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A 90-day safety study of genetically modified rice expressing Cry1Ab protein (*Bacillus thuringiensis* toxin) in Wistar rats

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Abstract

An animal model for safety assessment of genetically modified foods was tested as part of the SAFOTEST project. In a 90-day feeding study on Wistar rats, the transgenic KMD1 rice expressing Cry1Ab protein was compared to its non-transgenic parental wild type, Xiushui 11. The KMD1 rice contained 15 mg Bt toxin/kg and based on the average feed consumption the daily intake was 0.54 mg Bt toxin/kg body weight.

No adverse effects on animal behaviour or weight gain were observed during the study. Blood samples collected one week prior to sacrifice were analyzed and compared for standard haematological and biochemical parameters. A few parameters were significantly different, but all within the normal reference intervals for rats of this breed and age and not in relation to any other findings, thus not considered treatment related. Upon sacrifice a large number of organs were weighed, macroscopic and histopathological examinations were performed with only minor changes to report.

The aim of the study was to use a known animal model in performance of safety assessment of a GM crop, in this case KMD1 rice. The results show no adverse or toxic effects of KMD1 rice when tested in the design used in this 90-day study. Nevertheless the experiences from this study lead to the overall conclusion that safety assessment for unintended effects of a GM crop cannot be done without additional test group(s).

Keywords: Genetically modified rice; *Bacillus thuringiensis*; Cry1Ab protein; Animal study; Wistar rats; SAFOTEST; Food safety; Feeding trial

1. Introduction

Bt rice is rice that has been genetically modified to express insecticidal genes (*cry* genes) from *Bacillus thuringiensis* (Bt). The transgenic rice is resistant to major lepidopteran insect pests of rice and thus has the potential to significantly decrease yield losses, reduce the use of broad-spectrum chemical insecticides, and furthermore reduce levels of mycotoxins, one of the unexpected benefits of reducing larval attacks (Cheng et al., 1998; Papst et al., 2005). The Bt rice line, KMD1, since its development in 1998 has been characterized thoroughly at the molecular level, and in numerous field trials has shown evidence of

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affording the farmer a high level of resistance against at least eight different insect pest species (Shu et al., 2000; Ye et al., 2001, 2003).

In spite of the extensive research that has been conducted in developing this pest resistant rice, as summarized by High et al. (2004), Bt rice is not yet grown commercially. Other Bt crops, expressing a range of different *cry* genes, are commercially grown in many parts of the world including Bt corn, Bt cotton, Bt canola and Bt potatoes. Bt toxins (Cry proteins) have been used as microbial pesticides for many years and have a long history of safe use (Mendelsohn et al., 2003; Betz et al., 2000).

Cry proteins show highly species-specific toxicity against certain insects and only a few insect species are affected by each of the Cry proteins. The mode of action in the insect is through specific receptors in the gut, which is highly alkaline, with binding of the toxin resulting in pore-formation, osmotic imbalance, cell lysis and subsequently death of the insect (Betz et al., 2000).

The Cry proteins are regarded harmless or nontoxic to mammals, including humans, probably due to acidified gut pepsinolysis and the lack of Cry protein binding-sites on mammalian gut epithelial cells. Numerous data from toxicity studies show no significant adverse effects of the Cry proteins on body weight gain or clinical observations. Furthermore, no signs of pathogenicity to mammals, including humans, have been reported (McClintock et al., 1995).

Investigations on the effects of the Cry1Ab protein on mammalian cells have revealed no significant effect on bovine hepatocyte morphology or on albumin secretion *in vitro* (Shimada et al., 2003). In animal studies no significant differences were observed in general health or growth rate in pigs fed a Bt corn diet (Chowdhury et al., 2003), although in 1998 Fares and El Sayed observed fine structural microscopic changes in the ileum of mice fed Cry1 potato diet. Bt toxin released by the crop root or from the biomass of Bt corn has been found nontoxic to soil bacteria (Saxena and Stotzky, 2001).

This study is part of the EU-project SAFOTEST, designed to develop scientific methodologies for assessing the safety of genetically modified (GM) crops. The aim of the present 90-day study in Wistar rats was to perform a comparative safety assessment study of the genetically modified Bt rice, KMD1, expressing Cry1Ab in an animal model, when compared to the parental wild-type rice, Xiushui 11, and to furthermore monitor changes in major aerobe and facultative anaerobe bacterial populations in the intestines of the rats.

The study design includes two test groups given comparable diets containing 60% raw brown rice flour from parental and transgenic rice, respectively, to be tested in a directly comparative 90-day feeding study without spiking of the recombinant protein.

The objective was to have identical cohorts of male and female rats in a sub-chronic 90-day exposure to 60% rice diets, which contained realistic and meaningful levels of the transgene-expressed Cry protein, Cry1Ab. The focus was first and foremost on the tissues and organs in initial contact with the diets. These are the digestive tract and related organs, including a detailed veterinarian and pathological assessment of the whole animals' well-being and behaviour.

The rice materials tested in the 90-day toxicity study were subjected to comprehensive analytical characterization before the study so that the compositional data could provide the basis for the interpretation of any possible effects detected in the feeding studies. Every effort was made to provide a consistent and well-characterized GMO diet to the test animals, in a universally adoptable and approvable manner, for a study based on the OECD Guideline no. 408 (OECD, 1995).

2. Materials and methods

2.1. Test material

Bt rice KMD1 and the corresponding parental rice Xiushui 11, were accessed from University of Ottawa (Canada) and Zhejiang University (China), respectively. Seeds of KMD1 and its parental line, Xiushui 11, were produced in the late season of 2000 in Hangzhou, China. Wu et al. (2001) have described generation and selection of the transformant rice. During multiplication of rice seeds, the performance of these materials was consistent with previous years' observations. Neither leaf folders nor stem borers damaged plants of KMD1, while Xiushui 11 was infested by both, leading to curled leaves (caused by leaf folders), dead-hearts and white-heads (by stem borers at vegetative and heading stage, respectively) in the field. All shipping and handling was conducted to protect the freshness and quality of the rice grains. On arrival by air courier at the Danish Institute for Food and Veterinary Research (DFVF, Søborg, Denmark) the rice was stored at 5 °C, before dehulling, grinding and subsequent storage at −18 °C until use. Samples of intact rice grains representative for the bulk material were shipped to Technical University Munich (Germany).

2.2. Characterization of test material

Rice plants were generated by *Agrobacterium*-mediated transformation and positive transformants selected on the basis of hygromycin resistance (Wu et al., 2001). The presence of the Cry1Ab transformation cassette was confirmed by PCR and Southern blot analysis using standard protocols (Sambrook and Russell, 2002). Transgene expression of Cry1Ab in mature seeds of line KMD1 was verified by immunological assay (Western blotting after analysis of total protein by SDS-PAGE) using rabbit polyclonal antibodies raised against Cry1Ab as the primary antibody, with HRP-conjugated goat anti-rabbit IgG (Bio-Rad) as the secondary antibody. The protein was visualized using ECL (chemiluminescence) detection (Amersham) as previously described (Gatehouse et al., 1997) and quantified by densitometric scanning using Bio-Rad Molecular Analyst software. The final concentration of Cry1Ab in the animal diet was also determined by immunoassay.

2.3. Compositional analyses of test material

Intact rice grains were manually dehulled by means of a wooden rice dehuller and ground using a cyclone mill equipped with a 500-μm sieve. The rice flour obtained was immediately frozen and stored at −20 °C until analysis.

Proximates (moisture, starch, fibre, sugars, protein, fat, ash), amino acids, fatty acid distribution and minerals were determined using validated standard protocols (VDLUFA, 1996; VDLUFA, 1997). The content of

protein was calculated using nitrogen to protein conversion factor of 5.95. Vitamin B_1 was measured by the AOAC method (AOAC, 2000). Extraction and HPLC analysis of vitamin B_6 were performed according to Reitzer-Bergaentzle et al. (1993). To measure total vitamin B_6 contents including pyridoxol glucosides, extracts were treated with β-glucosidase (Bognar and Ollilainen, 1997). Niacin was extracted according to Ward and Trenerry (1997) and determined via HPLC analysis (Wills et al., 1977). Folate vitamers and total pantothenic acid were quantified by stable isotope dilution assays based on LC/MS/MS (Freisleben et al., 2003; Rychlik, 2003). A method using on-line coupled liquid chromatography–gas chromatography was used for determination of γ-oryzanol contents and steryl ferulate distributions (Miller et al., 2003). Phytic acid was measured using a colorimetric method (Latta and Eskin, 1980). Heavy metals (cadmium, lead, mercury) were measured by AAS (VDLUFA, 1996). Analysis for mycotoxins included aflatoxins (B_1, B_2, G_1, G_2), ochratoxin A, zearalenon and deoxynivalenol (VDLUFA, 1997). Bacterial and fungal counts were measured using validated standard protocols (VDLUFA, 1997). Pesticides were determined according to DFG, 1991.

2.4. Animals and housing

Sixty-four SPF Wistar rats [mol:Wist] (32 male and 32 female) were obtained from M&B Breeding Center, Ll. Skensved, Denmark. The rats were 6–7 weeks old at the initiation of the study. The animals were housed pair wise in stainless steel wire cages at 22 ± 1 °C, relative humidity $55 \pm 5\%$, air change 10 times/h and electric light from 09.00 to 21.00. Animal experiments and housing procedures were performed in accordance to the Danish Animal Experimentation act on a license granted by the Ministry of Legal Affairs and the Convention ETS 123 of the Council of Europe and the Danish Animal Experimental Inspectorate approved the study.

2.5. Diet formulation and feeding

The purified, or semi-synthetic, rat diet used in the study is produced in house (Poulsen et al., 2002), based on the rodent diet AIN-93 (Reeves et al., 1993). The purified diet is based on cornstarch and does not contain rice. In this study, both test diets contained 60% ground rice flour, either Xiushui 11 for the controls or KMD1 expressing Cry1Ab protein from the *cry1Ab* gene. Mixing procedures were performed as described by Poulsen et al. (2006); see Table 1 for diet composition.

Both diets were adjusted identically to assure an adequate supply of macronutrients and vitamins after substitution with 60% rice, but no adjustments were made to outbalance the differences in the constitution of the rice, observed by the compositional/chemical analyses. The rats were allowed free access to both food and water.

Table 1
Composition of diets

Ingredients (%)	Group 1	Group 2
Control rice (Xiushui 11)	60	0
Bt rice (KMD1)	0	60
Corn starch	5.2	5.2
Sucrose	6.8	6.8
Soybean oil	5	5
Cellulose	5	5
Mineral mixture[a]	2.8	2.8
Vitamin mixture[b]	1.2	1.2

[a] In mg/kg diet: Ca: 5000, P: 3100, K: 3600, S: 300, Na: 2500, Cl: 1500, Mg: 600, Fe: 34, Zn: 30, Mn: 10, Cu: 7, I: 0.20, Mo: 0.15, Se: 0.15, Si: 2.5, Cr: 1.0, F: 1.0, Ni: 0.5, B: 0.5, Li: 0.1, V: 0.1, Co: 0.07.

[b] In mg/kg diet: Vit. A.: 5000 (IU); Vit. D_3.: 1000 (IU); Vit. E.: 50 (IU); Thiamin: 5; Riboflavin: 6; Pyridoxol: 8; Folic acid: 2; D-biotin: 0.3; Vit. B_{12}.: 0.03; Panthothenate: 20; Cholinhydrogentartrat: 2600; Inositol: 400; Nicotinic acid: 40; Phylloquinine: 1; p-aminobenzoic acid: 40; Methionine: 1000; L-cystine: 2000.

2.6. Experimental design

Animals were randomly assigned to two experimental groups of 16 males and females, based on body weight means. The animals were observed twice daily; body weight, food and water consumption were measured once weekly. During the last week of treatment, blood samples were taken from the tail vein and collected in EDTA and heparin coated tubes for hematology and blood biochemistry, respectively. Blood samples were taken under Hypnorm–Dormicum anaesthesia and the animals were fasted overnight to minimize fluctuations in the parameters measured.

At terminal sacrifice, the animals were anaesthetized by CO_2 inhalation and killed by decapitation and exsanguination followed by examination for gross and histopathological changes.

2.7. Blood biochemistry and haematology

Following biochemical parameters were measured in plasma: urea (BUN), alanine aminotransferase (ALAT), sodium, potassium, cholesterol, protein, albumin, creatinine and glucose. All analyses on blood plasma were performed on a Cobas Mira S analyzer (Roche Diagnostic Systems, Switzerland) using the relevant kits for each parameter.

Haematology characteristics were assessed using a Twincounter 187 Hematology Analyser (Analysis Instruments AB, Stockholm, Sweden) on the following parameters: White blood cells (WBC), red blood cells (RBC), platelets (PLT), haemoglobin (HGB), haematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The differential count was performed manually on blood smears for neutrophilic, eosinophilic and basophilic granulocytes, lymphocytes, monocytes and large unstained cells (LU). The smears were stained with May-Grünwald and Giemsa and two times 100 cells were counted.

2.8. Bacterial counts

During the experimental period, fresh faecal samples were taken for microbial analysis from ten animals (5 males and 5 females) of each of the two groups by provoked defecation at day 30 and 60 of the experiment, and at termination of the study. Furthermore, at terminal sacrifice samples from ileum and duodenum were taken from the same ten animals of each group. The faecal and intestinal samples were treated as described by Poulsen et al. (2006).

2.9. Gross necropsy and histopathology

A complete necropsy was performed and the following organs were excised and weighed: adrenals, brains, epididymis, heart, kidneys, liver, mesenterial lymph nodes, ovaries, pancreas, small intestine, spleen, stomach, testes, thyroid gland and uterus. Paired organs (adrenals, epididymides, kidneys, ovaries and testis) were weighed as a total of left and right. Sections from the above organs including the axillary lymph nodes, skin with mammary glands, bones, spine and other organs and tissues with macroscopically visible lesions were fixed for a minimum of 24 h in 4% buffered formaldehyde before histological processing. Tissue samples were embedded in paraffin and sections, 4–6 μm thick, were then stained routinely with hematoxylin–eosin (H&E) for light microscopy.

The main focus of the histopathological examination was on the intestinal tract and the related organs. From a total of 10 males and females per group the following tissues were selected for histological examination: forestomach, glandular stomach, duodenum, jejunum and ileum of the small intestine, cecum, colon, rectum, the mesenterial lymph node, liver (sections from both right and left lateral lobes), pancreas (exocrine and endocrine), adrenal cortex and medulla, kidneys, axillary lymph node, heart, skeletal muscle (*m. biceps femoris*), spleen, and thymus.

2.10. Statistical analysis

Compositional data are presented as means ± confidence intervals ($p < 0.05$). Means are considered as statistically significantly different if their confidence intervals are not overlapping. All statistical calculations on data obtained from the feeding study were carried out using SAS release 8.1 (SAS Institute Inc., Cary, NC). Homogeneity of variance among groups and normality distribution were investigated. Two-way analysis of variance with repeated measures on one factor was used to analyze food and water consumption, body weight and faecal and intestinal microflora. A Least Significant Difference test, or Duncan multiple-range test if significant, followed the analyses. Organ weights, data on hematology and blood biochemistry were analyzed by ANOVA (general linear model), and where the overall *F*-test was significant, least square means was used to compare the exposed group to the control group.

In cases where data was not normally distributed a non-parametrical test was performed, using Kruskal–Wallis test followed by Wilcoxon Two-Sample test. $P \leqslant 0.05$ was in all cases considered significant, data on males and females were always analyzed separately. Data is mainly presented as group mean values ± SEM (standard error of the mean).

3. Results

3.1. Compositional analysis

Bt (KMD1) brown rice and parental (Xiushui 11) brown rice tested in the 90-day feeding study were subjected to comprehensive analytical characterization. More than fifty rice constituents were measured including proximates, amino acids, fatty acids, minerals, vitamins, steryl ferulates and phytic acid. In addition the material was screened for contaminants (heavy metals, pesticides), and the microbiological quality was evaluated by screening for mycotoxins and bacterial/fungal counts. Compositional data were compared to data reported for brown rice (Juliano, 1985; Latta and Eskin, 1980; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004; Kitta et al., 2005) and differences between the lines were assessed for statistical significance ($p < 0.05$).

Contents of proximates are presented in Table 2. Compared to the parental rice, KMD1 exhibited a statistically significantly higher protein content (+8%) and a statistically significantly lower fat content (−18%). However, data for both lines are within literature range (Juliano, 1985; Scherz and Senser, 2000; Møller et al., 2002; USDA, 2004; OECD, 2004). No statistically significant difference between the lines was detected for contents of moisture, starch, fibre, sugars, and ash.

The difference between KMD1 and Xiushui 11 in protein content is also reflected in the amino acid levels (Table 3). The transgenic rice exhibited statistically significantly higher contents of all amino acids except cystine and proline. In particular, levels of arginine and histidine were very high in KMD1 compared to Xiushui 11 (+98% and +123%, respectively). Whereas the level of arginine in KMD1 is within the data range reported in the literature (Scherz and Senser, 2000; USDA, 2004), the level of histidine in KMD1 significantly exceeds the data range reported for brown rice.

Statistically significant differences were observed for the fatty acid distribution (Table 4). A statistically significantly higher proportion of linoleic acid was found for the transgenic rice (+13%), whereas the parental rice exhibited a statistically significant higher proportion of oleic and stearic acid (+9% and +38%, respectively). For stearic acid the amount in KMD1 was below the minimum value reported in literature. For myristic and palmitic acid the proportions in both parental and GM material were slightly below the minima described in literature whereas for oleic acid the amount in the parental line was slightly above the maximum reported. Despite these minor differences, the overall fatty acid patterns of both lines with in agreement with the data reported for rice in the literature (Scherz and Senser, 2000; USDA, 2004; OECD, 2004; Kitta et al., 2005).

Contents of minerals are presented in Table 5. No statistically significant difference between the transgenic and the parental rice was observed for contents of calcium, magne-

Table 2
Proximate composition of brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 4$, $p < 0.05$)

Component (%)	Xiushui 11	KMD1	Literature data[a]
Moisture	12.5 ± 0.1	12.5 ± 0.4	9.1–14.1
Starch	72.5 ± 0.4	72.7 ± 0.2	57–77
Fibre	1.1 ± 0.2	1.2 ± 0.3	0.5–3.5
Sugars	0.6 ± 0.2	0.5 ± 0.1	0.6–1.3
Protein	8.7 ± 0.1	9.4 ± 0.1[b]	6.1–9.5
Fat	2.99 ± 0.05	2.46 ± 0.08[b]	1.4–2.9
Ash	1.30 ± 0.05	1.25 ± 0.03	0.9–1.5

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004; OECD, 2004).
[b] Statistically significantly different from parental line ($p < 0.05$).

Table 3
Amino acid levels in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (g/100 g; mean ± confidence interval, $n = 3$, $p < 0.05$)

Amino acid	Xiushui 11	KMD1	Literature data[a]
Alanine	0.48 ± 0.02	0.60 ± 0.02[b]	0.46–0.58
Arginine	0.44 ± 0.05	0.87 ± 0.02[b]	0.44–0.91
Aspartic acid	0.81 ± 0.04	1.00 ± 0.01[b]	0.74–0.87
Cystine	0.20 ± 0.01	0.22 ± 0.07	0.06–0.19
Glutamic acid	1.40 ± 0.03	1.76 ± 0.00[b]	1.52–1.76
Glycine	0.40 ± 0.00	0.47 ± 0.01[b]	0.39–0.49
Histidine	0.26 ± 0.01	0.58 ± 0.07[b]	0.12–0.27
Isoleucine	0.33 ± 0.03	0.39 ± 0.01[b]	0.26–0.57
Leucine	0.70 ± 0.01	0.85 ± 0.00[b]	0.50–0.93
Lysine	0.30 ± 0.01	0.35 ± 0.01[b]	0.10–0.42
Methionine	0.19 ± 0.01	0.27 ± 0.01[b]	0.05–0.31
Phenylalanine	0.44 ± 0.01	0.56 ± 0.02[b]	0.30–0.55
Proline	0.46 ± 0.02	0.50 ± 0.05	0.37–0.40
Serine	0.37 ± 0.01	0.44 ± 0.00[b]	0.41–0.50
Threonine	0.23 ± 0.00	0.32 ± 0.01[b]	0.19–0.62
Tryptophan	0.10 ± 0.00	0.12 ± 0.00[b]	0.03–0.11
Tyrosine	0.42 ± 0.02	0.56 ± 0.01[b]	0.21–0.47
Valine	0.48 ± 0.00	0.63 ± 0.01[b]	0.40–0.76

[a] Ranges from minimum to maximum reported values (Scherz and Senser, 2000; USDA, 2004).
[b] Statistically significantly different from parental line ($p < 0.05$).

Table 4
Fatty acid distribution in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 4$, $p < 0.05$)[a]

Fatty acid (%)	Xiushui 11	KMD1	Literature data[b]
Myristic acid	0.3 ± 0.1	0.3 ± 0.1	0.4–3.0
Palmitic acid	16.9 ± 0.2	16.2 ± 0.6	18–31
Stearic acid	1.8 ± 0.1	1.3 ± 0.1[c]	1.6–2.6
Oleic acid	42.2 ± 0.1	38.4 ± 0.5[c]	27–41
Linoleic acid	35.5 ± 0.1	40.1 ± 0.3[c]	31–40
Linolenic acid	1.5 ± 0.1	1.7 ± 0.1	0.9–1.7

[a] Proportions of total fatty acids (%).
[b] Ranges from minimum to maximum reported values (Scherz and Senser, 2000; USDA, 2004; OECD, 2004; Kitta et al., 2005).
[c] Statistically significantly different from parental line ($p < 0.05$).

Table 5
Contents of minerals in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 4$, $p < 0.05$)

Mineral	Xiushui 11	KMD1	Literature data[a]
Calcium (g/kg)	0.2 ± 0.0	0.5 ± 0.3	0.1–0.5
Copper (mg/kg)	5.1 ± 0.1	2.2 ± 0.3[b]	1–6
Iron (mg/kg)	22 ± 2	16 ± 1[b]	2–52
Magnesium (g/kg)	1.2 ± 0.1	1.2 ± 0.1	0.2–1.7
Manganese (mg/kg)	35.0 ± 0.4	21.6 ± 0.2[b]	2–37
Molybdenum (mg/kg)	0.6 ± 0.1	0.4 ± 0.1[b]	0.3–1.0
Phosphorous (g/kg)	3.0 ± 0.1	3.0 ± 0.1	1.7–4.4
Potassium (g/kg)	2.3 ± 0.1	2.4 ± 0.1	0.6–2.8
Zinc (mg/kg)	22.4 ± 0.3	15.7 ± 0.7[b]	6–28

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).
[b] Statistically significantly different from parental line ($p < 0.05$).

sium, phosphorous and potassium. However, the transgenic rice exhibited statistically significantly lower contents of copper (−57%), iron (−27%), manganese (−38%), molybdenum (−33%), and zinc (−30%). For both lines contents of minerals were in agreement with literature data (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).

Table 6 presents contents of important rice vitamins, which were in agreement with literature data (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).

Rice contains a mixture of steryl ferulic acid esters named γ-oryzanol (Xu and Godber, 1999). γ-Oryzanol was shown to exhibit antioxidative (Xu et al., 2001) and cholesterol-lowering properties (Rong et al., 1997). No statistically significant difference between transgenic and parental rice was observed for contents of total γ-oryzanol (Table 7). Data were within the range reported in literature (Miller et al., 2003). Despite minor but statistically significant differences, steryl ferulate distributions were similar in both lines. Except for the proportion of 24-methylenecycloartanyl ferulate in the transgenic rice, steryl ferulate distributions were in agreement with literature data (Miller et al., 2003).

Table 6
Contents of vitamins in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mg/kg; mean ± confidence interval, $p < 0.05$)

Vitamin	Xiushui 11	KMD1	Literature data[a]
B_1[b]	3.4 ± 0.4	3.8 ± 0.9	2.9–6.1
B_6[c]	1.1 ± 0.2	1.1 ± 0.1	2–10
Niacin[b]	54 ± 2	57 ± 3	35–58
Total pantothenic acid[d]	8.0	9.6	9–17
Total folic acid[a]	0.15	0.13	0.1–0.5
5-Methyl-H_4folate[a,e]	0.09	0.06	
5-Formyl-H_4folate[a,e]	0.06	0.07	

[a] Ranges from minimum to maximum reported values (Juliano, 1985; Møller et al., 2002; Scherz and Senser, 2000; USDA, 2004).
[b] $n = 3$.
[c] $n = 5$.
[d] $n = 2$.
[e] Calculated as folic acid.

Phytic acid is known as an anti-nutritive rice constituent. It has been shown to limit bioavailability of minerals (Saha et al., 1994). No statistically significant difference was detected between phytic acid content of the transgenic (0.90 ± 0.03%, mean ± confidence interval, $p < 0.05$, $n = 4$) and the parental rice (0.88 ± 0.04%). Data were in agreement with literature data (0.6–1.6%; Latta and Eskin, 1980).

To evaluate the microbiological quality of the rice material bacterial and fungal counts were measured and the materials were screened for mycotoxins. Analyses followed standard methods with limits of detection of 0.0003 mg/kg for aflatoxins (B_1, B_2, G_1, G_2), 0.00025 mg/kg for ochratoxin A, 0.0025 mg/kg for zearalenon and 0.025 mg/kg for deoxynivalenol (VDLUFA, 1997). No mycotoxins were detected in the material. Both materials exhibited similarly low bacterial ($<0.07 \times 10^6$/g) and fungal counts ($<0.1 \times 10^3$/g).

As regards contaminants, 149 pesticides from different classes were analyzed in each line following standard methods. They were shown to be below their respective detection limits ranging from 0.0025 mg/kg for PCBs (7) and chlorinated hydrocarbons (13) to 0.5 mg/kg for cymoxanil, pyridaben and thiobendazol; for the majority of the pesticides the detection limits were 0.005 mg/kg (59) and 0.05 mg/kg (42), respectively. Levels of heavy metals (lead, cadmium and mercury) were low. Contents of lead and cadmium were below limits set for rice by the Commission Regulation (EG) 466/2001 (Table 8).

The level of transgene expression of Cry1Ab in mature KMD1 rice seeds was shown to be 0.015–0.018% of the total soluble protein, while no Cry1Ab protein was detected in the parental control rice.

3.2. Clinical observation, body weight and food and water intake

Throughout the study, no adverse effects on animal behaviour were observed. The animals were observed twice

Table 7
γ-Oryzanol (steryl ferulates) in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mean ± confidence interval, $n = 3$, $p < 0.05$)

	Xiushui 11	KMD1	Literature data[a]
γ-Oryzanol (mg/100 g)	31 ± 1	32 ± 3	31–63
Steryl ferulate distribution[b]			
Campesteryl ferulate	19.3 ± 0.3	21.1 ± 1.4[c]	7–19
Campestanyl ferulate	6.8 ± 0.2	6.0 ± 0.2[a]	6–13
β-Sitosteryl ferulate	9.5 ± 0.3	10.5 ± 0.2[a]	5–10
Cycloartenyl ferulate	37.7 ± 0.6	44.2 ± 0.6[a]	33–47
24-Methylenecycloartanyl ferulate	26.6 ± 0.6	18.3 ± 0.4[a]	27–36

[a] Ranges from minimum to maximum values reported in Miller et al. (2003).
[b] Proportions of total γ-oryzanol content (%).
[c] Statistically significantly different from parental line ($p < 0.05$).

Table 8
Contents of heavy metals in brown rice material from transgenic KMD1 and its corresponding parental line Xiushui 11 (mg/kg; mean ± confidence interval, $n = 4$, $p < 0.05$)

Heavy metal	Xiushui 11	KMD1	Limit[a]
Lead	0.15 ± 0.09	0.14 ± 0.05	0.2
Cadmium	0.03 ± 0.02	0.02 ± 0.01	0.2
Mercury	0.006 ± 0.001	0.006 ± 0.001	–

[a] Commission Regulation (EG) 466/2001.

daily for well-being. Body weight, food and water consumption was measured weekly and the relative food consumption calculated. Statistically significant differences were seen on the food consumption in single weeks for the males, where the rats fed KMD1 had a slightly lower food intake, but no overall effect was seen when comparing the two groups (Table 9). There were no differences observed on water consumption (data not shown).

Growth curves are included for males and females in Fig. 1. They illustrate normal and similar growth patterns within and between the two groups. The slight reduction in body weight at week 12 in both groups was due to an overnight fasting period prior to blood sampling and possibly also stress, related to the blood sampling procedure.

3.3. Blood biochemistry and haematology

Male rats fed KMD1 had a significantly higher plasma concentration of urea (+10%; $p < 0.05$) and glucose (+13%, $p < 0.05$), whereas the concentration of protein was significantly reduced by 5% ($p < 0.05$) compared to the control group. For female rats fed KMD1, the only statistically significant observation was a 1% increase in plasma concentration of sodium ($p < 0.05$). See Table 10 for details on group values.

Regarding haematology only a few differences were observed between the two groups. In male rats statistically significant differences were observed on MCH, which was 3.5% lower in males fed on KMD1 ($p < 0.05$) and on the WBC, which was reduced by 17% in the same group ($p < 0.05$). See Table 11 for details about hematological measurements.

Table 9
Food consumption (in g/animal/week) presented as group mean values ± SD

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
Weeks 2–7	134 ± 13	134 ± 12	95 ± 5	97 ± 5
Weeks 8–13	141 ± 14	137 ± 8	102 ± 10	100 ± 9

3.4. Microbiology

For the faecal samples no significant differences in the bacterial micro flora could be found between the two groups ($p < 0.05$) (data not shown). Results of the significant microbiological findings in the small intestines are summarized in Table 12. In the samples from the duodenum a 13% decrease in the Bifidobacterial population was observed in the dosed group compared to the control group ($p < 0.05$). In samples from ileum an increase was observed in the coliform population, which was 23% higher in the KMD1 group ($p < 0.05$).

3.5. Organ weights

Only few significant differences in organ weights were observed in this study, namely on adrenal, testis and uterus weight. A statistically significantly reduced absolute weight of the adrenals (−15%) ($p < 0.05$) was detected in male rats fed the KMD1. The absolute weight of the testis from male rats fed the KMD1 was increased (+10%) ($p < 0.05$), as was the relative weight (+12%) ($p < 0.01$). The absolute weight of the uterus in KMD1 fed females was increased (+19%) ($p < 0.05$). There was no statistical difference in the relative weight of the uterus. Details regarding organ weights and minimum and maximum weight for relevant organs are summarized in Table 13.

3.6. Gross necropsy and histopathology

During the necropsy there were no gross pathological findings, nor did the histopathological examination reveal

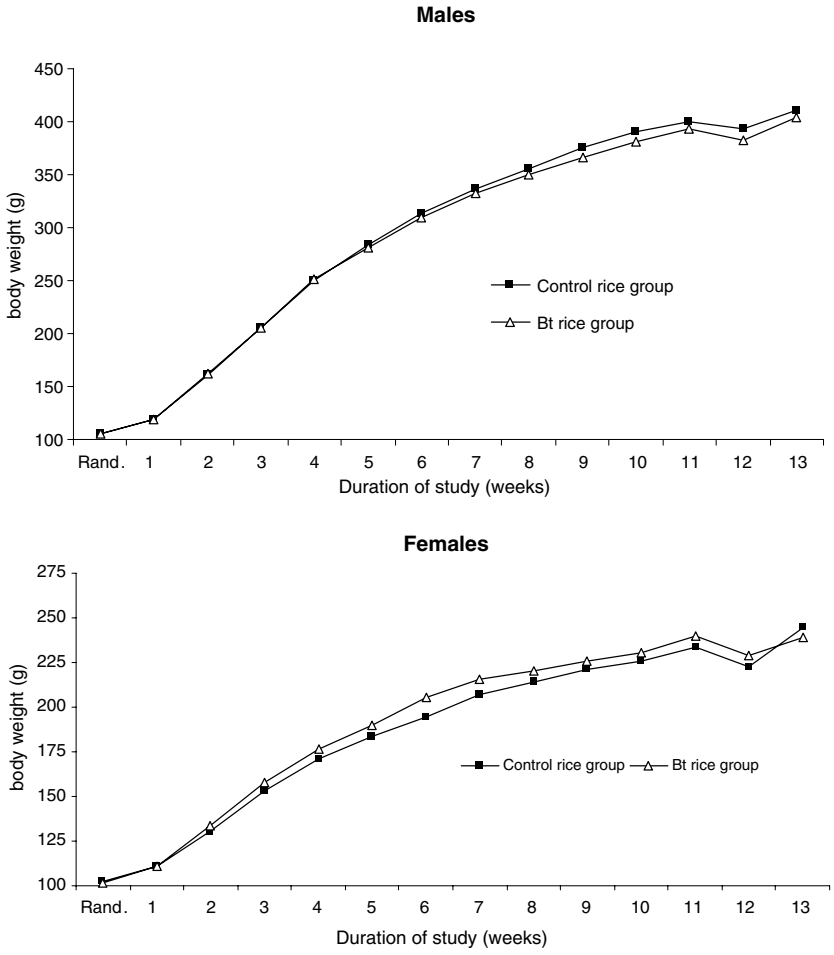

Fig. 1. Growth curves based on weekly measurements of body weight during the study. The curves show group means based on 16 rats/sex/group.

Table 10
Blood biochemical findings in rats fed on Xiushui 11 (control) diet and KMD1 diet

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
BUN (μmol/l)	5.4 ± 0.6	6.0 ± 0.5[a]	6.6 ± 0.7	6.7 ± 1.1
ALAT (U/l)	35.3 ± 4.7	32.4 ± 5.4	24.3 ± 8.1	25.3 ± 3.4
CREA (μmol/l)	33.5 ± 7.4	33.9 ± 6.8	40.1 ± 7.7	37.9 ± 7.5
CHOL (mmol/l)	1.7 ± 0.3	1.5 ± 0.3	1.3 ± 0.3	1.2 ± 0.3
PROT (g/l)	64.4 ± 2.2	61.2 ± 2.4[a]	63.9 ± 3.1	63.9 ± 3.5
ALB (g/l)	40.8 ± 1.5	39.9 ± 1.6	45.5 ± 3.2	45.2 ± 3.0
GLUC (mmol/l)	7.5 ± 0.8	8.6 ± 1.7[a]	10.5 ± 13.5	6.8 ± 0.6
Na^+ (mmol/l)	145.1 ± 1.3	145.9 ± 1.1	143.9 ± 1.3	145.5 ± 1.1[a]
K^+ (mmol/l)	4.3 ± 0.3	4.2 ± 0.3	4.1 ± 0.4	4.2 ± 0.5

The number of animals was 16 rats/sex/group; data is presented as group mean values ± SD.
[a] $p < 0.05$.

any dose-related changes in the intestinal tract or the related organs; in general no pathologically relevant changes were found to explain the identified differences in organ weights between the two groups.

Due to the observed difference in the weight of the testis a thorough histological examination was performed revealing unilateral testicular degeneration in different stages in both groups.

Macroscopically the testis were more or less swelled or atrophied which was directly related to the observed different stages of degeneration in the seminiferous tubules; 2 of 16 control males had mainly atrophic degeneration,

Table 11
Haematological findings in rats fed on Xiushui 11 (control) diet and KMD1 diet

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
WBC (10^9/l)	5.5 ± 0.2	4.7 ± 0.2[a]	3.0 ± 0.2	2.7 ± 0.2
RBC (10^{12}/l)	8.4 ± 0.1	8.7 ± 0.1	7.9 ± 0.1	7.8 ± 0.07
PLT (10^9/l)	640 ± 24	642 ± 19	663 ± 11	709 ± 32
HGB (mmol/l)	15.1 ± 0.1	15.1 ± 0.2	14.4 ± 0.2	14.0 ± 0.09
HCT (%)	45.5 ± 0.5	46.2 ± 0.6	43.5 ± 0.6	42.5 ± 0.3
MCV (fL)	53.9 ± 0.5	53.1 ± 0.4	54.9 ± 0.3	54.8 ± 0.5
MCH (fmol)	17.9 ± 0.2	17.3 ± 0.2[b]	18.2 ± 0.2	18.0 ± 0.2
MCHC (mmol/l)	33.1 ± 0.2	32.7 ± 0.2	33.2 ± 0.2	32.9 ± 0.2
Differential count				
Lymphocytes (%)	79.5 ± 1.5	79.1 ± 1.9	81.8 ± 1.5	77.6 ± 2.0
Neutrophils (%)	17.8 ± 1.6	17.3 ± 1.8	14.7 ± 1.4	18.8 ± 1.9
Eosinophils (%)	1.4 ± 0.3	1.5 ± 0.2	1.7 ± 0.3	1.5 ± 0.3
Basophils (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Monocytes (%)	1.4 ± 0.2	1.4 ± 0.2	1.4 ± 0.2	1.7 ± 0.2
Other cells (%)	0.4 ± 0.2	0.2 ± 0.06	0.4 ± 0.09	0.5 ± 0.1

The number of animals was 16 rats/sex/group; data is presented as group mean values ± SEM.
[a] *F*-value: 5.71, $p = 0.024$.
[b] $\chi^2 = 3.92$, $p = 0.048$ (non-par).

Table 12
Bacterial counts in the small intestine of rats (top: duodenum, bottom: ileum) fed Xiushui 11 (control) diet and KMD1 diet

	Xuishui 11	KMD1
Bacterial counts in duodenum (log_{10} cfu/g intestinal content)		
Total aerobe	5.75 ± 1.16 (9)	5.9 ± 1.05 (9)
Total anaerobe	6.18 ± 0.85 (9)	5.52 ± 1.34
Lactobacilli	6.87 ± 1.44 (8)	6.31 ± 0.66 (9)
Bifidobacteria	6.49 ± 0.87[a] (9)	5.72 ± 0.57[b] (9)
Coliforms	3.39 ± 0.69 (6)	2.85 ± 0.21 (2)
Enterococci	4.35 ± 1.15 (9)	4.45 ± 1.27 (8)
Bacterial counts in ileum (log_{10} cfu/g intestinal content)		
Total aerobe	6.78 ± 1.0 (9)	7.01 ± 0.78 (8)
Total anaerobe	7.53 ± 0.69	7.15 ± 0.59
Lactobacilli	7.55 ± 1.39 (9)	7.58 ± 1.25
Bifidobacteria	7.01 ± 0.96	7.02 ± 0.81
Coliforms	5.73 ± 0.93[a]	7.03 ± 0.59[b]
Enterococci	6.43 ± 0.98	6.04 ± 0.58 (9)

Data are presented as group mean values ± SEM for 10 animals; figures in brackets indicate the number of animals, if different from 10. Different superscripts in a row indicate significant difference in the bacterial counts between the two groups ($p < 0.05$).

whereas 3 of 16 males in the KMD1 group has more pronounced swelling associated with the degeneration.

4. Discussion

Compositional analysis of KMD1 and the parental rice Xiushui 11 tested in the feeding studies revealed statistically significant differences between the two. These significant differences were observed for contents of protein, fat, and minerals (copper, iron, manganese, molybdenum, zinc). The higher protein content in KMD1 was also reflected in higher levels of amino acids. Minor, although statistically significant differences were detected for the distribution of fatty acids and steryl ferulates. Compositional data on GM rice KMD1 and its parental rice Xiushui 11 has been published previously (Wang et al., 2002). The material investigated in that study had also been grown in Hangzhou (China), however at a different site and in another year. The spectrum of the constituents analyzed was more limited than in the present study and for some of the parameters investigated, the results were different from those found in the study presented here. However, Wang et al. (2002) reported no statistically significant differences between the GM and parental rice in their study. This indicates that the differences detected between KMD1 and Xiushui 11 in our study might be due to biological variability rather than to the genetic modification. Additional field trials would be necessary to confirm this conclusion, and field trial permission must be sought well enough in advance to have all the requisite rice lines grown in randomized block design in the same filed station sector in one optimal rice growing season (sow April–October harvest).

The concentration of Bt toxin present in mature rice seeds was estimated to 0.0165% of total soluble protein in the KMD1, which equates to approx. 15 mg Bt toxin/kg rice. With an estimated daily feed consumption of 15 g/rat/day and a mean body weight of 250 g, the daily feed consumption is approx. 60 g/kg body weight. In the 60% rice diet the Bt toxin concentration was 9 mg/kg feed giving a mean daily dosage of Bt toxin of 0.54 mg/kg body weight. For comparison sub-chronic oral toxicity studies have shown a NOEL (no-observed-effect-level) of up to 8400 mg Bt product/kg body weight/day when feeding Bt microbial toxins to rats (Betz et al., 2000). This means that possible toxicological findings in the present study with 0.54 mg/kg body weight/day most likely will derive from unintended changes introduced in the GM rice and not from toxicity of Bt toxin.

Table 13
Absolute and relative organ weights for rats fed on Xiushui 11 (control) diet and KMD1 diet

	Males		Females	
	Xiushui 11	KMD1	Xiushui 11	KMD1
Absolute weight (g)				
Body weight	419 ± 13	408 ± 7	249 ± 4	250 ± 6
Adrenals	0.0694 ± 0.003	0.0602 ± 0.002[a]	0.0611 ± 0.002	0.0673 ± 0.003
Brains	1.97 ± 0.03	1.87 ± 0.11	1.84 ± 0.02	1.89 ± 0.03
Epididymides	1.13 ± 0.03	1.15 ± 0.01		
Heart	1.15 ± 0.03	1.16 ± 0.02	0.813 ± 0.01	0.831 ± 0.03
Kidneys	2.27 ± 0.07	2.25 ± 0.04	1.53 ± 0.05	1.51 ± 0.03
Liver	13.04 ± 0.50	12.59 ± 0.25	8.18 ± 0.14	8.20 ± 0.25
Mesenterial ln.	0.222 ± 0.016	0.189 ± 0.017	0.183 ± 0.015	0.167 ± 0.014
Ovaries			0.0967 ± 0.007	0.1071 ± 0.007
Pancreas	1.43 ± 0.07	1.38 ± 0.05	1.06 ± 0.18	1.01 ± 0.26
Small intestine	7.79 ± 0.21	7.73 ± 0.18	6.16 ± 0.82	6.04 ± 0.94
Spleen	0.744 ± 0.03	0.714 ± 0.02	0.589 ± 0.105	0.532 ± 0.080
Testis	3.57 ± 0.09	3.94 ± 0.10[b]		
Thymus	0.435 ± 0.04	0.413 ± 0.02	0.414 ± 0.067	0.392 ± 0.101
Uterus			0.437 ± 0.05	0.519 ± 0.04[d]
Length small int.	107.5 ± 1.4	109.4 ± 1.1	99.3 ± 1.1	99.3 ± 1.9
Relative weight (g/100 g BW)				
Adrenals	0.0167 ± 0.001	0.0148 ± 0.001	0.0248 ± 0.001	0.0270 ± 0.001
Brains	0.473 ± 0.01	0.461 ± 0.03	0.740 ± 0.01	0.760 ± 0.02
Epididymides	0.271 ± 0.01	0.283 ± 0.005		
Heart	0.275 ± 0.005	0.286 ± 0.015	0.327 ± 0.005	0.333 ± 0.010
Kidneys	0.542 ± 0.009	0.553 ± 0.023	0.612 ± 0.014	0.607 ± 0.012
Liver	3.10 ± 0.05	3.09 ± 0.05	3.29 ± 0.06	3.28 ± 0.08
Mesenterial ln.	0.054 ± 0.005	0.046 ± 0.004	0.073 ± 0.006	0.067 ± 0.006
Ovaries			0.0390 ± 0.003	0.0425 ± 0.002
Pancreas	0.342 ± 0.01	0.340 ± 0.01	0.429 ± 0.02	0.405 ± 0.03
Small intestine	1.87 ± 0.06	1.90 ± 0.04	2.48 ± 0.09	2.41 ± 0.08
Spleen	0.177 ± 0.003	0.176 ± 0.005	0.236 ± 0.009	0.213 ± 0.008
Testis	0.860 ± 0.03	0.967 ± 0.02[c]		
Thymus	0.102 ± 0.009	0.101 ± 0.004	0.167 ± 0.006	0.158 ± 0.011
Uterus			0.177 ± 0.021	0.212 ± 0.021
Length small int.	25.9 ± 0.6	26.9 ± 0.4	40.4 ± 1.0	39.9 ± 1.0

Small intestinal length and relative length is expressed in cm and cm/100 g BW. Data is presented as group mean values ± SEM.
[a] Adrenals: *F*-value: 5.89, $p = 0.0216 \sim$ reduced absolute weight. Xiushui 11: min. 0.055 g, max. 0.103 g; KMD1: min. 0.043 g, max. 0.073 g.
[b] Testis: *F*-value: 7.43, $p = 0.011 \sim$ increased absolute weight. Xiushui 11: min. 2.76 g, max. 4.18 g; KMD1: min. 3.44 g, max. 5.27 g.
[c] Testis: *F*-value: 8.94, $p = 0.006 \sim$ increased relative weight. Xiushui 11: min. 0.585 g/100 g BW, max. 0.999; KMD1: min. 0.878, max. 1.18.
[d] Uterus: *F*-value: 7.09, $p = 0.013 \sim$ increased absolute weight. Xiushui 11: min. 0.24 g, max. 1.14 g; *KMD1*: min. 0.32 g, max. 0.82 g.

On analysis of hematological parameters a significantly reduced amount of white blood cells was observed in the male rats of the group fed with KMD1. This could be indicative of immuno-suppression, but neither the differential count nor other significant findings on clinical observations, organ weights or pathology of the immune organs (thymus and spleen) support this possibility. With respect to blood biochemistry the observed differences in glucose, urea, protein and sodium were minor and the measured values were all within the normal reference intervals for rats of this breed and age. The observed differences are not related to other clinical or pathological findings, and were thus considered insignificant.

Only minor effects were recognized in samples taken from the small intestine for bacteriological quantification in the dosed group. The faecal samples did not reveal any differences in bacterial counts in the animals fed KMD1 compared to animals fed the wild type rice. A recently published study investigated bacterial changes in the rumen of cattle fed Bt corn, where no significant influence of Bt corn could be found on the composition of the microbial population (Einspanier et al., 2004). In the current study reduced amounts of Bifidobacteria in the duodenum and increased amounts of coliforms in the ileum were observed in the KMD1 group. The mechanisms behind these changes are unknown, and further studies are required to clarify whether these findings are biologically significant.

The adrenal weight was significantly reduced in the KMD1 group, but due to the lack of histopathological changes in the organ, this was considered an insignificant finding. As to the observed testicular degeneration, the difference in testis weight was explained by the finding of different stages of degeneration in the seminiferous tubules. The incidence of these findings is not significantly higher in the KMD1 group and thus found not to be related to the GM rice. The conclusion from the present study suggesting

that expression of Cry 1Ab in transgenic rice was not responsible for the observed changes in the testis are supported by Wang et al. (2002) who carried out a comparable feeding study on Sprague–Dawley rats without any observed changes in the testis. In addition, Brake et al. (2004) evaluated the effects of Bt corn on mouse testicular development and with special emphasis on the effect of Bt toxins on the germ cell population, neither short-term nor multigenerational studies showed any apparent toxic effects on the reproductive system.

The inclusion of an additional test group to the present study, where the rats were fed on a diet spiked with pure recombinant protein Cry1Ab would have been desirable and probably the most suitable model for testing the safety of the GM crop, since this would have enabled a more comprehensive assessment of the observed differences in organ weights and the minute pathological changes in the reproductive organs of the male rats. An additional group spiked with Cry1Ab could have increased the specificity of the study to detect specific compound-related effects in order to furthermore ascertain whether the pathological findings and increased organ weights were indeed insignificant or related to either the Bt toxin in the rice or unintended changes in the rice genome. As tested in this present 90-day study the genetically modified Bt rice, KMD1, exhibited no toxicological effects on Wistar rats when fed as 60% of the diet, in comparison with the wild type parental rice, Xiushui 11.

The design may be limited in its ability to detect unintended toxic or nutritional effects of the genetic modification but in large measure this 90-day feeding trial with male and female rats proved that such a model is feasible and successful for safety assessment purposes when the incoming GMO grain diet contains a verifiable level of Cry1Ab in mature KMD1 rice at 0.015–0.018% of the total soluble protein, with no Cry1Ab protein detected in the Xiushui 11 parental control. There were no adverse findings that led to the conclusion that Bt rice is not safe to eat, but nevertheless greater certainty could have been obtained by the inclusion of an additional test group.

In the actual situation where recombinant protein was not available in 100 g quantities sufficient to perform neither a 28-day toxicity study nor to add a spiked group to the 90-day study it is relevant to discuss different possibilities for addition of a group to the study. Additional groups with different levels of KMD1 rice would have been helpful to assess the observed differences between the groups that were found.

It is important to keep in mind that the original aim of this study was not to perform a safety assessment study on KMD1 *per se*, but to test the suitability of the well-known 90-day study for safety assessment of GM crops. Based on the results of this study as presented here, the conclusion to be drawn concerning the model is that in order to thoroughly assess the safety of the GM crop an additional group is desirable, not to say necessary.

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