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Subchronic Toxicity Study on Soy Isoflavones in Rats[1]

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Objective To investigate the subchronic toxicity of soy isoflavones (SIF) in male rats. **Method** Fifty Sprague-Dawley rats were randomly divided into 5 groups, 10 rats per group. SIF were given to rats in different groups by gavage at dose of 0, 0.2, 0.5, 1.5, and 4.5 g/kg bw, respectively for 13 weeks. Clinical manifestations, body weight, and food consumption were observed weekly. At the end of the study, urinalysis, hematology, clinical chemistry, total testosterone, and follicle-stimulating hormone were tested, and histopathological examinations were performed. **Results** No mortality, ophthalmic abnormalities or treatment-related clinical signs were identified during the study. As compared with the control group, significantly lower body weights and food consumption were observed in 1.5 and 4.5 g/kg bw groups. In clinical chemistry tests, triglyceride was significantly decreased and high-density lipoprotein cholesterol was significantly increased in all SIF-treated groups. Total testosterone levels were significantly lower in 0.50, 1.50, and 4.5 g/kg bw dose groups than in the control group. Microscopic examination showed that the mammary glands exhibited hyperplasia and excreted latex in rats of the 4.5 g/kg bw group. No changes attributable to treatment of SIF in other parameters were found. **Conclusion** SIF at high dosages caused significant endocrine disruption in male rats. The no observed adverse effect level (NOAEL) of SIF to male rats in this study is considered to be 0.20 g/kg bw.

Key words: Soy isoflavone; Rat; Subchronic toxicity

INTRODUCTION

Soy isoflavones (SIF) has become a common ingredient for many dietary supplements or functional foods. For example, foods rich in isoflavones have been consumed by people in Asia for a long time. SIF is also used to prevent some cancers in clinical practice. Recently, concerns over possible toxicity of SIF have been raised based on some animal data in which isoflavones have been shown to cause various reproductive and developmental problems in males and females[1]. Additionally, a sex discrepancy of response to isoflavones is possible.

In male animals, traditional toxicology studies of isoflavones showed no adverse effects. Faqi[2] fed isoflavones (mix in diet) to male Wistar-Unilever rats at a dose of 0.2 or 2.0 g/kg bw for 12-13 months. No adverse effects were observed on body weight, testis or epididymis weight, sperm morphology, homogenization-resistant spermatid number, daily sperm production, or testis histopathology. However, Guan[3] in his recent study showed that isoflavone exhibited hepatotoxicity at 0.6 g/kg bw, leading to larger relative testis and epididymis in male rats, and reduced not only total testosterone level but also sperm count, as compared with that in the control group.

Results of animal studies on isoflavones are controversial. The present study aims to investigate subchronic toxicity of SIF on male rats.

MATERIALS AND METHODS

Materials

SIF was bought from Wuhan Biochemistry Production Co. Ltd., (Wuhan, China). The purity of SIF was 84% (25.6% daidzein, 50.0% genistein, 8.4% glycetin, and others). Reagents used in hematology and serum/plasma chemical tests were

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bought from Zhongsheng Chemical Reagent Co. Ltd. (Beijing, China). The Enzyme-Linked Immuno Sorbent Assay (ELISA) kit for the determination of blood total testosterone (T), follicle-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4) were bought from ADL Co. Ltd. (USA).

Animals and Housing Environment

Male Sprague-Dawley rats were received from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). All animals underwent a physical examination for clinical signs of ill health and were observed for 7 days following their arrival. After acclimatization for 7 days, animals were re-examined and weighed. Animals were randomly assigned to five groups of 10 males on the basis of body weight. Rats whose weight variation exceeded 2 standard deviations of the mean weight were not selected. In each group, initial mean body weights and weight distributions were similar. Animals were individually housed in suspended stainless steel, open-mesh cages in environmentally controlled rooms (23±2℃, 12-h light/dark cycle, 30%-70% relative humidity, ventilation frequency of 10-15 times/hour). They were fed a diet of isoflavones and alfafa free diet (SAFD) (Vital River Laboratory Animal Technology Co. Ltd., Beijing, China). Food and tap water were provided *ad libitum* except for scheduled fasting periods, when food was removed. Animals were divided into five groups, which were gavaged with various doses of SIF (0, 0.2, 0.5, 1.5, and 4.5 g/kg bw/day).

Animal Observations

Animals were observed twice daily for possible illness and mortality. Food spillage was also assessed twice weekly by visual inspection of the amount of food in each cage. Body weights were measured prior to the treatment, twice weekly during the treatment, and at the end of the treatment after fasting for 16-18 hours. Food consumption was determined weekly and total food consumption for each animal was calculated at the end of the study. Weekly and total efficiency of food utilization during the study were also determined. Prior to the treatment, all animals were subjected to ophthalmic examination using an indirect ophthalmoscope following dilation of eyes with tropicamide. All animals also underwent ophthalmic examinations at week 13.

Hematology and Serum/Plasma Chemistry

On day 91, rats were anesthetized with 3% sodium pentobarbital following an overnight fast for 16-18 hours. Blood was collected from caval veins for hematological tests and serum chemical parameters.

Hematological measurements included: red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin concentration, mean corpuscular hemoglobin, platelet count, white blood cell count (WBC), differential blood cell count, and reticulocyte count. Hematological parameters were measured using an automated hematoanalyzer (MEK-7222J/K, NIHON KOHDEN).

To assess blood coagulation, activated partial thromboplastin time was determined with plasma sample, using sodium citrate as an anticoagulant. Coagulation parameters were analyzed by an automated hematocoagulation analyzer (MC-4000 PLUS, Medicine Devices GmbH & Co. KG).

In serum chemical analysis, blood was centrifuged and the serum was separated and analyzed. Serum chemical parameters included: glucose, urea nitrogen (BUN), creatinine, alanine aminotransferase (ALT), gamma glutamyltransferase (GGT), aspartate aminotransferase (AST), total protein, albumin, cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides (TG), total bilirubin, alkaline phosphatase (ALP), calcium, inorganic phosphorus, sodium, potassium, and chloride. Parameters were analyzed using an automatic clinical analyzer (7080, Hitachi High-Technologies Corporation).

Urinalysis

Urine was collected for 16-18 hours prior to blood collection, and was assessed for: appearance/color, specific gravity, pH, protein, glucose, ketones, bilirubin, blood, urobilinogen, nitrite, and leukocytes. Urinalysis was conducted using a urine analyzer (Combi SCAN 100, analyticon Biotechnologies AG, Germany). Urine chemistry measurements included: calcium, sodium, potassium, phosphorus, and chloride, which were measured using an auto-analyzer (7080, Hitachi High-Technologies Corporation).

Clinical Pathology and Histopathology

All rats were anesthetized with 3% sodium pentobarbital solution on day 91 following an overnight fast and body weight measurement. Complete necropsies were performed, and organ weights were obtained for the adrenal glands, brain, epididymis, heart, kidneys, liver, spleen, ovaries, stomach (without contents), testes, thymus, thyroid lobes, parathyroid, and uterus. Paired organs were weighed together. Organ-to-body weight ratios

(relative weight) were also calculated. In addition to the above-mentioned organs, several tissues were sampled and fixed in 10% neutral-buffered formalin. These included the aorta, cecum, colon, cervix, duodenum, esophagus, eyes, femur with bone marrow, ileum, jejunum, lacrimal gland, lung, lymph node, mammary gland, nasal turbinates, pancreas, Peyer's patches, pituitary gland, prostate, rectum, salivary gland, sciatic nerve, seminal vesicles, skeletal muscle (thigh), skin, spinal cord, sternum with bone marrow, trachea, urinary bladder, vagina, and Zymbal's gland. All stored organs and tissues from each animal in 0, 0.2, 0.5, 1.5, and 4.5 g/kg bw groups were embedded to paraffin, sectioned, stained with hematoxylin and eosin, and subjected to microscopic examination.

T, FSH, T3, and T4 Measurement

Concentration of T, FSH, T3, and T4 were determined by specific ELISA kit.

Statistical Analysis

SPSS 11.0 Statistical System was used to analyze data of chemical pathology, organ weight, food consumption and body weight, followed by testing for variance homogeneity. Parametric or non-parametric statistical tests were used, as appropriate, to analyze the significance of differences between the control and treatment groups. All statistical analysis were performed at the P<0.05 level of significance.

RESULTS

Animal Observations

No mortality, ophthalmic abnormalities or treatment-related clinical signs were found. Growth of rats in 0.5, 1.5, and 4.5 g/kg bw groups were delayed (P<0.05). Significantly lower body weights were observed in the 1.5 and 4.5 g/kg bw group (P<0.05), as compared with those in the control group (Fig. 1).

No differences in weekly and total efficiency of food utilization were observed between the treatment and control groups (Table 1).

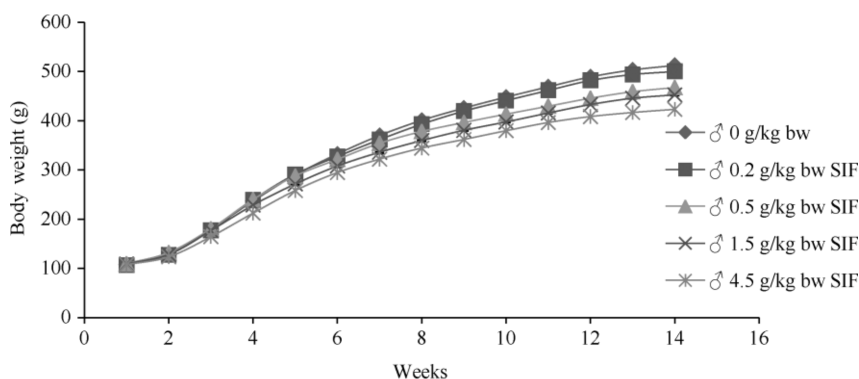

FIG. 1. Growth curve of rats in different groups.

TABLE 1

Efficiency of Food Utilization of Rats in Different Groups

Time	0 g/kg bw/day	0.2 g/kg bw/day	0.5 g/kg bw/day	1.5 g/kg bw/day	4.5 g/kg bw/day
Week 1	0.45±0.04	0.44 ±0.08	0.44 ±0.08	0.47 ±0.14	0.44 ±0.13
Week 2	0.38±0.04	0.38±0.05	0.40±0.03	0.43±0.05	0.37±0.16
Week 3	0.35±0.05	0.38±0.03	0.34±0.03	0.33±0.10	0.33±0.18
Week 4	0.25±0.09	0.22±0.10	0.20±0.13	0.25±0.15	0.26±0.04
Week 5	0.22±0.08	0.18±0.07	0.18±0.08	0.18±0.07	0.19±0.09
Week 6	0.19±0.09	0.17±0.08	0.15±0.08	0.15±0.09	0.14±0.09
Week 7	0.14±0.08	0.15±0.07	0.10±0.06	0.12±0.06	0.11±0.06
Week 8	0.13±0.09	0.12±0.04	0.09±0.05	0.11±0.03	0.11±0.02
Week 9	0.11±0.09	0.10±0.07	0.08±0.04	0.09±0.07	0.10±0.05
Week 10	0.11±0.06	0.10±0.06	0.09±0.06	0.10±0.08	0.08±0.03
Week 11	0.09±0.06	0.09±0.07	0.08±0.05	0.07±0.05	0.05±0.05
Week 12	0.05±0.05	0.04±0.05	0.04±0.05	0.05±0.03	0.03±0.04
Week 13	0.04±0.04	0.02±0.01	0.04±0.03	0.02±0.01	0.04±0.05
Total	0.17±0.02	0.16±0.02	0.15±0.02	0.16±0.02	0.15±0.02

Hematology and Serum/Plasma Chemistry

No statistically significant differences between the treatment and control groups were observed in hematological tests (Table 2) and coagulation parameters (data not shown). Clinical chemistry measurements showed that TG levels in the treatment groups decreased significantly and HDL-C levels in the treatment groups increased significantly, as compared with those in the control group (Table 3).

TABLE 2

Hematological Findings of Rats in Different Groups

SIF Dose (g/kg bw/day)	RBC ($\times10^{12}$/L)	Hgb (g/L)	HCT	MCV (fl)	MCH (pg)	MCHC (g/L)	PLT ($\times10^{9}$/L)	WBC ($\times10^{9}$/L)	Lymphocytes (%)	Monocytes (%)	Neutrophils (%)
0	7.31 ±0.50	151.30 ±7.35	0.41 ±0.02	56.91 ±2.25	20.81 ±0.75	363.10 ±7.06	1105.40 ±94.11	8.48 ±2.39	81.13 ±2.81	6.14 ±1.63	12.73 ±2.98
0.2	7.47 ±0.27	152.60 ±6.19	0.42 ±0.02	56.64 ±3.07	20.45 ±1.11	359.70 ±5.52	1150.30 ±139.15	9.50 ±2.65	80.98 ±3.48	6.42 ±1.54	12.60 ±2.56
0.5	7.40 ±0.32	150.00 ±5.96	0.41 ±0.02	56.04 ±1.96	20.28 ±0.65	361.70 ±4.57	1094.80 ±95.93	10.75 ±2.80	80.81 ±3.39	6.63 ±2.35	12.56 ±2.18
1.50	7.51 ±0.28	154.40 ±3.89	0.43 ±0.01	56.98 ±1.78	20.71 ±0.75	362.50 ±11.24	1135.90 ±86.69	9.45 ±3.33	80.66 ±2.85	6.43 ±1.13	12.91 ±3.19
4.50	7.35 ±0.16	151.20 ±5.20	0.43 ±0.01	58.17 ±1.81	20.56 ±0.57	358.20 ±12.40	1065.00 ±171.30	8.60 ±2.77	81.08 ±3.15	6.51 ±1.11	12.41 ±3.54

Note. RBC=red blood cell count; Hgb=hemoglobin; HCT=hematocrit; MCV=mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC=mean corpuscular hemoglobin concentration; PLT=platelet count; WBC= white blood cell count.

TABLE 3

Blood Clinical Chemistry Findings of Rats in Different Groups

Parameters	0 g/kg bw/day	0.20 g/kg bw/day	0.50 g/kg bw/day	1.50 g/kg bw/day	4.50 g/kg bw/day
ALT (U/L)	29.90±5.82	31.50±3.81	30.30±4.76	28.90±5.15	32.20±7.64
AST (U/L)	206.50±37.52	224.80±53.40	210.00±27.15	225.60±52.94	219.40±38.51
TP (g/L)	94.81±4.82	95.09±2.09	93.48±4.70	94.55±4.13	93.79±6.26
ALB (g/L)	46.46±2.02	46.65±1.91	46.12±2.16	46.89±1.52	46.06±3.40
ALP (U/L)	66.90±28.55	63.00±16.25	66.60±21.67	61.30±15.06	62.90±21.08
GLU (mmol/L)	4.23±0.61	3.89±0.99	4.20±0.94	3.57±0.82	3.63±0.74
BUN (mmol/L)	4.12±0.31	4.59±0.65	4.53±0.68	3.93±0.69	4.30±0.75
CRE (umol/L)	95.03±14.73	89.58±9.97	92.27±8.36	92.75±11.91	95.69±22.74
BCA (mmol/L)	2.74±0.22	2.70±0.20	2.74±0.19	2.77±0.14	2.73±0.14
BP (mmol/L)	1.93±0.14	1.76±0.35	1.69±0.30	1.91±0.33	1.65±0.28
CHO (mmol/L)	1.06±0.18	1.51±0.48	1.33±0.24	1.33±0.45	1.40±0.46
TG (mmol/L)	2.25±1.03	1.22±0.54*	1.13±0.40*	1.50±0.57$^\#$	1.06±0.69*
LDL-C (mmol/L)	0.14±0.08	0.13±0.04	0.10±0.02	0.13±0.05	0.15±0.07
LDH (IU/L)	22.82±4.58	23.36±5.85	22.59±1.77	24.07±5.32	22.35±5.28
BK (mmol/L)	8.05±0.75	7.98±0.77	8.33±0.70	7.80±0.99	8.19±0.57
BNA (mmol/L)	148.10±5.02	148.00±5.10	148.90±5.40	149.60±2.84	150.40±5.02
BCL (mmol/L)	105.90±0.99	106.20±0.92	106.60±1.51	106.20±1.03	105.80±1.03
HDL-C (mmol/L)	1.21±0.48	2.26±0.76*	2.06±0.47*	1.95±0.60*	2.04±0.68*

Note. Compared to the control group (0 mg/kg bw), $^*P<0.05$. ALT=alanine aminotransferase; AST=aspartate aminotransferase; TP= total protein; ALB=albumin; TBILL=total bilirubin; ALP=alkaline phosphotase; GGT=gamma glutamyltransferase; GLU=glucose; BUN= blood urea nitrogen; CRE=creatinine; CHO=cholesterol; TG=triglycerides; BCA=calcium; BP= inorganic phosphorus; BK=potassium; BNA =sodium; BCL=chloride.

Urinalysis

No glucose, bilirubin, blood, ketones or leukocytes were found in the urine samples of all animals, and there were no significant differences in protein and urobilinogen levels between the treatment groups and the control groups (data not shown).

Clinical Pathology and Histopathology

Significantly lower absolute weights of heart, liver, kidneys and spleen were observed in the 4.5 g/kg bw group (P<0.05), compared with those in the control group, so were the absolute weights of heart in the 1.5 g/kg bw group (P<0.05) (Table 4). However, there were no differences in the ratio of organ-to-body and organ-to-brain.

Microscopic examination showed that the mammary glands, in the 4.5 g/kg bw group, exhibited hyperplasia and excreted latex.

T, FSH, T3, and T4 Measurement

Concentration of serum testosterone decreased in 0.5, 1.5, and 4.5 g/kg bw groups (P<0.05); and no significant differences were found in FSH, T3, T4, and ratio of T3/T4 (Table 5).

TABLE 4

Weight of the Heart, Liver, and Kidney of Rats

SIF Dose (g/kg bw/day)	Heart (g)	Liver (g)	Kidney (g)
0	1.60±0.14	17.49±2.19	3.48±0.34
0.2	1.61±0.21	14.60±2.90	3.33±0.55
0.5	1.45±0.15	14.76±3.08	3.04±0.38
1.5	1.33±0.20*	14.71±2.92	3.17±0.57
4.5	1.26±0.21*	14.08±2.08*	2.41±0.44*

Note. Compared to the control group (0 mg/kg bw), *P<0.05.

TABLE 5

Serum Concentration of Testosterone, TSH, T3, T4, and T3/T4 of Rats in Different Groups

SIF Dose (g/kg bw/day)	T (ng/mL)	FSH (ng/mL)	T4 (μg/mL)	T3 (ng/dL)	T3/T4
0	9.89±1.63	0.66±0.98	9.02±3.89a	8.62±0.59	1.13±0.52
0.2	8.57±1.48	0.49±0.69	5.58±1.41	8.61±1.34	1.63±0.50
0.5	7.78±1.05*	0.66±1.13	7.06±1.72	8.77±1.30	1.32±0.40
1.5	7.34±0.83*	0.62±0.57	7.45±3.74	8.33±0.84	1.33±0.49
4.5	7.78±1.17*	0.59±0.81	10.71±3.97	10.77±2.06	1.11±0.35

Note. Compared to the control group (0 mg/kg bw), *P<0.05.

DISCUSSION

In this study, body weight and food consumption decreased in the 1.5 and 4.5 g/kg bw groups; but food utilization was not different between the treatment groups and the control group. Guan *et al.*[3] reported that SIF suppressed body weight gain in a dose-dependent manner in immature male rats of the 0.30, 150, 300, and 600 mg/kg bw groups, with no effect on food intake, but decreased food efficiency ratio. Both studies showed that SIF could decrease body weight; the inconsistencies in data on food intake and food efficiency may be due to different dose levels and difference in test materials.

SIF has been reported to have effects on lipid beneficial for fat metabolism[5]. Our study also showed that blood triglycerides decreased and HDL-C increased in all the treatment groups.

The study found that SIF was associated with endocrine disruption. Daily administration of SIF at a dose of 4.5 g/kg bw, caused hyperplastic and lactational mammary glands. Mcclain[6] reported significant estrogenic activities in female rats, with cytological changes in the uterus, squamous metaplasia at 50 and 500 mg/kg bw/day and hyperplasia at 500 mg/kg bw/day. Furthermore, hydrometra of the uterus and findings in the vagina consisting of anestric or diestrus vaginal mucosa with vaginal mucification, hyperplastic epithelium and multifocal cystic degeneration were noted at 500

mg/kg bw/day.

Genistein has been shown to down-regulate androgen receptor in androgen-dependent prostate cancer cell lines[7], and influences spermatogenesis and significantly inhibits Leydig cell steroidogenesis[8]. Another study showed that daidzein had an adverse effect on erectile function[9]. Our present study showed that the blood total testosterone levels decreased in the 0.5, 1.5, and 4.5 g/kg bw groups, although there were no dose-response relationship. This is consistent with results from other studies, in which daidzein decreased plasma testosterone and luteinizing hormone levels and SIF decreased total testosterone level[9-10]. Therefore, SIF had antiandrogenic effects on male rats with plausible mechanism.

Mcclain[6] in his study reported that after 52 weeks' treatment with genistein at doses of 5, 50, and 500 mg/kg bw/day in male rats, food consumption and body weight gain decreased, and hepatocellular hypertrophy and minimal bile duct proliferation were found at a higher incidence in animals in the 500 mg/kg bw/day group, while inflammation of the prostate were recorded at a higher incidence in the 50 and 500 mg/kg bw/day groups. Researchers also pointed out that most lesions were reversible. But Guan[3] in his study showed that SIF at a dose of 0.60 g/kg bw/day increased a relative liver weight, leading to larger relative testis and epididymis in male rats. In our study, except mammary glands and T levels, no pathological changes were found including macroscopic and/or microscopic examinations, as well as urinalysis, hematology and serum/plasma chemistry tests. The mammary glands lesion and changes of blood total testosterone are considered as functional and reversible.

Hampl's study[11] showed that 2 g/kg bw/day soy consumption affected thyroid hormone levels in healthy subjects, which was correlated with phytoestrogen level. Our study showed no changes in thyroid hormone levels, neither thyroid lesions in the animals.

Based on the functional changes in blood total testosterone in the 0.5, 1.5, and 4.5 g/kg bw groups, it is proposed that the no observed adverse effect level (NOAEL) of SIF is considered to be 0.2 g/kg bw/day.

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