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RESEARCH PAPER

Evaluation of the effects of feeding glyphosate-tolerant soybeans (CP4 EPSPS) on the testis of male Sprague-Dawley rats

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ABSTRACT. Glyphosate tolerant soybeans represent a large portion of soybeans grown and fed to farm animals around the world. Despite their widespread use for many years, some have raised questions regarding their safety because the soybeans were genetically modified. The CP4 EPSPS gene which imparts resistance to topical application of the herbicide glyphosate was introduced into soybeans. Application of glyphosate to soybean fields will reduce weed pressure and increase soybean yield. To assess their safety on the rat reproduction system, male Sprague Dawley rats were fed either glyphosate-tolerant (GM) soybean (40-3-2) or near-isogenic, non-GM (A5403) (control) soybean meal. The processed soybean meal was added to formulated rodent diets at 20% (w/w) and fed to rats for 90 days. Some rats from the control group were separately administered mitomycin C for 40 days and served as positive controls in the sperm abnormality test. Body weights and behavior were monitored daily, serum enzymes and histologic and EM appearance of the testis, and sperm morphology were also examined. After 90 days of feeding, no adverse effects were observed in rats fed glyphosate-tolerant soybeans.

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1. INTRODUCTION

Soybean is an important commercial crop with abundant protein and oil content and planted all over the world for human and animal consumption. The weed threat is a concern of planters, resulting in a lower yield of soybeans.[1] Use of herbicides according to label instructions can efficiently control weeds, while misuse of herbicides can lead to environmental pollution and increase residues on crops.[2] Genetically modified (GM) crops with herbicide-resistant traits can provide an effective means to improve crop yields by lowering weed pressure.[3]

GM technology has been widely applied in agriculture and food production. Most of the commercial GM crops planted worldwide have one or more genes introduced by modern biotechnology into their genomes to acquire desired properties including herbicide, insect or disease resistance, extreme temperature, and oxidative stress tolerance.[4,5] The expression of CP4 EPSPS protein confers the soybean tolerance to *Roundup*® agricultural herbicides. The *Roundup Ready* herbicide-tolerant GM soybeans have been commercially cultivated for over 20 years, of which GM soybean line 40-3-2 exhibits tolerance to glyphosate, as it contains the *Cp4 epsps* gene derived from *Agrobacterium sp*. strain CP.4[6] Globally, the *Roundup Ready* soybean has contributed 75% of total world soybean production in 2011.[7]

The safety of GM crops for human and animal consumption has been reviewed in many publications.[8–14] Since some have questioned the impact of GM crops on reproductive performance, the authors decided to assess the effects of glyphosate-tolerant soybeans on male rat health with a specific focus on reproductive organs. The male reproductive system involves complex biological processes which can be disrupted by many toxicants.[15] The testis examination has been utilized to monitor pollution by toxicants that can interfere with cellular proliferation and differentiation of the testis.[16,17] In the current study, we intend to assess the safety of a typical GM soybean by examining the possible toxicological effects on the reproductive system by feeding the Sprague-Dawley rats with the diet formulated with glyphosate-tolerant soybeans (line 40-3-2) and its non-GM near-isogenic line (A5403) for 90 days.

2. MATERIALS AND METHODS

2.1. Plant Materials

The GM soybean line 40-3-2 with exotic *Cp4-epsps gene* and non-GM soybean A5403 were identified by Ministry of Agriculture Genetically Modified Organisms Product Supervision and Inspection Center (Taiyuan, China) and were grown in parallel in the trial field.

2.2. Diet Formulation

The main nutrient contents that include moisture determination, protein, fat, crud fiber, ash, fatty acids, vitamin, amino acids, and iron were determined using the recommended methods of the State Standard of the People's Republic of China. The rat diet in this experiment contains either GM soybean line 40-3-2 or non-GM soybean A5403 at 20% (w/w). The other main ingredients include bean pulp, wheat flour, fishmeal, and yeast powder to ensure balanced nutrition, which meet the criteria of authorized standard GB14924.3-2010. All diets were vacuum-packed and irradiated with ^{60}Co by Experimental Animal Center of Shanxi Medical University and then kept in 4 ~ 8°C before use.

2.3. Animal Experiment

The rats were fed in the laboratory of Experimental Animal Center of Shanxi Agricultural University. The temperature and humidity were controlled from 20 ~ 24°C and 40 ~ 70%, respectively. Totally 45 male SPF Sprague-Dawley rats were purchased from the Experimental Animal Center of Shanxi Medical

TABLE 1. Animal experiment design.

Experiment	Grouping	Sampling timepoint	Tests
Experiment No. 1 Rats (4 weeks old)	CK (5 rats); GM (5 rats)	30 day, 60 day, and 90 day	Blood chemistry, body, testis and epididymis weighting, microscopic and electron micrographic examination
Experiment No. 2 Rats (7 weeks old)	CK (5 rats); GM (5 rats); CK (mitomycin C) (5 rats)	40 day	Sperm abnormality test

University (Shanxi, China) with the license number SCXK(Jin)2015001. After 7 days of acclimation, rats were randomly divided into 2 groups. The treatment group of rats were fed with the diet containing 20% GM soybean line 40-3-2 (GM group), while the control group were fed with 20% non-GM soybean A5403 (CK group). Two animal experiments were designed and the details are shown in Table 1. Water and diet were supplied *ad libitum* during the whole experiment. Two rats were housed in one cage. The animal study and handling procedures were carried out in compliance with the OECD Principles of Good Laboratory Practice.[18]

2.4. Behavior and Body Weight Gain

The rats were observed daily for their behavior, hair color, and sign of symptoms. Special attention was paid to the development and secretion of the external genitalia. The body weight was recorded weekly.

2.5. Bio-sample Collection and Examination

On day 30, 60, and 90 during the experiment, 5 rats were randomly selected from each group for biochemical analysis. The blood was drawn from the orbital sinus under anesthesia when the rats were fasted for 16 h. The epididymis and testis were collected and weighed, and the relative organ weight (organ weight/body weight in percentage) was calculated. For histopathological analysis, one testis was immersed in a 10% neutral buffered formalin solution and then embedded with paraffin. Paraffin section (5μm) was mounted onto glass sides, stained with hematoxylin and eosin (H&E), and examined under an optical microscope by pathologists. The other testis was stored in −80°C for further study.

2.6. Serum Clinical Analysis

The blood samples were centrifuged at 4000 × g for 15 min to obtain the serum. Alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), triglyceride (TG), cholesterol (CHO) were measured with an automatic Beckman Coulter AU5811 (Beckman, USA).

2.7. Electron Micrography

Part of the left testis was fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH7.4) for 14 hr, and post-fixed with 0.1% osmium tetroxide for 2 hr. The ultrathin section of the testis was photographed under a JEM-14 transmission electron microscope (JEOL, Japan).

2.8. Sperm Abnormality Test

Three groups were chosen in this experiment. Specifically, the CK (mitomycin C) group (five rats) were treated with mitomycin C (1.5 mg/kg·BW) by oral gavage for 5 days.[19] The CK group (five rats) and GM group (five rats) were treated with physiological saline. All the rats were fed with their corresponding diets for 35 days. On day 40, the rats were killed by cervical dislocation. The sperms from the left testis were fixed with methanol and stained with Eosin Y. Five hundred sperm cells were

counted, and the percentage of abnormal sperm in total was recorded for each rat. The sperm was analyzed by microscopy (Olympus, BX51, USA).

2.9. Statistical Analysis

All the data were presented as mean value ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA followed by a least squared differences model or Dunnett's test based on the homogeneity evaluation using SPSS v12.0 (SPSS Inc., Chicago, IL, USA). The significant level was set at p value <.05.

3. RESULTS

3.1. GM Soybean Showed No Observational Toxic Effect on Rats

No biologically significant differences were found in daily clinical signs, reproductive organ weight, serum chemical indices, hormone levels, and histopathology on selected reproduction organs between the GM and non-GM soybean groups of rats. All rats appeared healthy throughout the course of the feeding study with no death occurring until scheduled sacrifice. During the 90-day trial, no clinical evidence of toxicity was observed. During clinical examination, all rats had normal development on external genitalia and anus, penis and perianal corneal. The fur was smooth, with no abnormal secretions.

3.2. GM Soybean Did Not Affect Body Weight of Rats

The bodyweight of the rats in GM group during the feeding period were comparable to those of the CK group (Fig. 1). No difference was observed in body weight.

3.3. Biochemistry Evaluation

Only significant differences in AST and TP were observed in serum indices between the GM and non-GM group on 90-day ($P < .05$). Specifically, AST decreased by 3% and TP decreased by 5% after 90 days' feeding of GM soybean (Table 2). However, these differences were not observed in 30-day and 60-day. No other significant difference was found. As all the values were within the normal range, these effects were not considered to be treatment-related.

FIGURE 1. Bodyweight change of rats during 13 weeks. The weight did not significantly change between GM and CK groups. n = 5.

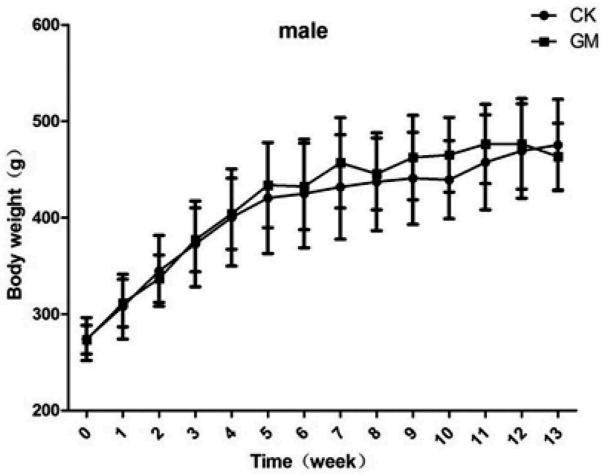


TABLE 2. Blood biochemical parameters of male rats.

	GM			CK		
	30 d	60 d	90 d	30 d	60 d	90 d
ALT(U/L)	32.60 ± 0.98	39.10 ± 1.11	40.70 ± 1.37	33.80 ± 0.52	37.50 ± 1.92	39.70 ± 0.89
AST(U/L)	152.40 ± 0.70	153.50 ± 0.51	155.80 ± 0.27	149.10 ± 0.77	150.70 ± 3.00	151.10 ± 0.09※
TP(g/L)	49.60 ± 0.51	47.50 ± 0.22	46.50 ± 0.76	47.80 ± 0.03	46.10 ± 0.10	44.10 ± 0.32※
ALP(U/L)	186.00 ± 0.16	130.20 ± 1.06	110.00 ± 0.06	193.00 ± 0.44	143.00 ± 0.85	112.00 ± 2.12
ALB(g/L)	15.00 ± 2.10	16.80 ± 0.52	18.90 ± 1.06	14.70 ± 0.35	16.40 ± 0.23	19.20 ± 0.22
GLB(g/L)	13.50 ± 1.01	13.70 ± 0.48	15.80 ± 0.97	11.70 ± 0.05	13.00 ± 0.64	16.30 ± 0.74
A/G	1.11 ± 0.20	1.23 ± 0.52	1.20 ± 0.24	1.26 ± 0.44	1.26 ± 1.92	1.18 ± 0.29
TG(mmol/L)	0.30 ± 0.85	0.40 ± 1.28	0.51 ± 2.22	0.28 ± 1.83	0.47 ± 0.13	0.54 ± 1.77
CHO(mmol/L)	1.09 ± 1.35	1.60 ± 1.21	1.86 ± 1.35	1.17 ± 0.20	1.55 ± 0.76	1.62 ± 1.42

※ $p < 0.05$ compared to the corresponding CK group, n = 5.

ALT, alanine aminotransferase; AST, aspartate aminotransferase; TP, total protein; ALP, alkaline phosphatase; ALB, albumin; GLB, globulin; A/G, albumin/globulin; TG, triglycerides; CHO, total cholesterol.

TABLE 3. Organ/body weight of rats.

		30 d	60 d	90 d
Testis	CK	0.930 ± 0.015	0.702 ± 0.040	0.769 ± 0.106
	GM	0.970 ± 0.058	0.650 ± 0.165	0.703 ± 0.012
Epididymis	CK	0.187 ± 0.003	0.225 ± 0.078	0.336 ± 0.025
	GM	0.201 ± 0.013※	0.220 ± 0.134	0.336 ± 0.035

※$p < 0.05$ compared to the corresponding CK group, n = 5.

3.4. Testis Index and Epididymis Index

There were no significant differences in epididymis or testis weight indices between groups (Table 3).

3.5. Histopathology Analysis and Electron Microscopy Examination

The histopathology test on the testis and epididymis was conducted on day 30, day 60, and day 90 (Fig. 2). A compact and regular arrangement of cells in seminiferous tubules was shown in all periods, in which nearly all stages of spermatogenesis were found in a cross-section of the seminiferous tubules. No histopathology changes were observed for the testes and epididymis in all groups. There was no noticeable chromatin condensation and fragmentation of spermatogenic cells. Elongated spermatids were detected within seminiferous tubules of testes in all rats. Some spermatids were closely embedded in cytoplasmic leydig cells, while some spermatids were poised to be released into the lumen.

FIGURE 2. Histopathology analysis of testis and epididymis in different periods.
A, testis and B, epididymis; 1, spermatogonia; 2, basement membrane; 3, leydig cell; 4, spermatocyte; 5, sertoli cell; 6, sperm; 7, epithelial Cells. The photos are presented at 200×.

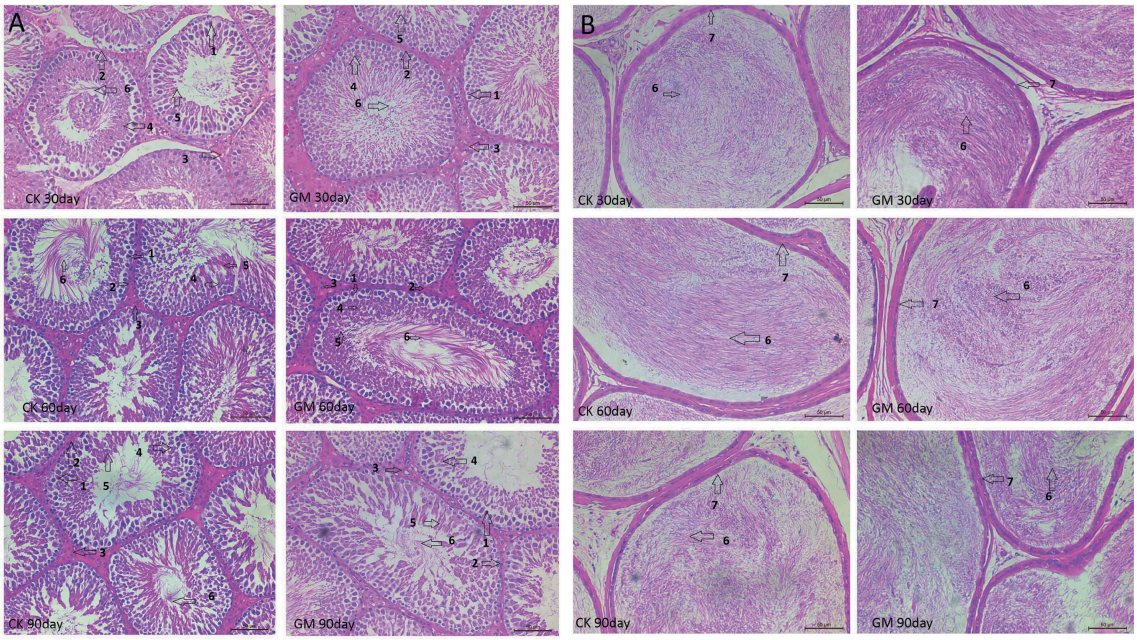
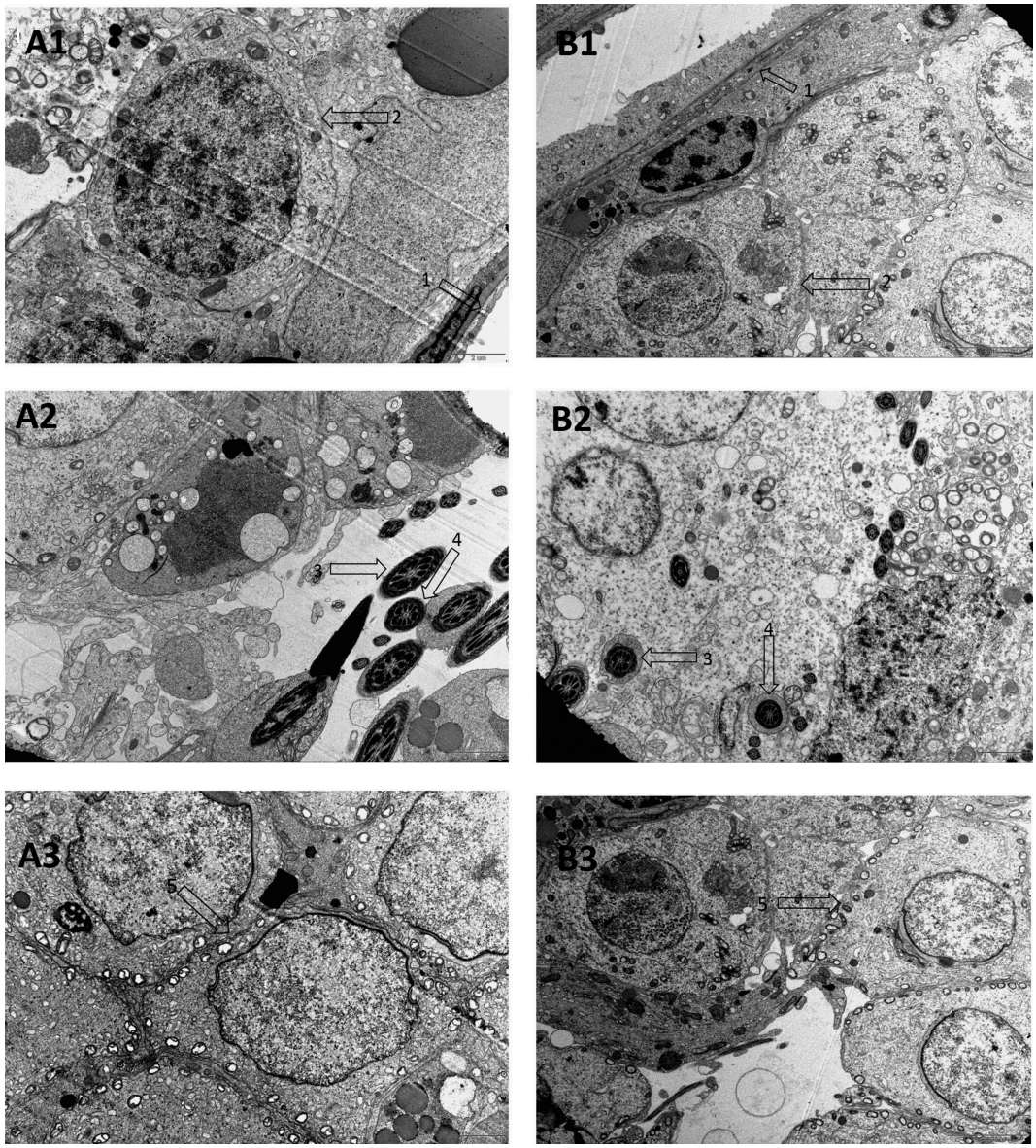


FIGURE 3. Electron micrographs of the testes.
A, CK group and B, GM groups; 1, basement membrane; 2, spermatogonia; 3, tail of sperm 4, the midpiece of spermatids; 5, spermatocyte. The photos are presented at 10,000×.



The sertoli cells, leydig cells, spermatogonia, spermatocyte cytoplasmic extensions between various classes of germinal cells were clearly detectable.

The electron microscopy examination was shown as Fig. 3. Spermatogonias (Fig. 3A1,B1) and spermatocytes (Fig. 3A3,B3) were clearly observed in CK and GM groups, there is no significant difference between these two groups. The spermatids were closely embedded in the cytoplasmic processes of Sertoli cells. It was possible to observe the structure of mitochondria wrapped around the outer dense fibers in the midpiece of spermatids (Fig. 3A2,B2). No

TABLE 4. Aberrant sperm rate of male rats.

	Sperm No.	Aberrant sperm No.	Sperm abnormalities (%)
CK	2500	35	1.4
GM	2500	50	2.0
CK(mitomycin C)	2500	135	5.4※

※$P < 0.01$, n = 5

different structure change was observed in the electron microscopy examination.

3.6. Sperm Morphology

The data of sperm morphology assay were presented in Table 4. The sperm abnormalities were comparable between rats in CK group and GM group after exposure to GM soybean line 40-3-2. No significant differences were detected in terms of sperm abnormalities between CK group and GM group ($P = .15$).

4. DISCUSSION

The male reproductive system has a complex developmental path. Any exposure to toxicants over the entire life span of testes may destroy the function of the reproductive system. High rate of cellular proliferation and differentiation within the testis makes it a very sensitive organ that cellular and molecular changes occur when exposed to a toxicant.[20]

Though long term feeding of registered genetic modified soy products to farm animals or human shows no evidence of adverse effects on reproduction after 20 years of commercial use, some researchers have still raised possibilities that these plants may cause unintended effect via different pathways which have to be systematically evaluated.[21,22] One of the possible unintended effects is dependent on the products from modified genes that might cause the generation of toxins to hurt human health. However, there is no solid evidence to date that the genes introduced which produce enzymes generate any additional products that are considered to be toxic. The modified genes are usually those that can confer the crops for reducing the attack from plant diseases and insect pests. The strategies include imbedding of the factors with the insecticidal function[23] or protecting the plants from herbicides.[24] Herbicide-tolerant crops including soybeans which are resistant to glyphosate have been widely cultivated for over 20 years. The glyphosate residues on glyphosate-tolerant crops can be controlled within tolerances when label instructions are followed. To solve the public concerns of the unintended effects, any genetic-modified plant or animals should undergo strict safety evaluation before entering the food market.[25,26]

Consumption of the GM soybean showed no observed toxicological effects on the reproductive function in male rats based on our 90-day feeding study. We conclude that the GM soybean carrying the exogenous *Cp4-epsps* gene is as safe as the non-GM soybean A5403 in the reproductive system.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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