

Safety assessment of transgenic *Bacillus thuringiensis* with VIP insecticidal protein gene by feeding studies

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Abstract

The aim of this study was to evaluate the toxicology safety of a genetically modified (GM) *Bacillus thuringiensis* with vegetative insecticidal protein (VIP) gene. Acute and subacute toxicity studies by using its powder preparation were conducted in Wistar rats. The result of the acute study showed the no-observable-adverse-effect level (NOAEL) of this GM *B. thuringiensis* powder preparation was greater than 5000 mg/kg body weight (BW). In the subacute study, the data analysis of body weight gain, food and water consumptions, clinical observations, haematology, serum biochemistry, organ weight ratios and histopathological findings did not show significant differences between control and treated groups. These results proved the NOAEL of this GM *B. thuringiensis* powder preparation in subacute test was greater than 5000 mg/kg BW. Since both the acute and subacute oral toxicity were not detected at the highest dose recommended by OECD guidelines, this GM *B. thuringiensis* could be generally regarded as safe for use in bio-pesticide industry.

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1. Introduction

Bacillus thuringiensis is a ubiquitous gram-positive soil bacterium, and produces insecticidal crystal proteins (ICPs) which have active against certain insect species among the orders Lepidoptera, Diptera, Coleoptera,

Hymenoptera, Homoptera, Orthoptera, Mallophaga, nematodes, mites, and protozoa (Schnepf et al., 1998). So far, *B. thuringiensis* has been extensively used as bio-pesticide to control lots of crop pests, and it is also a key source of genes for transgenic expression to provide pest resistance (Hofte and Whiteley, 1989; Schnepf et al., 1998).

The vegetative insecticidal proteins (VIPs) from *B. thuringiensis* have toxicity against a wide spectrum of lepidopteran insects (Estruch et al., 1996; Donovan et al., 2001; Selvapandiyan et al., 2001; Liao et al., 2002; Loguercio et al., 2002; Franco-Rivera et al., 2004) and have a different action mode to pests compared with that of crystal proteins (Yu et al., 1997; Lee et al., 2003), so they have been extensively used in bio-pesticide industry to control crop pests (Warren, 1997), and will become a potentially useful tool in the prevention or management of pest resistance to ICPs. A novel VIP gene *vip83* (GenBank: Accession No. AY044227) was isolated from *B. thuringiensis* strain YBT-833 (Cai et al., 2002). The *vip83* under the control of the promoter of insecticidal protein gene *cry3Aa*

Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; Alb, albumin; *B. thuringiensis*, *Bacillus thuringiensis*; BW, body weight; BUN, blood urea nitrogen; CN, creatinine; EDTA-2K, ethylenediamine-*N,N,N',N'*-tetraacetic acid, dipotassium salt; Glu, glucose; GM, genetically modified; GMOs, genetically modified organisms; HB, hemoglobin concentration; HT, hematocrit; ICPs, insecticidal crystal proteins; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; NOAEL, no-observable-adverse-effect level; OECD, Organization of Economic Cooperation and Development; PLT, platelets count; RBC, red blood cell count; SPF, specified pathogen free; TP, total protein; TC, total cholesterol; VIPs, vegetative insecticidal proteins; WBC, white blood cell count.

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(De Souza et al., 1993) was subcloned into a resolution vector pBMB5401 derived from *B. thuringiensis* transposons Tn5401 (Baum, 1995), the recombinant plasmid was transformed and expressed in a commercialized *B. thuringiensis* strain YBT-1520, and then, a novel genetically modified *B. thuringiensis* strain BMB696B was obtained. The indoor bioassay and the field trial result showed BMB696B was highly toxic against *Heliocoverpa armigera* and *Spodoptera exigua* (Zhu et al., 2006). These result indicated that BMB696B can be potentially used as an attractive insecticide.

However, since the generation of genetically modified organisms (GMOs), its bio-safety has been given serious consideration. It is well known that several kinds of GMOs have been proved to be safe according to the concepts of “substantially equivalent”, and some of them have been commercialized. Syngenta Seeds Incorporated proved that the LD₅₀ for pure VIP3A protein is great than 3675 mg/kg body weight, that is no toxicity to rodents. What’s more, they demonstrated that the VIP3A protein in both the microbial and plant derived test substance was determined to be “substantially equivalent” to VIP3A produced in event COT102 derived cotton plants (U.S.A.EPA Notice, 2003). Based on this result and according to the concept of “substantially equivalent”, it can deduce that the VIP3A protein derived from the GM *B. thuringiensis* BMB696B is safe to rodents. But just by right of this fact, we cannot prove the BMB696B is safe to rodents. On one hand, genetic engineering introduces new genes, new genetic information, and new constituents into the cells of producing organism. The foreign proteins could themselves cause allergies or be toxic or could alter the cellular metabolism of the producing organism in unintended and unanticipated ways, and, in turn, these alternations in metabolism could cause allergens or toxins. Though VIP3A protein is safe to rodents, when expressed in YBT-1520, it would cause unexpected changes in the cellular metabolism of YBT-1520, whether these alternations would cause allergens or toxins or unintended adverse changes are not clear. On other hand, GMO is an organism, and contains many components just like protein, sugar, nucleic acid, fattiness and so on. To some certain, it is a new species. So when we to explain its toxicity, we should establish the direct toxicological data of the whole product BMB696B, but not to evaluate one or some parts of its components, after all, the simple added toxicology data of the components are not equal to that of the whole GMO. So, in the safety assessment of BMB696B, the concept of “substantially equivalent” can give us some advice, but not enough, the final evidence is the result of short time or long-term animal feeding studies. What’s more, as a bio-pesticide, the GM *B. thuringiensis* BMB696B would not directly but can enter into the food chain by certain ways, so we need realize its safety. While, in order to evaluate the safety of this GM *B. thuringiensis* bio-pesticide, we should establish the direct toxicological data of the whole GMO, not one part or some parts of its component. So in the present study, the acute

and subacute oral toxicity by using BMB696B powder preparation was investigated. Male and female Wistar rats were given BMB696B powder preparation which was made from this GM *B. thuringiensis*, at doses of 0, 500, 1250, 2500 or 5000 mg/kg bw by gavage for 14 in acute test and at doses of 0, 500, 1000 or 5000 mg/kg bw by gavage for 28 consecutive days in subacute test. Changes in the following parameters were analyzed: mortality, clinical observations, body weights and body weight gain in acute test while mortality, clinical observations, body weights, body weight gain, food and water consumptions, hematological and serum biochemical parameters, relative organ weights and gross and microscopic observations in subacute test.

By the way, all the test programs mentioned in this paper were prescribed and performed by some experienced participators, and all the study protocols were in accordance with the Organization of Economic Cooperation and Development (OECD) guidelines for materials of low toxicity.

2. Materials and methods

2.1. The GM *B. thuringiensis* BMB696B powder preparation

The GM *B. thuringiensis* BMB696B powder preparation used for all safety studies were provided by our lab and produced in the same manner as its production scale.

2.2. Animals and husbandry

Specified pathogen free (SPF) Young adult (five to six-weeks-old) Wistar rats of both sexes were purchased from the Laboratory Animals Center of Tongji Medical College, Huazhong University of Science and Technology, (Wuhan City, China) and adapted for one week to laboratory conditions: controlled temperature ($23 \pm 2^\circ\text{C}$), and 12 h light–dark cycles. All animal manipulations were performed according to the ethical principles for animal care and management. The animals were caged in plastic cages with chipped hardwood bedding and received solid diet (purchased from Hubei Centers for Disease Prevention and Control, Hubei Province, China) and tap water ad libitum during the experiments.

All the tested animals’ husbandry in the single safety studies was conducted under the supervision of some experienced researchers in the related field.

2.3. Oral acute toxicity study

An acute oral toxicity study was performed in accordance with the Organization of Economic Cooperation and Development (OECD) guidelines (OECD, 1995).

Fifty young adult (five to six-weeks-old) Wistar (25 males and 25 females) rats with a body weight ranging from 187 to 229 g were used in this study. All the fifty rats were randomly divided into five groups, and each group contains five females and five males. The test material, BMB696B powder preparation was suspended in distilled water, and administered orally (1 ml/100 g body weight) at doses of 500, 1250, 2500, and 5000 mg/kg bw by oral gavage to single female and male Wistar rats lasted 14 consecutive days, control groups received distilled water by 1 ml/100 g bw like that of treated groups. This study in order to yielded information on the dose-toxicity relationship and provide a does standard for the subacute study.

All the tested rats were observed shortly after dosing, and then each rat was observed daily for a period of 14 consecutive days. All animals were examined for general behavior signs of toxicity and mortality twice daily,

and all the data were recorded systematically. The general behavior signs of toxicity observations included changes in the skin and fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as behavioral pattern. The number of survivors was noted after dosing immediately and then they were maintained for a further 14 days with a twice daily's observation.

The body weights were recorded on days 0, 4, 7, 10, 14. On the day 15, all the tested animals were killed and the vital organs were separated and processed for routine gross and microscopically examination.

2.4. Oral subacute toxicity study

The subacute toxicity studies involved oral administration of BMB696B powder preparation at doses of 0, 200, 1000 and 5000 mg/kg bw per day to both male and female Wistar rats (five six-week old and weighing female 199–218 g, male 215–246 g) for a consecutive 28 days. Forty Wistar rats were randomly assigned to groups for a total of four groups (each group contains five females and five males). The test material, BMB696B powder preparation was suspended in distilled water, and administered orally (1 ml/100 g BW) to animals at a dose of 200, 1000 and 5000 mg/kg bw per day lasted 28 days; control groups received distilled water by 1 ml/100 g bw. During the period of administration, the animals were observed twice daily and weighed weekly to detect signs of toxicity. Daily visual observations were made and recorded systematically similar those performed as in the case of acute toxicity study. Rats died during the test period were examined pathologically. At the end of the experiment, all surviving animals were fasted overnight before anesthetization. Then blood samples were collected from a common carotid into EDTA-2K as an anticoagulant and dry non-EDTA-2K centrifuge tubes. The EDTA-2K blood was used for hematological study, and the non-EDTA-2K blood was used to separate the serum, which was used for serum biochemical examination. After blood collection all the animals were killed and necropsy for tissue studies and histological examinations.

To say concretely, during this subacute study, the observations and measurements contains below aspects.

2.4.1. Clinical observations, mortality and body weight

All animals were examined for general behavior, signs of toxicity and mortality, twice daily, as that in the case of acute toxicity study. Body weights were measured at the initiation of the experiment, and then at weekly intervals. Before were killed, all rats' final body weights were also determined following overnight fasted, and then all the tested rats' body weight gain was calculated and recorded systematically.

2.4.2. Food and water consumptions

Total food and water consumptions per cage were measured at the end of every week throughout the study and the results were recorded systematically. At the termination of this study, food and water consumptions were finally calculated and shown as a daily mean per rat in the results.

2.4.3. Hematology and serum biochemical examination

At the end of the experiment, all surviving animals were fasted overnight before anesthetization. Blood samples were collected from a common carotid into EDTA-2K as an anticoagulant and dry non-EDTA-2K centrifuge tubes. The EDTA-2K blood was used for hematological study. The hematological parameters, measured with an automated hematology analyzer, consist of white blood cell count (WBC), red blood cell count (RBC), hemoglobin (Hb), clotting time (CT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), and white blood cell differential count. The non-EDTA-2K blood was used for serum biochemical examination. The clinical biochemical parameters, measured with an automated biochemical analyzer were: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), blood urea nitrogen (BUN), total protein (TP), albumin (Alb), globulin (Gb), total cholesterol (TC), creatinine (Cr) and glucose.

2.4.4. Organ/body weights and histopathological examination

All the tested animals were killed by exsanguinations at the termination of the treatment period. Gross observations were made at autopsy and then recorded. At necropsy, all the animals from oral administration of BMB696B powder preparation groups and the control group were anatomized and following the next histopathological examination: all the organs/tissues were carefully examined macroscopically and the lungs, heart, spleen, liver, adrenals and kidney were weighted and their organ weight per 100 g body weight (relative weight) was calculated based on the fasted animals' body weight. All organs/tissues, including lungs, heart, spleen, liver, adrenals and kidney were fixed and preserved in 10% phosphate buffered formalin. Fixed tissues were routinely processed for embedding in paraffin, sectioned and stained with hematoxylin and eosin. Collected tissues were grossly and microscopically examined during histopathological examination.

2.5. Statistical analyses

Continuous variables such as body weight, hematology and serum biochemical parameters and relative organ weight percent were processed by variance analysis, pre- and post-treatment data were analyzed using paired samples *t*-tests; meanwhile, categorical data such as mortality, clinical signs and histopathological lesions were comparatively analysed using the Fisher's Exact Probability Test. Unless otherwise noted, all results are presented as means \pm S.D., and a probability level of $P < 0.05$ was considered significant.

3. Results

3.1. Acute oral toxicity in rats

During this study, no deaths were observed; as judged by clinical signs of toxicity during 14 consecutive days, except for a reduced locomotion of one female rat in dose 2500 mg/kg bw group and one male rat in dose 5000 mg/kg bw group, all the other tested rats did not show any significant abnormality in the skin, fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as behavioral pattern, and also had no obvious signs of toxicity or change in other physiological activities.

The body weight, body weight gain of the rats treated with the BMB696B powder preparation had no significant change compared with that of the control group (shown in Table 1). At necropsy, the pathological examination of the internal organs related showed that no gross histopathological alterations were found in all the tested animals and the control group.

Since a high dose of 5000 mg/kg BW did not induce any deaths or significant toxic symptoms in this study, the oral acute toxicity of BMB696B powder preparation can be considered as unclassified.

3.2. Subacute oral toxicity in rats

3.2.1. Mortality, clinical observations, and body weight

During the experiment period, there are no animals died from BMB696B powder preparation administration. The hairs of one male rat in does 5000 mg/kg bw and one female rat in does 1000 mg/kg bw were found wetted, while one female rat in does 5000 mg/kg bw was found a reduced in

Table 1
Acute toxicity study of BMB696B powder preparation in rats-body weight values (g, mean \pm SD)

Sex	Level (mg/kg)	Day					Body weight gain
		0	4	7	10	14	
Female	0	198 \pm 8	209 \pm 7	220 \pm 7	230 \pm 5	242 \pm 5	44.2 \pm 3.3
	500	206 \pm 11	219 \pm 12	229 \pm 11	240 \pm 11	252 \pm 10	45.4 \pm 3.1
	1250	199 \pm 8	210 \pm 7	220 \pm 5	231 \pm 5	241 \pm 6	42.0 \pm 4.6
	2500	202 \pm 9	212 \pm 8	222 \pm 7	231 \pm 6	243 \pm 5	41.4 \pm 3.9
	5000	200 \pm 10	211 \pm 8	222 \pm 8	233 \pm 8	245 \pm 6	44.8 \pm 4.1
Male	0	214 \pm 7	235 \pm 5	260 \pm 6	285 \pm 4	307 \pm 5	94.2 \pm 6.2
	500	214 \pm 8	237 \pm 7	261 \pm 8	287 \pm 8	308 \pm 9	93.6 \pm 6.3
	1250	214 \pm 5	234 \pm 4	259 \pm 5	283 \pm 7	310 \pm 5	95.8 \pm 8.4
	2500	215 \pm 7	237 \pm 8	258 \pm 8	279 \pm 7	305 \pm 8	91.8 \pm 3.8
	5000	219 \pm 9	240 \pm 7	265 \pm 7	285 \pm 5	311 \pm 8	93.8 \pm 7.3

Table 2
Subacute toxicity study of BMB696B powder preparation in rats-body weight values (g, mean \pm SD)

Sex	Level (mg/kg)	Week					Body weight gain
		0	1	2	3	4	
Female	0	193 \pm 7	216 \pm 6	237 \pm 6	250 \pm 5	263 \pm 8	72.2 \pm 3.7
	200	192 \pm 8	216 \pm 8	238 \pm 7	251 \pm 7	263 \pm 6	71.0 \pm 3.4
	1000	195 \pm 9	216 \pm 8	238 \pm 8	252 \pm 7	262 \pm 6	69.8 \pm 4.8
	5000	194 \pm 7	216 \pm 8	237 \pm 6	251 \pm 7	263 \pm 5	67.4 \pm 2.3
Male	0	217 \pm 10	268 \pm 11	308 \pm 12	344 \pm 7	374 \pm 8	153.0 \pm 5.7
	200	217 \pm 12	261 \pm 11	307 \pm 13	343 \pm 8	373 \pm 9	154.2 \pm 4.8
	1000	217 \pm 8	263 \pm 13	308 \pm 12	342 \pm 8	371 \pm 9	157.0 \pm 4.7
	5000	213 \pm 9	257 \pm 11	300 \pm 10	338 \pm 6	367 \pm 7	156.8 \pm 4.7

locomotion, except for those, all the other tested rats did not show any significant abnormality in the skin, fur, eyes and mucous membranes, and also respiratory, circulatory, autonomic and central nervous system as well as behavioral pattern, and also had no obvious signs of toxicity or change in other physiological activities (The data were not shown.). In addition, animals from all BMB696B powder preparation treated groups gained weight for the duration of the study, and no statistically significant changes were observed compared to the control group (shown in Table 2).

3.2.2. Food and water consumptions

Tables 3 and 4 corresponding show the results of food and water consumptions. A tendency toward reduced food consumption values was observed in the 200 mg/kg BW female group (shown in Table 3) in week one compared to

Table 3
Subacute toxicity study of BMB696B powder preparation in rats-food consumption values (g/animal/day, mean)

Sex	Level (mg/kg)	Week			
		1	2	3	4
Female	0	15.67	14.87	13.93	14.97
	200	13.67	15.14	14.93	14.77
	1000	14.87	15.27	15.03	14.83
	5000	14.69	14.10	15.13	15.10
Male	0	20.43	19.15	20.03	20.14
	200	19.69	19.97	19.74	20.37
	1000	19.36	20.08	20.33	19.87
	5000	19.63	18.90	19.65	20.53

Table 4
Subacute toxicity study of BMB696B powder preparation in rats-water consumption values (g/animal/day, mean)

Sex	Level (mg/kg)	Week			
		1	2	3	4
Female	0	21.29	21.52	22.07	21.63
	200	23.43	22.83	23.13	23.30
	1000	22.74	22.59	23.20	23.00
	5000	23.03	23.17	23.10	22.27
Male	0	26.13	26.53	25.78	26.43
	200	27.03	26.66	26.29	27.10
	1000	29.00	27.83	27.17	26.98
	5000	26.02	27.09	25.31	24.37

the control and other groups. Except for that, the food consumption in other groups was similar to the corresponding control group value throughout this study. In addition, a tendency toward increased water consumption values was observed in the 1000 mg/kg BW male group in week one, while in the 5000 mg/kg BW male groups in week four, a tendency toward reduced water consumption values was observed. Likewise, except for those changes, water consumption in other groups was similar to the corresponding control group value throughout the experiment.

3.2.3. Hematology and serum biochemical examination

The hematology and serum biochemistry results after four-week administration of BMB696B powder preparation show in Tables 5–7.

Table 5
Subacute toxicity study of BMB696B powder preparation in rats-hematology values (mean \pm SD)

Sex	Level (mg/kg)	No. of rats examined	RBC ($10^{12}/l$)	HB (g/l)	HT (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT ($10^9/l$)
Female	0	5	7.9 \pm 0.1	157.0 \pm 5.0	41.5 \pm 1.6	55.5 \pm 1.4	19.2 \pm 0.8	35.3 \pm 0.7	1096 \pm 130
	200	5	7.7 \pm 0.2	154.8 \pm 5.3	40.9 \pm 1.8	54.5 \pm 1.5	19.8 \pm 0.7	35.1 \pm 0.8	844 \pm 82*
	1000	5	7.9 \pm 0.1	154.3 \pm 5.0	39.4 \pm 1.3	56.0 \pm 1.1	20.2 \pm 0.8	36.0 \pm 0.3	1084 \pm 133
	5000	5	7.8 \pm 0.2	155.9 \pm 5.0	42.3 \pm 1.2	56.3 \pm 0.4	19.2 \pm 0.8	35.2 \pm 0.5	1170 \pm 68
Male	0	5	7.8 \pm 0.3	157.8 \pm 3.4	42.9 \pm 1.4	55.2 \pm 1.4	19.6 \pm 0.4	35.2 \pm 0.9	1078 \pm 119
	200	5	7.7 \pm 0.1	155.5 \pm 3.6	44.0 \pm 1.2	54.3 \pm 1.8	19.5 \pm 0.6	34.7 \pm 0.5	1166 \pm 63
	1000	5	7.7 \pm 0.3	155.2 \pm 4.8	43.6 \pm 1.4	55.3 \pm 1.6	19.1 \pm 0.5	36.1 \pm 0.3	1092 \pm 86
	5000	5	7.8 \pm 0.2	155.7 \pm 5.2	43.3 \pm 1.1	55.5 \pm 1.1	18.9 \pm 0.5	35.4 \pm 0.7	1102 \pm 85

RBC = red blood cell count, HB = hemoglobin concentration, HT = hematocrit, MCV = Mean corpuscular volume, MCH = Mean corpuscular hemoglobin, MCHC = Mean corpuscular hemoglobin concentration, PLT = platelets count.

* Significantly different from control group at $P < 0.05$.

Table 6
Subacute toxicity study of BMB696B powder preparation in rats-hematology values-WBC and differential count of WBC (mean \pm SD)

Sex	Level (mg/kg)	No. of rats examined	White blood cell count ($10^9/l$)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)
Female	0	5	9.9 \pm 1.0	73.1 \pm 3.9	17.0 \pm 1.7	9.6 \pm 3.1
	200	5	10.3 \pm 1.1	73.6 \pm 3.4	16.4 \pm 1.0	10.4 \pm 1.7
	1000	5	10.1 \pm 1.1	73.0 \pm 2.2	15.6 \pm 1.2*	10.2 \pm 1.4
	5000	5	10.5 \pm 1.4	73.2 \pm 1.5	17.5 \pm 1.3	9.0 \pm 2.1
Male	0	5	10.5 \pm 1.0	72.6 \pm 3.6	17.2 \pm 1.4	9.9 \pm 1.9
	200	5	10.9 \pm 1.2	72.6 \pm 1.8	17.4 \pm 0.8	7.9 \pm 2.7*
	1000	5	10.8 \pm 0.7	72.3 \pm 2.5	17.5 \pm 1.3	10.0 \pm 1.5
	5000	5	10.5 \pm 1.0	72.4 \pm 2.9	17.0 \pm 0.7	10.4 \pm 1.4

WBC = White blood cell count.

* Significantly different from control group at $P < 0.05$.

Table 7
Subacute toxicity study of BMB696B powder preparation in rats-relevant serum biochemical values (mean \pm SD)

Sex	Level (mg/kg)	No. of rats examined	AST (U/l)	ALT (U/l)	ALP (IU/l)	BUN (mg/dl)	TP (mg/dl)	Alb (mg/dl)	TC (mg/dl)	CN (mg/dl)	Glu (mg/dl)
Female	0	5	159.5 \pm 9.8	40.2 \pm 4.7	35.9 \pm 2.2	6.07 \pm 0.51	67.4 \pm 5.1	40.0 \pm 2.6	43.9 \pm 3.0	0.64 \pm 0.09	104 \pm 21
	200	5	152.5 \pm 11.4	41.3 \pm 2.9	35.6 \pm 2.1	6.53 \pm 0.52	67.2 \pm 2.2	38.4 \pm 2.1	43.1 \pm 1.9	0.70 \pm 0.06	95 \pm 14
	1000	5	153.6 \pm 10.3	41.3 \pm 3.4	34.7 \pm 1.4	7.09 \pm 0.65	67.2 \pm 3.1	37.7 \pm 1.3	45.4 \pm 2.0	0.57 \pm 0.05	120 \pm 11
	5000	5	155.8 \pm 10.6	40.9 \pm 1.3	37.4 \pm 1.6*	6.52 \pm 0.64	68.3 \pm 1.6	40.0 \pm 2.0	45.0 \pm 2.3	0.67 \pm 0.07	106 \pm 13
Male	0	5	151.8 \pm 9.0	42.2 \pm 3.4	35.6 \pm 2.0	6.54 \pm 0.88	70.6 \pm 3.0	38.5 \pm 1.9	45.4 \pm 1.5	0.64 \pm 0.10	105 \pm 19
	200	5	153.2 \pm 11.7	42.3 \pm 2.9	36.2 \pm 2.2	7.04 \pm 0.58	66.6 \pm 2.4	39.1 \pm 1.5	44.2 \pm 1.8	0.55 \pm 0.05	116 \pm 18
	1000	5	118.2 \pm 10.3*	41.8 \pm 2.4	34.4 \pm 2.3	6.49 \pm 0.42	67.7 \pm 1.6	38.7 \pm 1.5	45.2 \pm 1.7	0.71 \pm 0.05	78 \pm 10*
	5000	5	155.3 \pm 7.6	39.4 \pm 3.9	35.4 \pm 2.1	6.90 \pm 0.44	67.5 \pm 2.3	38.6 \pm 1.6	46.5 \pm 1.7	0.65 \pm 0.07	96 \pm 13

AST = aspartate aminotransferase, ALT = alanine aminotransferase, ALP = alkaline phosphatase, BUN = blood urea nitrogen, TP = total protein, Alb = albumin, TC = total cholesterol, CN = creatinine, Glu = glucose.

* Significantly different from control group at $P < 0.05$.

For hematology, PLT (platelets count value) in the 200 mg/kg BW female group was significantly lower than corresponding control groups (shown in Table 5). In addition, in differential counts of white blood cells, the monocytes ratio in 1000 mg/kg BW female group was significantly lower and the granulocytes ratio in 200 mg/kg BW male group was significantly higher (shown in Table 6). Except for above changes, there were no significant differences found in other hematological parameter in both sexes compared to the control group (shown in Tables 5 and 6).

For serum biochemical parameters, the AST (aspartate aminotransferase) and Glu (glucose) in 1000 mg/kg bw

male group were significantly lower than control group, while, in 5000 mg/kg bw female group, the ALP (alkaline phosphatase) was significant higher. Except for those, no other differences were found in all of the BMB696B powder preparation treatment groups in both sexes compared to the control group (shown in Table 7).

3.2.4. Organ/body weights and histopathological examination

Table 8 shows the results of relative organ weights after the period of 4 weeks of BMB696B powder preparation administration. No treatment-related variation was observed for relative organ weights. At autopsy,

Table 8
Subacute toxicity study of BMB696B powder preparation in rats-final body and relative organ weight (g% relative to body weight) (g, meanSD)

Sex	Level (mg/kg)	No. of rats examined	Final bw. g	Lungs %	Heart %	Spleen %	Liver %	Adrenals %	Kidney %
Female	0	5	263 ± 8	0.586 ± 0.09	0.332 ± 0.017	0.280 ± 0.03	3.10 ± 0.39	0.020 ± 0.004	0.588 ± 0.05
	200	5	263 ± 6	0.538 ± 0.06	0.316 ± 0.014	0.272 ± 0.03	3.02 ± 0.29	0.021 ± 0.005	0.608 ± 0.03
	1000	5	262 ± 6	0.572 ± 0.07	0.346 ± 0.059	0.252 ± 0.02	2.84 ± 0.23	0.019 ± 0.003	0.592 ± 0.05
	5000	5	263 ± 5	0.538 ± 0.05	0.334 ± 0.024	0.278 ± 0.02	3.04 ± 0.28	0.024 ± 0.003	0.594 ± 0.03
Male	0	5	374 ± 8	0.586 ± 0.07	0.334 ± 0.020	0.268 ± 0.03	3.04 ± 0.41	0.018 ± 0.004	0.598 ± 0.05
	200	5	373 ± 9	0.582 ± 0.06	0.316 ± 0.014	0.248 ± 0.01	3.00 ± 0.29	0.019 ± 0.005	0.608 ± 0.04
	1000	5	371 ± 9	0.618 ± 0.05	0.340 ± 0.010	0.270 ± 0.02	3.16 ± 0.29	0.021 ± 0.004	0.600 ± 0.03
	5000	5	367 ± 7	0.634 ± 0.04	0.350 ± 0.011	0.270 ± 0.02	2.99 ± 0.23	0.023 ± 0.004	0.596 ± 0.03

macroscopic observation of the organs did not show any change due to the treatment with BMB696B powder preparation. Gross necropsy and pathological examination of treated rats did not reveal any abnormality in morphology of lungs, heart, spleen, liver and kidney.

4. Discussion

The safety assessment of transgenic *B. thuringiensis* with VIP insecticidal protein gene has been done.

In acute study, a reduced locomotion of one female rat in dose 2500 mg/kg BW group and one male rat in dose 5000 mg/kg BW group were found. But those changes were not found in other similar treated group. So it had no dose-dependence, and also was not considered to have any observable physiological significance. Since a dose of 5000 mg/kg did not induce any deaths and significant toxic symptoms in this study, the oral acute toxicity of BMB696B powder preparation can be considered as unclassified.

In subacute study, there were slight changes in food and water consumption found in some treated group, but this change is not dose-related. In hematological parameters, the monocytes ratio in 1000 mg/kg female group was significantly lower, while the granulocytes ratio in 200 mg/kg male group was significantly higher, but these changes did not demonstrate dose-dependence, and also not considered to have any observable physiological significance. The decrease in PLT (platelets count value) of the 200 mg/kg female group has statistical significance, but it was similarly considered to be of no dose-dependence and toxicological or physiological significance, because the magnitude of change was small, and a similar effect was not observed in males and other doses of female groups. In the serum biochemical analysis, a significant decrease in AST was observed in 1000 mg/kg male group. AST and ALT are considered to be sensitive indicators of hepatocellular damage and within limits can provide a quantitative assessment of the degree of damage sustained by the liver. Except for AST, there were no significant alterations in other liver function parameters including total protein, bilirubin, and other liver enzymes including alkaline phosphatase and ALT. Furthermore, there were no gross or microscopic pathological changes found in the liver. So,

the decrease in AST in 1000 mg/kg male group is not indicative of toxic to hepatocytes. Besides, the ALP (alkaline phosphatase) in 5000 mg/kg female group was significantly higher, and the Glu (glucose) in 1000 mg/kg male group were significantly lower than control. For ALP value, the change was caused by one of the five rats significantly higher, and for Glu value, the change was caused by two of the five rats significantly lower, other than all the members had significant change, what's more, similar changes were not discovered in other doses, so these changes did not have dose-dependence and can be regarded to be incidental or haphazard. Therefore, it has no toxicological significance. All the above results suggest that the oral subacute toxicity of vip3Aa7 GM *B. thuringiensis* BMB696B powder preparation was estimated to be greater than 5000 mg/kg BW per day, and it can be considered as unclassified class.

Even BMB696B was administered to rats at the dose of 5000 mg/kg BW per day both in the oral acute and oral subacute studies, there were no significant toxicological changes. Based on these results, it can be concluded that the no adverse effect level of the vip3Aa7 GM *B. thuringiensis* BMB696B is higher than 5000 mg/kg BW per day. On the basis of these, and according to the highest dose levels required by OECD guidelines for materials of low toxicity, it can be drawn that the vip3Aa7 GM *B. thuringiensis* is safe to rodents and could be considered as a safe bio-pesticide for future use. On other hand, since the whole product vip3Aa7 GM *B. thuringiensis* showed no significant effect in the acute and subacute studies, the host cell YBT-1520 can be considered as a safe production microorganism. What's more, in this production system, the foreign gene was subcloned into a resolution vector pBMB5401 and transformed into host cell. It also can be proved that this resolution vector system is safe in GM *B. thuringiensis* construction.

The transgenic *B. thuringiensis* is the same with the transgenic plants, are all produced by genetic manipulation. Although the GM *B. thuringiensis* would not directly enter into the food chain, but it can be touched by certain animal even human being when using as a pesticide. So, if we cannot realize and manage its safety, it will bring harmful to human being. But by far, the reports about the safety of transgenic *B. thuringiensis* are very limited, not like the

transgenic plants have obtained galactic recognition. We feel that it is important to establish an internationally harmonized framework for the safe handling of GMOs within a few years, and we hope that the safety of the GM *B. thuringiensis* would gained more recognition.

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