.58
	CR/(μmol•L^{-1})	59.91±6.00	59.40±5.01	58.30±5.12	57.51±4.20
	GLU/(mmol•L^{-1})	4.07±0.53	4.02±0.48	4.24±0.45	4.41±0.69
	CHO/(mmol•L^{-1})	1.88±0.17	1.89±0.08	1.89±0.17	1.91±0.09
	TG/(mmol•L^{-1})	0.61±0.07	0.59±0.07	0.59±0.07	0.58±0.07
	Potassium/(mmol•L^{-1})	4.89±0.51	4.91±0.70	4.85±0.52	4.92±0.41
	Sodium/(mmol•L^{-1})	141.8±2.4	138.9±3.9	141.2±2.9	139.1±3.1
Female	ALT/(U•L^{-1})	53.5±7.4	59.7±8.4	60.7±10.9	59.8±5.0
	AST/(U•L^{-1})	164.6±11.1	169.3±11.8	167.1±17.3	163.9±9.0
	TP/(g•L^{-1})	64.7±2.4	64.1±3.0	65.0±3.5	64.2±3.3
	ALB/(g•L^{-1})	34.6±1.4	34.6±1.1	34.3±1.1	34.0±1.1
	BUN/(mmol•L^{-1})	6.25±0.39	5.91±0.41	5.99±0.53	6.09±0.31
	CR/(μmol•L^{-1})	59.3±5.3	58.6±3.9	60.0±6.0	57.3±4.4
	GLU/(mmol•L^{-1})	4.32±0.45	3.90±0.37	4.29±0.59	4.04±0.57
	CHO/(mmol•L^{-1})	1.85±0.12	1.94±0.10	1.87±0.09	1.89±0.09
	TG/(mmol•L^{-1})	0.60±0.10	0.62±0.06	0.56±0.07	0.59±0.06
	Potassium/(mmol•L^{-1})	4.8±0.6	4.9±0.7	4.8±0.5	4.9±0.6
	Sodium/(mmol•L^{-1})	142.8±2.1	139.9±1.9	141.7±3.2	139.2±3.0

0: The negative control. Abbreviation of biochemistry parameters: alanine aminotransferase [ALT], aspartate aminotransferase [AST], total protein [TP], albumin [ALB], blood urea nitrogen [BUN], creatinine [CR], blood glucose [GLU], cholesterol [CHO], triglyceride [TG], potassium, sodium.

As shown, hematologic and clinical chemistry indexes were not affected by KMOS significantly.

2.3 Organ weights and histopathology examination

After 90-day of treatment with KMOS, there were no significant differences in the organ/body weight ratios in comparison with the concurrent untreated group (Table 4).

At necropsy, there were no macroscopic findings suggestive of adverse effects. However, minor microscope examination differences in some organs including cecum, liver and kidney between the treated and untreated group were noted. The cecum sections of rat fed 7.50 g/kg daily KMOS presented more regular morphology (Figure 3), and its villi length increased from (118.8±5.8) μm in the control group to (135.0 ±8.8) μm ($P < 0.05$). In addition, slight fatty degeneration of hepatocytes in the liver (1 male in the control group) as well as low-grade edema of renal pelvis mucosa epithelial cells accompanied by atrophy of renal collecting tubule in the kidney (1 female rat in the 7.50 g/kg daily group) were observed in both of control and 7.50 g/kg daily group. However, the findings in the liver and kidney were consistent with normal background lesions in clinically normal rats of the age and strain used in this study, hence, the differences were considered spontaneous and incidental in nature and unrelated to KMOS-treatment.

Table 4 Effect of sub-chronic oral administration of LMW KMOS on relative organ weights of male and female rats (mean ± SD)

Parameters			Doses(g•kg^{-1}•d^{-1})			
			0	2.25	5.25	7.50
Male	Body weight		474.8±14.9	461.8±23.8	477.7±13.1	470.3±16.7
	Relative/%	Brain	0.39±0.03	0.41±0.02	0.39±0.04	0.40±0.02
		Heart	0.31±0.03	0.30±0.02	0.31±0.02	0.30±0.03
		Liver	2.71±0.22	2.63±0.26	2.76±0.22	2.82±0.27
		Kidney	0.62±0.05	0.65±0.07	0.60±0.04	0.55±0.20
		Spleen	0.17±0.05	0.17±0.02	0.17±0.03	0.18±0.02
		Lung	0.31±0.01	0.32±0.03	0.30±0.06	0.32±0.02
		Adrenals	0.048±0.006	0.047±0.011	0.041±0.007	0.042±0.005
		Thymus	0.068±0.005	0.068±0.009	0.062±0.005	0.063±0.006
		Testis	0.83±0.06	0.80±0.07	0.74±0.08	0.79±0.10
		Epididymis	0.33±0.04	0.29±0.03	0.30±0.07	0.36±0.07
Female	Body weight		289.7±28.0	303.4±10.8	273.7±19.8	288.0±23.5
	Relative/%	Brain	0.70±0.05	0.66±0.06	0.75±0.03	0.70±0.07
		Heart	0.36±0.09	0.35±0.08	0.39±0.13	0.36±0.10
		Liver	2.63±0.17	2.53±0.31	2.75±0.28	2.82±0.17
		Kidney	0.59±0.07	0.52±0.06	0.58±0.07	0.52±0.02
		Spleen	0.16±0.03	0.18±0.03	0.18±0.03	0.19±0.02
		Lung	0.44±0.03	0.42±0.03	0.43±0.05	0.48±0.06
		Adrenals	0.0210±0.0061	0.0241±0.0050	0.0212±0.0032	0.0234±0.0043
		Thymus	0.0811±0.0127	0.0823±0.023	0.0821±0.033	0.0851±0.0125
		Ovaries	0.0310±0.0021	0.0298±0.0043	0.0312±0.0023	0.0290±0.0030
		Uterus	0.32±0.03	0.36±0.07	0.29±0.06	0.30±0.02

0: The negative control.

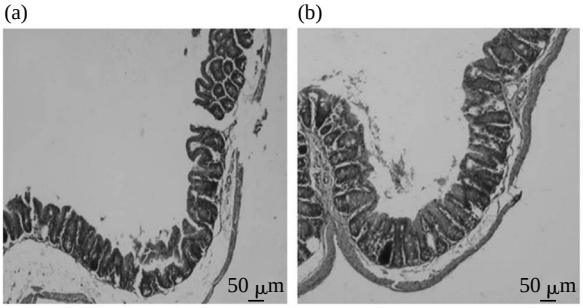

Fig. 3 Microscope graphs of cecum villi of rats (×100)
(a) Control. (b) 7.50 g/kg Daily KMOS.

2.4 Ames test

The result of Ames test was showed in Table 5. The positive controls (with or without S9) induced revertant colonies/plate more than two folds compared to that of negative control group, implying their mutagenic effects. There was no biologically significance and dose-dependence in the number of revertant colonies in the five strains (TA 1535, TA97, TA98, TA100 and TA102) no matter with or without S9 mixture at any tested KMOS dose. It can be concluded that, the Ames test result for KMOS was negative.

2.5 Micronucleus assay

Table 6 showed the result of micronucleus test of erythrocytes in mice. As shown, micronuclei rate was significantly higher in the positive control group (19.8% for male and 19.0% for female) compared with that of the negative group (1.4% for male and 1.6% for

female) ($P < 0.05$). However, the experimental groups at three dose levels, regardless of gender, did not show any statistically significant difference compared with the negative control ($P > 0.05$). Therefore, the mouse micronucleus test for KMOS was negative.

Table 5 The results of Ames test on KMOS

Chemical	Dose/(μg/plate)	S9	Revertant colonies/plate				
			TA97	TA98	TA100	TA102	TA 1535
KMOS	8	–	117 ± 16	37 ± 3	147 ± 18	287 ± 12	15 ± 3
	40	–	116 ± 15	38 ± 1	148 ± 17	287 ± 15	11 ± 5
	200	–	115 ± 16	39 ± 2	146 ± 13	289 ± 19	14 ± 4
	1000	–	117 ± 13	37 ± 3.	146 ± 9	285 ± 15	17 ± 4
	5000	–	118 ± 10	38 ± 3	150 ± 17	288 ± 15	16 ± 3
Negative control		–	114 ± 15	39 ± 2	145 ± 11	288 ± 18	15 ± 4
Solvent control		–	116 ± 8	40 ± 2	149 ± 15	292 ± 17	16 ± 4
Positive control		–	1320 ± 71*	1886 ± 65*	1278 ± 59*	1245 ± 83*	350 ± 15*
KMOS	8	+	122 ± 16	40 ± 3	151 ± 15	281 ± 17	15 ± 2
	40	+	126 ± 12	42 ± 3	152 ± 17	291 ± 19	17 ± 3
	200	+	123 ± 9	41 ± 2	157 ± 9	290 ± 16	18 ± 4
	1000	+	123 ± 12	41 ± 2	154 ± 11	290 ± 13	14 ± 2
	5000	+	121 ± 16	40 ± 2	154 ± 11	291 ± 12	11 ± 4
Negative control		+	120 ± 15	40 ± 3	151 ± 15	289 ± 18	15 ± 2
Solvent control		+	122 ± 15	41 ± 4	154 ± 17	293 ± 8	15 ± 1
Positive control		+	1344 ± 58*	1893 ± 81*	1293 ± 71*	884 ± 73*	314 ± 20*

* Compared with the negative control, $P < 0.05$.

Table 6 Effect of sub-chronic oral administration of KMOS on bone narrow cell micronucleus of male and female rats

Sex	Dose/(g•kg^{-1})	Cell	Micronucleus	Micronuclei Rate/‰	PCE/NCE
Female	0	1000×5	7	1.4	1.08
	2.5	1000×5	8	1.6	1.02
	5.0	1000×5	6	1.2	1.10
	10.0	1000×5	9	1.8	1.11
	Endoxan	1000×5	99	19.8*	1.02
Male	0	1000×5	8	1.6	1.05
	2.5	1000×5	7	1.4	1.11
	5.0	1000×5	5	1.0	1.18
	10.0	1000×5	10	2.0	1.09
	Endoxan	1000×5	95	19.0*	1.10

0: The negative control; *$P < 0.05$, compared with the negative control.

2.6 Sperm malformation test

Table 7 showed the results of the mouse sperm malformation experiment. Morphological examination revealed that sperm morphological alterations including indefinite form, banana head, no hook head and fat head occurred in each group. The positive control gave a statistically significant elevation of abnormal sperm (3.96%) compared with the negative control (1.00%) ($P < 0.05$). The frequencies of sperm abnormalities for all doses KMOS treated groups (1.14% for 2.5 g•kg^{-1} group, 1.06% for 5.0 g•kg^{-1} group and 1.16% for 10.0 g•kg^{-1} group) were not significant different from the negative control ($P > 0.05$).

Table 7 Sperm abnormality rate of mice administration orally with KMOS

Dose/(g•kg^{-1})	No. sperm	Types of abnormal sperm						Sperm abnormality rate/%
		Amorphism	Banana shape	No hook	Fat head	other	total	
0	5000	17	4	17	12	0	50	1.00 ± 0.19
2.5	5000	18	5	18	16	0	57	1.14 ± 0.30
5.0	5000	17	3	15	18	0	53	1.06 ± 0.21
10.0	5000	20	4	19	15	0	58	1.16 ± 0.18
Endoxan	5000	67	12	62	57	0	198	3.96 ± 0.13*

0: The negative control; *P < 0.05, compared with the negative control.

3 Discussion

KMOS is a type of neutral oligosaccharide extracted from konjac, which is consisted of D-mannopyranosyl and D-glucopranosyl units linked together by β-D-1, 4-linkages. It is poorly digested carbohydrate that reaches the colon followed by being fermented by the gut microflora. Being a time- and dose- dependent course, the fermentation pattern of KMOS is obviously influenced by its structure (defined as monomer composition, degree of polymerization and type of glycosidic linkage) [26]. Al-Ghazzewi [4] reported that KMOS produced with cellulose enzymes (hydrolyze β-1, 4-linked glucose residues) promote the growth of lactic acid bacteria more effectively than that hydrolysed by mannase (hydrolyse randomly β-1, 4-linked mannose residues). Besides, several studies have confirmed that higher degree of hydrolysis results in higher physiological activity of KMOS [12, 27]. For instance, KMOS with DP (12 ± 1) promoted a greater inhibitory effect on lipid peroxidation than KM with higher DP[12]. Also, Yeh[27] demonstrated that KMOS with DP 4 exerted the greater protective against DNA damage of fecal water-treated Caco-2 cells than that of DP 8. Consequently, in this study, low molecular mass KMOS was adopted, and for the first time, KMOS (DP =2 ～7) was prepared by enzymatic hydrolysis followed by organic solvent precipitation method. In order to fully understand its potential toxicological in mammals,especially at higher doses and under long-term exposure, 90-day subchronic toxicity and genotoxicity assessments were performed.

The repeated-dose toxicity test conducted on rats for 90-day could provide information regarding the cumulative toxic effects of test chemical. Oligosaccharide are not digested in the small intestinal but fermented in the large intestine, producing gas, lactate and SCFA. Excessive SCFA and lactate level increase intraluminal osmotic pressure and passage rate in the large intestine, resulting in the production of the softer feces and flatulence [28]. In consistent with that, high moisture and soft feces were found after KMOS fed. Besides, microscopic change in cecum villi was noted after KMOS feeding, which showed more regular structure and higher length. Nevertheless, it was common findings in oligosaccharide treated animals and was beneficial for the health status of growing animals [29]. Beyond those, microscopic changes in the liver and kidney, but these symptoms were commonly occurred in untreated group and of comparable incidence and severity between untreated and treated groups. Additionally, there were no correlative changes in biochemistry indexes of liver (ALT, AST, TP and ALB) and kidney (BUN and CR). Therefore, the change in the histology was considered toxicologically insignificant. It was noteworthy that the previous studies found hypoglycemic and antilipidemic effect of KMOS in mice by incorporation 5% (w/w) KMOS in drinking water for a period of 14 weeks[14]. Inconsistent with that, no significant decline in the blood glucose and cholesterol was observed in our study. It was possible that the differences in KMOS properties, such as DP or molecular structure due to different manufacturing process and sources[27, 30], were responsible for the discrepancy in the blood glucose and cholesterol result.

The Ames mutagenicity assay is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of that chemical substances, which can result in genetic damage, and further leads to gene mutations [31]. The micronucleus assay is considered as preferable method for measuring chromosome damage as it allows the determination of

both chromosomal loss and breakage[32-33]. The mouse sperm morphology assay has been widely adopted for evaluating mammalian germ mutagens. In our experiment, the Ames test, micronucleus test and sperm abnormality test revealed that all the positive control substance in the three tests produced the expected increase and all criteria for all valid study were met. Overall, all these results revealed that KMOS had no genotoxicity effect under our experiment conditions.

In conclusion, the results observed following 13 weeks of feeding showed that KMOS up to 7.50 g/kg daily was safe to rats. The genotoxicity studies *in vitro* and *in vivo* indicated that administration of KMOS up to 10.0 g/kg had no mutagenicity potential.

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低分子质量魔芋甘露寡糖的长期毒性与遗传毒性研究 *

蒋 敏[1)] 李 恒[1)] 王 苗[1)] 罗春勤[2)] 许正宏[1)] 史劲松[1)**] 马延和[3)]

([1)] 江南大学药学院制药工程实验室，无锡 214000；[2)] 成都永安缘和药业生物科技有限公司，成都 610000；
[3)] 中国科学院微生物研究所，北京 100101)

摘要 魔芋甘露寡糖是一种具有肠道菌群调节作用的新型食品添加剂．本研究首次通过酶解与有机溶剂沉淀法制备了低分子质量的甘露寡糖(聚合度 2～7)，并对这类寡糖进行了长期毒性与遗传毒性评价．在长期毒性试验中，以大鼠为实验对象，分低、中、高(2.25，5.25，7.50 g/kg)药物剂量组和阴性对照组，连续灌胃给药 90 天．一般状况观察、生化指标、血液学指标、病理学等与对照组比较均无显著性差异，而大体解剖观察发现，部分大鼠的肝脏与肾脏形态发生变化，但这些变化均在正常范围内，且其他各项指标差异均无统计学意义．此外，一系列实验包括小鼠骨髓微核实验、Ames 试验、小鼠精子畸变试验均未发现低分子质量甘露寡糖有明显的遗传毒性．试验结果提示，本研究方法获得的低分子质量甘露寡糖在本实验条件下未发现长期毒性与遗传毒性．

关键词 魔芋甘露寡糖，长期毒性，Ames 试验，骨髓微核试验，精子致畸试验

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** 通讯联系人．

Tel: 0510-85327353, E-mail: shijs@163.com

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