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A 90-day safety study of genetically modified rice expressing rhIGF-1 protein in C57BL/6J rats

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Abstract Genetically modified plants expressing disease resistance traits offer new treatment strategies for human diseases, but at the same time present a challenge in terms of food safety assessment. The present 90-day feeding study was designed to assess the safety of transgenic rice expressing the recombinant human insulin-like growth factor-1 (rhIGF-1) compared to its parental wild rice. Male and female C57BL/6J rats were given a nutritionally balanced purified diet with 20% transgenic rhIGF-1 rice or 20% parental rice for 90 days. This corresponds to a mean daily rhIGF-1 protein intake of approximately 217.6 mg/kg body weight based on the average feed consumption. In the animal study a range of biological, biochemical, clinical, microbiological and pathological parameters were examined and several significant differences were observed between groups, but none of the effects were considered to be adverse. In conclusion, no adverse or toxic effects on C57BL/6J rats were observed in the design used in this 90-day study. These results will provide valuable information for the safety assessment of genetically modified food crops.

Keywords Genetically modified rice · rhIGF-1 protein · Safety assessment · Animal study

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Introduction

Human insulin-like growth factor-1 (hIGF-1) is essential for normal fetal growth and development and has broad potential therapeutic applications. However, little attempt has been reported about therapeutic applications of hIGF-1 yet, partly due to the shortage of adequate supplies (Savage et al. 2004). In a previous study, Xie et al. (2008) developed transgenic rice plants that could accumulate recombinant hIGF-1 fused to the C-terminus of an ER luminal binding protein (BipC) in seeds, which can reduce blood glucose of diabetic mice via oral delivery.

The mature hIGF-1 is a single-chain peptide of 70 amino acid residues that shares 50% homology with

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human insulin (Daughaday and Rotwein 1989). Clinically, a high dose of IGF-I results in hypoglycemia, decreased serum levels of free fatty acids (FFA), and increased lipogenesis, effects which are similar to those of insulin (Guler et al. 1987; Moses 1997; Schmitz et al. 1991). Therefore, hIGF-1 can be effectively used in treating patients with type 1 and type 2 diabetes mellitus, or severe insulin resistance syndromes (Clemmons 2007).

However, several effects of IGF-I therapy are not insulin-like, including increased lipolysis in adipose tissue (Hussain et al. 1994), decreased lipoprotein lipase activity in adipose tissue (Oscarsson et al. 1999), and decreased body fat mass (Guler et al. 1988; Laron et al. 1995; Tomas et al. 1993). Furthermore, insulin-like growth factor-1 stimulates ventricular myocyte hypertrophy (Cittadini et al. 1996; Decker et al. 1995) and may be involved in the regulation of tumor growth (Neuberg et al. 1997). Therefore, the safety assessment of rhIGF-1 transgenic rice is very necessary considering its extensive effects mentioned above.

Genetically modified (GM) crops represent a challenge in terms of food safety assessment. The present study is part of a Chinese research project 'Transgene major projects', one of whose objectives is to assess the safety of genetically modified foods.

The study design includes two test groups fed comparable diets containing 20% raw brown rice flour from transgenic and parental rice, respectively, to be tested in a subchronic 90-day feeding study in C57BL/6J rats; this duration is considered to be sufficient to provide data for use in evaluating safety or determining whether further studies are required (Howlett et al. 2003).

Prior to the 90-day feeding study the two rice lines were subjected to a comprehensive analytical characterization so that the compositional data could provide the basic information for the interpretation of any possible effects that would be observed in the feeding studies. A total of more than 45 parameters, including major constituents and amino acids, fatty acids, minerals vitamins and phytic acid, were measured in the compositional analysis.

The purpose of the 90-day feeding study was to compare the safety of transgenic rice expressing the recombinant human insulin-like growth factor-1 to its parental rice. This study may add new information to the biosafety assessment of genetically modified foods and provide valuable lessons for the future safety assessment of genetically modified food crops, especially for rhIGF-1 gene transfer.

Materials and methods

Production and characterization of transgenic rice seed

Transgenic rice expressing recombinant human insulin-like growth factor-1 was generated by fusion to the C-terminus of a rice luminal binding protein. Transgene expression levels in mature seeds were estimated by immunoassay using western blots (Gatehouse et al. 1997). The average rhIGF-1 content of mature seeds from the transgenic line was estimated to be $6.8 \pm 0.5\%$ of total seed protein, which was equivalent to 136 ± 10 μg per seed. Southern blot analysis of the selected transgenic line revealed the presence of a single insertion with 2–3 copies of the transformation construct in the rice genome (Xie et al. 2008).

Seeds of the rhIGF-1 transgenic rice and its parental line TP309 to be used in the animal studies were grown in the Experimental Farm of Wuhan University at Wuhan city, Hubei Province of China. After rice seed harvest, the rice seed was sent to the Beijing Inspection Test Center for Biosafety of Genetically Modified Animal and Feed of the Ministry of Agriculture of China (GMAF).

Compositional analysis

Proximates (moisture, starch, fiber, sugars, protein, fat, and ash) and amino acids were determined using validated standard protocols (VDLUFA 1996, 1997). Amino acids were measured using a HITACHI Amino Acid Analyzer L-8900.

Quantification of fatty acids was carried out using a gas chromatograph (GC, Agilent 6890, Agilent Technologies China, Inc., Beijing, China) equipped with a flame ionization detector (FID), automatic sample injector HP 7683, and using a HP-88 J&W fused silica capillary column (100 m, 0.25 mm i.d., 0.2 μm film thickness, Agilent Technologies China, Inc., Beijing, China). The method was adopted from a published method as described by Juárez et al. (2008).

ISO method (ISO 6869: 2000) based on HITACHI atomic absorption spectrophotometer was used for the determination of calcium, copper, iron, magnesium, manganese, potassium, and zinc concentrations. Furthermore, phosphorous and molybdenum determinations were performed according to AOAC 1995 and AOAC 1984, respectively.

Vitamin B1 and B6 was detected by the AOAC 2000 and AOAC 1995, respectively. In addition, the other vitamins including niacin, folate vitamers and total pantothenic acid were quantified according to national standard protocols GB/T 7300-2006, GB/T 7302-2008 and GB/T 18397-2001, respectively. Phytic acid was measured using a colorimetric method (Latta and Eskin 1980).

Intact rice grains were manually dehulled by means of a wooden rice dehuller and were 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.

Animals and housing

Sixty-four male and female C57BL/6J rats (SPF) were obtained from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (CAMS). The rats were 4–5 weeks old at the initiation of the study. The animals were kept in stainless steel wire cages (two/cage) at 22 ± 1°C, relative humidity 55 ± 5%, air change 10 times/h, and electric light from 0900 to 2100. Animal experiments and housing procedures were performed in accordance to the Beijing Laboratory Animal Management Ordinance and the China Animal Experimental Inspectorate approved the study.

Experimental design

The animals were randomly sorted into two experimental groups each comprising 16 males and 16 females. The rats were fed diets of defined composition containing either 20% rhIGF-1 rice or control rice for 13 weeks (Table 1). The rice flour was added directly and thoroughly mixed into the purified diet to ensure homogeneity. All ingredients were ground to a similar particle size to ensure a homogeneous mixture. The purified rat diet used in the study is produced by a feed company, based on the rodent diet standard GB 14924.3-2001. Diets and acidified water (adjusted to pH 3.5 by citric acid to prevent growth of microorganisms) were provided ad libitum. During the experimental period all animals were observed clinically twice daily. Body weight, food and water consumption were recorded weekly. During the last week of treatment, blood samples were taken from the tail vein and collected in heparin and EDTA tubes for blood biochemistry and hematology, respectively. Blood samples were taken under Hypnorm-Dormicum anaesthesia and the animals were fasted overnight to minimize fluctuations of the parameters measured. Faecal samples for microbiological examinations were taken three times during the study (see below).

Table 1 Composition of diets

Ingredients (%)	Group 1	Group 2
rhIGF-1Transgenic rice	20	0
TP309 rice	0	20
Soybean meal	23	23
Flour	10	10
Fish meal	5	5
Corn	20	20
Bran	12	12
Grass meal	2	2
Yeast powder	2	2
Vegetable oil	1.5	1.5
Additive	1	1
Calcium hydrogen phosphate	1.6	1.6
Mountain flour	1.3	1.3
Salt	0.6	0.6

Containing in mg/kg diet: Ca: 1,110, P: 760, K: 500, Na: 200, Mg: 200, Fe: 150, Zn: 30, Mn: 75, Cu: 10, I: 0.5, Se: 0.1. Vitamin A: 15,000 (IU); Vitamin D: 1,500 (IU); Vitamin E: 120 (IU); Vitamin K: 5 (IU); Thiamin: 15; Riboflavin: 15; Folic acid: 6; D-biotin: 0.2; Vitamin B6: 15; Vitamin B12: 0.02; Pantothenate: 30; Bilineurine: 1,250; Nicotinic acid: 60

At the termination of the study, all animals were anaesthetised by carbon dioxide inhalation and killed by exsanguinations for gross and histopathological examination. In addition, samples of intestinal contents were collected for microbiological determination. Referring to method that adopted by Schrøder et al. (2007) and Poulsen et al. (2007), we combined male and female fecal samples and small intestine samples together to test the differences between rhIGF-1 transgenic rice group and wild type rice group.

Blood biochemistry

The following plasma biochemical parameters were measured: urea (BUN), alanine aminotranferase (ALAT), sodium, potassium, cholesterol, protein, albumin, triglyceride, 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

Haematology characteristics were assessed using a Vet ABC, Animal Blood Counter (Analysis instruments AB, Stockholm, Sweden) on the following parameters: White blood cells (WBC), red blood cells (RBC), haematocrit (HCT), haemoglobin (HGB), mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), platelets (PLT), and mean corpuscular haemoglobin concentration (MCHC). The differential count was performed for neutrophils (N), lymphocytes (L), eosinophils (E), basophils (B), and monocytes (M).

Bacteriological quantification

During the experimental period, fresh faecal samples were taken for microbial analysis from twelve animals (6 males and 6 females) of each of the two groups by provoked defecation at day 30 and 60 of the experiment, and at the termination of the study. Furthermore, at terminal sacrifice, samples from ileum and duodenum were taken from the same twelve animals of each group. Both ends of intestine were tied up with sutures in order to avoid the contents being exposed to the air. The intestinal and faecal samples were homogenized in saline supplemented with 0.1% peptone to 10^{-1} dilution. Ten-fold serial dilutions of the processed samples were prepared in the same buffer and were then applied to appropriate selective media on Petri dishes. Total aerobic and anaerobic populations were enumerated on Luria–Bertani medium and on CDC Anaerobion Blood Agar respectively, after incubation for 72 h aerobically and anaerobically, respectively. Lactobacillus, Bifidobacteria and Streptococcus were detected by Lactobacillus selector agar, TPY plates and KF streptococcal medium, respectively, and were counted after anaerobic growth for 72 h. Bile Esculin Azide medium and Eosin methylene blue agar medium were used to determine the number of Enterococcus and Coliform respectively and the above-mentioned two kinds of aerobic bacteria were incubated aerobically for 72 h. All the anaerobic bacteria were cultured in the atmosphere generation system contained in plastic anaerobic jars. For comparison, each species of bacteria in the same tissue from the experimental group and the control were simultaneously cultured and analyzed. All plates were incubated at 37°C.

Organ weights, gross necropsy and histopathology

A thorough 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, testes, thymus and uterus. Paired organs (adrenals, epididymides, kidneys, ovaries and testis) were weighed as a total of right and left. Sections from the above organs including the skeletal muscle, quadriceps femoris, lymph nodes, skin with mammary glands and tissues with macroscopically evident lesions were fixed in 4% buffered formaldehyde for histological processing. Tissue samples were embedded in paraffin sections, 4–6 μm thick, and were then stained with standard hematoxylin-eosin for light microscopy. Microscopy observations were performed with a Vanox-S microscope (Olympus, Japan). In addition, for the 90-day study, the intact small intestine was flushed with a 0.09% NaCl solution and its length was measured.

Statistical analysis

Compositional data are presented as means ± 95% confidence intervals. Means are considered as statistically significantly different if their confidence intervals are not overlapping.

Data obtained from the animal studies were analyzed separately for each sex and presented as mean ± SD where appropriate. Statistical comparisons of body weight, food and water consumption, bacterial counts, blood biochemistry, haematology and organ weights between groups were performed by the Student t test and $P < 0.05$ was considered as statistically significant. All statistical analyses were carried out using SAS release 8.1 (SAS Institute Inc., Cary, NC).

Results

Compositional analysis

The transgenic (rhIGF-1) and the parental (TP309) rice materials tested in the 90-day feeding study were subjected to comprehensive analytical characterization. Compositional data were compared to data reported for brown rice (Latta and Eskin 1980; Juliano 1985; Møller et al. 2002; Scherz and Senser 2000; 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 S1 (Supplementary Table S1 online). No statistically significant differences between transgenic and parental rice were observed for starch, fiber, sugars, fat and ash content. Compared to the parental line, rhIGF-1 rice exhibited a statistically significant higher protein content (+9%) and a statistically significant lower moisture content (−8%). However, for both lines, contents of proximates are within literature range (Juliano 1985; Scherz and Senser 2000; Møller et al. 2002; USDA 2004; OECD 2004).

The difference between rhIGF-1 and TP309 in protein content is also reflected in the amino acid levels (Supplementary Table S2 online). The transgenic rice exhibited statistically significantly higher contents of amino acids including serine, glutamic acid, proline, cysteine, leucine, histidine and lysine compared to the control rice (mean ± 95% confidence interval). The data for rhIGF-1 rice is within the data range reported in the literature except for aspartic acid, alanine, cysteine and glutamic acid (Scherz and Senser 2000; USDA 2004).

Only minor but statistically significant differences were observed for the fatty acid distributions between the two lines (Supplementary Table S3 online). The proportion of myristic acid and linoleic acid were statistically significantly higher in the transgenic rice. Data of palmitic acid, oleic acid and linoleic acid of both lines was less than data reported for rice in the literature (Scherz and Senser 2000; USDA 2004; OECD 2004; Kitta et al. 2005).

Mineral compositions are presented in Table S4 (Supplementary Table S4 online). No statistically significant difference between the transgenic and the parental rice was detected for contents of calcium, copper, iron, magnesium and molybdenum. However, the transgenic rice exhibited statistically significantly higher contents of manganese, phosphorous, potassium and zinc. For the rhIGF-1 line, the content of potassium exceeded the range reported in the literature, and the contents of other minerals were in agreement with literature data (Juliano 1985; Møller et al. 2002; Scherz and Senser 2000; USDA 2004).

No statistically significant difference was observed for contents of important rice vitamins (Supplementary Table S5 online). Both lines exhibited similar levels of B1, B6, Niacin, pantothenic acid and folic acid. Except for folic acid, contents of important rice vitamins measured were slightly lower than the range obtained from the literature (Juliano 1985; Møller et al. 2002; Scherz and Senser 2000; USDA 2004).

Phytic acid is known as an anti-nutritive rice constituent. It has been shown to limit the bioavailability of minerals (Saha et al. 1994). No statistically significant difference was detected between phytic acid content of the transgenic (0.94% ± 0.03%, mean ± confidence interval, $P < 0.05$, $n = 4$) and the control rice (0.91% ± 0.05%). Data were in agreement with literature data (0.6–1.6%; Latta and Eskin 1980).

Clinical observation, body weight and food and water intake

In the course of the experiment, no treatment-related signs of adverse effects in clinical appearance of the animals were observed. The animals were observed twice daily for well-being. Body weight, food and water consumption was measured weekly and the relative food consumption calculated. There was no statistically significant difference in food consumption between groups, although consumption was slightly higher in both male and female rats given the rhIGF-1 rice (Supplementary Table S6 online).

Growth curves are included for males and females in Fig. S1 (Supplementary Fig. S1 online). They illustrate normal and similar growth patterns within and between the two groups. The body weights of male rats fed rhIGF-1 rice and parental rice were comparable throughout the study period, whereas female rats fed the diet containing rhIGF-1 rice tended to have a lower body weight compared to the control group. The reduction in body weight seen in week 12 was due to

an overnight fasting and blood sampling stress. There were no differences observed in water consumption (data not shown).

Blood biochemistry

Data on blood biochemistry are presented in Table 2. Male rats given rhIGF-1 rice had a significantly higher plasma concentration of protein and albumin, whereas levels of sodium were significantly lower compared to the control group. Furthermore, statistically significantly higher plasma activity of alanine aminotransferase (ALAT) and a significantly lower plasma concentration of creatinine were observed in the female rats given rhIGF-1 rice compared with the control group.

Haematology

Regarding haematology only a few statistically significant differences were observed between the two groups (Table 3). In male rats the platelet count (PLT) was slightly higher in the group fed rhIGF-1 rice, whereas the mean cell haemoglobin (MCH) was slightly lower compared to the control group. Female rats given rhIGF-1 rice had a higher number of neutrophil cells (NEU) compared to the control group.

Microbiology

For the faecal samples no significant difference in the bacterial micro flora could be found between the group fed rhIGF-1 rice and the control group (Table 5). However, in samples from the duodenum a statistically significant increase in the Lactococcal population was observed in the rhIGF-1 rice group compared to the control group (Table 4). This was not observed in the ileum samples, where an increase in the total amount of anaerobe and Enterococci population was detected for the rhIGF-1 rice group compared to the control group ($P < 0.05$).

Organ weights, gross necropsy and histopathology

The absolute and relative mean organ weights are presented in Table 6. Only few significant differences in organ weights were detected in this study, namely on adrenal, small intestine and pancreas. The absolute weights and relative weights of the adrenals and the small intestine were significantly higher in the male group fed rhIGF-1 rice compared to the control group. Furthermore, a statistically significant increase in the absolute and relative weight of the pancreas was observed in female rats fed rhIGF-1 rice compared to the control group ($P < 0.05$).

During the necropsy there were no gross pathological findings, nor did the histopathological examination reveal any changes in the intestinal tract or the

Table 2 Blood biochemical findings in rats fed on rhIGF-1Transgenic rice and control rice

	TG-M	C-M	TG-F	C-F
Urea (mmol/l)	17.3 ± 1.6	16.2 ± 1.9	16.5 ± 0.8	15.7 ± 1.1
ALAT (IU/l)	53.4 ± 1.8	54.8 ± 1.2	55.4 ± 2.4[a]	51.2 ± 1.9
Creatinine (μmol/l)	48.2 ± 8.6	51.5 ± 7.3	43.2 ± 6.7[a]	53.5 ± 4.4
Cholesterol (mmol/l)	5.1 ± 0.2	4.9 ± 0.4	5.4 ± 0.3	5.2 ± 0.5
Protein (g/l)	106 ± 8.7[a]	96.5 ± 9.4	98.6 ± 4.3	95.2 ± 6.4
Albumin (g/l)	49.8 ± 4.8[a]	43.6 ± 3.5	46.5 ± 2.8	42.4 ± 5.4
Triglyceride (mmol/l)	1.9 ± 0.5	2.2 ± 0.3	1.8 ± 0.2	1.6 ± 0.4
Glucose (mmol/l)	5.1 ± 1.4	5.4 ± 1.2	5.3 ± 1.1	5.5 ± 1.3
Sodium (mmol/l)	215 ± 6.4[a]	224 ± 9.6	209 ± 12.4	212 ± 8.5
Potassium (mmol/l)	8.56 ± 1.6	9.85 ± 1.2	8.68 ± 1.1	7.15 ± 1.8

The number of animals was 16 rats/sex/group; data is presented as group mean values ± SD

TG-M rhIGF-1Transgenic rice-males, *C-M* control rice-males, *TG-F* rhIGF-1Transgenic rice-females, *C-F* control rice-females

[a] Statistically significantly different from control group within same sex when a Students *t* test was performed ($P < 0.05$)

Table 3 Blood biochemical findings in rats fed on rhIGF-1Transgenic rice and control rice

	TG-M	C-M	TG-F	C-F
WBC (10^9/l)	5.39 ± 0.3	5.14 ± 0.5	4.17 ± 0.4	4.24 ± 0.2
RBC (10^{12}/l)	7.95 ± 0.1	7.84 ± 0.3	7.87 ± 0.2	7.73 ± 0.3
PLT (10^9/l)	943 ± 167[a]	714 ± 186	829 ± 113	756 ± 212
HGB (g/l)	116 ± 6.5	117 ± 7.3	105 ± 4.4	103 ± 3.8
HCT (%)	0.4 ± 0.2	0.38 ± 0.3	0.46 ± 0.3	0.42 ± 0.5
MCV (FL)	49.81 ± 0.4	49.13 ± 0.8	48.25 ± 0.5	48.54 ± 0.3
MCH (pg)	13.85 ± 0.2[a]	15.13 ± 0.5	13.82 ± 1.2	13.69 ± 1.4
MCHC (g/l)	294 ± 18	307 ± 23	312 ± 26	286 ± 42
Differential count				
LYM (%)	91.3 ± 2.4	92.8 ± 3.3	91.5 ± 2.9	90.3 ± 3.5
NEU (%)	6.5 ± 0.4	5.9 ± 0.8	7.5 ± 0.3[a]	5.7 ± 0.6
EOS (%)	0.1 ± 0.04	0.1 ± 0.02	0.2 ± 0.03	0.1 ± 0.08
BAS (%)	0.3 ± 0.06	0.4 ± 0.05	0.3 ± 0.07	0.2 ± 0.1
MON (%)	1.2 ± 0.4	0.8 ± 0.7	1.6 ± 0.6	1.9 ± 0.3

The number of animals was 16 rats/sex/group; data is presented as group mean values ± SD

TG-M rhIGF-1Transgenic rice-males, *C-M* control rice-males, *TG-F* rhIGF-1Transgenic rice-females, *C-F* control rice-females

[a] Statistically significantly different from control group within same sex when a Students *t* test was performed ($P < 0.05$)

Table 4 Bacterial counts in the small intestine of rats fed rhIGF-1Transgenic rice and control rice

Group	Total aerobe	Total anaerobe	Lactobacilli	Bifidobacteria	Enterococci	Coliforms	Streptococci
Bacterial counts in duodenum ($\log_{10}$ *cfu* g^{-1} *intestinal content*)							
TG	5.68 ± 1.21	6.35 ± 0.76	6.22 ± 0.36[a]	4.55 ± 0.82	3.89 ± 1.24	2.96 ± 0.38	3.85 ± 0.42
C	5.93 ± 1.15	5.85 ± 1.21	5.15 ± 0.24	4.86 ± 0.52	4.15 ± 0.86	3.46 ± 0.75	3.38 ± 0.56
Bacterial counts in ileum ($\log_{10}$ *cfu* g^{-1} *intestinal content*)							
TG	7.68 ± 1.28	7.25 ± 0.24[a]	7.62 ± 0.38	6.85 ± 0.76	6.72 ± 0.55[a]	5.85 ± 0.87	6.35 ± 0.82
C	7.15 ± 0.83	6.32 ± 0.18	7.23 ± 0.42	6.24 ± 0.62	5.65 ± 0.32	6.24 ± 0.72	5.87 ± 0.69

Data are presented as group mean ± SD for 12 animals

Group TG: rhIGF-1 rice, Group C: control rice

[a] Statistically significantly different from the control group ($P < 0.05$)

related organs; in general no pathologically relevant changes were found to explain the identified differences in organ weights between the two groups.

Discussion

Compositional analysis of rice material tested in the 90-day feeding study revealed a number of statistically significant differences between transgenic and parental rice. Differences were detected for proximates (moisture and protein), amino acids (serine, glutamic acid, proline, cystine, leucine, histidine and lysine), fatty acids (myristic acid and linoleic acid) and minerals (manganese, phosphorous, potassium and zinc). Further field trials would be necessary to determine whether the differences detected are due to the genetic modification or due to biological variability in the field. However, it is important to keep it in mind that existing food composition databases do not necessarily reflect the complete natural variation (Burlingame 2004). Thus, in order to assess the overall relevance of statistically significant differences in the light of natural variability within species, more comprehensive databases for the different plant species are necessary, which include samples

Table 5 Bacterial counts in the faecal samples of rats fed rhIGF-1Transgenic rice and control rice

Group	Total aerobe	Total anaerobe	Lactobacilli	Bifidobacteria	Enterococci	Coliforms	Streptococci
Bacterial counts at day 30 ($\log_{10}$ *cfu* g^{-1} *faeces*)							
TG	8.42 ± 0.36	8.63 ± 0.47	8.07 ± 0.76	8.39 ± 0.53	7.74 ± 0.43	6.82 ± 0.51	8.33 ± 0.47
C	8.55 ± 0.56	8.51 ± 0.92	8.18 ± 0.89	8.23 ± 0.78	7.82 ± 0.55	6.94 ± 0.63	8.45 ± 0.69
Bacterial counts at day 60 ($\log_{10}$ *cfu* g^{-1} *faeces*)							
TG	8.69 ± 0.43	8.45 ± 0.38	8.19 ± 0.59	8.27 ± 0.48	7.33 ± 0.84	7.12 ± 0.53	7.75 ± 0.68
C	8.77 ± 0.64	8.58 ± 0.53	8.12 ± 0.66	8.15 ± 0.72	7.45 ± 0.78	7.29 ± 0.46	7.62 ± 0.84
Bacterial counts at day 90 ($\log_{10}$ *cfu* g^{-1} *faeces*)							
TG	8.56 ± 0.32	8.39 ± 0.51	7.86 ± 0.75	8.07 ± 0.42	7.45 ± 0.52	7.03 ± 0.38	7.63 ± 0.54
C	8.66 ± 0.41	8.48 ± 0.72	7.69 ± 0.83	8.12 ± 0.55	7.58 ± 0.84	7.11 ± 0.45	7.72 ± 0.62

All results are presented as group mean ± SD for 12 animals

Group TG: rhIGF-1 rice, Group C: control rice

with different genetic and/or environmental backgrounds (Poulsen et al. 2007).

The current study was performed based on the results of previous studies, which suggested that rhIGF-1 transgenic seeds reduced blood glucose of diabetic mice by enhancing islet cells survival and increasing insulin secretion (Xie et al. 2008). The main purpose of the present study was to assess whether the diet containing rhIGF-1 transgenic seeds can bring any adverse effect on normal subjects by oral delivery, and to see if the transgenic rice could reduce the blood glucose of these normal animals. Some people thought that oral delivery of small peptides or proteins was generally not feasible because of its pre-systemic enzymatic degradation and poor penetration through the intestinal membrane (Hamman et al. 2005; Mahato et al. 2003). Recently, several vaccines (Takaiwa 2007; Wu et al. 2007) and pharmaceutical peptides (Arakawa et al. 1998; Yamada et al. 2008) expressed in transgenic plant seeds were proven to be orally effective.

The concentration of rhIGF-1 present in mature rice seeds from the transgenic line was estimated to be 6.8 ± 0.5% of total seed protein. Since the previous in vitro studies have already shown that rhIGF-1 from transgenic rice seeds could efficiently reduce the blood glucose of diabetic mice by consuming a diet containing 10% rhIGF-1 transgenic seeds (Xie et al. 2008), for the sake of caution, diet containing 20% rhIGF-1 rice was chosen in this safety study of genetically modified rice. The inclusion level of 20% rhIGF-1 transgenic rice in the diet corresponds to a mean daily rhIGF-1 protein intake of approximately 217.6 mg/kg body weight based on the average feed consumption. In addition, the diet in the present study was balanced out to ensure an adequate supply of macro- and micro-components (Table 1).

In the analysis of hematological parameters the observed differences in alanine aminotranferase, creatinine, albumin, protein, sodium, platelets, mean corpuscular haemoglobin and neutrophils were minor and the measured values were all within the normal range for rats of this breed and age. The decrease in plasma creatinine seen with the added rhIGF-1 in the diets could be due to increased amounts of creatinine excreted in the urine or to depression of N absorption in the gut; the reason behind it is not clear. The ingested rhIGF-1 protein might disturb the absorption of N by changing the metabolic nutrition system. The minor but significantly higher level of ALAT seen in the female group given rhIGF-1 rice could be indicative of some kind of liver damage (Moss and Henderson 1994). However, no weight difference and histopathological findings on the corresponding organ were observed to explain the above identified differences, hence, the observed differences were considered insignificant.

In the present experiment five species of bacteria were used as indicating microbes. These same bacteria were studied in the model experiment of safety assessment of transgenic material conducted in animals (Schrøder et al. 2007; Poulsen et al. 2007) and were also the predominant microflora in the animal digestive tract (Mitsuoka et al. 1977). Thus, to inspect the population changes of these bacteria in animals given rhIGF-1 rice is meaningful. Of course, we were

Table 6 Absolute and relative organ weights for rats fed on rhIGF-1Transgenic rice and control rice

	TG-M	C-M	TG-F	C-F
Absolute weight (g)				
Body weight	30.13 ± 2.47	30.38 ± 2.94	23.20 ± 2.63	23.32 ± 2.56
Adrenals	0.0135 ± 0.0024[a]	0.0099 ± 0.0016	0.0104 ± 0.0004	0.0092 ± 0.0002
Brains	0.44 ± 0.27	0.44 ± 0.02	0.47 ± 0.01	0.46 ± 0.04
Epididymis	0.0847 ± 0.006	0.0938 ± 0.13	–	–
Heart	0.17 ± 0.03	0.18 ± 0.02	0.14 ± 0.04	0.13 ± 0.03
Kidneys	0.36 ± 0.05	0.37 ± 0.04	0.28 ± 0.03	0.26 ± 0.02
Liver	1.51 ± 0.12	1.46 ± 0.19	1.19 ± 0.16	1.25 ± 0.09
Mesenterial ln.	0.21 ± 0.07	0.19 ± 0.07	0.11 ± 0.03	0.11 ± 0.05
Ovaries	–	–	0.011 ± 0.004	0.010 ± 0.004
Pancreas	0.17 ± 0.04	0.21 ± 0.05	0.21 ± 0.01[a]	0.15 ± 0.02
Small intestine	1.47 ± 0.05[a]	1.36 ± 0.10	1.25 ± 0.10	1.24 ± 0.11
Spleen	0.14 ± 0.03	0.13 ± 0.01	0.13 ± 0.04	0.13 ± 0.04
Testes	0.19 ± 0.01	0.20 ± 0.04	–	–
Thymus	0.03 ± 0.01	0.04 ± 0.01	0.062 ± 0.008	0.065 ± 0.006
Uterus	–	–	0.07 ± 0.01	0.06 ± 0.01
Length small int.	36.2 ± 5.26	36.7 ± 4.53	31.38 ± 4.17	31.25 ± 4.69
Relative weight				
Adrenals	0.045 ± 0.009[a]	0.033 ± 0.005	0.045 ± 0.006	0.040 ± 0.004
Brains	1.45 ± 0.11	1.48 ± 0.16	2.03 ± 0.19	1.99 ± 0.22
Epididymis	0.28 ± 0.03	0.31 ± 0.05	–	–
Heart	0.56 ± 0.06	0.58 ± 0.03	0.61 ± 0.11	0.57 ± 0.08
Kidneys	1.18 ± 0.08	1.21 ± 0.06	1.22 ± 0.14	1.14 ± 0.05
Liver	5.02 ± 0.08	4.78 ± 0.32	5.11 ± 0.30	5.38 ± 0.26
Mesenterial ln.	0.69 ± 0.22	0.65 ± 0.21	0.46 ± 0.12	0.47 ± 0.23
Ovaries	–	–	0.05 ± 0.02	0.04 ± 0.02
Pancreas	0.58 ± 0.15	0.68 ± 0.10	0.89 ± 0.13[a]	0.64 ± 0.04
Small intestine	4.90 ± 0.26[b]	4.48 ± 0.18	5.47 ± 0.91	5.38 ± 0.87
Spleen	0.46 ± 0.07	0.42 ± 0.07	0.58 ± 0.12	0.56 ± 0.09
Testes	0.63 ± 0.03	0.67 ± 0.09	–	–
Thymus	0.11 ± 0.03	0.13 ± 0.04	0.27 ± 0.04	0.29 ± 0.05
Uterus	–	–	0.29 ± 0.05	0.26 ± 0.03
Length small int.	1.20 ± 0.13	1.21 ± 0.05	1.36 ± 0.24	1.35 ± 0.28

Relative organ weights expressed as g/100 g body weight. Small intestinal length and relative length is expressed in cm and cm/g body weight. Data is presented as group mean values ± SD

TG-M rhIGF-1Transgenic rice-males, *C-M* control rice-males, *TG-F* rhIGF-1Transgenic rice-females, *C-F* control rice-females

[a] Statistically significantly different from control group within same sex ($P < 0.05$)

[b] Statistically significantly different from control group within same sex ($P < 0.01$)

very interested in knowing whether feeding the transgenic rice that containing foreign rhIGF-1 protein would lead to any changes of bacterial flora in the intestinal tract and feces of experimental animals.

Only minor effects were observed in samples taken from the small intestine for bacteriological quantification in the rhIGF-1 group. The faecal samples did not reveal any differences in bacterial counts in the animals given rhIGF-1 rice compared to animals fed the control rice. A previous study showed that no significant influence could be found in the composition of the microbial population in the rumen

of cattle fed Bt corn (Einspanier et al. 2004). Schrøder et al. (2007) found that feeding rats with Cry1Ab protein for 90 days had minor effects on the number of bacteria flora in the small intestine. In the current study, increased amounts of lactobacilli in the duodenum and increased amounts of total anaerobe and enterococci in the ileum were observed in the rhIGF-1 group. The mechanisms behind these changes are unknown, and further studies are required to clarify whether these findings are biologically significant.

The absolute and relative increase in the adrenal and small intestine weight after intake of rhIGF-1 rice was observed in the male group, but due to the lack of histopathological changes in the corresponding organ, this was considered an insignificant finding. A significantly higher absolute and relative weight of the pancreas was observed in female rats given rhIGF-1 rice, but was not seen in the male group. At the same time, as no macroscopic and histopathological effect was observed on the pancreas, the increased absolute and relative weight of pancreas was not considered an adverse effect.

The results of the present 90-day study demonstrated that several significant differences were observed between groups fed diets with genetically modified rice and parental rice, but none of the effects were considered to be adverse. Additional experiments with rats given a parental rice diet spiked with pure rhIGF-1 (with parental rice as the control) may determine if the observed differences in specific organs in this study were due to genetic changes in the transgenic rice or resulting from the expression of rhIGF-1. Mark et al. (2003) reported that biological variation becomes more problematic as drug testing progresses into in vivo animal testing. Great biological variation including histological, cellular, biochemical, molecular and functional aspects can be seen between and within different mdx mouse colonies, between littermates and even between 2 legs of an individual mouse (Miranda et al. 2008). Therefore, a deeper understanding of the many reasons for biological variation will help to initiate practices to minimize this problem.

High blood glucose and insulin β cell damage are the main features of streptozotocin-induced diabetic mice. In a previous study, the transgenic rice seed-derived rhIGF-1 fusion could efficiently reduce blood glucose of streptozotocin-induced diabetic mice by enhancing β cell regeneration and increasing insulin secretion (Xie et al. 2008). But for these normal rats here, no difference was observed on their blood glucose after fed with rhIGF-1 rice compared with the control group. A similar case was reported by Sheng et al. (2005) that the blood glucose level was significantly lower in the selenite-treated diabetic group compared to the diabetic control group, but the blood glucose level was similar between the selenite-treated normal group and the normal control. So it is speculated that the rhIGF-1 rice feeding and selenite treatment had similar blood glucose-reducing mechanisms, by which their blood glucose-reducing role did not work in normal subjects but acted in diabetic groups. Further confirmation is needed.

There were no adverse findings detected in this study, leading to the conclusion that no toxicological effects on C57BL/6J rats were observed when fed diets with 20% genetically modified rhIGF-1 rice, in comparison with the parental rice.

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