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A generational study of glyphosate-tolerant soybeans on mouse fetal, postnatal, pubertal and adult testicular development

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Abstract

The health safety of transgenic soybeans (glyphosate-tolerant or Roundup Ready) was studied using the mammalian testis (mouse model) as a sensitive biomonitor of potential toxic effects. Pregnant mice were fed a transgenic soybean or a non-transgenic (conventional) diet through gestation and lactation. After weaning, the young male mice were maintained on the respective diets. At 8, 16, 26, 32, 63 and 87 days after birth, three male mice and an adult reference mouse were killed, the testes surgically removed, and the cell populations measured by flow cytometry. Multi-generational studies were conducted in the same manner. The results showed that the transgenic foodstuffs had no effect on macromolecular synthesis or cell growth and differentiation as evidenced by no differences in the percentages of testicular cell populations (haploid, diploid, and tetraploid) between the transgenic soybean-fed mice and those fed the conventional diet. Additionally, there were no differences in litter sizes and body weights of the two groups. It was concluded that the transgenic soybean diet had no negative effect on fetal, postnatal, pubertal or adult testicular development.
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Keywords: Glyphosate-tolerant soybeans; Roundup Ready soybeans; Transgenic crops; Testicular cell populations; Flow cytometry; Fetal/pubertal testicular differentiation

1. Introduction

The use of biotechnology in the development of glyphosate-tolerant plants greatly contributes to efficiency of crop production, though there are public concerns about the ecological risks and the health safety of the byproducts of these transgenic crops (Conner and Jacobs, 1999). Substantial equivalence of a conventional parental line and a transgenic line is established using phenotypic characteristics and compositional analysis including nutrients, toxicants and allergens (Martens, 2000). Other parameters, such as feeding value and protein characteristics, are used to further appraise the safety of new products.

Substantial equivalence has been established between conventional and untreated glyphosate-tolerant soybeans (GTS; Padgette et al., 1996) and with those that have been treated with glyphosate at manufacturer suggested rates for cropland (Taylor et al., 1999). Feeding value, as measured by growth, feed conversion, milk production and composition and other variables, of two GTS lines fed to rats, chickens, catfish and dairy cattle was found to be comparable to the parental line (Hammond et al., 1996). Physical and functional characteristics of the expressed protein that imparts glyphosate tolerance to plants have been used to evaluate safety. According to Harrison et al. (1996), the protein readily degrades in simulated gastric fluids, is not toxic to acutely gavaged mice and does not contain characteristics of known allergenic proteins.

The primary concern addressed in this study was the health safety of glyphosate-tolerant soybeans fed during pregnancy, early development and growth into adulthood as measured by flow cytometry of acridine orange-stained testicular cell populations in the mouse. The high rate of cell proliferation and the unique cellular differentiation in the mammalian testis make it a very sensitive organ that can detect cellular and molecular changes that occur when exposed to a toxicant (Evenson et al., 1985). Our hypothesis stated

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that glyphosate-tolerant soybeans have no short- or long-term negative effects on fetal, postnatal, pubertal and adult testicular development.

2. Materials and methods

2.1. Glyphosate

Glyphosate (the active ingredient in Monsanto's Roundup) is a broad-spectrum, post-emergence herbicide that non-selectively kills annual and perennial grasses and broadleaves. It plays a crucial role in farm production practices in maintaining weed control, while being on the favorable end of the spectrum of farm chemicals in regard to environmental qualities. Glyphosate degrades to harmless products, is inactivated rapidly in soil and has low toxicity to animals (Hetherington et al., 1999). It inhibits the action of the key enzyme 5-enolpyruvyl shikimate 3-phosphate synthase (EPSPS) that is required for plants to synthesize necessary aromatic amino acids, vitamins and lignin. In glyphosate-tolerant soybeans, the gene encoding a glyphosate-insensitve EPSPS from *Agrobacterium* sp. Strain CP4 (CP4 EPSPS) is inserted into the soybean genome enabling the synthesis of the necessary amino acids even after glyphosate application.

2.2. Sub-chronic mouse study

2.2.1. Feed

Soybeans were obtained from the 2000 crop from a seed dealer who identified an isolated conventional field and a transgenic soybean field in eastern South Dakota. Samples were taken directly from the combine from the middle of each large field. All soybeans were transported to North Dakota State University for extrusion and drying of meal. South Dakota State University Chemistry Analytical Lab analyzed grain samples for crude protein, crude fiber, crude fat (ether extract), $Ca+$ and $K+$. Purina Test Diet (Richmond, IN) formulated rations using transgenic soybeans and conventional soybeans and corn. Chemical composition of each ration is shown in Table 1. The transgenic ration contained 21.35% glyphosate-tolerant soybeans by weight. This percentage was used because it is the highest percentage allowed that still maintained a balanced rodent diet. The diet is certified by the Purina Company.

2.2.2. Experimental design

A complete randomization method was used throughout this experiment. Ten randomly selected C57Bl/6J female mice and six C3H/HeJ male mice were fed either the transgenic or conventional soybean diets. Following breeding, gestation and parturition, three male progeny of the same age (by day) were chosen at random for each of six time points (8, 16, 26, 32, 63, and 87 days after birth). Time points were selected to show the various stages of cellular proliferation and differentiation that occur during the development of the testes and ongoing cycles of spermatogenesis. These time points were selected based on an in depth study by Janca et al. (1986), that characterized the development of the testicular cell populations using the techniques employed here. Three mice were killed for each time point, both testes removed and processed together, two sub-samples of each pooled testicular suspension were measured by flow cytometry, and 5000 cells of each sample were analyzed. This equals a total of six testicular measurements or a total of 30,000 testicular cells measured and analyzed at each time point.

2.2.3. Experimental animals

Five-week-old C57BL/6J female and C3H/HeJ male breeding mice were obtained from Jackson Laboratories (Bar Harbor, ME). These two strains of breeding mice were used so that the F1 progeny were of the crossbred strain used in previous studies (Janca et al., 1986; Evenson et al., 1985, 1993b; Evenson & Jost, 1993; Sailer et al., 1997). The mice were allowed to acclimate to the housing conditions for a 3-week period during which they were fed Purina Mouse Chow 5002. Animals were housed in plastic cages with wire tops and pine shavings for bedding. The mice were maintained on an ad libitum diet of their respective chows and deionized water in a 22 ± 2 °C room on a 12-h light/dark schedule. At the time of breeding, the specially formulated transgenic or conventional diets were started and fed through gestation and lactation. Litter size was measured within 24 h of parturition and we noted no deaths to the progeny during this study. Animals were monitored two times daily for general well-being and care. After weaning, the young male mice were continued on the diets of their respective dams until measurement. Female offspring were removed and sacrificed. All male offspring without 2 other males of the same age (in days) were removed and sacrificed. Adult reference mice (F1) were maintained on Purina Mouse Chow 5002 and used to ensure standardization of the flow cytometer and reagents. The experiment was approved and monitored by the Institutional Animal Care and Usage Committee of South Dakota State University.

2.3. Tissue preparation

At 8, 16, 26, 32, 63, and 87 days after birth, three male mice on the conventional soybean diet, three male mice on the transgenic soybean diet and an adult reference mouse were weighed, killed by cervical dislocation, and the testes surgically removed. Testes were placed in 60 mm Petri dishes containing 1–2 ml Hank's Balanced Salt Solution (HBSS), minced with curved surgical

Table 1
Chemical composition[a] of modified certified rodent diet 5002 with conventional corn and soybean diet (C) and transgenic soybean diet (T)

	C	T		C	T
Nutrients[b]			Physiological fuel value[e] (kcal/g)	3.47	3.49
Protein (%)	21.0	21.0	Metabolizable energy (kcal/g)	3.19	3.19
Arginine (%)	1.23	1.24	*Minerals*		
Cystine (%)	0.29	0.29	Ash (%)	6.0	6.0
Glycine (%)	0.93	0.93	Calcium (%)	0.92	0.92
Histidine (%)	0.53	0.53	Phosphorus (%)	0.60	0.60
Isoleucine (%)	1.10	1.11	Phosphorus (non-phytate) (%)	0.36	0.34
Leucine (%)	1.68	1.69	Potassium (%)	0.99	0.99
Lysine (%)	1.26	1.27	Magnesium (%)	0.20	0.20
Methionine (%)	0.43	0.43	Sulfur (%)	0.21	0.21
Phenylalanine (%)	0.98	0.98	Sodium (%)	0.30	0.30
Tyrosine (%)	0.64	0.64	Chlorine (%)	0.54	0.54
Threonine (%)	0.83	0.84	Fluorine (ppm)	6.7	6.7
Tryptophan (%)	0.25	0.25	Iron (ppm)	205	205
Valine (%)	1.11	1.12	Zinc (ppm)	74	74
Servine (%)	0.61	0.61	Manganese (ppm)	69	69
Aspartic Acid (%)	1.30	1.30	Copper (ppm)	12	12
Glutamic Acid (%)	2.78	2.78	Cobalt (ppm)	0.80	0.79
Alanine (%)	0.88	0.88	Iodine (ppm)	0.96	0.96
Proline (%)	1.05	1.05	Chromium (ppm)	2.48	2.48
Taurine (%)	0.02	0.02	Selenium (ppm)	0.24	0.24
Fat (ether extract) (%)	6.2	6.6	VITAMINS		
Fat (acid hydrolysis) (%)	3.2	3.2	Carotene (ppm)	3.7	3.7
Cholesterol (ppm)	153	153	Vitamin K (menadione) (ppm)	0.4	0.4
Linoleic acid (%)	12.41	12.61	Thiamin hydrochloride (ppm)	15	15
Linolenic acid (%)	0.07	0.07	Riboflavin (ppm)	8.0	8.0
Arachidonic acid (%)	0.01	0.01	Niacin (ppm)	90	90
Omega-3 fatty acids (%)	0.20	0.20	Pantothenic acid (ppm)	18	18
Total saturated fatty acids (%)	0.59	0.59	Choline chloride (ppm)	1800	1800
Total monounsaturated fatty acids (%)	0.66	0.66	Folic acid (ppm)	3.0	3.0
Fiber (max) (%)	5.1	5.2	Pyridoxine (ppm)	7.32	7.32
Neutral detergent fiber[c] (%)	15.3	15.3	Biotin (ppm)	0.1	0.1
Acid detergent fiber[d] (%)	7.2	7.2	Vitamin B-12 (mcg/kg)	20	20
Nitrogen-free extract (difference) (%)	51.7	51.2	Vitamin A (IU/g)	18	18
Starch (%)	29.13	28.91	Vitamin D-3 (added) (IU/g)	2.2	2.2
Glucose (%)	0.20	0.20	Vitamin E (IU/kg)	71	71
Fructose (%)	0.25	0.24	Ascorbic acid (ppm)	0.0	0.0
Sucrose (%)	2.59	2.59			
Lactose (%)	1.34	1.34	*Calories provided by*		
Total digestible nutrients (%)	77.6	77.5	Protein (%)	24.241	24.098
Gross energy (kcal/g)m	4.11	4.14	Fat (ether extract) (%)	16.109	17.139
			Carbohydrates (%)	59.650	58.762

[a] Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will also differ accordingly.
[b] Expressed as % of ration except where indicated, Moisture content is assumed to be 10.0% for the purpose of calculations.
[c] NDF = approximately cellulose, hemi-cellulose and lignin.
[d] ADF- = approximately cellulose and lignin.
[e] Physiological fuel value (kcal/gm)—sum of decimal fractions of protein, fat and carbohydrate (nitrogen free extract)×4, 9, 4 kcal/gm respectively.

scissors, and placed into conical tubes (12×75 mm). Tissue fragments were allowed to settle, and the supernatant was gravity filtered through 53 μm Nitex filters. All tissues were kept on crushed ice (4 °C) from dissection until measurement by flow cytometry.

2.4. Cell staining and flow cytometry

Dual parameter flow cytometry (FCM) was used to characterize testicular cell populations. Cells were stained with acridine orange (AO), a metachromatic dye that intercalates into double-stranded nucleic acid and produces green fluorescence when excited by blue (488 nm) laser light. AO interaction with single-stranded DNA or RNA exhibits red fluorescence with the same excitation (Darzynkiewicz et al., 1983). The relative content of red and green fluorescence is used to identify the haploid (1n), diploid (2n) and tetraploid (4n) cell types in the testicular cells (Evenson and Melamed, 1983).

Two hundred microliter aliquots of each testicular cell suspension were mixed with 400 μl of an acid-detergent solution containing 0.08 N HCl, 0.15 M NaCl and 0.1% Triton X-100 (pH 1.2). This causes permeabilization of the cell membrane and pH-induced H1 histone dissociation from testicular cells, which allows the acridine orange (AO) stain access to the cellular nucleic acids (Evenson et al., 1980). Thirty seconds later, 1.2 ml of the staining solution containing 0.20 M Na_2HPO_4, 0.1 M citric acid buffer (pH 6.0), 1 mM EDTA, 0.15 M NaCl and 6.0 μg/ml chromatographically purified AO (Polysciences, Inc., Warrington, PA) was added to the sample (Darzynkiewicz et al., 1976; Evenson et al., 1985). Immediately after staining with the AO approximately 70 cells/s were passed through the quartz flow channel of a flow cytometer interfaced to a PC Cicero Unit (Cytomation, Fort Collins, CO). Recorded measurements were begun 3 min after staining, and fluorescence data were collected on 5000 cells/sample. Red ($F_{\geqslant 630}$) and green fluorescence ($F_{515-530}$) emitted from each individual cell were separated optically and stored on the computer. Testicular files were analyzed using the Cyclops PC computer program.

An adult reference mouse was used each time of measurement as an internal control. The information provided by this reference ensured that the instrument, techniques and reagents were all working properly because the testicular population percentages from this adult male mouse were always the same.

There were 18 animals measured from the transgenic soybean diet, 18 from the conventional soybean diet, six adult reference mice and three positive control mice.

2.4.1. Statistical analysis

Data were statistically analyzed using the General Linear Model procedure of the Statistical Analysis System (SAS for Windows, v. 6.12). *P* values less than 0.05 were considered significant. Results were graphed using Sigma Plot, 2000 software, v. 6.00 (SPSS, Inc).

2.5. Multigenerational mouse study

2.5.1. Feed

Soybean harvest and preparation and ration formulation followed the same procedure as for the sub-chronic mouse study. Extra feed for the long-term study was stored in airtight bags at −20 °C.

2.5.2. Experimental design

A complete randomization method was also used throughout this experiment. Transgenic or conventional diets were fed to eight randomly selected male and eight randomly selected female mice. For each diet (transgenic or conventional soybean) the following breeding scheme took place: Generation 1—two C57Bl/6J males and two C57Bl/6J females were bred to keep that strain pure. Two C3H/HeJ males and two C3H/HeJ females were bred to keep that strain pure. Generation 2—following breeding, gestation, parturition, and weaning, six females and three males from each strain (C57Bl/6J or C3H/HeJ) were randomly selected for growth, development and breeding for the next generation. Offspring not randomly chosen for the next generation parents were removed and sacrificed. Generation 3—when all 3rd generation mice were at least 6 weeks old, six C57Bl/6J females and three C3H/HeJ males were randomly chosen and bred to obtain 4th generation cross-bred progeny; the same crossbred strain used in all former studies (Janca et al., 1986; Evenson et al., 1985, 1993b, Evenson & Jost, 1993; Sailer et al., 1997). Offspring not randomly chosen for the next generation parents were removed and sacrificed. Generation 4—three male progeny of the same age (by day) were chosen at random for each of five time points. Time points and sampling followed the same procedure as the sub-chronic study (8, 16, 26, 32, and 63 days after birth), except that the extended adult time point at 87 days postpartum was deleted. Female offspring were removed and sacrificed. All male offspring without two other males of the same age (in days) were removed and sacrificed.

2.5.3. Experimental animals

Five-week old C3H/HeJ males and females and C57Bl/6J males and females were obtained from Jackson Laboratories, allowed to acclimate to the facilities, and bred. Care of the mice followed the sub-chronic procedure above. Three generations of the respective lines were fed the transgenic diet or the conventional diet. In all generations we noted no deaths of the progeny. The fourth generation was a cross between C57Bl/6J females and C3H/HeJ males of each diet in order to maintain the use of the crossbred animal as the measured population as in the sub-chronic study above and previous toxicology studies.

2.5.4. Tissue preparation

At 8, 16, 26, 32, and 63 days after birth three male mice on the transgenic soybean diet, three male mice on the conventional soybean diet, and an adult reference mouse were weighed, killed by cervical dislocation, and the testes surgically removed. Tissue preparation for flow cytometry followed the same procedure as above in the sub-chronic study.

2.5.5. Cell staining and flow cytometry

Cell staining and flow cytometry followed the same procedure as above in the sub-chronic study.

2.5.6. Statistical analysis

Statistical analysis followed the same procedure as above in the sub-chronic study.

2.5.7. Positive control

For the positive control, F1 (C57BL/6J×C3H/HeJ) males were injected at 26 days after birth with 0.2 ml of phosphate-buffered saline (PBS) containing 300 mg/kg bw hydroxyurea (Sigma Chemical Co., St. Louis, MO) for 5 consecutive days. Three mice were killed 6 days after the last injection of hydroxyurea (HU), and the testicular cells were measured by flow cytometry. This was to demonstrate that the assay does detect changes in testicular cell kinetics.

3. Results

3.1. Testicular cell kinetics of sub-chronic study

Testicular cell populations reflect the kinetics of the developing testes. Table 2 shows the ratios of the three major cell populations in the testes. If the percentage of one population increases or decreases, the relative percentages of the others will be affected. The 8-day old mouse is at the end of the spermatogonial proliferation stage (Janca et al., 1986). Mitotic activity of the diploid cells occurs as they evolve from spermatogonia to primary spermatocytes. 88.0%±1.5 of the control and 87.4%±0.4 of the transgenic-tested cells are at the diploid stage. At day 16, mouse testicular development is in the meiosis stage (Janca et al., 1986) that reduces the primary spermatocytes (2n) to haploid spermatids (1n). Spermatogonial mitotic division has slowed at this stage, so the relative decrease in diploid cells (55.1%±9.0—conventional, 57.0%±4.2—transgenic) and increase in tetraploid cells (36.4%±10.5—conventional, 35.0%±4.1—transgenic) reflects the meiotic activity of the spermatocytes. The cytograms of a conventional and a transgenic soybean-fed mouse at 16 days after birth show the diploid, tetraploid and active S-phase populations (Fig. 1A and a, respectively). Spermiogenesis initially occurs from 18 to 30 days after birth. Significant temporal changes occur in the cell populations as round spermatids are biochemically and morphologically transformed to elongated spermatids (Janca et al., 1986). Results at 26 days demonstrate this extremely variable time period of development. Percentages of haploid, diploid and tetraploid populations for the conventional soybean-fed animals average 52.8±2.8, 23.7±2.2 and 21.2±4.5 respectively, while the transgenic soybean-fed animals have means of 46.5±2.7, 24.0±1.1 and 26.7±1.7. Two of the three transgenic-tested mice measured at 26 days were younger than the control by up to 18 h, which caused a significant difference in the haploid population at this very rapid developmental time-point. By day 32, cell population proportions are approaching adult levels (Janca et al., 1986) and becoming more stable. Percentages of 63- and 87-day mouse testicular populations (Table 2) show that adult levels have been reached and that no significant differences occur between the conventional and the transgenic soybean-fed mice as ongoing cycles of spermatogenesis ensue. Bar graphs of the tetraploid (A), diploid (B) and haploid (C) populations (Fig. 2) of transgenic and conventional soybean-fed

Table 2
Percentages of testicular cell populations of conventional and transgenic soybean-fed mice from postnatal to adult development in the sub-chronic study

	8-day	16-day	26-day	32-day	63-day	87-day
Transgenic haploids	2.4±0.8	3.9±0.4	46.5±2.7*	69.6±1.3	83.6±1.9	81.3±1.6
Conventional haploids	2.2±0.1	4.3±2.0	52.8±2.8*	69.3±1.8	82.2±1.3	82.2±0.4
Transgenic diploids	87.4±0.4	57.0±4.2	24.0±1.1	13.7±1.4	7.2±0.9	7.7±0.3
Conventional diploids	88.0±1.5	55.1±9.0	23.7±2.2	15.0±1.8	7.2±0.3	8.6±0.5
Transgenic tetraploids	5.9±0.6	35.0±4.1	26.7±1.7	14.5±1.4	7.7±0.9	9.5±1.6
Conventional tetraploids	5.9±0.9	36.4±10.5	21.2±4.5	13.4±0.9	9.1±1.3	7.7±0.6

* $P<0.05$; small but significant differences seen during this rapidly changing time point are due to age difference (in hours) of mice measured.

Table 3
Mean body weights (gms)±standard deviation of both the sub-chronic (SC) and multigenerational (MG) mice fed transgenic or conventional soybeans (3 mice/time point/treatment)

Age (days)	SC Transgenic	SC Conventional	MG Transgenic	MG Conventional
8	3.7±0.1	3.7±0.2	4.2±0.2	4.1±0.1
16	7.1±0.2	7.3±0.3	7.3±0.2	7.5±0.4
26	15.5±1.9	14.2±0.1	17.3±0.6	12.4±1.3
32	19.9±2.0	19.1±0.2	22.2±0.9	21.4±0.5
63	29.9±0.1	27.7±0.7	29.2±0.3	28.3±0.4
87	32.5±0.8	32.0±1.5		

mice and HU-injected mice over time show the relative proportions of the cell types in the testes of the developing and adult male mouse. Note that the tetraploid population graph is scaled to 50%, while the diploid and haploid graphs are scaled to 100%. This enables comparisons to be made more clearly among the three treatments at the lower percentage levels of the tetraploid population.

3.2. General health parameters of mice in sub-chronic study

Mice fed the transgenic soybean diet showed no differences in body weight (Table 3; $P=0.3391$) from the conventional soybean-fed mice. Average litter sizes between the two groups were comparable [7.3±2.1—transgenic ($n=9$), 7.3±0.9—conventional ($n=9$)]. This is an important parameter in toxicology studies where DNA damage can cause embryo death and resorption in the uterus (Sega and Owens, 1983; Evenson et al., 1993b).

3.3. Testicular cell kinetics of multigenerational mice

Four generations of mice were fed the transgenic soybean diet or the conventional diet, and testicular cell population measurements were taken on the 4th generation mice at the time periods used in the short-term study. Cytograms of adult (63-day old), 4th generation mice fed the conventional diet and the transgenic diet (Fig. 1B and b) depict the distribution of the adult testicular cell populations. Percentages for haploid, diploid and tetraploid populations for the control mice averaged 80.8±2.5, 9.3±1.5 and 9.2±1.1, respectively, while the percentages of the respective populations for the transgenic soybean-fed mice averaged 81.7±2.1, 8.4±0.8 and 9.4±1.2. Fig. 3 is the graph of testicular cell populations over time from the multigenerational mice. Significant differences are seen in the haploid and diploid populations ($P<0.0001$) at 26 days. This demonstrates the extremely variable time period of normal developmental spermiogenesis that can evoke significant changes in a matter of hours.

3.4. General health parameters of mice in multigenerational study

Mean body weights (Table 3) showed no differences between conventional and transgenic soybean-fed mice except at 26 days where the control littermates were significantly lower in weight than the transgenic-tested littermates ($P<0.0001$). Average litter sizes between the two groups were comparable [7.2±2.3—transgenic ($n=5$), 6.6±2.1—conventional ($n=5$)].

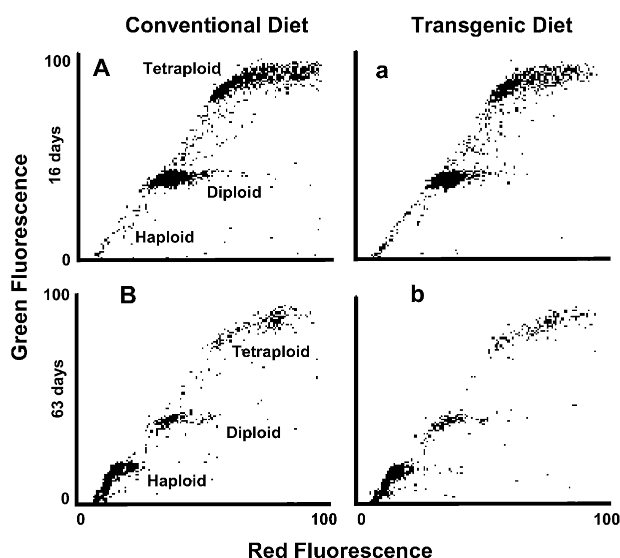

Fig. 1. Green vs. red fluorescence cytograms derived from 2000 AO-stained testicular cells from 16-day old mice fed (A) a conventional soybean diet and (a) a transgenic soybean diet. Diploid and tetraploid populations are apparent. Cytograms from (B) conventional and (b) transgenic soybean-fed 4th generation mice at 63 days after birth. Haploid cells comprise approximately 80% of total cells. There were no differences between treatments on the respective days.

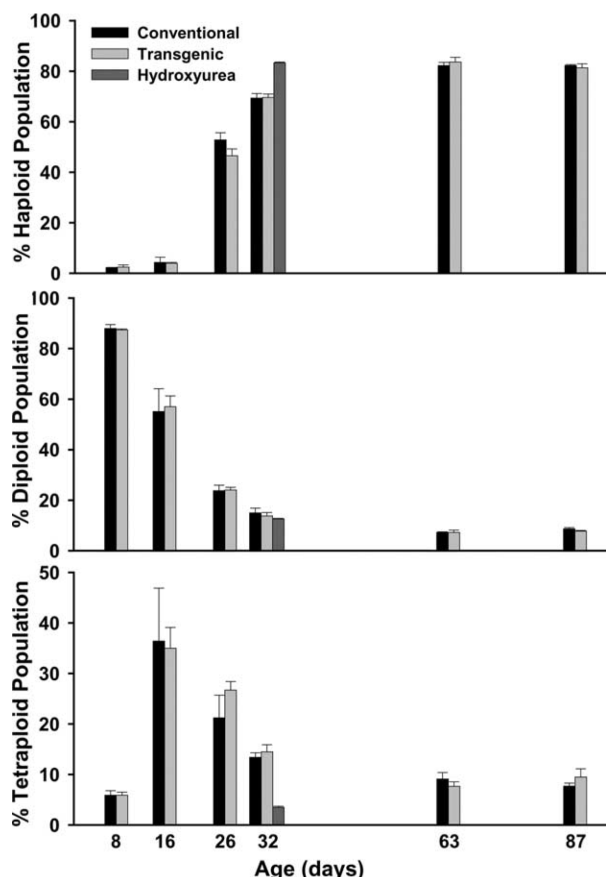

Fig. 2. Time course of tetraploid (A), diploid (B) and haploid (C) testicular populations of conventional diet, transgenic soybean diet, and positive control (hydroxyurea) mice. The effects of hydroxyurea were compared at the 32-day time point only. Each bar of the graph represents the mean±standard deviation of three mice.

3.5. Positive control

Hydroxyurea (HU) was used as a positive control showing a reduction in certain testicular cell populations due to exposure. Hydroxyurea inhibits DNA synthesis by inactivating the ribonucleotide reductase enzyme that converts ribonucleotides to deoxyribonucleotides (Yarbro, 1992). This well-absorbed, rapidly cleared metabolic inhibitor reduces the tetraploid population of testicular cells and subsequent haploid cells by its stage-specific and dose-dependent effects (Evenson and Jost, 1993). The dual parameter FCM cytogram in Fig. 4A depicts the fluorescence of 2000 AO-stained testicular cells from a 32-day old conventional soybean-fed mouse and the distinct populations of 1n, 2n and 4n cells. Fig. 4B is the cytogram of a 36-day old mouse previously injected with hydroxyurea. The tetraploid (upper right quadrant) and S-phase (cells between 2n and 4n) populations are depleted. The bar graphs in Fig. 2 at 32 days show the percentages of each major population for conventional and transgenic soybean-fed mice and HU-injected mice. The percent diploid cell population (B) in the HU mice is less ($P = 0.0056$) than the conventional and transgenic mice. The HU mice showed a very significantly decreased tetraploid (A) population ($P < 0.0001$) and a concurrent increase in percent haploid (C) cells ($P < 0.0001$). The positive control demonstrates that the FCM results of AO-stained testicular cells can depict abnormal cellular and molecular changes.

4. Discussion

The health safety of glyphosate-tolerant soybeans that have been treated with commercial levels of glyphosate and utilized in a nutritionally balanced diet has been studied using the mammalian testes as the sensitive biomonitor of potential toxic effects. DNA synthesis, cellular differentiation and other molecular functions are involved in the complex development of the testes and ongoing cycles of spermatogenesis. Precise measurement of normal development in the growing mammalian testis (Janca et al., 1986) allows for comparisons in toxicology studies (Evenson et al., 1993b). FCM can detect alterations in cell growth and differentiation due to deficient dietary zinc levels (Evenson et al., 1993a) and chemical (Evenson and Jost, 1993) and thermal (Sailer et al., 1997) insults to the testicular cells and epididymal sperm.

Janca et al. (1986), followed mouse testicular development by measuring the cellular populations from birth to adulthood. The standard deviations of the mouse measurements at each time point were extremely tight. Because of this homogeneity of proportions of testicular populations from this strain of mice in previous studies (Janca et al., 1986; Evenson et al., 1985, 1993b; Evenson and Jost, 1993; Sailer et al., 1997) three mice per experimental time point has become an acceptable number of mice to use. Also, on measurements of hundreds of adult male mice from this particular F1 progeny, the percentage of each testicular population for control mice remains consistent from mouse to mouse, study to study and year to year. Adult mice (~48 days of age) consistently have the same basic ratio of testicular populations until old age. Ten thousand cells per animal were measured which is a large statistical sample population. Light microscopy constraints allow for only several hundred cells to be measured.

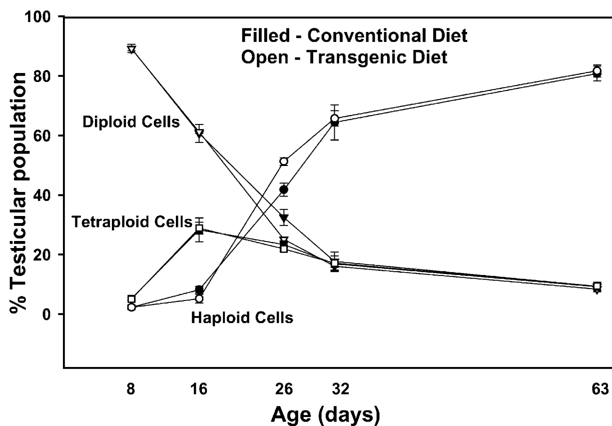

Fig. 3. Multigenerational study plots of mean values ± standard deviation ($n = 3$) for proportions of tetraploid, diploid and haploid testicular cells from 8 to 63 days after birth, comparing transgenic soybean-fed mice with mice fed a conventional diet. Significant differences seen at 26 days are expected during this extremely variable time period.

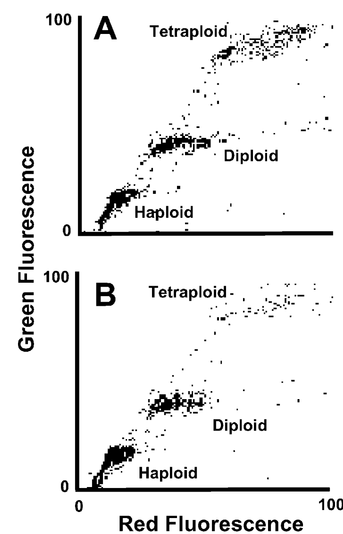

Fig. 4. Green vs. red fluorescence cytograms showing 2000 AO-stained testicular cells from (A) a 32-day conventional soybean-fed mouse and (B) a 36-day mouse previously injected with 300 mg/kg hydroxyurea (B) as a positive control. Note the depleted tetraploid population in cytogram B.

This study, along with a previous study by Janca et al. (1986), has shown that changes during the first round of spermiogenesis (18–30 days after birth) occur very rapidly, where a number of hours can make a statistically significant difference in the percentages of cellular populations in the testes. This study has also shown that beyond these time-of-birth related changes at 26 days, there were no differences in the percentages of haploid, diploid and tetraploid testicular cell populations between the conventional soybean-fed mice and those fed the transgenic soybean diet in short- and long-term studies. Ingestion of glyphosate-tolerant soybeans in a nutritionally balanced diet by the mother during pregnancy and lactation and later by the young developing male mouse caused no differences in the precise cellular and molecular changes that occur within the mouse testes. In conclusion, glyphosate-tolerant soybeans have no negative effect on fetal, postnatal, pubertal or adult testicular development or body growth.

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