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Three-generation reproduction toxicity study of genetically modified rice with insect resistant genes

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ABSTRACT

In the present work, we evaluated the three generation reproductive toxicity of the genetically modified rice with insectresistant cry1Ac and sck genes. 120 Sprague-Dawley (SD) rats were divided into three groups which were fed with genetically modified rice diet (GM group), parental control rice diet (PR group) and AIN-93 control diet (both used as negative control) respectively. Bodyweight, food consumption, reproductive data, hematological parameters, serum chemistry, relative organ weights and histopathology for each generation were examined respectively. All the hematology and serum chemistry parameters, organ/body weight indicators were within the normal range or no change to the adverse direction was observed, although several differences in hematology and serum chemistry parameters (WBC, BUN, LDH of male rat, PLT, PCT, MPV of female rats), reproductive data (rate of morphologically abnormal sperm) were observed between GM rice group and two control groups. No macroscopic or histological adverse effects were found or considered as treatment-related, either. Overall, the three generation study of genetically modified rice with cry1Ac and sck genes at a high level showed no unintended adverse effects on rats's reproductive system.

1. Introduction

Rice (*Oryza Sativa.* L.), cultivated in more than 120 countries (Yu, 2009), is the second largest crop in the world, and it feeds nearly 1/2 of the entire population. The rice acreage of China has reached 2.87–3.00 million hectares and the yield reached 1.80–1.90 million tons (Liao et al., 2015). However, many rice production areas are suffering from the decline in rice production (OECD, 2004). Pest is one of the main reasons for the reduction of rice production. There are many kinds of rice pests in China, and the annual yield loss caused by pest accounts for 3.0–5.0% of the total rice production despite the prevention and control measures on the pest (Liao et al., 2015). Moreover, the use of pesticides is the most important way to control rice pests at present. The environmental pollution, pest resistance, increased production costs, pesticide residue and other issues caused by chemical control have become increasingly prominent. In view of this, genetically modified rice with exogenous insect resistance genes might be a way to resolve these problems.

Global acreages of the GM plants increased from 1.7 million hectares in 1996 to 179.7 million hectares in 2015 (Clive, 2015). The first GM rice was produced in 1988 (Toriyama et al., 1988; Zhang and Wu, 1988), and GM rice with various traits, such as herbicide tolerance and insect resistance, or nutritional components, were developed since then.

Given the significance of rice as staple food for human worldwide, safety assessment of GM rice has become particularly stringent before its commercialization. Till now, GM rice has not been approved for commercial cultivation in the world. In this study, the GM rice carrying *Bacillus thuringiensis* (*Bt*)-derived gene and *Cowpea Trypsin Inhibitor* (*CpTI*)-derived gene, namely *cry1Ac* gene and *sck* gene, respectively, for the control of major insect pests in the main rice-growing areas of China, such as Chilo suppressalis, Cnaphalocrocis medinalis, and Scirpophaga incertulas. The GM rice was developed by Institute of Genetics and Developmental Biology, Chinese Academy of Sciences in collaboration with Fujian Academy of Agricultural Sciences through an Agrobacterium-mediated

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transformation method. The parental control rice, Minghui 86 (*Oryza Sativa L.* ssp. *indica* M86) was used as the host and the traditional control. The cultivation process has been described by previous works (Deng et al., 2003; Zhou et al., 2012). The GM rice combining different insecticidal mechanisms of *cry1Ac* and *sck* genes exhibits higher resistance than GM rice transformed with single sck gene (Zhang et al., 2007). Some short term feeding studies have been performed to demonstrate the possible effects of GM rice with *cry1Ac* and *sck* genes on animal health, including acute toxicity test, subchronic toxity test, induced mutation test, immunotoxicologic test (Liu et al., 2008, 2012; Xing et al., 2012), and no adverse effects have been observed.

GM rice has higher consumption exposure than other GM products, and consumers will pay more attention to the safety of it. Snell et al. (Snell et al., 2012) stated that chronic and multigenerational studies should be conducted in a case-by-case approach if some reasonable doubt remains after a 90-day feeding trails (subchronic toxicity test). In our earlier study, we have performed a chronic toxicity experiment with GM rice at high levels in diets and evaluated the potential adverse effects of the GM rice carrying *cry1Ac* and *sck* genes on rats (Zhang et al., 2014), which was focusing on mortality rates, tumor incidences and pathological findings after 78 weeks feeding. However, no reports on the possible health effects of the GM rice through multiple generations in rats were obtained. Therefore, we performed a three generation study in rats fed with GM rice with *cry1Ac* and *sck* genes in this study, which was designed to study the safety of multi-generation insect resistant rice consumption. This study had passed the inspection of the Animal Experimental welfare & ethical committee, National Institute for Nutrition and Health, China CDC, and was conducted by China National Standard (Chinese Standard GB15193.15–2003) combined with OECD Test Guideline 416: Two-Generation Reproduction Toxicity Study, (OECD, 2001), and OECD Good Laboratory Practice guidelines.

2. Methods

2.1. Materials

The genetically modified rice with two insect-resistant genes *cry1Ac* and *sck* was cultivated by the scientists of Institute of Genetics and Developmental Biology, Chinese Academy of Sciences through *Agrobacterium*-mediated transformation method. The parental rice was an indica rice restorer line Minghui 86, both the genetically modified (GM) rice and its parental rice were simultaneously cultivated in two adjoining plots in the experimental field of Fujian province and they were planted and harvested under identical conditions. And then both rice were hulled and stored in the same way.

The presence of the *cry1Ac* and *sck* transformation cassette was confirmed by PCR using standard protocols (FINNZYMES, Part of Thermo Scientific, Product code:F-130). Transgene expression of *Bt* and *CpTI* in mature seeds was verified by ELISA assay. *Cry1Ac* was detected by a commercially available kit (Envirologix Inc., USA). *CpTI* was detected by the method described by Wang et al., 2005. The *Cry1Ac* content was about 6.1 μg per gram rice, and the *CpTI* content was below 0.035 μg per gram (the detectable threshold).

2.2. Animals, housing and diets

One hundred and twenty weaning Sprague-Dawley rats (aged 7–8 weeks), half male rats and half female rats, weighed 50–70 g were obtained from Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). After 5 days' acclimatization, the rats were randomly assigned to 3 groups, genetically modified rice group (GM), traditional parental control rice group (PR) and AIN-93G control group (AIN). Five rats of the same sex were kept in one cage with free access of food and water. The animal room were provided appropriate environmental conditions (22 ± 2 °C room temperature, 12 h light/dark cycle and 50% ± 10% relative humidity). Nutritional components (protein, fat, moisture, ash and fiber) of GM rice and PR rice were analyzed according to standard methods (Chinese Standard GB 5009. 3–2010, GB 5009. 4–2010, GB 5009. 5–2010, GB/T 5009. 6–2003, GB/T 5009. 88–2008). Carbohydrate levels were estimated by the formulation as follows.

$$\text{carbohydrate}(\%) = 100 - \text{protein}(\%) - \text{fat}(\%) - \text{ash}(\%) - \text{moisture}(\%)$$

The diets of the three groups were genetically modified rice (GM), parental rice (PR) and AIN-93G (as the control), repectively. By taking maximum protein addition as the principle, protein content of each diet was formulated up to 20%, while the insufficient part was supplemented by casein. All the other ingredients were added according to the formulation recommended by AIN-93G (Reeves et al., 1993). According to the nutrient composition of the two rice, the mixed rice in diets of GM group and PR group were 73.2% and 73.8%, respectively. All the animals were fed with experimental diets after weaning. The diets were produced every two months.

2.3. Study design

Study design was depicted in Fig. 1. The experiment began with 20 male and 20 female rats per group as P0 generation. The corresponding diet and water were adaptation fed to P0 animals during prebreeding, mating, gestation and lactation period. After 10 wks prebreeding period, a female rat mated with a single male in the same group for 2 weeks (wks) and produced F1a litter. After weaning of the F1a litter, the P0 animals mated again and produced F1b litter. 8 pups (with equal sex ratio, if possible) from the F1 litters were selected on post-natal day (PND) 4. Each litter was weighed and the number of stillbirths, live births and sex of pups were recorded. After each litter adjusted to 8 pups on PND 4, each pup was weighed on PND 4, 7, 14 and 21. Randomly selected 12 male and 12 female weaning F1a pups in each group and fed with AIN93G diet for 91 days. Randomly selected 20 male and 20 female weaning F1b pups in each group as P1 parent generation and were treated as described above for the P0 animals. The offsprings of P1 animals i.e. F2a and F2b were also treated as described above for F1a and F1b rats, respectively. The P2 (F2b) animals were also treated as P0 and P1 parent adults. The F3a animals were administrated as F1a and F2a rats. The F3b were maintained for corresponding diets for 91 days. Male and female siblings were not used as breeding pairs.

2.4. Experimental evaluation

2.4.1. Parental animals (P0, P1, P2)

A general observation were conducted and recorded every day throughout the course of the study. Body weight and food consumption were measured every week until mating. The body weights of the F0 and F1 parental male mice were recorded initially and weekly through mating. Feed consumption was not measured during the period of cohabitation. The observation of copulation plugs was taken daily during mating period. The presence of a copulation plug in the vaginal tract was considered evidence of successful mating, and the date of a vaginal plug was observed was designated gestation day 0. Once the vaginal plug was observed, the male and female from that mating pair were individually housed.