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« Long term toxicity of a ROUNDUP herbicide and a ROUNDUP-tolerant genetically modified maize »

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Report

A 104-Week Feeding Study of Genetically Modified Soybeans in F344 Rats

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A chronic feeding study to evaluate the safety of genetically modified glyphosate-tolerant soybeans (GM soybeans) was conducted using F344 DuCrj rats. The rats were fed a diet containing GM soybeans or Non-GM soybeans at a concentration of 30% in basal diet. Non-GM soybeans were a closely related strain to the GM soybeans. These two diets were adjusted to an identical nutrient level. In this study, the influence of GM soybeans in rats was compared with that of the Non-GM soybeans, and furthermore, to assess the effect of soybeans themselves, the groups of rats fed GM and Non-GM soybeans were compared with a group fed a commercial diet (CE-2). General conditions were observed daily and body weight and food consumption were recorded. At termination (104 weeks), animals were subjected to hematology, serum biochemistry, and pathological examinations. There were several differences in animal growth food intake, organ weights, and histological findings between the rats fed the GM and/or Non-GM soybeans and the rats fed CE-2. However, body weight and food intake were similar for the rats fed the GM and Non-GM soybeans. In pathological observation, there was neither an increase in incidence nor any specific type of non-neoplastic or neoplastic lesions in the GM soybeans group in each sex. These results indicate that long-term intake of GM soybeans at the level of 30% in diet has no apparent adverse effect in rats.

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Key words: Rat; genetically-modified soybean, 104-week feeding study; toxicity test

Introduction

Many of the genetically modified soybeans currently available in Japan are tolerant to glyphosate, a chemical herbicide.

Regarding the safety of genetically modified soybeans, neither the effect of the proteins produced from the inserted gene nor the genetically modified soybeans themselves have been reported in short-term administration studies using animals[1-4]. However, many genetically modified foods are consumed over a long period of time, consumer interest in genetically modified foods is high, and the issue of possible adverse health effects due to long-term consumption has not been settled. We conducted a 104-week feeding study of genetically modified soybeans (GM soybeans) using rats to investigate whether there are any biological effects caused by long-term consumption of GM soybeans. A previous study showed that, at week 26 and 52 of feeding, rats fed GM soybeans do not show any particular GM-soybean-related abnormalities compared to rats that had been fed non-GM soybeans[5]. In this report, we present the results of the 104-week feeding study of GM soybeans conducted as a continuation of the 52-week feeding study.

Materials and Methods

1. Soybeans For genetically modified soybeans (GM soybeans) in this study, w

e used Pioneer Brand soybeans (Lot No. B3WAH11301-00-0018, variety 90B72) with Roundup Ready gene (glyphosate-tolerant) that were harvested in the USA in 2000. For non-genetically modified soybeans (non-GM soybeans), we used soybeans seeds (non-GM type, no batch specified, variety: 9071) harvested in the USA in 2000. These two varieties are related to each other, and they possess similar properties in terms of growth cycle, morphology, and composition. All soybeans were stored at 10ºC until they were used. Regarding pesticide and plant hormone levels in both types of soybeans, aside from the quantitation limit of 0.1 ppm of glyphosate being detected in the GM soybeans, there were no significant differences[5].

2. Animals Four-week old male and female F344 rats were purchased from Charles River Laboratories Japan, Inc., and after rearing them for 1 week in preparation for the study, animals showing good growth were selected for the study. The rearing conditions were identical to those described in the previous report[5], and the rats were given *ad libitum* access to food and filtered tap water during the study period.

3. Feed and administration method For test feed, growth purified diet AIN-93G[6] was provided during the rearing period from the beginning of administration to week 26, and for subsequent rearing weeks, AIN-93M[6] for adult rats (both manufactured by Oriental Yeast Co., Ltd.) were given as basic feed. For both basic feeds, the composition was partially modified, and powdered soybeans were added to a proportion of 30% of the feed by dry weight. This is the highest concentration at which a well-formed solid feed can be created without upsetting the nutritional balance. Detailed nutrition composition for both soybean-supplemented feeds is described in the previous report[5]. To ensure that protein and fat content were identical in both soybean groups, casein and non-genetically modified corn oil were added. Also, similar to our previous study, a group fed CE-2 (CLEA Japan, Inc.), which is a frequently used feed for rearing and breeding rodents, was set up [as a control] in addition to the two soybean groups to better understand the effects common to the soybean-supplemented feed groups. The ingredients of CE-2 are given in the previous report[5].

4. Administration period and number of animals

The feeding period was set to 104 weeks. Fifty male and fifty female rats were used for the GM group and the non-GM group, respectively, and 35 rats were used for the CE-2 group. During the feeding period, two male rats in the non-GM group and one female rat in the GM group died. Although the cause is unknown, it is believed that they died from asphyxia due to food getting stuck between their throat and trachea. In this study, these animals were excluded from each study group as cases of accidental death, and the number of animals at the start of administration was 48 male rats in the non-GM group and 49 female rats in the GM group. The animals were handled in this study in accordance with our centre's animal experimentation regulations.

Test Items and Methods

Animals that were alive at week 104 were etherized and exsanguinated by collecting their blood from the jugular vein using a syringe, after which they were used for tests.

1. General conditions and weights The general condition of all animals was observed daily. In addition, whether or not an animal had died was also checked every day. The animals were weighed once every 4 weeks from the start of administration.

2. Food intake and soybean intake Food intake was measured once every 8 weeks from week 6 after the start of administration until week 94. The food intake measurement method is described in the previous report[5], and the amount of soybean intake was calculated from the amount of food intake (g/rat/day and g/kg b.w./day).

3. Haematological analysis For animals that were still alive at the end of the feeding period, white blood cell count (WBC), red blood cell count (RBC), hemoglobin level (Hgb), hematocrit value (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet count (PLT) were measured using a multi-channel blood cell counter (TOA Medical Electronics E-400).

4. Biochemical analysis Total protein (TP), albumin (Alb), alkaline phosphatase activity (ALP), total cholesterol (TCHO), creatinine (CRE), and transaminase activity (AST, ALT) in blood serum prepared from blood samples collected using the abovementioned method were measured using an automatic analyser (Hitachi 7150).

5. Histopathological analysis

Animals that were exsanguinated after collecting blood were dissected and observed macroscopically. Of the extracted organs, the brain, heart, lungs, liver, kidneys, spleen, testes, epididymis, prostate gland, ovaries, adrenal glands, and thymus were weighed. In addition to the weighed organs, the pituitary, thyroid, salivary glands, pancreas, stomach, intestine, bladder, seminal vesicle, preputial glands, uterus, cervix, clitoris, mammary glands, and femur were fixed in buffered formaldehyde, after which paraffin-embedded sections were prepared using a conventional method, and stained with hematoxylin-eosin staining (HE staining). In addition, pathological specimens were prepared in the same way for moribund animals that were killed, as well as those that died during the feeding period.

6. Statistical analysis

For weekly weight and food intake, as well as organ weights, hematological and blood biochemical analysis results, a Student's *t*-test was used to compare the GM and non-GM groups. A Bartlett test was also used to compare the GM and non-GM groups with the CE-2 group for homogeneity of variance. If the variance was uniform, a multiple comparison test was performed using one-way analysis of variance and the Scheffé method. If the variance was non-uniform, a Kruskal-Wallis test and Scheffé's rank-sum test were performed. As for the number of tissue-change incidents, a χ^2-test or Fischer's exact test was performed. The significance level was set to 5% for all tests.

Results

1. General condition and survival rate

During the study period, symptoms that were specific to the GM-soybean intake group were not observed in either male or female rats. For male rats, the survival rates were 76% in the GM group, 73% in the non-GM group, and 80% in the CE-2 group. For female rats, the survival rates were 80% in the GM group, 70% in the non-GM group, and 74% in the CE-2 group. There was no significant difference between the male and female rats among these groups. Also, there were no notable differences in the progression of survival curves for each group (Table 1, Fig. 1). The first fatal case during the feeding period was a female rat in the GM group, which occurred during week 67.

2. Weight and food intake

Weight increase in the GM group and the non-GM group was the same for both male and female rats during the administration period (Fig. 2). Furthermore, there was no significant difference in the weights of male and female rats in either soybean group at the time of necropsy (Table 1).

Regarding weights during the administration period, when the soybean groups were compared to the CE-2 group, the weights of the male rats in both soybean groups were significantly higher compared to the CE-2 group throughout most of the administration period. However, there was no difference in the female rats (Fig. 2, significant difference mark omitted).

Table 1. Final body weights, food intake, soybean intake and survival rate of F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

Group	Final body weight	Food intake	Soybean intake	Survival rate
	g	g/rat/day	g/kgBW/day	%
Male				
GM	267±30[a)]	12.6±0.7	11.4±1.7	76
Non-GM	353±35	12.6±0.6	11.7±1.9	73
CE-2	342±37	13.2±0.8	—	80
Female				
GM	237±21	9.1±1.4[*]	14.6±1.2	80
Non-GM	232±22	8.7±1.0[*]	14.3±1.4	70
CE-2	238±23	10.5±0.9	—	74

[a)]Values are mean±SD.
[*]: Significantly different from CE-2 group, $p<0.05$

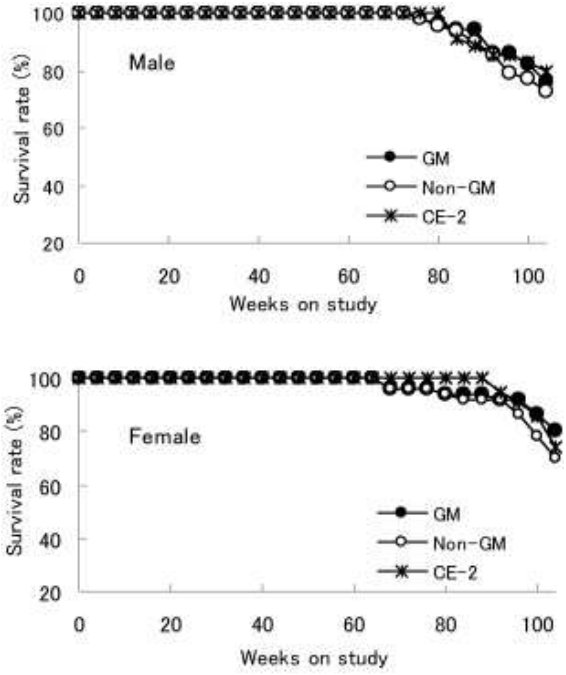

Fig. 1. Survival curves of male and female F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

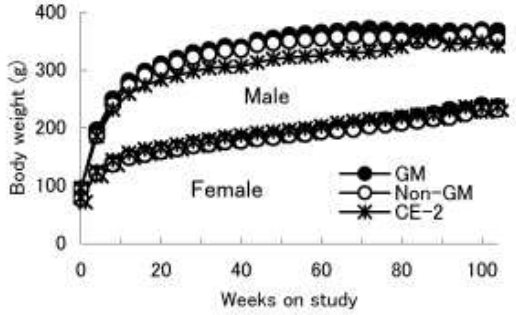

Fig. 2. Growth curves of male and female F344 rats fed diet containing GM and No-GM soybeans for 104 weeks

Initial number of rats; GM, Non-GM; $n=50$, CE-2; $n=35$

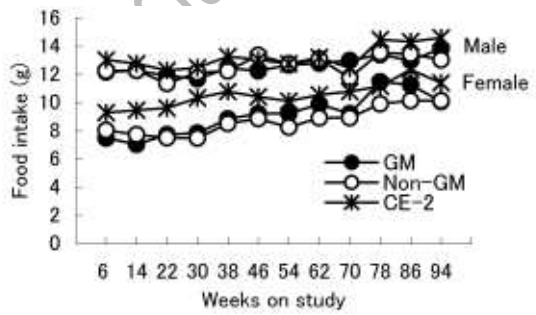

Fig. 3. Food intake curves of male and female F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks ($n=10$)

Regarding food intake, the male rats in both soybean groups consumed 11 to 14 g and the female rats in both soybean groups consumed 7 to 11 g of food throughout the administration period. There was no difference between male or female rats in the soybean groups. When the soybean groups were compared to the CE-2 group, the change in food intake among the male rats in the soybean groups was similar to that in the CE-2 group, but the change in food intake tended to be lower among the female rats in the soybean groups compared to that in the CE-2 group throughout the administration period, and the average food intake was significantly lower (Fig. 3, Table 1).

3. Hematological analysis

When the GM group and the non-GM group were compared for RBC-related analyses, the male rats in the GM group had significantly lower MCHC and the female rats in the GM group also had significantly lower Hgb and Hct compared to the non-GM group. However, the differences in each analysis were minimal, and because there was no difference compared to the CE-2 group, it was believed that these were random fluctuations and not related to any underlying issues. When the soybean groups were compared to the CE-2 group, Hgb and Hct were lower in the male GM group compared to the CE-2 group (Table 2). The differences in these test values were minimal, and because no uniform tendency related to the fluctuations of the test values in the tissue findings was observed in the GM group, these were probably random fluctuations and not related to any underlying issues.

4. Blood serum biochemical analyses

There were no differences in the values between the GM and non-GM groups for either male or female rats. When the soybean groups and the CE-2 group were compared, CRE was significantly higher among the male rats in the soybean groups, and ALT was significantly lower in both male and female rats in the soybean groups (Table 3). The abovementioned differences between the soybean groups and the CE-2 group were minimal, and because no uniform tendency was observed in the histological changes of organs in any group, the observed differences were probably caused by random fluctuations.

Table 2. Hematological data of male and female F344 rat fed diet containing GM and Non-GM soybeans for 104 weeks

Parameter		GM	Non-GM	CE-2
Male		(27)[a]	(25)	(15)
WBC	$\times 10^3/\mu L$	6.59±1.69[b]	6.76±1.43	5.67±1.04
RBC	$\times 10^6/\mu L$	9.3±0.88	9.42±0.83	9.83±0.67
Hgb	g/dL	14.43±1.94‡	14.93±1.47	15.89±0.73
Hct	%	45.08±4.58‡	45.62±3.55	48.69±2.13
MCV	fl	48.53±2.60	48.52±2.32	49.63±1.75
MCH	pg	15.52±1.33	15.83±1.10	16.21±0.72
MCHC	g/dL	31.93±1.52*	32.67±1.17	32.63±0.65
PLT	$\times 10^3/\mu L$	760±143	763±143	651±69
Female		(29)	(32)	(11)
WBC	$\times 10^3/\mu L$	3.34±0.98	3.52±1.10	3.65±1.92
RBC	$\times 10^6/\mu L$	8.38±0.66	8.71±0.35	8.78±0.60
Hgb	g/dL	14.67±1.52*	15.29±0.42	15.09±1.17
Hct	%	44.21±3.63*	45.98±1.71	46.20±2.04
MCV	fl	52.77±1.83	52.79±0.83	52.71±2.10
MCH	pg	17.48±1.09	17.56±0.45	17.20±0.82
MCHC	g/dL	33.12±1.28	33.26±0.74	32.62±1.26
PLT	$\times 10^3/\mu L$	554±119	593±54	526±84

[a]Number of rats examined. [b]Values are mean±SD.
*: Significantly different from Non-GM, $p<0.05$
‡: Significantly different from CE-2, $p<0.05$

Table 3. Biochemical data of male and female F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

Parameter		GM	Non-GM	CE-2
Male		(33)[a]	(32)	(11)
ALB	g/dL	2.34±0.18[b]	2.37±0.19	2.38±0.17
ALP	IU/L	322±71	318±61	374±80
TCHO	mg/dL	134±27	128±26	136±40
CRE	mg/dL	0.54±0.04‡	0.54±0.05‡	0.49±0.04
AST	U/L	107±22	103±19	117±33
ALT	U/L	49.9±19.8‡	50.7±16.9‡	68.6±23.6
TP	g/dL	6.59±0.36	6.71±0.37	6.73±0.37
Female		(33)	(33)	(9)
ALB	g/dL	2.71±0.25	2.65±0.22	2.54±0.23
ALP	IU/L	261±69	282±106	298±43
TCHO	mg/dL	123±23	121±17	112±22
CRE	mg/dL	0.57±0.04	0.56±0.05	0.58±0.05
AST	U/L	116±31	101±21.0	120±26
ALT	U/L	45.0±11.3‡	42.9±10.2‡	70±16
TP	g/dL	6.81±0.53	6.71±0.36	6.53±0.35

[a]Number of rats examined. [b]Values are mean±SD.
‡: Significantly different from CE-2, $p<0.05$

5. Organ weights

There were no significant differences in organ weights in either male and female rats between the soybean groups. When the soybean groups were compared to the CE-2 group, the absolute weights and relative weights of the kidneys were lower among male rats in both soybean groups, and although there was no difference in the absolute weights of the livers, their relative weights were lower. Furthermore, the testes were larger in terms of their absolute weights, and they also tended to be larger in terms of their relative weights. Among the female rats in the soybean groups, the liver, kidneys, uterus, and adrenal glands were smaller in terms of their absolute weights and their relative weights compared to the CE-2 group (Tables 4, 5).

6. Macroscopic findings and histological findings

During necropsies, aside from isolated incidents of outgrowths such as tumours and changes associated with naturally-occurring lesions, changes characteristic of the GM soybean group were not observed. When the soybean groups and the CE-2 group were compared, no clear difference in any of the organs was observed. Below are our histological findings of lesions that were found in the organs,

categorized as either non-tumour involvement or tumour involvement.

1) Non-tumour involvement

The main lesions that were observed in both soybean groups and the CE-2 group were: proliferation of the bile duct in the liver as well as fibrosis and altered cell foci of the area surrounding the bile duct, calcification of the cortico-medullary junction in the kidneys, myocardial inflammation of the heart muscles, calcification of the vascular intima in the lungs, atrophy and hypertrophy of the acinar cells in the pancreas, and fatty infiltration of the parotid glands. These lesions had already been observed in the 52-week study[5]. In the 104-week study, the number of incidents and severity of these lesions increased, and furthermore, in addition to the abovementioned lesions, localized necrosis and granuloma in the liver, inflammatory changes in the heart muscles, inflammatory lesions of the preputial and clitoral glands, and pituitary cysts were observed in each group. Also, in terms of hyperplastic lesions, hyperplasia of the alveolar epithelium, anterior lobe of the pituitary gland, C-cell of the thyroid gland, adrenal medullary cells, pancreatic ancinus, interstitial cells of the testes, prostate epithelial cells, endometrial epithelial cells, and bone-marrow stromal cells, as well as enlargement of the spleen and the parotid ancinar cells, were observed in each of the groups. All of these lesions have been reported as naturally occurring lesions due to the aging of the rats[7-9]. Out of the non-tumour involvement observations, the foci of cellular alterations can be classified into basophilic, clear cell, eosinophilic, and mixed in HE tissues[10]; however, of these, the vacuolar foci of cellular alterations are difficult to distinguish from localized steatosis, and both were systematically listed as vacuolization in this report (Table 6). Also, in the kidneys, in addition to the renal tubular degeneration observed in week 52, the number of incidents and severity of thickening of the tubule basement membrane and atrophy of the tubular epithelium, as well as eosinophilic pigmentation, also increased, and interstitial fibrosis as well as cellular infiltration were also observed. In the 104-week study, these lesions were collectively referred to as chronic nephropathy[11,12].

Comparison between the GM group and the Non-GM group

As shown in Table 6, lesions that were characteristic of GM soybeans were not observed. Also, there was no significant difference between the GM group and the non-GM group in terms of the lesions' incidence rates. Among the changes that were observed in all or the majority of the male and female rats in both soybean groups, proliferation of the bile duct in the liver, foci of cellular alterations in the liver, and chronic nephropathy were compared in terms of their severity, incidence rate and the number of lesions per histological specimen in each rat. However, there were no significant differences between the soybean groups in either male or female rats (Tables 7-9).

Comparison between the soybean groups and the CE-2 group

Lesions that occurred significantly more frequently in the soybean groups than in the CE-2 groups were cystic growth of the small bile duct, vacuolization and spongiosis of the hepatic cells, as well as hyperplasia of the acinar cells in the pancreas among the male rats, proliferation and fibrosis of the bile duct among the female rats, and mixed foci of cellular alterations in both male and female rats. Although basophilic cell foci in the liver were observed in almost all female and male rats in both the soybean groups and the CE-2 group, the number of lesions per histological specimen was higher in the soybean groups than in the CE-2 group for both male and female rats (Table 8).

In addition, lesions that occurred significantly less frequently in the soybean groups than in the CE-2 group were localized enlargement of the parotid acinar cells in male rats, and chronic nephropathy as well as calcification of the cortico-medullar junction in female rats. Granuloma of the liver and atrophy of the pancreatic acinar cells occurred less in both male and female rats. Chronic nephropathy tended to occur less often among male rats in the soybean groups.

Table 4. Organ weights of male F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

Organ		Group					
		GM		Non-GM		CE-2	
Body weight	g	368±30[a]	(38)[b]	353±35	(34)	343±37	(28)
Absolute weight							
Brain	mg	2,154±46	(38)	2,121±65	(34)	2,092±59	(28)
Heart	mg	1,043±133	(38)	990±77	(34)	964±76	(28)
Lungs	mg	1,145±113	(38)	1,084±74	(34)	1,065±84	(28)
Liver	g	9.97±1.05	(38)	9.56±1.11	(34)	10.05±1.63	(28)
Kidneys	mg	2,176±121*	(38)	2,187±127*	(34)	2,337±204	(28)
Spleen	mg	690±117	(37)	622±98	(33)	691±133	(28)
Testes	mg	3,011±536*	(38)	3,071±307*	(33)	2,551±609	(20)
Adrenals	mg	42.0±5.3	(37)	42.1±5.4	(33)	44.6±5.5	(27)
Relative weight (weight/100 g body weight)							
Brain	mg	589±49		606±62		617±63	
Heart	mg	285±43		282±22		283±22	
lungs	mg	313±36		309±32		314±39	
Liver	g	2.71±0.21*		2.71±0.20*		2.93±0.31	
Kidneys	mg	594±49*		624±70*		685±48	
Spleen	mg	207±99		177±32		212±70	
Testes	mg	823±159		878±109		759±161	
Adrenals	mg	11.4±1.7		12.0±2.0		13.2±1.8	

[a] Values are mean±SD. [b] Number of rats examined.
*: Significantly different from CE-2 group, $p<0.05$

Table 5. Organ weights of female rats fed diet containing GM and Non-GM soybeans for 104 weeks

Organ		Group					
		GM		Non-GM		CE-2	
Body weight	g	237±21[a]	(39)[b]	232±19	(34)	238±23	(26)
Absolute weight							
Brain	mg	1,969±36	(39)	1,966±60	(34)	1,937±37	(26)
Heart	mg	714±68	(39)	675±52	(34)	714±56	(26)
Lungs	mg	824±99	(39)	794±101	(34)	804±50	(26)
Liver	g	6.84±0.79*	(39)	6.70±0.55*	(34)	7.42±0.75	(26)
Kidneys	mg	1,543±127*	(39)	1,492±94*	(34)	1697±95	(26)
Spleen	mg	459±103	(39)	439±112	(34)	505±162	(26)
Ovaries	mg	63.5±16.5	(39)	62.8±12.3	(34)	61.4±11.6	(25)
Uterus	mg	711±120*	(32)	707±118*	(33)	823±197	(20)
Adrenals	mg	43.5±4.2*	(38)	39.9±2.6*	(34)	53.47±5.48	(26)
Relative weight (weight/100 g body weight)							
Brain	mg	838±82		852±74		820±74	
Heart	mg	302±29		292±20		302±30	
Lungs	mg	350±52		343±36		340±35	
Liver	g	2.89±0.26*		2.90±0.22*		3.12±0.28	
Kidneys	mg	624±58*		646±52*		720±63	
Spleen	mg	194±43		190±50		212±61	
Ovaries	mg	27.5±5.0		27.2±5.7		26.0±4.6	
Uterus	mg	296±50*		308±66		358±107	
Adrenals	mg	18.7±2.6*		17.3±1.6*		22.5±2.8	

[a] Values are mean±SD. [b] Number of rats examined.
*: Significantly different from CE-2, $p<0.05$

Table 6. Nonneoplastic lesions in male and female F344 rats fed diets containing GM soybeans for 104 weeks

		Male/Group			Female/Group		
		GM	Non-GM	CE-2	GM	Non-GM	CE-2
Number of rats initially used[a]		50	48	35	49	50	35
Number of surviving rats at termination of study		38	35	28	39	35	26
Number of rats examined microscopically[b]		38	35	28	39	35	26
Organs	Lesions						
Liver	Bile duct, proliferation	38[c] (100)[d]	35 (100)	28 (100)	33 (85)*	28 (80)*	7 (27)
	fibrosis	38 (100)	35 (100)	28 (100)	20 (51)*	12 (34)*	1 (4)
	cyctic dilatation	15 (35)*	15 (43)*	4 (14)	3 (8)	2 (6)	0 (0)
	Altered cell foci, basophilic	38 (100)	35 (100)	28 (100)	39 (100)	34 (97)	26 (100)
	clear cell	27 (71)	27 (77)	14 (50)	13 (33)	14 (40)	5 (19)
	eosinophilic	7 (18)	11 (31)	12 (43)	6 (15)	8 (23)	1 (4)
	mixed	25 (66)*	15 (43)*	7 (25)	10 (26)*	7 (20)*	0 (0)
	Necrosis, focal	4 (11)	3 (9)	5 (18)	6 (15)	6 (17)	4 (15)
	Vacuolation, zonal	18 (47)*	16 (46)*	4 (14)	8 (21)	6 (17)	3 (12)
	focal	6 (16)	3 (9)	3 (11)	5 (13)	5 (14)	6 (23)
	Spongiosis	11 (29)*	5 (14)	2 (7)	2 (5)	1 (3)	1 (4)
	Microgranuloma	4 (11)*	4 (11)*	24 (86)	7 (18)*	7 (20)*	26 (100)
Kidney	Nephropathy	21 (55)	18 (51)	21 (75)	8 (21)*	5 (14)*	11 (42)
	Calcification, cortico-medullary junction	0 (0)	2 (6)	0 (0)	11 (28)*	8 (23)*	23 (88)
Heart	Myocardial inflammation	28 (74)	21 (60)	21 (75)	30 (77)	20 (57)	21 (81)
	Myocardial fibrosis	8 (21)	6 (17)	5 (18)	10 (26)	12 (34)	11 (42)
Lung	Calcification, vascular intima	33 (87)	31 (89)	23 (82)	20 (51)	20 (57)	18 (69)
	Inflammation, focal, chronic	1 (3)	2 (6)	3 (11)	1 (3)	2 (6)	0 (0)
	Foamy cell aggregation, focal	1 (3)	3 (9)	0 (0)	0 (0)	1 (3)	0 (0)
	Hyperplasia, alveolar epithelium	4 (11)	6 (17)	2 (7)	2 (5)	5 (14)	1 (4)
Pituitary	Cyst, anterior lobe	8 (21)	13 (37)	6 (21)	21 (54)	16 (46)	15 (58)
	Angiectasis, anterior lobe	6 (16)	3 (9)	5 (18)	5 (13)	5 (14)	7 (27)
	Cyst, Rathke's cleft	2 (5)	1 (3)	2 (7)	16 (41)	10 (29)	6 (23)
	Hyperplasia, anterior lobe	10 (26)	7 (20)	9 (32)	10 (26)	8 (23)	5 (19)
Thyroid gland	C-cell, hyperplasia	11 (29)	11 (31)	5 (18)	10 (26)	8 (23)	10 (38)
	Ultimobranchial body	0 (0)	1 (3)	1 (4)	0 (0)	1 (3)	0 (0)
Parathyroid gland	Hyperplasia	0 (0)	0 (0)	2 (7)	2 (5)	1 (3)	1 (4)
Adrenal gland	Cortex, vacuolation, focal,	7 (18)	4 (11)	7 (25)	7 (18)	7 (20)	5 (19)
	hyperplasia, focal	2 (5)	2 (6)	1 (4)	2 (5)	2 (6)	1 (4)
	accessory adrenal nodule	3 (8)	2 (6)	8 (29)	4 (10)	6 (17)	4 (15)
	Medulla, hyperplasia	2 (5)	5 (14)	2 (7)	2 (5)	2 (6)	3 (12)
Spleen	Pigmentation, hemosiderin	0 (0)	0 (0)	0 (0)	2 (5)	0 (0)	0 (0)
	Extramedullary hematopoiesis	2 (5)	2 (6)	2 (7)	2 (5)	0 (0)	3 (12)
	Congestion	2 (5)	4 (11)	1 (4)	3 (8)	1 (3)	0 (0)
Bone marrow	Atrophy	0 (0)*	0 (0)*	5 (18)	6 (16)	4 (11)	5 (19)
	Hyperplasia, stromal cell	2 (5)	1 (3)	1 (4)	5 (13)	3 (9)	5 (19)
Pancreas	Atrophy, acinar cell, lobular	7 (18)*	10 (29)*	16 (57)	3 (8)*	2 (6)*	7 (27)
	Hypertrophy, acinar cell, focal	1 (3)	2 (6)	3 (11)	2 (5)	3 (9)	3 (12)
	Hyperplasia, acinar cell	9 (24)*	6 (17)*	1 (4)	0 (0)	0 (0)	2 (8)
	Hyperplasia, islet cell	0 (0)	0 (0)	4 (14)	1 (3)	2 (6)	1 (4)
Parotid gland	Hypertrophy, acinar cell, focal	2 (5)*	1 (3)*	8 (29)	4 (10)	2 (6)	5 (19)
	Atrophy, acinar cell, focal	3 (8)	1 (3)	3 (11)	2 (5)	6 (17)	3 (12)
Testes	Atrophy, seminiferous tubules	12 (32)	7 (20)	11 (39)	—	—	—
	Interstitial cell hyperplasia	16 (42)	15 (43)	17 (61)	—	—	—
Prostate	Inflammation, suppurative	1 (3)	1 (3)	1 (4)	—	—	—
	Inflammation, chronic	0 (0)	1 (3)	0 (0)	—	—	—
	Hyperplasia	6 (16)	6 (17)	10 (36)	—	—	—
Mammary gland	Ectasia	3 (8)	4 (11)	1 (4)	3 (8)	1 (3)	2 (8)
	Adenosis (hyperplasia)	0 (0)	0 (0)	1 (4)	6 (15)	3 (9)	0 (0)
Preputial/Clitral gland	Inflammation, suppurative	2 (5)	3 (9)	2 (7)	0 (0)	0 (0)	3 (12)
	Inflammation, chronic	30 (79)	28 (80)	22 (79)	3 (8)	6 (17)	1 (4)
	Hyperplasia	1 (3)	0 (0)	0 (0)	1 (3)	1 (3)	1 (4)
Uterus	Hyperplasia	—	—	—	2 (5)	3 (9)	3 (12)
	Cystic change	—	—	—	3 (8)	3 (9)	3 (12)

[a] Two rats in the male Non-GM group and 1 rat in the female GM group were excluded from the 50 rats initially used in the study because of accidental early deaths. [b] Nonneoplastic lesions were observed in the rats alived at the termination of the study. [c] Number of rats with lesions. [d] Incidence of lesions (%). *: Significantly different from CE-2 group, $p < 0.05$

Table 7. Grade and incidence of bile duct proliferation in F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

Grade	Male/Group			Female/Group		
	GM 38[a]	Non-GM 35	CE-2 28	GM 39	Non-GM 35	CE-2 26
	(100)[b]	(100)	(100)	(85)	(80)	(27)
−	0[c] (0)[d]	0 (0)	0 (0)	6 (15)	7 (20)	19 (73)
+	0 (0)	0 (0)	0 (0)	28 (72)	27 (77)	7 (27)
++	22 (58)	18 (51)	12 (43)	5 (13)	1 (3)	0 (0)
+++	16 (42)	17 (49)	16 (57)	0 (0)	0 (0)	0 (0)

[a]Number of rats observed. [b]Total incidence of lesion (%). [c]Number of rats with lesion. [d]Incidence of lesion with each grade (%). Grade of lesions: − none, + slight, ++ moderate, +++ marked

2) Tumour involvement

Tumours that were commonly found across all groups were interstitial cell adenoma in the testes and pancreatic acinar cell adenoma among male rats, endometrial polyps among female rats, and anterior lobe adenoma in the pituitary gland and C-cell adenoma in the thyroid gland among both male and female rats. All of these tumours are known to frequently occur naturally due to the aging process of F344 rats[7,8,13]. Mononuclear cell leukemia also occurred slightly more frequently in female rats than in male rats (Table 10). Although other adenoma and carcinoma were observed in various organs, all of them occurred sporadically. For the mononuclear cell leukemia, although not indicated in Table 10 the infiltration of leukemia cells into the liver, lungs, and other organs was observed.

Table 8. Number of basophilic, clear and eosinophilic altered hepatocellular foci in F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

Sex	Type of foci	Group		
		GM	Non-GM	CE-2
		(38)[a]	(35)	(28)
Male	Basophilic cell foci	4.21±2.25[b]‡	3.78±1.90‡	1.69±1.26
	Clear cell foci	1.17±1.09	0.87±0.80	0.71±1.00
	Eosinophilic foci	0.18±0.46	0.21±0.41	0.3±0.42
		(39)	(35)	(26)
Female	Basophilic cell foci	9.08±3.79‡	7.56±2.90‡	3.53±2.77
	Clear cell foci	0.22±0.38	0.31±0.46	0.09±0.20
	Eosinophilic foci	0.08±0.18	0.14±0.29	0.02±0.10

[a]Number of rats observed. [b]Values represent mean ±SD (No. of foci/liver section).
‡: Significantly different from CE-2, $p < 0.05$

Table 9. Severity and incidence of chronic nephropathy in F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

Grade	Male/Group			Female/Group		
	GM 38[a]	Non-GM 35	CE-2 28	GM 39	Non-GM 35	CE-2 26
	(55)[b]	(51)	(75)	(21)‡	(14)‡	(42)
−	17[c] (45)[d]	17 (48)	7 (25)	30 (77)	30 (86)	15 (58)
+	18 (47)	17 (49)	15 (54)	8 (21)	5 (14)	10 (38)
++	2 (5)	1 (3)	6 (21)	1 (2)	0 (0)	1 (4)
+++	1 (3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

a) Number of rats observed. b) Total incidence of lesion (%). c) Number of rats with lesion. d) Incidence of lesion with each grade (%). Grade of lesions: − none, + slight, ++ moderate, +++ marked
‡: Significantly different from CE-2 group, $P < 0.05$.

Table 10. Neoplastic lesions in F344 rats fed diet containing GM and Non-GM soybeans for 104 weeks

		Group/Male			Group/Female		
		GM	Non-GM	CE-2	GM	Non-GM	CE-2
Number of rats initially used in study[a]		50	48	35	49	50	35
Number of surviving rats at termination of study		38	35	28	39	35	26
Number of rats examined microscopically[b]		50	48	35	49	50	35
Number of rats with tumor		39	33	28	35	32	28
Organs	Type of tumors						
Testes	Interstitial cell, adenoma	6[c] (12)[d] ‡	4 (8)‡	20 (57)	—	—	—
Preputial/Clitoral gland	Adenoma	3 (6)	0	0	1 (2)	2 (4)	1 (3)
	Carcinoma	1 (2)	0	0	1 (2)	0	1 (3)
Ovary	Granulosa cell tumor	—	—	—	1 (2)	0	0
	Granulosa-theca cell tumor	—	—	—	1 (2)	1 (2)	0
Uterus	Polyp, endometrial stromal	—	—	—	8 (16)	7 (14)‡	12 (34)
	Adenoma, endometrial	—	—	—	1 (2)	2 (4)	0
	Carcinoma, endometrial	—	—	—	2 (4)	0	0
	Sarcoma, endometrial	—	—	—	0	1 (2)	1 (3)
	Leiomyoma	—	—	—	1 (2)	0	0
Vagina	Polyp	—	—	—	0	0	1 (3)
Mammary gland	Fibroadenoma	1 (2)	0	0	1 (2)	0	0
	Adenoma	0	0	0	1 (2)	1 (2)	0
	Adenocarcinoma	0	0	1 (3)	1 (2)	0	0
Pituitary gland	Adenoma, anterior lobe	13 (26)	11 (23)	5 (14)	7 (14)	7 (14)	11 (31)
Thyroid gland	Adenoma, c-cell	8 (16)	12 (25)	6 (17)	10 (20)‡	9 (18)‡	1 (3)
	Carcinoma, follicular cell	1 (2)	0	1 (3)	0	1 (2)	0
Adrenal gland	Pheochromocytoma, benign	2 (4)	3 (6)	2 (7)	1 (2)	1 (2)	0
	Pheochromocytoma, malignant	1 (2)	0	0	0	0	0
Pancreas islet	Adenoma	3 (6)	4 (8)	4 (11)	0	1 (2)	0
Pancreas	Adenoma	4 (8)‡	3 (6)‡	0	0	1 (2)	0
Intestine	Adenocarcinoma	0	1 (2)	0	0	0	0
Lung	Alveolar/bronchiolar adenoma	2 (4)	2 (4)	1 (3)	0	1 (2)	2 (6)
	Adenocarcinoma	1 (2)	0	0	0	0	0
	Adenocarcinoma, metastatic (uterus)	0	0	0	1 (2)	0	0
	Squamous cell carcinoma, metastatic (skin)	1 (2)	0	0	0	0	0
Heart	Fibroma	1 (2)	0	0	0	0	0
Kidney	Lipoma	0	1 (2)	1 (3)	0	0	0
Urinary bladder	Papilloma	1 (2)	2 (4)	0	0	0	0
Abdominal cavity	Mesothelioma	4 (8)	2 (4)	2 (6)	0	0	0
	Lipoma	0	1 (2)	0	1 (2)	0	0
Skin/Subcutis	Fibroma	3 (6)	1 (2)	1 (3)	2 (4)	0	0
	Squamous cell carcinoma	1 (2)	0	0	0	0	0
	Basal cell adenoma	1 (2)	0	0	0	0	0
	Carcinoma, Zymbal's gland	0	0	0	0	1 (2)	0
Bone	Osteosarcoma	1 (2)	0	0	0	0	0
Spleen	Fibroma	1 (2)	0	0	0	0	0
Brain	Oligodendroglioma	0	0	0	0	1 (2)	0
	Astrocytoma	0	0	1 (3)	0	0	0
Systemic	Mononuclear cell leukemia	3 (6)	2 (4)	1 (3)	7 (14)	4 (8)	4 (11)

a) Two rats in the male Non-GM group and 1 rat in the female GM group were excluded from the 50 rats initially used in the study because of accidental early deaths. b) Number of rats examined include the number of deaths before the termination of the study. c) Number of rats with tumor. d) Incidence of tumor (%). ‡: Significantly different from CE-2 group, $p<0.05$

Comparison between the GM group and the Non-GM group

As shown in Table 10, there was no onset of tumours that was characteristic of the GM group in either male or female rats. Also, the incidence of tumours was not significantly different between the soybean groups for either male or female rats.

Comparison between the soybean groups and the CE-2 group

Compared to the CE-2 group, the incidence of interstitial cell adenoma was significantly lower in

the soybean groups, but the incidence of pancreatic acinar cell adenoma in male rats and C-cell adenoma in the thyroid gland of female rats were higher.

Discussion

1) Overview of the 104-week study

A diet containing 30% GM soybeans was given to male and female rats for 104 weeks to study whether there are biological effects from consuming GM soybeans. The rats fed GM soybeans were compared to rats fed non-GM soybeans in terms of their survival rates, body weights, organ weights, hematological/blood serum biochemical analysis and histological analysis results. The results indicate that there was no apparent adverse effect caused by consuming GM soybeans. There are reports of animal testing for observing biological effects caused by GM soybeans in short-term feeding studies. However, 104 weeks (2 years) is a long study period that covers the entire rat lifespan. Since there are no reports of such long-term studies to date, we believe that ours is the first long-term study to be conducted thus far.

2) Validity as a long-term feeding study

In carcinogenicity studies and other long-term studies, the survival rate in the control group or the low-dose group at the end of the study must be at least 50% to be able to assess the study results. Also, if 10% or more of the animals were lost due to cannibalism and other rearing issues, the study is considered invalid for assessment[14].

In this study, the survival rates of the CE-2 group, which was set up as the comparative control group, were 80% for the male rats and 74% for the female rats. Based on this, the rearing conditions of the animals in this study were considered to be good, and we conclude that the study was conducted under appropriate testing conditions for assessing the biological effects of GM soybeans in a long-term feeding study.

3) Feed supplemented with 30% of soybeans

In this study, the AIN-93G growth purified feed was supplemented with soybeans at a concentration of 30%. Although up to 13-week feeding studies using rats and feed with soybean concentrations of 30, 60, and 90% have been reported, no changes in weights or food intake by up to 60% Soybean (ref 3) and no adverse effects have been observed[3]. Since there were no clear differences between the soybean groups and the CE-2 group in terms of weight and other analysis results, long-term consumption of a diet with a soybean concentration of 30% does not appear to cause adverse effects, particularly those related to soybeans, on the growth of animals.

4) Comparison between the GM group and the non-GM group

There were no differences in terms of body weights, survival rates and food intake between the soybean groups for male or female rats. In the 52-week study[5], the body weights of male rats in the GM group increased after week 36 compared to the non-GM group, and there was a significant difference at the time of necropsies carried out at week 52. However, there was no clear difference between the two soybean groups in the present study, and because there was no difference in food intake, we believe that what we observed in the 52-week study was not related to the consumption of GM soybeans. In the hematological analyses, MCHC was lower among the male rats in GM group, and Hgb and Hct were lower among the female rats in the GM group compared to those in the non-GM group. However, their variances compared to the non-GM group were slight and we believe that they were random fluctuations and not caused by GM soybean-specific effects on the hematopoietic system.

As for blood serum biochemistry, in the 52-week study[5], AST and ALT were higher among male rats at week 26 and ALT was higher at week 52 in the GM group compared to the non-GM group, and these results suggested hepatic dysfunction. However, in the 104-week study, there were no differences in the AST and ALT levels between the soybean groups in either male or female rats, and because there were no clear differences in terms of liver masses and histological changes, GM soybeans do not appear to affect hepatic functions.

Regarding organ weights, there were no differences between the GM group and the non-GM group for either male or female rats. This result reflects the fact that there were no differences in the hematological and blood serum biochemistry analysis results between the two soybean groups, as well as the fact that there were no large differences between the two soybean groups in terms of histological changes, particularly in the onset of non-tumour involvement.

In the histological analyses, the various non-neoplastic and neoplastic lesions that were observed

across all groups were naturally-occurring lesions that were caused by the aging process in rats[7-9,13]. There were no notable differences in either male or female rats in terms of the incidence of these lesions between the two soybean groups. The foci of cellular alterations in the liver, chronic nephropathy and other naturally-occurring lesions that were commonly found are known to become severe or occur more frequently as a result of the adverse effects in the affected organs due to chemical substances, etc.[9,15,16] We therefore investigated whether or not the consumption of GM soybeans could cause adverse effects in the organs based on the differences in the number of lesions and the severity of the lesions in the tissue of each rat for the proliferation of the bile duct, foci of cellular alterations, and chronic nephropathy as representative lesions of those that occurred commonly across all groups; however, there were no notable differences in how the lesions manifested themselves in the two soybean groups. Based on these results, GM soybeans do not have characteristic effects that differ from the properties of non-GM soybeans in terms of causing lesions. The results also indicate that GM soybeans do not have any adverse effects on organs that may affect the onset of naturally-occurring lesions. The fact that there were no obvious differences between the two soybean groups in terms of organ weights and blood serum biochemistry also probably reflects the lack of differences in the abovementioned histological analyses. The incidence of neoplastic lesions that were observed frequently in both soybean groups was within the scope of incidence reported in long-term rat rearing [13], and there were no significant differences in the incidence rate between the soybean groups for either male or female rats. These results indicate that GM soybeans do not possess specific tumour-inducing properties or modify the onset of naturally-occurring tumours that are specific to rats.

For the non-GM soybeans that were used for comparison with GM soybeans in this study, a variant related to the GM soybean was used. In addition to the presence of a transgene and a genetically derived protein (CP4-EPSPS), GM soybeans differ somewhat from non-GM soybeans in regard to their protein and fat content, but the difference is minute, and there were no significant differences in their properties. Both soybean-containing diets were prepared so that protein, fat, starch, and sugar content were practically identical and they had the same caloric profile. There was also no significant difference between the two soybean groups in terms of plant sex hormone levels[5].

With regard to the safety of conventional GM soybeans, the issue of allergenicity of the genetically derived protein (CP4-EPSPS) has been raised. However, because the CP4-EPSPS protein is digested quickly in artificial gastric and intestinal juices, it is believed that it will be easily digested as a food ingredient intended for humans. Furthermore, CP4-EPSPS is not considered to possess any allergenic characteristics[1].

5) Comparison between the soybean groups and the CE-2 group

In this study, to understand the effects that are characteristic of and common to GM and non-GM soybean-supplemented diets, the study results were compared to those obtained from rats that were fed a standard rodent feed (CE-2) typically used for toxicity tests.

When the soybean groups and the CE-2 group were compared, there were significant differences in body weight among male rats, food intake among female rats, and some organ weights in both sexes. Histologically, there were significant differences between the two soybean groups and the CE-2 group in the incidence and severity of naturally-occurring lesions that mainly involved the liver and kidneys. Furthermore, in the soybean groups, the incidence of tumours in the testes and uterus were lower compared to the CE-2 group, and the incidence of pancreatic and thyroid tumours were higher. Because the differences in these test results were not significant between the GM and the non-GM groups, the differences between the soybean groups and the CE-2 group likely reflect differences in the ingredients of the diets.

Various biological effects of soybeans have been reported, including extended survival period and suppression of chronic nephropathy when casein — usually used in test diets— is replaced with soybean protein[17,18], enlarging and proliferating effects on pancreatic acinus caused by trypsin inhibitors in soybeans[19], suppression of weight gain, suppression of accumulating body fat, and reduced food consumption in ovariectomized rats and mice due to soybean isoflavones and genistein, which is an isoflavone[20,21], and effects on hepatic functions associated with histological changes, such as

proliferation of the bile duct and enlargement of both male and female sexual organs[22]. All of these are believed to be caused by female-hormone-like effects caused by isoflavones. Furthermore, female hormones are known to affect proliferative lesions of the liver, the development of endocrine organs, and the onset of proliferative lesions[23-26]. The difference in the incidence of interstitial cell adenoma in the testes and pituitary adenoma that were observed in the present study between the soybean groups and the CE-2 group may be due to the effects of plant sex hormones in the soybeans, such as genistein. However, because there were no differences in body weights, uterus mass or histological changes, further studies are necessary to assess whether or not genistein affected these results. As for non-soybean dietary ingredients, a high-sucrose diet is known to enhance the foci of cellular alteration and tumours, as well as the onset of fatty liver[27]. In the soybean-supplemented diet used in the present study, sucrose was added at a concentration of 10% as a source of carbohydrates. This is not a particularly high concentration, but because many cases of vacuolization believed to be steatosis were observed among male rats in the GM group, the histological differences in the livers observed between the soybean groups and the CE-2 group may be due to long-term consumption of sucrose, which is not found in CE-2.

The differences between the soybean groups and the CE-2 group observed in this study may be caused by non-soybean nutrition differences in the diets. For this reason, it is necessary to study this subject from nutritional and endocrinological perspectives in the future to identify the cause of the differences observed between different diets.

Conclusion

GM soybeans and non-GM soybeans were added to diets at a concentration of 30% and fed to rats continuously for 104 weeks to observe the biological effects caused by GM soybeans. The fact that there were no differences in body weight and other analysis results indicates that GM- and non-GM-soybean-added diets are not significantly different in their nutritional and chemical properties. For this reason, we believe that long-term consumption of GM soybeans does not cause any adverse effects.

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