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HEALTH SAFETY ASSESSMENT OF RICE GENETICALLY MODIFIED WITH BOTH GENES OF *BT* AND *EPSPS* USING A MOUSE (*MUS MUSCULUS*) MODEL

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ABSTRACT

Genetically modified (GM) rice has huge potential in rice production, however, its safety as food or feed remains debatable. In this study, the effects of rice modified with both genes of *Bt* and *epsps* on mice were determined by feeding tests and *in vitro* cytotoxicity assays in order to evaluate the safety of GM rice. The results showed that the survival rate of mice fed with GM rice diet for 245 days was 90% for the male and 100% for the female, not lower than that of mice fed with non-GM rice diet or the formula diet. The average body weight gain of the mouse fed with GM rice diet for 30 days was 20.9 g for the male and 11.6 g for the female, not significantly different from that of the mouse fed with non-GM rice diet or the formula diet. The internal organ indices, blood parameters, and allergenic indicators of mice fed with GM rice diet for 245 days were not significantly different from those of mice fed with non-GM rice diet or both non-GM rice diet and the formula diet. The survival rates of mouse lymphocytes exposed to different doses of whole protein from both GM and non-GM rice grains for 2-24 hours *in vitro* were greater than 90%, not significantly different from each other. These results suggest that GM rice, similar to its non-GM rice counterpart, is a safe feed for mice.

Key words: Genetically modified rice; *Mus musculus*; Biosafety assessment; Feeding; Cytotoxicity.

INTRODUCTION

With the growth of global population and income, the demand for foods also increases (United Nations, 2013; Alexandratos *et al*., 2012). To meet this increasing demand, the global agricultural production in 2050 must be 60% higher than that in 2005/07 (Alexandratos *et al*., 2012). Plant biotechnology, emerged in early 1980s (Herrera-Estrella *et al*., 1983; Bevan *et al*., 1983; Fraley *et al*., 1983; Murai *et al*., 1983), has recently advanced dramatically. New varieties of corn, soybean, and other plants with tolerance or resistance to herbicides and insects have been created by the plant molecular breeding, which is difficult or impossible to be developed by the traditional breeding method. Since GM crops were approved to be planted commercially in 1996, its adoption has been expanded rapidly, as the global planting area of GM crops in 2013 is about 175.2 million hectares (James, 2014) producing huge economic, ecological and social benefits (James, 2014, 2013; Brookes and Barfoot, 2006). It seems now that the greater adoption of GM crops is the only promising way to make a substantial contribution to meet the sustainable increasing demand for food, although it is not a panacea.

Rice is the staple food for about half of the world's population, and it's also one of the sources of food and feed raw materials for livestock. The global planting area of rice (160 million hectares) ranks the second, only behind that of wheat (220 million hectares) (Alexandratos *et al*., 2012). In 1999 and 2000, the first two herbicide-resistant GM rice varieties LLRice62 and LLRice06 were approved for commercial cultivation for food and feed in the United States. In the following ten years, the import permits of LLRice62 for food or food and feed were approved in Canada, Mexico, and other countries. In 2004, the insect-resistant transgenic rice Tarom Molaii + CryIAb for commercial cultivation as food and feed was approved in Iran (http://www.isaaa.org/gmapprovaldatabase/default.asp). However, none of these approvals results in the commercialization. In 2009, the biosafety certificates of GM hybrid rice BT Shanyou 63 and its paternal Huahui-1 with pest resistance were issued in China, the world's largest rice producing country, however, they haven't been commercialized because their commercial cultivation were not approved (JAB, 2010). As the rice is widely planted in the world, especially in Asia countries, GM rice has huge potential for solving the food issue; therefore, it has attracted much attention all over the world. A lot of GM rice varieties or hybrids with herbicides-tolerance, insects or diseases resistance, and other outstanding agronomic traits have been developed via the molecular breeding method (Yang *et al*., 1989; Fujimoto *et al*., 1993; Nayak *et al*., 1997; Tu *et al*., 2000; Xiao *et al*., 2007; Ye *et al*., 2000; He *et al*., 2011; Deka *et al*., 2010). These transgenic rice varieties/hybrids have great potential in terms of increasing rice grain yield, improving rice quality, reducing labor intensity and environmental pollution, etc.. However, whether GM rice affect human and animal health as food or feed ingredients is still debatable. Most of the public hold the rejection attitude toward GM rice due to the lack of sufficient knowledge, which hindering its

commercialization. Many scholars have performed the health biosafety assessment of different GM rice varieties. Most of them believe the GM rice varieties are safe and substantially equivalent to their non-GM rice counterparts (Yuan *et al*., 2013; Wang *et al*., 2013; Chen *et al*., 2012; Liu *et al*., 2012 a, b; Liu, *et al*., 2008; Wang *et al*., 2000; Wang *et al*., 2002; Momma *et al*., 2000), while the others believe GM rice varieties are unsafe or not currently considered safe (Zhang *et al*., 2010; Xu *et al*., 2011; Poulsen *et al*., 2007; Schrøder *et al*., 2007; Kroghsbo *et al*., 2008). Unsafe of other GM crops like corn has been reported (Séralini, *et al*., 2013, 2011; Spiroux de Vendômois , *et al*., 2009; Malatesta *et al*., 2002). Séralini *et al*. (2011) believes that most of reported studies are not independent, the animal feeding test time is not long enough, long-term assessment of inter-generations is lack, and the contents of the assessment are too extensive; therefore, the assessment system needs to be improved. Obviously, there are differences among the scholars on the health safety of GM rice and GM crops, so it is necessary to continue independent studies (Zheng, 2013; Verma *et al*, 2011; Séralini *et al*, 2011). In addition, as GM crops may produce unintended effects due to the position effects of insert gene(s), it is necessary to evaluate the health biosafety for each independent transgenic event based on case assessment rule. For these reasons, the health biosafety of rice genetically modified with *Bt* and *epsps* genes was evaluated in this study. The aim is to provide a scientific foundation for the applications of this GM rice and other GM crops.

MATERIALS AND METHODS

Materials: The substance for test is seeds of an *indica* rice variety 93-11 genetically modified with both genes of *Bt* and *epsps*. The seeds of GM rice and its corresponding non-GM rice variety 93-11 were provided by Yahua Zhongye Agriculture Academy of Hunan, Yuan Long Ping High-Tech Agriculture CO., Ltd., Hunan Province, China. The GM rice plants have both agronomic traits of stem borer resistance and glyphosate herbicide resistance.

The animals for experiment are six-week-old SPF-glade mice (*M. musculus*), purchased from Hunan Slca Jingda Experimental Animal Center CO., Ltd. [production license number: SCXK (Xiang) 2009-0004]. A total of 60 male and female (30 pairs) mice weighing about 20 g each (The variation in weight is ≤10%) were randomly divided into three groups numbered Group I, Group II, and Group III with 10 pairs for each group.

The feeds for mice were divided into three categories: 1) the feed containing 60% GM rice grains; 2) the feed containing 60% of non-GM rice grains; and 3) the commercial formula feed without adding rice grains. The feeds with 60% GM rice grains and 60% non-GM rice grains were commissioned to be made by the Animal Feed Manufacturer of Central South University, Hunan Province, China, and the commercial formula feed was purchased from Hunan Slca Jingda Experimental Animal Center CO., Ltd. [production license number: SCXK (Xiang) 2009-0009]. All three feeds were made according to China national standards GB14924.3-2010 (Laboratory animals-Nutrients for formula feeds), whose composition and content can be found in Liu's paper (Liu *et al*., 2012 a, b), and they are all in line with the nutritional requirements of mouse for feeding trial.

Method of feeding mice with GM rice grains: The feeding of mice was conducted in a mice breeding room in the College of Life Science, Hunan Normal University, Hunan Province, China. In the room, the temperature is 22 - 25 °C, the relative humidity is 50% - 60%, and the light length is 13 h (the illumination time is from 6: 00 - 19: 00) with the light intensity of 2-3 $\mu mol.s^{-1}.m^{-2}$. The mice were housed in steel cages (two male mice or two female mice per cage) with free access to food and water. The daily diet was provided with the quantity of 10% mouse body weight, and the water was sufficiently supplied by an 80 ml water-giving tube device per cage. Before the beginning of feeding test, the mice were fed the commercial formula feed for three days for acclimation. Then the mice were fed with the feed containing 60% GM rice grains (Group I), with the feed containing 60% non-GM rice grains (Group II), or with the commercial formula feed (Group III), respectively, until the end of the experiment which lasted 245 days or 35 weeks.

Observation and measurement of morphological indicators of mouse's growth and development: The external morphology, behavior, and survival of mice were observed daily to determine whether they are normal or not, and the mortality rates of mice of three groups were investigated after the mice were fed 245 days.

The body weights of mice were measured on the day(s) of 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31 after starting the feeding trial (fasting 12 h before weighing), and the growth curves were drawn according to the body weights of mice.

For internal organ indices, the body weights of fasting mice were measured first after fed 245 days, then the mice were killed and their eyes and blood removed. Autopsy of their viscera was used to see if there was an exception. Then, the livers, kidneys, spleens, hearts, lungs, testes / ovaries, stomachs, small intestines, and brains of mice were removed and cleaned, and their fresh weights were measured. The internal organ index was calculated as the ratio of an organ weight / body weight.

Measurement of hematological and serum biochemical parameters: At the end of the feeding trial, five female mice and five male mice of each group were

randomly selected to measure their hematological and serum biochemical parameters. The selected mice were killed; their eyes removed; and their bloods immediately taken from the damaged eyes with a pipette. One part of blood from each mouse was transferred into an anticoagulant tube for the detection of hematological indices, while the other part was transferred into an EP tube in which the serum was extracted from blood following Liu's Method (2012) for the determination of serum biochemical parameters. Hematological indicators including red blood cell count, hemoglobin content, white blood cell count, platelet count, etc. were measured with Mindray BC-5800 Auto Hematology Analyzer (Mindray Medical International Limited, Shenzhen, Guangdong Province of China) at the fourth hospital of Changsha City, Hunan Province of China. Serum biochemical parameters including serum total protein content, albumin content, alanine aminotransferase, etc. were measured with Mindray BS-300 Chemistry Analyzer (Mindray Medical International Limited, Shenzhen, Guangdong Province of China) at the Hospital in Hunan Normal University, Hunan Province , China.

Analysis of serum IgG and IgE: The IgG and IgE levels in serum were analyzed by ELISA with IgG and IgE kits purchased from Huisong Science & Technology Co., Ltd., Shenzhen, Guangdong Province of China, following the manufacturer's protocol. The light absorbance (A value) of the sample was measured at 450 nm with DG5033A ELISA Analyzer (Nanjing Huadong Electronics Group Medical Equipment Co., Ltd., Nanjing, Jiangsu Province, China). The level of IgG and IgE were evaluated according to the A values.

Detection of toxicity of whole protein extracted from dehulled GM rice grains on mouse lymphocytes: The toxicity of whole protein extracted from dehulled GM rice grains on mouse lymphocytes was detected according to Chen's Method (2012) with some modification. The detection target cells of mouse spleen lymphocytes were provided by Cardiac Development Research Lab., Life Science College, Hunan Normal University, and the whole protein concentrations of dehulled GM and non-GM rice grains exposed to lymphocytes were 200, 100, 50, 25 μg/ml with exposure times of 2, 6, 24 h for each concentration.

Statistical analysis: The values in all the tables and figures are means ± SD. Among them, the values in Fig. 1, Fig. 2, and Table 1 are means from 9 or 10 independent samples; in Table 2, Table 3, Fig. 3, and Fig. 4 from 5 independent samples; and in Table 4 and Table 5 from 3 independent samples. One way ANOVA and Duncan's multiple range tests were used to determine the differences among means obtained from 3 groups of mice. The p-value < 0.05 was considered significant difference. All calculations were performed using statistical software of IBM SPSS Statistics 19.

Bioethics: All experimental procedures were approved by the College of Life Science, Hunan Normal University, Changsha, Hunan province, China.

RESULTS

The effects of GM rice on the signs, behavior, and survival of mice: The signs, behavior, and mortality rates of three groups of mice fed different kinds of feeds for 245 days were observed and measured comparatively. The results showed that the growth and development of mice of three groups were well-maintained with no abnormal signs and behaviors. and almost all the mice survived. Only one mouse died in each group of male mice and the Group II of female mice, therefore, the mortality rate of mice fed GM rice grains was not higher than either that of mice fed non-GM rice grains or that of mice fed commercial formula feed, indicating that feeding mice with GM rice grains does not influence their survival.

The effects of GM rice on the growth and development of mouse: The weights of the fasting mice of Group I, Group II, and Group III fed with GM rice feed, non-GM rice feed, or the commercial formula feed, respectively, were measured once every three days for one month. The results showed that there were no significant differences ($P \geq 0.05$) of body weights among the mice of three groups at each time (Fig.1 and Fig. 2). Therefore, there was no any side effect on the growth rates of mice fed GM rice grains.

Mouse organ index is an important indicator to reflect whether the growth and development of its organs are normal, and it is widely used to evaluate the impact of the test substance on organ growth and development. The ratios of liver weight / body weight (BW), kidney weight / BW, spleen weight / BW, heart weight / BW, lung weight / BW, brain weight / BW, small intestine weight / BW, stomach weight / BW, and ovary or teste weight / BW of Group I, Group II, and Group III mice were determined after they were fed with three kinds of feeds for 245 days. The results showed that except the ratio of male lung weight / body weight and the ratio of female kidney weight / body weight of Group I mice (fed with GM rice feed) were significantly less than those of Group III mice (fed with formula feed), the organ indices of all others of male and female mice tested showed no any significant differences among the three groups of mice (Table 1). Although there were significant differences of above two organ indices between Group I and Group III mice, there were no any significant difference of the two organ indices between Group I and Group II mice (fed with non-GM rice feed), indicating that there is no

obvious adverse effect on growth and development of mice fed GM rice grains.

The effects of GM rice on the hematological and serum biochemical parameters in mice: The hematological parameters like red blood cell count (RBC), hemoglobin (HGB) content, white blood cell count (WBC), platelet count (PLT), etc., and serum biochemical parameters such as activities or contents of alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine (CREA), total protein (TP), etc. of mice of three groups were analyzed after they were fed three kinds of feeds for 245 days. The results showed that all the indicators were not significantly different from each other among the mice of three groups (Table 2 - Table 3), suggesting that feeding mice with GM rice has no adverse effect on the structure and function of their tissues and organs such as livers, kidneys, bone marrows.

Analysis of allergenicity of feed containing genetically modified rice grains to mice: The IgG and IgE levels in serum are two important indicators to evaluate the allergenicity of the test substance. If a mouse is allergic to the test substance, the levels of either or both IgG and IgE in its serum will responsively increase. The analysis of serum IgG and IgE in mice of Group I, Group II and Group III showed that the serum IgG and IgE levels in mice among the three groups were not significantly different from each other (Figure 3, Figure 4) after they were fed three kinds of feeds for 245 days, indicating that the feed containing GM rice grains has no allergenicity to mice.

Cytotoxicity of whole protein extracted from the dehulled genetically modified rice grains to mouse lymphocytes *in vitro*: The cytotoxicity of whole protein extracted from the dehulled GM rice grains and non-GM rice grains on mouse spleen lymphocytes was detected by Cell Counting Kit-8 and the Neutral Red Uptake assay *in vitro* with the exposure protein concentrations of 25, 50, 100, and 200 μg/ml and incubation time of 2 h, 6 h, 24 h. The results showed that the survival rate of lymphocytes exposed to whole protein of the dehulled GM rice grains was not significantly different from that of exposed to whole protein of the dehulled non-GM rice grains (Table 4, Table 5), indicating that the whole protein of GM rice grains has no toxicity to mouse lymphocytes.

Table 1. Organ indices of mice after fed with GM rice feed for 245 days

Organ indices	Group I GM rice feed	Group II Non-GM rice feed	Group III Formula feed
BW (g) of male mouse	44.5870 ± 3.0411 a	43.2145 ± 4.0456 a	45.1964 ± 2.9497 a
Liver / BW (%)	4.66 ± 0.47 a	4.69 ± 0.43 a	4.90 ± 0.22 a
Kidneys / BW (%)	1.85 ± 0.45 a	1.92 ± 0.18 a	1.93 ± 0.20 a
Spleen / BW (%)	0.24 ± 0.06 a	0.18 ± 0.06 a	0.23 ± 0.07 a
Heart / BW (%)	0.56 ± 0.09 a	0.60 ± 0.08 a	0.62 ± 0.13 a
Lungs / BW (%)	0.60 ± 0.09 a	0.67 ± 0.16 ab	0.77 ± 0.14 b
Brain / BW (%)	1.00 ± 0.11 a	0.97 ± 0.05 a	1.02 ± 0.07 a
Testes / BW (%)	0.53 ± 0.10 a	0.59 ± 0.06 a	0.54 ± 0.11 a
Stomach / BW (%)	1.58 ± 0.54 a	1.80 ± 0.28 a	1.58 ± 0.40 a
Small intestine / BW (%)	3.49 ± 0.58 a	3.79 ± 0.60 a	3.86 ± 0.46 a
BW (g) of female mouse	50.0650 ± 3.7440 a	46 .9190 ± 4.7930 a	46.6380 ± 3.5000 a
Liver / BW (%)	5.85 ± 1.10 a	6.12 ± 0.90 a	6.90 ± 1.51 a
Kidneys / BW (%)	1.13 ± 0.16 a	1.31 ± 0.19 ab	1.43 ± 0.28 b
Spleen / BW (%)	0.37 ± 0.17 a	0.37 ± 0.12 a	0.30 ± 0.11 a
Heart / BW (%)	0.50 ± 0.06 a	0.55 ± 0.06 a	0.50 ± 0.07 a
Lungs / BW (%)	0.58 ± 0.14 a	0.67 ± 0.15 a	0.70 ± 0.12 a
Brain / BW (%)	0.98 ± 0.15 a	0.96 ± 0.10 a	0.98 ± 0.12 a
Testes / BW (%)	0.04 ± 0.02 a	0.03 ± 0.02 a	0.05 ± 0.03 a
Stomach / BW (%)	1.71 ± 0.34 a	1.63 ± 0.47 a	1.88 ± 0.26 a
Small intestine / BW (%)	4.01 ± 0.92 a	4.04 ± 1.59 a	4.19 ± 1.39 a

Note: The values in the table are means ± SD. The means with the same letters on a line indicate no significant difference ($P \geqslant 0.05$) among the groups, while the means with the different letters indicate significant difference ($P < 0.05$). These are also applied to Table 2 and Table 3 below. BW, body weight

Table 2. Hematological indices of mice after fed with GM rice feed for 245 days

Hematological parameters	Group I GM rice feed	Group II Non-GM rice feed	Group III Formula feed
Male RBC（$\times10^{12}$/L）	10.054 ± 1.304 a	9.858 ± 1.491 a	10.482 ± 0.638 a
HGB (g/L)	164.6 ± 20.0 a	158.0 ± 21.5 a	162.8 ± 6.9 a
HCT(%)	51.06 ± 6.47 a	48.94 ± 5.71 a	50.86 ± 2.29 a
MCV(fL)	50.88 ± 3.47 a	49.86 ± 2.65 a	48.60 ± 2.22 a
MCH(pg)	16.40 ± 0.97 a	16.06 ± 0.30 a	15.56 ± 0.63 a
MCHC(g/L）	322.6 ± 9.8 a	322.4 ± 14.0 a	320.4 ± 11.3 a
RDW (% CV)	14.24 ± 0.53 a	13.60 ± 0.51 a	13.94 ± 0.52 a
PLT（$\times10^9$/L）	995.2 ± 133.0 a	1056.0 ± 125.8 a	1140.4 ± 133.7 a
PCT(%)	0.4750 ± 0.0776 a	0.4816 ± 0.0612 a	0.5106 ± 0.0667 a
PDW(% CV)	14.56 ± 0.17 a	14.48 ± 0.13 a	14.42 ± 0.26 a
MPV(fL)	4.76 ± 0.24 a	4.56 ± 0.18 a	4.50 ± 0.31 a
WBC($\times10^9$/L）	4.272 ± 1.001 a	3.920 ± 0.834 a	3.798 ± 1.082 a
Lymphocyte (%)	70.92 ± 3.57 a	71.82 ± 5.05 a	70.70 ± 7.51 a
Neutrophil (%)	21.88 ± 2.83 a	21.56 ± 4.79 a	22.18 ± 6.41 a
Eosinophil (%)	0.28 ± 0.11 a	0.52 ± 0.41 a	0.36 ± 0.18 a
Basophil (%)	0.10 ± 0.07 a	0.12 ± 0.18 a	0.10 ± 0.17 a
Monocyte (%)	6.82 ± 1.44 a	6.04 ± 2.25 a	6.62 ± 1.73 a
Female RBC（$\times10^{12}$/L）	9.674 ± 0.544 a	9.818 ± 0.847 a	10.508 ± 1.101 a
HGB (g/L)	155.0 ± 9.5 a	154.4 ± 19.0 a	164.6 ± 18.8 a
HCT(%)	44.86 ± 2.28 a	44.96 ± 3.63 a	47.50 ± 4.41 a
MCV(fL)	45.52 ± 1.35 a	44.98 ± 0.52 a	44.20 ± 1.72 a
MCH(pg)	16.00 ± 0.75 a	15.72 ± 1.06 a	15.70 ± 1.14 a
MCHC(g/L）	351.2 ± 7.7 a	349.4 ± 25.7 a	355.6 ± 26.3 a
RDW (% CV)	13.40 ± 1.0 a	13.16 ± 0.70 a	14.28 ± 1.01 a
PLT（$\times10^9$/L）	988.0 ± 463.2 a	1035.4 ± 234.2 a	1112.2 ± 392.0 a
PCT(%)	0.5012 ± 0.0581 a	0.5070 ± 0.0816 a	0.5128 ± 0.0756 a
PDW(% CV)	14.54 ± 0.59 a	14.62 ± 0.75 a	14.02 ± 0.70 a
MPV(fL)	5.78 ± 0.65 a	5.60 ± 0.89 a	5.54 ± 0.63 a
WBC($\times10^9$/L）	4.96 ± 0.71 a	4.70 ± 1.01 a	4.56 ± 0.87 a
Lymphocyte (%)	70.26 ± 6.09 a	68.74 ± 4.92 a	69.18 ± 5.46 a
Neutrophil (%)	22.12 ± 3.68 a	22.44 ± 3.32 a	22.84 ± 5.27 a
Eosinophil (%)	0.34 ± 0.22 a	0.46 ± 0.43 a	0.5 ± 0.37 a
Basophil (%)	0.08 ± 0.08 a	0.20 ± 0.28 a	0.10 ± 0.22 a
Monocyte (%)	7.20 ± 2.58 a	8.16 ± 1.41 a	7.38 ± 0.45 a

Note: RBC, red blood cell count; HGB, hemoglobin; HCT, hematocrit; MCV, mean cell volume; MCH, mean cell hemoglobin; RDW, red blood cell volume distribution width; PLT, platelet; PCT, plateletcrit ; PDW, platelet volume distribution width; MPV, mean platelet volume; WBC, white blood cell (leukocyte) count.

Table 3. Blood Biochemistry indices of mice after feeding with GM rice feed for 245 days

Biochemical compositions		Group I GM rice feed	Group II Non-GM rice feed	Group III Formula feed
Male	ALT（U/L）	50.34 ± 10.76 a	61.06 ± 14.19 a	53.42 ± 8.89 a
	AST（U/L）	99.1 ± 18.78 a	105.2 ± 18.74 a	102.2 ± 24.32 a
	γ-GT(U/L)	34.66 ± 13.47 a	39.32 ± 5.10 a	40.52 ± 9.58 a
	ALP（U/L）	132.6 ± 17.56 a	129.54 ± 17.94 a	139.04 ± 21.46 a
	TP（g/L）	62.58 ± 6.57 a	59.60 ± 5.59 a	58.44 ± 4.93 a
	Alb（g/L）	28.80 ± 4.32 a	25.26 ± 3.65 a	24.26 ± 2.28 a
	G（g/L）	33.8 ± 3.8 a	34.4 ± 5.1 a	34.2 ± 5.4 a

A/G	0.82 ± 0.11 a	0.72 ± 0.13 a	0.68 ± 0.13 a
TB (μ Mol/L)	4.316 ± 1.277 a	4.546 ± 1.924 a	5.134 ± 1.496 a
CREA（μ Mol/L）	52.60 ± 11.67 a	47.72 ± 13.93 a	50.52 ± 8.53 a
Urea (m Mol/L)	6.44 ± 2.17 a	6.94 ± 1.23 a	5.62 ± 2.61 a
UA（μ Mol/L）	63.60 ± 8.39 a	61.80 ± 5.21 a	58.16 ± 15.41 a
Glu (m Mol/l)	2.50 ± 0.35 a	3.66 ± 1.15 a	3.68 ± 1.29 a
TG(m Mol/l)	1.92 ± 0.45 a	1.84 ± 0.78 a	2.54 ± 0.34 a
TCH(m Mol/l)	3.72 ± 0.94 a	3.04 ± 0.59 a	3.62 ± 0.56 a
Female ALT（U/L）	40.57±12.74 a	45.22±7.23 a	53.80±10.28 a
AST（U/L）	93.38±12.24 a	89.24±10.90 a	98.44±22.59 a
γ-GT(U/L)	30.64±8.15 a	38.99±12.99 a	33.61±4.87 a
ALP（U/L）	123.60±14.70 a	134.04±22.16 a	137.68±21.67 a
TP（g/L）	56.88±4.99 a	59.74±18.47 a	65.48±4.50 a
Alb（g/L）	25.29±4.36 a	26.98±9.07 a	28.82±2.89 a
G（g/L）	32.00±4.23 a	32.56±9.55 a	36.22±2.88 a
A/G	0.73±0.17 a	0.76±0.06 a	0.76±0.09 a
TB (μ Mol/L)	5.134±1.096 a	5.468±0.570 a	5.736±0.763 a
CREA（μ Mol/L）	44.64±7.74 a	39.08±8.92 a	39.88±9.99 a
Urea (m Mol/L)	6.402±2.196 a	6.021±1.379 a	8.276±2.335 a
UA（μ Mol/L）	65.98±18.68 a	61.89±10.80 a	57.3±17.17 a
Glu (m Mol/l)	4.078±0.359 a	4.218±0.713 a	3.775 ±0.506 a
TG(m Mol/l)	1.58±0.37 a	1.73±0.75 a	1.67±0.45 a
TCH(m Mol/l)	3.492±0.877 a	2.544±0.771 a	2.904±0.423 a

Note: Data in the table are mean ± SD. Alb: Albumin, G: Globulin, A/G: Ratio of Albumin/globulin, TP: Total protein, ALT: Alanine aminotransferase, AST:Aspertate amino transferase, γ-GT: γ-glutamine transferase, ALP: Alkaline phosphatase, TB:Total bilirubin, CREA:creatinine, TG: Triglycerides. TCH: Total cholesterol, UA:Uric acid, Glu: Glucose..

Table 4. The survival % of mice lymphocytes detected by Cell Counting Kit-8 (CCK-8) assay after exposed to GM rice protein and non-GM rice protein *in vitro*

Test substances		**Time of lymphocytes exposed to test substances（h）**		
Category	Concentration（μg/ml）	2	6	24
Blank control	0.000	100.00±0.00 a	100.00±0.00 a	100.00±0.00 a
Positive control (Vincristine)	0.025	81.52±1.36 b	76.48±1.45 b	67.36±1.29 b
Whole protein of GM dehulled rice grains	25.0	94.53±3.49 a	92.43±3.54 a	93.25±4.19 a
	50.0	92.68±4.43 a	91.59±3.35 a	94.48±3.31 a
	100.0	92.53±3.41 a	93.46±4.29 a	93.39±4.32 a
	200.0	93.21±3.27 a	92.51±3.58 a	92.37±2.65 a
Whole protein of Non-GM dehulled rice grains	25.0	93.75±4.21 a	94.25±3.76 a	93.59±4.43 a
	50.0	92.53±3.29 a	93.78±2.43 a	94.24±3.32 a
	100.0	94.16±4.24 a	92.36±4.12 a	93.75±3.52 a
	200.0	92.65±3.47 a	92.46±4.36 a	92.74±4.16 a

Note: 1) Blank control: the lymphocytes were not exposed to any test substances ; 2) The values in the table are means ± SD. The means with the same letters on a column indicate no significant difference ($P \geqslant 0.05$), while the means with the different letters indicate significant difference ($P < 0.05$).

Table 5. The survival % of mice lymphocytes detected by neutral red uptake (NRU) assay after exposed to GM rice protein and non-GM rice protein *in vitro*

The test substances		**Time of lymphocytes exposed to test substances（h）**		
Category	Concentration（μg/ml）	2	6	24
Blank control	0.000	100.00±0.00 a	100.00±0.00 a	100.00±0.00 a
Positive control (Vincristine)	0.025	81.52±1.36 b	76.48±1.45 b	67.36±1.29 b
Whole protein of GM dehulled rice grains	25.0	94.25±4.16 a	93.87±3.52 a	91.87±3.34 a
	50.0	93.77±3.36 a	93.56±4.34 a	92.36±4.41 a
	100.0	94.53±5.12 a	94.47±3.36 a	92.52±3.54 a
	200.0	92.35±4.23 a	91.82±4.19 a	93.74±4.43 a
Whole protein of Non-GM dehulled rice grains	25.0	92.52±3.45 a	92.45±2.48 a	91.58±3.42 a
	50.0	93.38±5.02 a	91.75±3.52 a	93.38±5.40 a
	100.0	93.76±4.56 a	95.14±4.26 a	92.67±4.28 a
	200.0	92.84±3.49 a	93.51±5.39 a	94.10±3.26 a

Note: 1) Blank control: the lymphocytes were not exposed to any test substances ; 2) The values in the table are means ± SD. The means with the same letters on a column indicate no significant difference (P≥0.05), while the means with the different letters indicate significant difference (P<0.05).

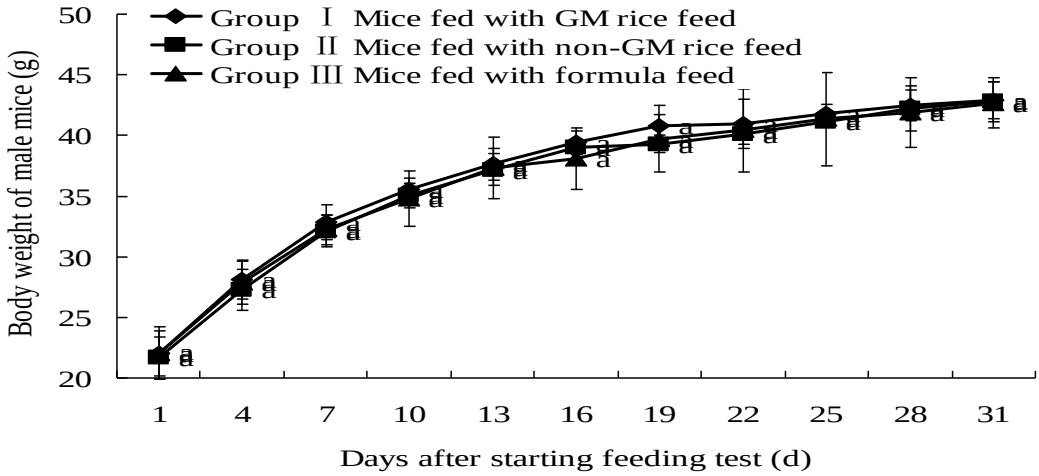

Fig. 1. Body weight of male mice fed with GM rice feed in one month after starting feeding test. The values in the figure are means ± SD. The means with the same letters on the same day indicate no significant difference (P≥0.05) of body weight among the mice of three groups. This is also applied to Fig. 2.

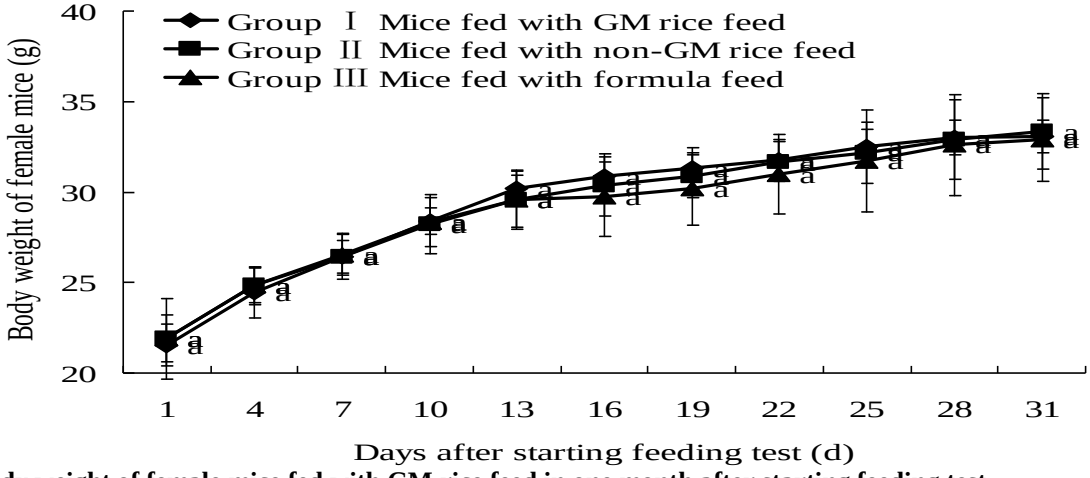

Fig. 2. Body weight of female mice fed with GM rice feed in one month after starting feeding test

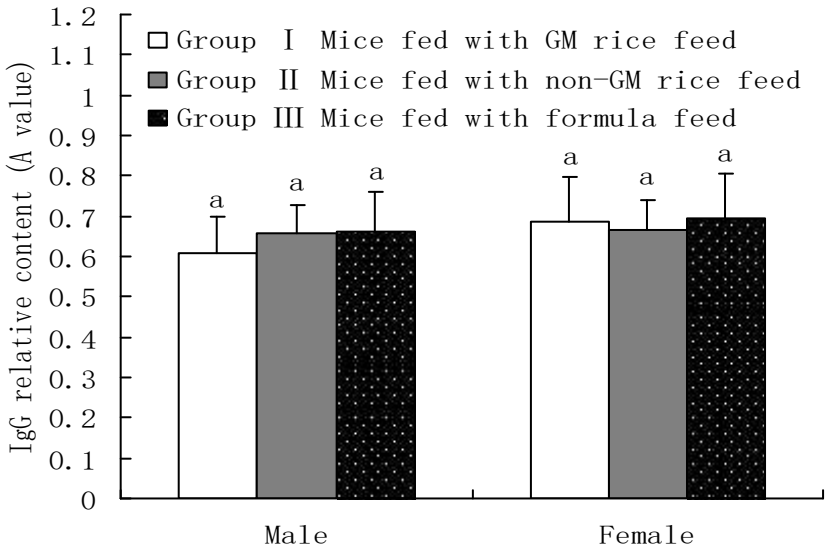

Fig. 3. IgG relative content (A value) in blood of mice after fed with GM rice feed for 245 days. The values in the figure are means ± SD. The means with the same letters indicate no significant difference (P⩾0.05) of IgG among the mice of three groups.

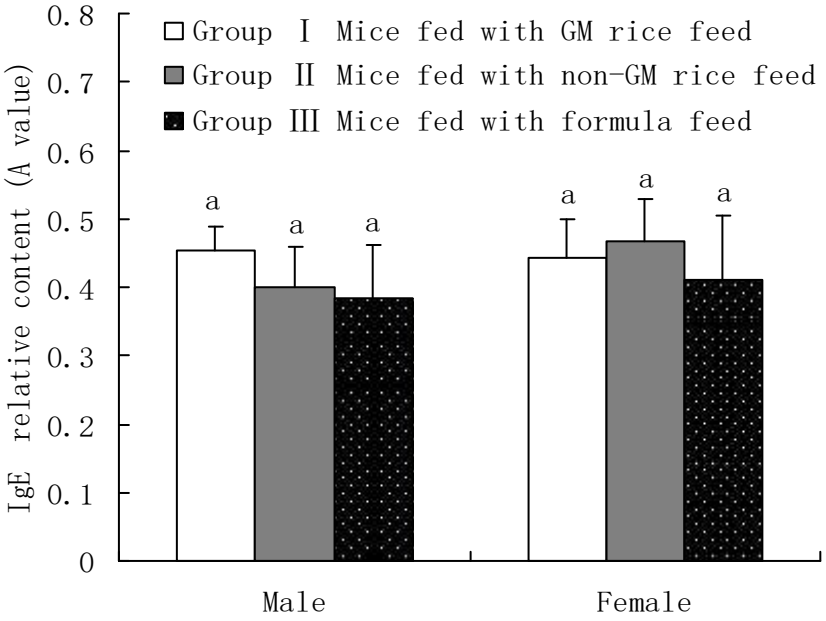

Fig. 4. IgE relative content (A value) in blood of mice after fed with GM rice feed for 245 days. The values in the figure are means ± SD. The means with the same letters indicate no significant difference (P⩾0.05) of IgE among the mice of three groups.

DISCUSSION

Rice is one of the world's major food crops. Although GM rice varieties or strains with elite or special agronomic traits like anti-pest resistance, herbicide tolerance have been developed to improve rice yields and quality, as well as to reduce the environmental pollution, however, they haven't been commercialized due to concerns about their biosafety. This study has analyzed the effects of GM rice genetically modified with both genes of *Bt* and *epsps* on the health of mice. The results show that although there are significant differences of the ratio of male lung weight / body weight and the ratio of female kidney weight / body weight between the mice fed GM rice feed and the mice fed formula feed, for which

the reason is undetermined, there are no significant differences of the two organ indices between the mice fed GM rice feed and the mice fed non-GM rice feed. And there are no significant differences of the other test indicators regarding the mouse health between the mice fed GM rice feed and the mice fed non-GM rice feed. Similarly, there are no significant differences between the survival percentage of lymphocytes exposed to whole protein of GM rice grains and that of lymphocytes exposed to whole protein of non-GM rice grains *in vitro*. Those results suggest that the GM rice has no adverse effect on the health of mice. This conclusion is consistent with those of most studies with other GM rice varieties using mice or rats as feeding animals (Yuan *et al*., 2013; Wang *et al*., 2013; Chen *et al*., 2012; Liu *et al*., 2012 a, b; Liu *et al*., 2008; Wang *et al*., 2000; Wang *et al*., 2002; Momma *et al*., 2000). It provides a scientific foundation for the biosafety of GM rice, which is critical for the consideration of the approval of the commercial cultivation of GM rice. Additionally, it will help the production and consumption of this GM rice.

Since the report that the transgenic pollen grains of BT corn harmed non-target monarch larvae, which resulted in wide concerns about the biosafety of GM crops, a lot of studies regarding the health safety of GM crops or GM rice have been done. However, most of those studies were conducted for sub-chronic toxicity test of 90 days feeding or acute toxicity test of 2 weeks feeding using the mouse or rat model. Only a few studies were performed for sub-chronic feeding trials of longer than 90 days, with the longest feeding time of 600 days for GM crops and 180 days for GM rice (Séralini, *et al*., 2013; Liu *et al*., 2012 a, b). Séralini *et al*. (2011) believes that the 90 days feeding time of sub-chronic toxicity test and 2 weeks feeding time of acute toxicity test are not long enough to evaluate the cumulative effect of the test substances, and the feeding time should be prolonged more for the chronic toxicological test to evaluate new drugs. Compared with most previous studies, the feeding time of this study is much longer, almost three times of the time for 90 days feeding of sub-chronic toxicity test, and is the longest so far in the safety assessment of GM rice. These results will undoubtedly be more reliable and valuable. Due to the limitation of some conditions, the chronic toxicity test up to 2 years' feeding failed to be performed in this study, but this test is still worth undertaking in the future for a more comprehensive understanding of the health safety of GM rice.

Molecular breeding of crops or rice with transgenic complex traits has become the major developing trend. A lot of studies have been done on the health safety assessment of GM crops with single transgenic trait, but rarely with multiple transgenic traits. As the GM crops may exhibit undesired effects, the conclusion of the health safety assessment of GM crops with single transgenic trait may not apply to those with polygenic transgenic traits; therefore, the health safety assessment of GM crops or rice with polygenic transgenic traits must be conducted. In this study, the health safety assessment of *indica* GM rice variety 93-11 with *Bt* and *epsps* genes is first carried out. It provides a safety evaluation reference for the applications of two foreign genes together in rice molecular breeding and has important reference value for the molecular breeding and application of GM rice with transgenic complex traits.

Due to the restrictions of ethics and complexity, it is infeasible to conduct health safety assessment of GM rice in human beings, but it is feasible to do it with human lymphocytes, which is simple and direct. Rodents and human beings are different species, although they are all mammals. Therefore, the effects of GM rice on human heath can't completely rely on the evaluation results of the animal experiments. So *in vitro* toxicology studies with human lymphocytes provide much relavent functional values and these studies needs to be further strengthened. Of course, we need more volunteers as the subjects involved in the health safety assessment in order to have a complete understanding of the health safety of GM rice and to make the GM rice beneficial for the mankind.

Conclusion: Feeding mice with the diet containing 60% rice grains genetically modified with genes of both *Bt* and *epsps* for 245 days does not influence their survival. There is no obvious adverse effect on growth and development of mice fed with the GM rice diet. There is no side effect on the hematology and serum physiological and biochemical indices of mice fed with the GM rice diet for 245 days. The content of serum IgG and IgE (allergenicity indicators) in mice fed with the GM rice diet for 245 days was not significantly different ($P \geq 0.05$) from that of mice fed with the non- GM rice diet and formula diet. The mouse lymphocyte exposed to whole protein of dehulled GM rice grain can normally survive *in vitro*. In overall, GM rice genetically modified with genes of both *Bt* and *epsps*, similar to its non-GM rice counterpart, is a safe feed for mice.

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