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Developmental immunotoxicity is not associated with the consumption of transgenic *Bt* rice TT51 in rats

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ABSTRACT

TT51 is a transgenic strain of *Bt* rice generated by fusing a synthetic *CryAb/Ac* gene into MingHui rice. In this study, rats from F0, F1, and F2 generations were fed a diet with 60% TT51 rice, MingHui rice, or nominal-origin rice. The study focused on developmental immunotoxicity in F1 and F2 offspring after long-term consumption of TT51. A wide range of immunological parameters was monitored in this two-generation study on reproductive toxicity. The experiments were performed on F1 and F2 offspring at postnatal days 21 and 42. No adverse clinical effects were observed in any of the experimental groups. In addition, histopathology observations and immunotoxicity tests, including hematological indicators, spleen lymphocyte subsets, natural killer cell activity, lymphoproliferative response, and plaque-forming cell assay, revealed no significant difference between the groups. These results indicated that developmental immunotoxicity was not associated with a diet of transgenic *Bt* rice TT51, compared to the parental MingHui rice.

1. Introduction

Rice is a staple cereal for over 3 billion people worldwide, and provides 21% of calories for the global population (Zhou et al., 2011). However, it is often severely damaged by pests; about 2–10% of the annual rice yield from Asia is lost to insect pests, such as those belonging to the order Lepidoptera (High et al., 2004). Transgenic rice research has made significant progress towards the development of genetically modified strains to control pest damage. Transgenic *Bt* rice strains have been successfully employed worldwide. *Bacillus thuringiensis* (*Bt*) is a Gram-positive soil bacterium that, during sporulation, produces a crystal insecticidal δ-endotoxin protein, which is highly specific to target insects. Transgenic *Bt* rice strains that express the Bt protein are highly resistant to lepidopteran pests. TT51 is a novel strain of transgenic *Bt*rice generated by fusing a synthetic *CryAb/Ac* gene into MingHui rice. It has been certified by the Chinese Department of Agriculture as safe.

A number of studies have been conducted on transgenic *Bt* rice, including evaluations for nutrition, allergenicity, and toxicology. Although most of these studies indicated no adverse effects after *Bt* rice/protein exposure (Tan et al., 2015), the safety of *Bt* rice/protein is still doubtful in the public mind, and therefore, a matter of national concern.

Developmental immunotoxicity (DIT) occurs due to exposure to risk factors (chemical, biological, physical, or physiological factors) prior to adulthood, which alters the development of the immune system (DeWitt et al., 2012). Testing for DIT is currently not included in regulatory toxicology assessments. The Office of Economic Co-operation and Development (OECD) guideline 443 for extended one-generation reproductive toxicity studies includes the assessment of potential impact of chemical exposure on the developing immune system of a cohort of rats (OECD, 2012). In OECD 443, only one functional immune parameter, T-dependent antigen response (TDAR) in sheep red blood cells (SRBC) or keyhole limpet hemocyanin (KLH), is recommended. Apart from TDAR, which is considered one of the most sensitive indicators of immune integrity (Brake and Evenson, 2004), two additional tests were also included in our study: the natural killer (NK) cell activity and the lymphoproliferative response to ConA; these tests could detect even minimal changes in the immune response. The DIT tests in our laboratory have been previously validated, ensuring the reliability of our data.

The aim of this study was to strengthen the evidence for the safety of transgenic *Bt* rice TT51. It focused on potential DIT in F1 and F2 offspring after two-generation consumption of TT51 rice. A wide range of immunological parameters was monitored in this two-generation experiment on reproductive toxicity. The results of reproductive toxicity have been published previously (Wang et al., 2014).

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2. Materials and methods

2.1. Test materials

TT51 rice was produced by introducing the *CryAb/Ac* gene for insect resistance into MingHui rice. To eliminate the species difference, variations in nutritional composition, and environmental impact, we included two control groups: a parent-control (MingHui) group and anominal-origin rice control group. The TT51 and MingHui rice were provided by the Huazhong Agricultural University (Wuhan, China), and the nominal-origin rice was obtained from a supermarket (Beijing, China).

2.2. Confirmation of CryAb/Ac protein expression

An ELISA was performed using Quickstix kit for libertylink rice and Quickstix kit for Cry1Ab in corn leaf & seed (EnviroLogix Inc., Portland, ME), according to the manufacturer's protocol, to quantitatively and qualitatively analyze the three types of rice and diets (Zhu et al., 2015).

2.3. Compositional analysis of rice

To obtain the substantial equivalence of TT51 and MingHui rice, the three types of rice were submitted to the Beijing Institute of Nutritional Resources for testing their compositions. The parameters tested included crude protein, crude fat, crude fiber, crude ash, and amino acid.

2.4. Experimental diets

Based on the results of the composition analysis, rice was introduced into the diet at 60% of the total mass (Ib and Morten, 2007). The mix ratio for the feed formulation was chosen to maintain a balanced diet. Rats in the TT51 group were on a diet of 60% TT51 rice, those in the MingHui group were on a diet of 60% MingHui rice, and those in the control group were fed a formulation of 60% nominal -origin rice. All the maintenance- and growth-purified diets were in accordance with AIN-93M and AIN-93G (Reeves et al., 1993). A growth-purified diet contained more protein than a maintenance-purified diet in order to ensure sufficient nutrients for pregnant rats and growing pups. The feed was compounded at the Beijing HuaFuKang biological technology company.

2.5. Animal husbandry

The in-life parts of the study were carried out at the animal facilities in the Institute for Occupational Safety & Health, China CDC. Female and male Wistar rats, purchased from Vital River Laboratories (Beijing, China), were acclimatized for 7 days before the start of the study. All animals were housed alone, on a 10–14-h light/dark cycle. They were maintained in an environment of constant temperature (23 ± 3 °C) and humidity (50–65%).

All animals were treated humanely, and all the animal study protocols were approved by the Office of Laboratory Animal Welfare, National Institute for Nutrition and Food Safety (Beijing, China).

2.6. Study design

The two-generation study was performed based on OECD 416 (OECD, 2001) and in compliance with good laboratory practices (GLP). The scheme of the study was showed in Fig. 1.

Ninety female Wistar rats (30 rats/group) were mated with forty-five male rats (15 rats/group). The mating period for the animals was 2 weeks. Before mating, the different groups of parental rats were administered their corresponding diets for 10 weeks. Generation day 0 (GD0) was defined as the day when a vaginal plug was detected. At post-natal day (PND) 21, 30 female weanlings/group and 15 male weanlings/group were randomly selected as the F1 generation. The F1 rats were also placed on the same diets as their parents for 10 weeks pre-mating. The offspring from different dams of each generation were mated between themselves. Dams and their offspring were fed with corresponding diets during the periods of mating, gestation, lactation, offspring care, and pubescence.

The study focused on the effects of TT51 exposure on F1 and F2 offspring. Therefore, the F0 and F1 rats used for mating were excluded from the study at PND 21.

Physiological developmental indices of the F1 and F2 offspring, such as eye opening, auricle separation, vaginal opening, balano-preputial separation, cliff avoidance reflex, surface righting reflex, auditory startle reflex, and forelimb hanging were tested. The body weights of F1 and F2 pups at PND 0, 4, 7, 14, and 21 were recorded.

2.7. Necropsy

A senior pathologist assisted by a trained team performed complete necropsy and histopathology examination on 10 female and 10 male offspring from different litters of F1 and F2 at PND 21 and 42. The thymus, liver, and spleen were trimmed and weighed (paired organs were weighed together) immediately after sacrifice. Relative organ weights (percentage of final body weight) were then calculated. Thymus, liver, spleen (a half), Peyer patch (PP), and mesenteric lymph nodes (MLN) were placed in 4% formalin for 24 h, embedded in paraffin, sectioned to 5-μm pieces, and stained with hematoxylin and eosin for microscopic observation. The splenocyte from spleen (the other half) was prepared and suspended in 2 mL of an RPMI 1640 medium (Solarbio, Beijing, China) supplemented with 5% fetal bovine serum (FBS, Biochrom AG, Germany).

2.8. Immunotoxicity assessment

Developmental immunotoxicity in offspring was assessed at PND 21 and 42. At each assessment, 10 female and 10 male pups from different litters were sacrificed. The immunological endpoints included hematology, flow cytometric analysis of spleen lymphocyte subsets, natural killer (NK) cell activity, lymphoproliferative response to ConA, and plaque-forming cell (PFC) assay.

2.8.1. Hematology

As rats at PND 21 were too small to collect tail blood sufficient for hematological analyses, only blood from rats at PND 42 was analyzed. Whole blood samples from the rat tail tip were collected with an anticoagulant. The samples were evaluated for white blood corpuscles (WBC), red blood cell (RBC) count, hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), blood platelet count (PLT), and red cell distribution width (RDW). The analyses were performed using a Mek-6813K cell counter (MEK-6813K, NIHON KOHDEN, Japan).

2.8.2. Flow cytometric analysis of spleen lymphocyte subsets

Single-cell suspensions were prepared by passing the spleen tissue through a 70-μm sterile nylon mesh (Falcon, Franklin lanes, NJ). After three washes, the cells were resuspended in an RPMI 1640 medium with 5% FBS. The cells (100 μl) were then probed with CD4/CD8/CD3 and T/B/NK antibodies (10 μL). After 10-min incubation in dark place, 1 mL stain buffer was added to the mixture, and then it could be analyzed by flow cytometry (BD FACS Calibur, America).

2.8.3. NK cell activity

Splenic NK cell activity was assessed using the in vitro CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, America). After adjusting the concentration of spleen single-cell suspensions to 5×10^6 cells/mL, the procedure was performed according to the