

Original Article



Effects of Parental Dietary Exposure to GM Rice TT51 on the Male Reproductive System of Rat Offspring*

WANG Er Hui^{1,2,3}, YU Zhou³, JIA Xu Dong³, ZHANG Wen Zhong³, and XU Hai Bin^{3,#}

1. Department of Nutrition and Food Hygiene, College of Public Health, Xinxiang Medical University, Xinxiang 453003, Henan, China; 2. Henan Collaborative Innovation Center of Molecular Diagnosis and Laboratory Medicine, Xinxiang Medical University, Xinxiang 453003, Henan, China; 3. China National Center for Food Safety Risk Assessment, Beijing 100021, China

Abstract

Objective To evaluate the health effects of parental dietary exposure to GM rice TT51 on the male reproductive system of rat offspring.

Methods Rice-based diets, containing 60% ordinary grocery rice, MingHui63, or TT51 by weight, were given to parental rats (15 males/30 females each group) for 70 days prior mating and throughout pregnancy and lactation. After weaning, eight male offspring rats were randomly selected at each group and fed with diets correspondent to their parents' for 70 days. The effects of exposure to TT51 on male reproductive system of offspring rats were assessed through sperm parameters, testicular function enzyme activities, serum hormones (FSH, LH, and testosterone levels), testis histopathological examination, and the relative expression levels of selected genes along the hypothalamic-pituitary-testicular (HPT) axis.

Results No significant differences were observed in body weight, food intake, organ/body weights, serum hormone, sperm parameters, testis function enzyme ACP, LDH, and SDH activities, testis histopathological changes, and relative mRNA expression levels of GnRH-R, FSH-R, LH-R, and AR along the HPT axis.

Conclusion The results of this study demonstrate that parental dietary exposure to TT51 reveals no significant differences on the reproductive system of male offspring rats compared with MingHui63 and control.

Key words: Genetically modified rice; Sperm parameter; Hypothalamic-pituitary-testicular axis; Reproductive toxicity

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INTRODUCTION

Genetically modified (GM) crops are now under rapid development and commercial use worldwide. Knowing if GM food affects human and animal health before putting it on the market is a question of great importance. However, we are still uncertain whether GM food can exert potential reproductive toxicology

on humans or animals^[1]. Until now, only a small number of studies have reported the health effects of GM crops on animal reproductive system^[2-6]. Thus, the current scientific data are considered by many scientists as inadequate to prove whether GM food affects reproductive health; some studies even have reported apparently harmful effects. In the study performed by Vecchio^[2], the authors fed pregnant Swiss mice and male litters on a standard laboratory

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#Correspondence should be addressed to XU Hai Bin, Tel: 86-10-52165554, E-mail: hbxu1231602@vip.sina.cn

Biographical note of the first author: WANG Er Hui, male, born in 1985, lecturer, majoring in toxicology.

chow containing 14% GM soybean. By means of immunoelectron microscopy, they focused their attention on Sertoli cells, spermatogonia, and spermatocytes at 2, 5, or 8 months of age. The results of their study indicated that the immunolabelling for Sm antigen, hnRNPs, SC35, and RNA Polymerase II decreased in 2 and 5 month-old GM-fed mice and was restored to normal at 8 months. Moreover, in GM-fed mice of all considered ages, the number of perichromatin granules was higher and the nuclear pore density was lower. The author further found enlargements in the smooth endoplasmic reticulum of Sertoli cells in GM-fed mice. A three-generation study reported minimal histopathological changes in the liver and kidney of F3 female offspring rats fed on *Bt* maize diet^[6]. The appearance of these apparently harmful effects inevitably aroused suspicion and public anxiety for the safety of GM food and caused extensive debate among scientists.

Rice is one of the most important world widely consumed crops, which is severely damaged by insects. The development of pest-resistant GM crops provides to farmers an effective method for the prevention of pest. TT51 was recently created by inserting a synthetic gene *CryAb/Ac* into parental rice MingHui63. Field tests indicated that TT51 could reduce pesticide application and increase the efficiency of rice production. For its excellent performance, TT51 was granted with the biosafety certificate by China's Ministry of Agriculture in 2009. It means that China is on the threshold of becoming the first country to allow the commercialization of GM rice. However, considering that rice is the most widely consumed staple food, if the product was toxic; the outcome would be quite serious. Therefore, it is not difficult to understand why an acute debate on the safety of this GM rice never stopped since the release of its biosafety certificate in China.

The function of the male reproductive system depends upon various complex biological processes that can be disrupted by many extraneous factors. This system is at risk during fetal development, postnatal period during puberty, and even over the entire life span; the targets include testes and accessory organs. In addition, the high rate of cellular proliferation and the unique cellular differentiation within the mammalian testis make it a very sensitive organ that is able to detect cellular and molecular changes when exposed to a toxicant^[1].

In our previous 90-day feeding study, we found

no harmful effects on the male reproductive system in rats after dietary exposure to TT51^[5]. Considering that rice is a widely consumed staple food and serious debate continues on the safety evaluation of GM crops to protect a large number of people from the risk of potential adverse effects of the GM rice, it would be better to finish the debate by conducting extensive toxicological evaluations. One can consider that if GM food/feed exerts reproductive toxicology on humans or animals after long time-consumption, it is most likely that it would not appear in the parental generation but in the offspring generation. Gene expression index is considered more highly sensitive compared with general toxicological parameters. Until now, the influence of dietary exposure to GM food on the expression levels of male reproductive regulation-related genes has rarely been investigated. Due to this unclear situation, the relative expression levels of reproductive-related genes GnRH-R, FSH-R, LH-R, and AR along the HPT axis of male offspring rats were firstly investigated in this study.

MATERIALS AND METHODS

Materials

Genetically modified rice TT51 and its none-transgenic counterpart MingHui63 were cultivated in the experimental field of Central China Agricultural University in adjoining plots, under identical environmental conditions. After harvest, the paddies were dried under sunshine and then stored in a dry warehouse. Additional common rice purchased at the supermarket was selected as the negative control. Detailed description of the process of compositional analysis and results of the test materials, as well as preparation of the experimental diets, were presented in our previous article^[5]. Testosterone (T), folliclestimulating hormone (FSH), and luteinizing hormone (LH) radioimmunoassay (RIA) assay kits were purchased from Beijing North Institute of Biological Technology. Total protein (TP), acid phosphatases (ACP), lactic dehydrogenase (LDH), and succinate dehydrogenase (SDH), were bought from Nanjing Jiancheng Bioengineering Institute (China). Genomic RNA purification kits, cDNA synthesis kit, and SYBR Green PCR Master Mix were bought from BIO-LAB, China.

Animals and Treatment

Four-week-old Wistar rats, purchased from Vital

River Laboratory Animal Technology Co. Ltd (Beijing, China)-license numbers: SCXK (Jing) 2007-0001-were housed in stainless steel wire-mesh cages at a room temperature of 22-23 °C, 40%-55% relative humidity, with 12 h light/dark cycle, and air change of 10 times/h. All animals were provided with unlimited tap water and food. After 1 week of acclimation, the animals were randomly assigned into three experimental groups according to the body weight with 30 female and 15 male rats in each group. The animals were fed in a self-feeding manner with diets of control, MingHui63, and TT51. Before mating, all parental rats were treated with corresponding diets for 10 weeks. Next, male rats were housed together with a female rat from the same group for mating. In total, 90 female Wistar rats (30 rats/each group) were mated with 45 male rats (15 rats/each group) overnight for 2 weeks of mating period. After weaning, 8 randomly selected male offspring rats were individually housed and fed with diets correspondent to their parents' for 70 days. During the study, body weight and food intake were weekly measured. At the end of the experiment, the rats were sacrificed under sodium pentobarbital anesthesia (60 mg/kg, i.p.), and their testes, epididymis, and accessory glands (seminal vesicles and prostate) were carefully dissected out and weighed. This study was conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology and approved by the Ethics Committee of National Center for Food Safety Risk Assessment.

Serum Hormone Analysis

On the last day of the study, blood samples were collected and immediately stored in a refrigerator at 4 °C and then centrifuged at 3000 rpm in a low temperature high-speed centrifuge (Beckman Allegra X-22R, Brea, CA, USA) for 10 min to separate serum. Serum was then collected and stored at -20 °C until analysis. Serum levels of FSH, LH, and T were measured by RIA using hormone specific kits purchased from Beijing North Institute of Biological Technology.

Sperm Motility, Head Counts, and Sperm Morphology

Following the euthanasia, the left epididymitis was quickly detached from the animals, adherent fat and connective tissues were cut away and placed on clean plates. The head of the fresh removed epididymis was cut into several pieces and incubated

with 3 mL of pre-warmed DMEM at 37 °C for 10 min to allow the sperm release. The sperm suspensions were evaluated for sperm motility, head counts, and morphology following the procedure described below. The sperm suspension was stirred and one drop was placed on a microscope slide and covered with a 22 mm×22 mm coverslip. At least 10 microscopic fields were observed at ×400 magnification using a phase-contrast microscope (Nikon Eclipse E200); a stage warm maintained the sample at 37 °C during the examination, and the percentage of motile sperm was recorded^[7]. The sperm head counts were determined with a hemocytometer. A sample of 0.5 mL of the sperm suspension was diluted with 9.5 mL of physiological saline. Approximately 10 µL of diluted sperm suspension was transferred to each counting chamber and kept for 5 min and then counted under a light microscope (LEICA DM 1000) at ×400 magnification^[8]. The results of sperm head counts were expressed as sperm/g of epididymis. For sperm morphology, sperm smears were coated on histological slides and then air dried and stained with 1% Eosin Y. Sperm morphology was described by a senior pathologist using a microscope (LEICA DM 1000). The morphological abnormalities of sperm cells were determined from 200 sperms per animal. The percentage of the sperm with abnormal morphology was counted, and the mean percentage of all the slides was calculated and recorded^[7,9-10].

Assay of Testicular Function Enzyme ACP, LDH, and SDH Activity

Part of the left testis after ice-water washing, and after being minced with scissors, was homogenized in 9 volumes of 0.9% saline solution and centrifuged at 3000 rpm at 4 °C for 15 min. The supernatant was used for the assay of TP, ACP, LDH, and SDH based on the methodology of assay kits.

Histopathology of Testis

A complete gross visual pathology inspection was conducted by a senior pathologist, assisted by a trained team, on the testes of all animals during the necropsy. The testes were trimmed of extraneous fat and immediately weighed. The testis weight ratio related to terminal body weights was calculated. Tissue sections from the testes were fixed with 4% formalin for 24 h, embedded in paraffin, sectioned to 5 µm, and stained with hematoxylin and eosin for microscopic observation.

Relative Expression Levels of Selected Genes Along HPT Axis

Following the sacrifice, the pituitary and testes were rapidly removed and frozen in liquid nitrogen (approximately -196 °C) until use. The total RNA was isolated from the pituitary and testes using a genomic RNA purification kit (BIO-LAB, China) and reverse transcribed using a cDNA synthesis kit (BIO-LAB, China), as described by the product characterization. Real-time PCR was performed in duplicate for the gonadotropin-releasing hormone receptor (GnRH-R), follicle-stimulating hormone receptor (FSH-R), luteinizing hormone receptor (LH-R), and androgen receptor (AR) genes in a 40 μ L reaction volume of 2 μ L template DNA, 20 μ L SYBR Green Master Mix (BIO-LAB, China), and 16 μ L of distilled water. The target relative gene expression was detected using a comparative cycle threshold (Ct)^[11]. All samples were tested in triplicate. The mRNA transcripts of all selected genes were normalized with rat β -actin (ribosomal protein) mRNA transcripts in the same sample. The sequences of primers used for real-time PCR are listed in Table 1.

Statistical Analysis

The data are expressed as means \pm SD (standard deviation). Statistical analyses were performed using one-way ANOVA to evaluate the homogeneity of the data, followed by a least squared differences model or Dunnett's multiple comparison tests if the homogeneity evaluation indicated significant deviation variances. Analyses were performed in

SPSS for windows version 11.5 (SPSS Inc., Chicago, IL, USA). The differences were considered statistically significant at $P < 0.05$.

RESULTS

Body Weights Gain, Food Consumption, and Reproductive Organ/Body Weights

No significant differences were observed in the mean weekly body weight gain (Table 2) or food consumption (Table 3) among rats in different groups ($P > 0.05$). There were no significant differences in testis, epididymis, seminal vesicle, and prostate weights (Table 4) between groups ($P > 0.05$).

Serum Hormone Analysis

The influences of TT51 on serum hormone levels are listed in Table 5. No significant differences were observed in the serum levels of the FSH, LH, and T among the test groups ($P > 0.05$).

Sperm Motility, Sperm Head Count, and Morphology

The data of sperm motility, sperm head count, and morphology assay are presented in Table 6. The sperm parameters were comparable between rats of different groups after exposure to TT51-based diet, MingHui63-based diet, and the control diet, respectively. No significant differences were detected in terms of sperm motility; sperm head counts, and morphology of epididymis sperm between groups ($P > 0.05$).

Table 1. Primer Sequences Used for Real-time PCR

Primer Name	Gene Bank ID	Sequences (5'-3')	Tm (°C)	Product Size (bp)
GnRHR-F	NM_012767.2	gggggaagagaataactgaaca	59.5	157
GnRHR-R		tctcaatcagacgttccagag		
FSHR-F	NM_199237.1	tactttctggcgtttg	52	246
FSHR-R		tcctactctggtgcttga		
LHR-F	NM_012978.1	acccatagttctcttctctg	61.5	107
LHR-R		aaggtcacaggtcattggtg		
AR-F	NM_012502	aggcaggagccacaacacg	52	247
AR-R		atttgaaaccttaataacc		
β -actin-F	NM_017008.3	cagtgttgaccctgcta	65	208
β -actin-R		tggtgtccctgtatcct		

Table 2. Mean Weekly Body Weight of the Rats (mean±SD, n=8)

Group	Control (g)	MingHui63 (g)	TT51 (g)
0 Week	56.50±3.89	54.24±7.46	51.99±5.72
1 Week	84.83±6.52	95.41±14.25	90.39±10.80
2 Week	153.95±12.68	156.21±17.01	142.03±22.45
3 Week	216.84±14.53	212.30±25.41	210.25±18.19
4 Week	282.98±21.37	279.53±26.40	266.22±24.47
5 Week	346.89±21.92	332.17±33.36	329.09±25.88
6 Week	393.89±24.48	390.33±37.75	378.48±26.75
7 Week	444.59±27.84	433.45±39.35	420.97±29.11
8 Week	476.79±31.34	478.12±32.88	457.59±35.30
9 Week	512.98±35.69	502.58±51.50	485.59±35.90
10 Week	542.19±35.11	533.43±51.88	513.28±38.30

Note. There were no significant differences ($P>0.05$).

Table 3. Mean Weekly Feed Consumption of the Rats (mean±SD, n=8)

Group	Control (g)	MingHui63 (g)	TT51 (g)
1 Week	9.70±1.93	11.11±2.58	10.23±2.13
2 Week	19.16±2.95	20.31±6.54	18.66±4.80
3 Week	24.39±6.67	23.56±6.63	25.00±4.45
4 Week	33.73±3.91	34.96±5.24	33.21±4.24
5 Week	35.22±3.23	33.62±5.86	35.05±4.83
6 Week	33.24±1.89	33.05±3.08	33.01±2.30
7 Week	33.16±1.81	32.44±2.87	32.94±2.49
8 Week	33.72±1.41	34.19±1.77	33.93±1.60
9 Week	31.33±3.19	30.06±3.19	30.55±2.34
10 Week	30.01±3.98	29.59±2.09	30.19±2.55

Note. There were no significant differences ($P>0.05$).

Table 4. Organ/body Weight Ratios of the Rats (mean±SD, n=8)

Group	Prostate	Seminal Vesicle	Epididymis	Testis
Control	0.17±0.06	0.28±0.10	0.22±0.05	0.67±0.11
MingHui63	0.20±0.09	0.28±0.10	0.20±0.05	0.70±0.11
TT51	0.17±0.06	0.28±0.09	0.20±0.05	0.67±0.14

Note. There were no significant differences ($P>0.05$).

Table 5. Serum Hormones of the Rats (mean±SD, n=8)

Group	FSH (mIU/mL)	LH (mIU/mL)	T (ng/mL)
Control	1.67±0.56	1.33±0.29	2.52±0.77
MingHui63	1.58±0.57	1.42±0.44	2.09±0.85
TT51	1.66±0.51	1.33±0.29	2.35±1.16

Note. There were no significant differences ($P>0.05$).

Table 6. Sperm Parameters of the Rats (mean±SD, n=8)

Group	Sperm Heads ($10^6/g$)	Sperm Motility (%)	Sperm Abnormalities (%)
Control	185.15±27.89	85.29±10.27	6.25±2.11
MingHui63	173.81±16.49	83.10±7.05	6.63±1.93
TT51	185.49±20.35	86.02±8.72	6.38±2.11

Note. There were no significant differences ($P>0.05$).

Assay of Testicular Function Enzyme ACP, LDH, and SDH Activity

The toxic effects of TT51 on the activity of testicular marker enzymes are presented in Table 7. The results indicated that the activity of three enzymes had no significant differences as compared with MingHui63 and the control group ($P>0.05$).

Histopathology of Testis

A compact and regular arrangement of cells in the seminiferous tubules was demonstrated in all test groups, and nearly all stages of spermatogenesis were found in a cross-section of the seminiferous tubules.

No group-related histopathology changes were observed for the testes (Figure 1A-C) in the control group, MingHui63 group, and TT51 group after a complete necropsy was conducted on all animals.

Relative Expression Levels of Selected Genes Along HPT Axis

The relative expression level of selected GnRH-R, FSH-R, LH-R, and AR genes along HPT axis were assessed. The relative expression levels of GnRH-R in the pituitary, FSH-R, LH-R, and AR genes in the testis were unaffected by the consumption of different diets among individuals of the control group, MingHui63 group, and TT51 group (Figure 2).

Table 7. Activity of Testicular Marker Enzymes of the Rats (mean \pm SD, $n=8$)

Group	ACP (U/g prot)	LDH (U/g prot)	SDH (U/g prot)
Control	101.14 \pm 20.31	6095.66 \pm 1138.60	7.60 \pm 1.76
MingHui63	101.02 \pm 16.26	5729.54 \pm 1277.19	7.37 \pm 1.77
TT51	103.55 \pm 15.55	6010.51 \pm 2136.97	7.64 \pm 2.48

Note. There were no significant differences ($P>0.05$).

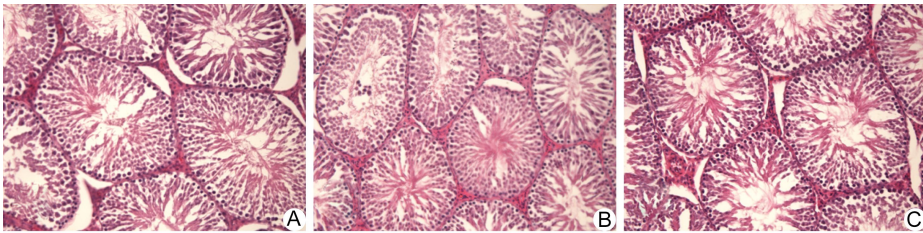


Figure 1. Histopathological changes in testicular tissue (A-C, control group, MingHui63 group, and TT51 group) of rats stained with H&E \times 200.

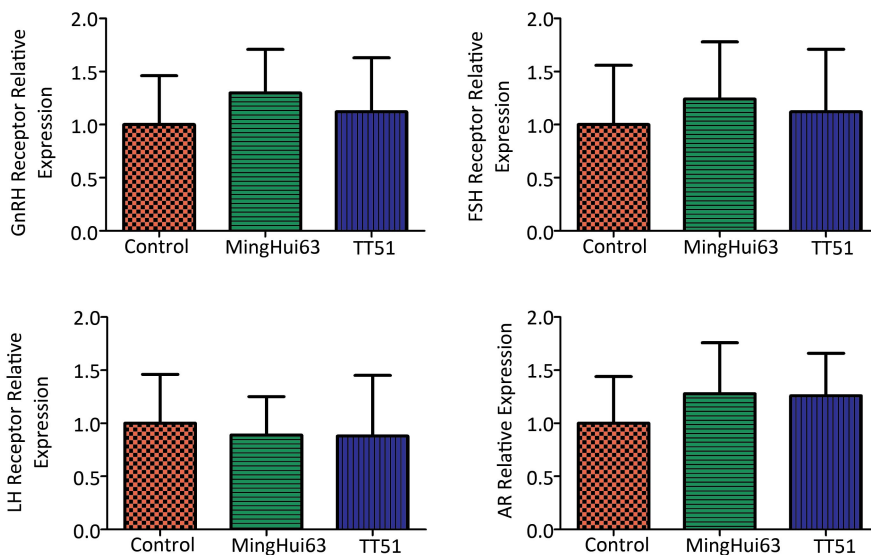


Figure 2. Relative mRNA expression levels of GnRH, FSH, LH, and AR along the HPT axis in the control group, MingHui63 group, and TT51 group after parental dietary exposure to TT51 in offspring rats ($n=8$).

DISCUSSION

The rapid and widespread development of the commercial use of various foods derived from GM technology has been a source of concern. Presently, it is still uncertain whether GM food consumption exerts potential reproductive toxicology on humans or animals^[1]. Therefore, many research groups are concerned about whether GM food exerts negative effects on the male reproductive system, trying to ensure the safety of GM food by numerous methods over many species of animals^[11]. However, most animal feeding studies are acute and sub-chronic and focused mainly on the following parameters: body weight gain, feed consumption, hematology, serum chemistry, relative organ/body weights and histopathology changes, serum hormones, and sperm parameters. Although there were almost no harmful effects observed in these short-term studies, serious debate still surrounds long-term and multigenerational feeding studies. There has been speculation that GM foods produce unintended outcomes that affect the reproductive function of human beings or animals^[12]. However, long-term animal feeding studies with highly sensitive indexes as gene expression level were rarely employed in GM food safety assessment. Therefore, long-term feeding studies with GM rice TT51 on rats and other species, in collaboration with new improving technologies, would be essential to strengthen the safety evaluation process^[5]. Therefore, health effects of parental dietary exposure to GM rice TT51 on the male reproductive system of offspring rats were evaluated in this study through the assessment of sperm parameters, testicular function enzyme activities, and serum concentration of reproductive hormone FSH, LH, and testosterone (T) levels, testis histopathological changes, and the relative expression levels of selected genes along the HPT axis.

A complex mechanism under the regulated functioning of the HPT axis is responsible for the initiation and maintenance of spermatogenetic activity. Initially, by the secretion of GnRH from the hypothalamus, FSH and LH are released from the pituitary gland. In the testes, under the stimulatory action of LH, the Leydig cells, located in interstitial tissue, produce and secrete testosterone. Simultaneously, FSH supports the function of Sertoli cells, which are mediators for the effects of testosterone and FSH on germ cells for a successful spermatogenesis in the seminiferous tubules^[13]. It is

well understood that the spermatogenesis and the physiological functions of Sertoli cells in mammals depend largely on T production by the Leydig cells in response to stimulation by FSH and LH^[14-15]. In the present study, there were no biologically significant differences between groups on serum hormones T, FSH, and LH levels. Similar results were also found previously^[5].

According to the Environmental Protection Agency (EPA) and Food and Drug Administration (FDA) guidelines, the following parameters are considered the most predictive ones for the fertilizing capacity of male rats: sperm motility, sperm head counts, and morphology^[7]. The evaluation of the count and abnormality rate of spermatozoa is considered useful to quantitatively detect the adverse effects on spermatogenesis^[16]. Consistent with the results of the histopathological examination, the present results demonstrated that sperm motility, sperm head counts, and morphology demonstrated no significant differences.

Testes are the most important organs in the male reproductive system, which are involved in the spermatogenesis and T secretion functions. In male animals, many types of enzymes are closely correlated with the functions of testes, and their activity in keeping the germ cell growing normally is a very important endpoint to assess reproductive toxicity^[17]. To evaluate the toxic effects of TT51 on the biochemical metabolism of testes, the levels of ACP, LDH, and SDH in the testes of offspring rats were detected in this study. In testis, the ACP, mainly detected in the cytoplasm of Sertoli cells, is associated with the denaturation of seminiferous epithelium and phagocytosis of Sertoli cells. LDH and SDH, widely distributed in the seminiferous tubules and germ cells, are associated with the maturation of spermatogenic cells and spermatozoa, as well as the energy metabolism of spermatozoa. The results from this study indicated that feeding with TT51 caused no significant differences in testicular enzyme ACP, LDH, and SDH activities in rats between the studied groups.

In all groups, the arrangements of ordered and intercellular connections were compact, and the junction between Sertoli and germ cells were normal (Figure 1). In this study, the weight and structure of the testes did not change in the experimental groups, as compared with the normal group, which resulted in normal testosterone level and also sperm concentration and motility, which are two critical parameters for male fertility.

The male reproductive system is strictly regulated by the HPT axis, which involves the GnRH-R, FSH-R, LH-R, and AR. In this study, the relative expression levels of these selected genes along the HPT axis of the offspring rats were unaffected (Figure 2).

Unlike other crops, as corn or soybean, rice is directly and widely consumed worldwide. If unintended harmful effects to human or animal reproduction health happen, the outcome can be quite serious. Therefore, proposals for studies that attend to the effects of long-term and multigenerational reproduction feeding with this GM rice TT51 on rats and other species in collaboration with new improving technologies need more attention and seem to be necessary.

CONCLUSION

In this study, there were no significant differences observed in the reproductive system of offspring male rats between groups after parental exposure to diet formulated with GM rice TT51 compared with diets that were formulated with MingHui63 rice or with the control diet. The combined data of this study indicates that GM rice TT51 is as safe as the conventional non-transgenic rice MingHui63 rice for rat consumption under the given experimental conditions.

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