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ACUTE AND LONG-TERM SAFETY EVALUATION OF A NOVEL IH636  
GRAPE SEED PROANTHOCYANIDIN EXTRACT

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*Abstract*

Grape seed proanthocyanidins are known to possess a broad spectrum of pharmacological, medicinal and therapeutic properties. Previous studies in our laboratories have demonstrated the various protective abilities of a novel IH636 grape seed proanthocyanidin extract (GSPE) against various pathologic conditions. However no extensive safety studies have been conducted on grape seed proanthocyanidins to date. This study demonstrates the acute and chronic safety studies on GSPE. Acute oral toxicity, dermal toxicity, dermal irritation and eye irritation studies have been conducted. The LD<sub>50</sub> of GSPE was found to be greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats. The LD<sub>50</sub> of GSPE was found to be greater than 2000 mg/kg when administered once for 24 hr to the clipped, intact skin of male and female albino rats. In addition, 2000 mg/kg was found to be the no-observed-effect level (NOEL) for systemic toxicity under the conditions of the study. In a dermal irritation study, GSPE received a descriptive rating classification of moderately irritating. Extensive chronic studies were also conducted. We have assessed the effects of chronic administration of 100 mg GSPE/kg/day for twelve months and its effect on seven vital target organs, namely, brain, heart, intestine, kidney, liver, lung and spleen, and on serum chemistry changes in male B6C3F1 mice. Furthermore, the dose-dependent chronic effects of GSPE in female B6C3F1 mice were evaluated. Mice were fed 0, 100, 250 or 500 mg GSPE/kg/day for six months and the effects of GSPE exposure were examined on brain, duodenum, heart, kidney, liver, lung, pancreas and spleen, and on serum chemistry changes in female mice. These acute studies demonstrated that GSPE is safe and did not cause any detrimental effects *in vivo* under the conditions investigated in this study.

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*Introduction*

Proanthocyanidins are naturally occurring compounds widely available in fruits, vegetables, nuts, seeds, flowers and bark (Hocman, 1989; Masquelier *et al.*, 1979; Rice Evans *et al.*, 1996). These compounds are a group of polyphenolic bioflavonoids diverse in chemical structure, pharmacology and characteristics (Chen *et al.*, 1996; Masquelier *et al.*, 1979; Shahidi and Wanasundara, 1992). Proanthocyanidins have been reported to exhibit a wide range of biological effects (Jovanovic *et al.*, 1994; Kolodziej *et al.*, 1995; Chen *et al.*, 1996). Proanthocyanidins are known to be non-toxic, and if absorbed and biologically active *in vivo*, may prevent free radical mediated cytotoxicity and lipid peroxidation as well as oxidation of low density lipoproteins (LDL) (Frankel *et al.*, 1993). Proanthocyanidins have been shown to prevent the growth of breast cancer cells and to inhibit the enzyme involved in the replication of rhinoviruses (common cold) and HIV viruses (Hocman, 1989).

Substantial documentation exists showing that a number of phenolic/bioflavonoid compounds have the ability to protect against drug and chemically-induced toxicities in diverse model systems. Recent research suggests that these proanthocyanidins may play a crucial role in reducing various types of heart disease and cancer, and have anti-inflammatory, anti-hyperlipidemic, anti-ulcer, anti-proliferative, skin tumor prevention and immunomodulatory effects (Bagchi *et al.*, 2000; Rice-Evans and Packer, 1997; Sadzuka *et al.*, 1997). Besides demonstrating a broad spectrum of therapeutic efficacy, proanthocyanidins exhibit potent antioxidant and free radical scavenging properties (Rice-Evans and Packer, 1997, Bagchi *et al.*, 2000).

Our laboratory has been primarily interested in exploring various cellular, organellar, subcellular, and molecular targets of various drugs, chemicals, and cytoprotective agents, including studies on grape seed proanthocyanidins. Interestingly, most studies using plant extracts emphasize the positive influences of the product on a particular process, and have largely ignored any toxic effects of the product. To date, the potential adverse effects of grape seed proanthocyanidin extract (GSPE) have not been closely examined. Majority of the past *in vitro* and *in vivo* studies mostly found GSPE-induced cytoprotection since the period of exposure was very limited (ranging from hours

to days up to weeks). In the present study, we have examined dose and chronic long-term exposure effects of GSPE on multiple target organs in mice and rats.

*Materials and Methods*

*Chemicals:* IH636 Grape seed proanthocyanidin extract (GSPE, commercially known as ActiVin) was obtained from InterHealth Nutraceuticals, Inc. (Benicia, CA). It is a standardized water-ethanol extract from red grape seeds. High performance liquid chromatographic and gas chromatography-mass spectrometric analyses demonstrated that GSPE contains oligomeric proanthocyanidins (OPC) including 54% dimeric-, 13% trimeric and 7% tetrameric OPCs, and a small amount of catechin derivatives, flavonoids and other oligomeric proanthocyanidins. All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) or Gibco (Grand Island, NY) and were of analytical grade or the highest grade available.

*Animals and Treatment:* The protocol was designed and the study was conducted in compliance with the Environmental Protection Agency Guidelines for Registering Pesticides in the U.S. (Pesticide Assessment Guidelines, Subdivision F, Hazard Evaluation: Human and Domestic Animals Section 81-5), the Toxic Substances Control Act (SCA) Health Effects Test Guidelines, 40 CFR 798.4470 and the Japanese Agricultural Chemicals Laws and Regulations Testing Guidelines for Toxicology Studies published by the Society of Agricultural Chemical Industry, under the auspices of MAFF (Ministry of Agriculture, Forestry and Fisheries).

According to Institutional Animal Care and Use Committee (IACUC) regulations, the guidelines were strictly followed for conducting animal experiments as indicated in the following publications:

1. Guide for the Care and Use of Laboratory Animals. by Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council. National Academy Press, Washington D.C. 1996
2. 1993 Report of the AVMA Panel on Euthanasia. *JAVMA* 202(2): 231-249 (1993).

3. Rodents and Rabbits: Current Research Issues. Proceedings of a conference sponsored by Scientists Center for Animal Welfare (SCAW) and Working for Animals in Research, Drugs and Surgery (WARD) held in Washington, D.C. on May 21, 1993. By SCAW (Greenbelt, MD) and WARD (Washington, D.C.)
4. Public Health Service Policy on Humane Care and Use of Laboratory Animals. Health Research Extension Act of 1985. Public law 99-158, November 20, 1985 "Animals in Research". Office for Protection from Research Risks, National Institutes of Health (Rockville, MD 20892-7507), Rev. September 1986, Rep. March 1996.

*Oral Toxicity:* Male and female albino rats (weighing 225-280 g) were obtained from Charles River Breeding Laboratories, Inc. (Portage, MI) and were allowed free access to lab chow (Purina Certified Rodent Chow, No. 5002, St. Louis, MO) and tap water *ad libitum*. Animals were acclimated to laboratory conditions for a minimum of seven days prior to initiation of dosing. The animal room was kept at a constant temperature (71.5-72.2°F), humidity (36.7-52.6%), and light (12 h light/12 h dark). Experiments began after the animals were young adults, and all animal treatment and protocols received prior approval by the IACUC, and met or exceeded any local, state, or federal standards.

Amount of GSPE administration was based on body weights taken just prior to dosing and a dose volume of 10 ml/kg. GSPE was administered orally via gastric intubation with ball-tipped oral dosing needles, which were affixed to approximate size syringes. The rats were fasted approximately 18-20 h prior to dosing and returned to feed 3-4 h after dosing. One group of five male and five female rats was administered a single dose at a level of 5,000 mg/kg.

*Dermal Toxicity:* Male and female albino rats (weighing 225-280 g) were obtained from Charles River Breeding Laboratories, Inc. (Portage, MI) and allowed free access to lab chow (Purina Certified Rodent Chow, No. 5002, St. Louis, MO) and tap water *ad libitum*. Animals were acclimated to laboratory conditions for a minimum of seven days prior to

initiation of dosing. The animal room was kept at a constant temperature (71.6-72.6°F), humidity (41.9-59.5%), and light (12 h light/12 h dark).

The route of GSPE administration was direct application to clipped, intact skin. This is a potential route of exposure in humans and is the accepted route of administration for evaluation of acute dermal toxicity. Individual doses of GSPE were calculated based on body weights taken just prior to dosing and a dose level of 2000 mg/kg was applied to individual animals. On the day prior to dosing, hair was removed from the backs and flanks of the rats using a small animal hair clipper. Individual doses of GSPE were moistened with approximately 0.3 ml of deionized water and applied to the dorsal skin using a microspatula. The doses covered approximately 5-6% of the total body surface. GSPE was held in contact with the skin using gauze bandaging. The bandage was secured with non-irritating tape. Collars were applied and remained on the rats for the duration of the exposure to prevent the ingestion of GSPE and/or wrappings during the 24 h exposure period. Upon completion of the exposure period, the collars and bandages were removed and the application sites were wiped with disposable paper towels moistened with tepid water.

*Dermal Irritation:* Young adult male and female albino New Zealand White rabbits weighing 2044-2223 g were obtained from Hazleton Research Products, Inc (Denver, PA) and were allowed free access to lab chow (Purina Certified Rabbit Chow, No. 5322, St. Louis, MO) and tap water *ad libitum*. Animals were acclimated to laboratory conditions for a minimum of six days prior to initiation of dosing. The animal room was kept at a constant temperature (65.0-67.6°F), humidity (35.5-54.2%), and light (12 h light/12 h dark). The route of GSPE administration was direct application to clipped, intact skin. This route of administration is standard for assessment of local dermal irritative potential. On the day prior to dosing, the hair was removed from the backs and flanks of the rabbits using a small animal hair clipper. Each individual 0.5 gm dose was moistened with approximately 0.3 ml deionized water and applied to an area of skin approximately 1" x 1" under a secured 2-ply gauze patch, that was overwrapped with a gauze binder and secured with tape. Plastic restraint collars were applied and remained on the animals for the duration of the exposure period.

The dosage level was 0.5 g/site. One group of six rabbits (three male and three female) were used with one intact site per rabbit. Each animal received a single, four-hour, semi-occluded exposure. At the end of four hours, the collars and bandages were removed and the sites were wiped with disposable towels moistened with deionized water. Body weights were obtained and recorded on study day 0 (initiation) and at each rabbit's termination from the study. The application sites were observed for erythema, edema and other dermal findings approximately 30-60 min and 24, 48 and 72 h after bandage removal and once daily thereafter through day 12, if irritation persisted. Dermal irritation was graded in accordance with the method of Draize (Draize, 1965). In order to facilitate dermal observations, the areas of application were clipped free of hair approximately one h prior to collecting 72 h dermal scores. The rabbits were observed twice daily (morning and afternoon) for mortality for the duration of the study.

The Primary Dermal Index was calculated from the scores recorded at 30-60 min, 24, 48 and 72 h after patch removal. The mean scores for erythema and edema were calculated separately to the nearest tenth and added together. Based on this value, the Draize grading system was used to arrive at the primary dermal irritation descriptive rating (Draize, 1965).

*Eye irritation:* Female New Zealand white rabbits weighing 4202-4759 g obtained from Hazleton Research Products, Inc (Denver, PA), were allowed free access to lab chow (Purina Certified Rodent Chow, No. 5322, St. Louis, MO) and tap water *ad libitum*. Animals were acclimated to laboratory conditions for a minimum of six days prior to initiation of dosing. The animal room was kept at a constant temperature (66.4-68.7°F), humidity (25.4-64.5%), and light (12 h light/12 h dark).

The route of GSPE administration was direct conjunctival instillation. This route of administration is standard for assessment of local ocular irritative potential. GSPE, 85 mg, was placed directly into the cupped lower conjunctival sac of the right (test) eye of six rabbits. The eyelid was held closed for approximately one second after instillation. The left eye was manipulated in an identical manner to simulate the dosing of the right eye. A group of six rabbits received a single, unwashed exposure. The rabbits were observed twice daily (morning and afternoon) for mortality for the duration of the study.

Both eyes of the rabbits were examined for ocular abnormalities prior to initiation of dosing. The pre-initiation examination included the use of sodium fluorescein and ultraviolet light for detection of corneal abnormalities. Rabbits assigned to the study had no pre-existing abnormalities. Both eyes of the rabbits were examined macroscopically for ocular irritation using a handheld pen light in accordance with the method of Draize at approximately one, 24, 48 and 72 h after dosing and on days 4, 7 and 14 (Draize, 1965). In addition, both eyes were further examined at 72 h and on days 7 and 14 with sodium fluorescein and ultraviolet light. Body weights were obtained and recorded on study day 0 (initiation) and on day 14 (termination). Upon termination, the rabbits were euthanized by intravenous injection of sodium pentobarbital solution and discarded.

*Long-Term Chronic (12 month) Study in Mice:* Male B6C3F1 mice (15-20 gms) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN), and given access to lab chow (Purina Laboratory Rodent Chow, St Louis, MO) and tap water *ad libitum*. Animals were allowed to acclimate in an environment of controlled temperature (22-25°C), humidity and light/dark cycle for four weeks prior to study. All animal procedures received prior approval by the University Laboratory Animal Care and Use Committee and met or exceeded current local, state, and federal standards. GSPE was mixed with a lab chow, Purina #5001 base, providing an average daily intake of approximately 100 mg/kg/b.w. Groups of animals were sacrificed at 90 day intervals for 12 mon to monitor serum chemistry and histopathological changes. Blood and organ samples (brain, heart, intestine, kidney, liver, lung and spleen) were collected upon sacrifice. Tissue samples were sectioned and collected in 10% buffered formalin for histopathological evaluation.

*Dose-dependent Effects of GSPE Exposure on Eight Organs in Mice for Six Months:* Female B6C3F1 mice (15-20 gms) were obtained from National Cancer Institute (Fredrick, MD), and given access to lab chow (Herlan Teklad, Madison, WI) and tap water *ad libitum*. Animals were allowed to acclimate in an environment of controlled temperature (22-25°C), humidity and light/dark cycle for four weeks prior to study. All animal procedures received prior approval by the University Laboratory Animal Care and Use Committee and met or exceeded current local, state, and federal standards. GSPE

was mixed with lab chow and groups of mice received approximately 0, 100, 250 or 500 mg GSPE/kg/day. Animals were sacrificed after 180 days (six months) on the respective diets, and serum chemistry and histopathological changes were examined. Blood and organ samples (brain, lung, liver, spleen, heart, kidneys, duodenum and pancreas) were collected upon sacrifice. Tissue samples were sectioned and collected in 10% buffered formalin for histopathological evaluation.

*Serum alanine aminotransferase (ALT) activity:* The effect of GSPE on the liver was inferred from the serum levels of ALT activity [EC 2.6.1]. ALT activity was monitored using a Sigma kit (# 59-UV).

*Serum blood urea nitrogen (BUN) level:* The effect of GSPE on the kidneys was inferred from serum blood urea nitrogen levels. BUN levels were monitored using a Sigma kit (# 640).

*Creatine kinase (CK) activity:* The effect of GSPE on the heart (plus other tissues) was inferred from the increase in serum creatine kinase activity. CK activity was determined using a Sigma kit (#47-UV).

*Intact Tissue Morphology:* A 2- to 3-mm section of the respective tissue was collected at the time of sacrifice and preserved in 10% buffered formalin. Sections were sent to Pathology Associates (Frederick, MD) for further processing, sectioning and PAS (periodic Acid Schiff: a purple color specific for glycogen) and/or hematoxylin and eosin (H&E) staining. Normal, apoptotic and necrotic cells were identified from 5 micron stained tissue sections using a Carl-Zeiss brightfield microscope (Axioskop 20 equipped with a camera). Our previously published guidelines were followed to characterize normal, apoptotic and necrotic cells (Ray *et al.*, 1996). The same guidelines were followed to distinguish normal areas from the damaged areas.

*DNA Fragmentation:* Frozen tissue samples were homogenized in lysis buffer at 4–8°C (5 mM Tris HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0). Homogenates were

centrifuged at 27,000 g for 20 min (8°C) to separate intact chromatin in the pellets from fragmented DNA in the supernatant fractions. Pellets were resuspended in 0.5 N perchloric acid and 5.5 N perchloric acid was added to supernatant samples to reach a final concentration of 0.5 N. Samples were heated at 90°C for 15 min and centrifuged at 1,500 g for 10 min to remove protein. Resulting supernatant fractions were reacted with diphenylamine reagent for 16-20 h at room temperature, and absorbances were measured at 600 nm. DNA fragmentation in control samples is expressed as percent of total DNA appearing in the supernatant fraction. Treatment effects are reported as percent of control fragmentation (Ray *et al.*, 1999).

**Statistics:** The data were analyzed using standard statistical analysis, i.e., ANOVA and Scheffe's post-hoc test. All values are reported as mean  $\pm$  SE from three to four samples. Statistical significance was set at  $p<0.05$ .

#### *Results*

**Oral Toxicity:** The rats were observed at approximately 1, 3, and 4 h after receiving a single oral 5000 mg/kg dose of GSPE on the first day of treatment and twice daily (morning and afternoon) thereafter for fourteen days both for mortality and clinical observations. Body weights were obtained and recorded on study days -1, 0 (first day of treatment), 7 and 14 (termination). Upon termination, all rats were euthanized by carbon dioxide asphyxiation. For scheduled necropsy, the major organ systems of the cranial, thoracic and abdominal cavities were examined for all animals. Stomach, external surface and adrenal glands, brain, intestine, esophagus, eyes, heart, kidneys, liver, mesenteric lymph node, lungs, mammary gland, ovaries, pancreas, pituitary, salivary gland, skin, spleen, thymus gland, thyroid gland, trachea, urinary bladder and uterus were carefully examined.

Of the ten animals, one female rat died on day 1. Red matting, proximal tail and brown material adhered to the nonglandular portion of stomach were observed in the dead female animal. No significant changes were observed in the adrenal glands, application site, brain, intestine, esophagus, eyes, heart, kidneys, liver, mesenteric lymph node, lungs, mammary gland, ovaries, pancreas, pituitary, salivary glands, seminal

**TABLE 1. Acute Oral Toxicity Study of GSPE in Albino Rats: Summary of Clinical Findings: Total Occurrence/No. of Animals**

Group	Male		Female	
	Day 0 to Day 14	1	Day 0 to Day 14	1
<b>Acutes</b>				
-Appeared Normal	75	5	54	5
-Mucoid Feces	3	2	11	5
-Wet yellow urogenital staining	3	1	1	1
-Wet brown urogenital staining	2	1	10	5
-Clear wet matting around mouth	1	1	0	0
-Dried yellow material around mouth	2	1	0	0
-Dried yellow urogenital staining	2	2	1	1
-Dried brown urigenital staining	0	0	3	3
-Found dead	0	0	1	1
-Clear ocular discharge	0	0	2	1
-Hypoactive	0	0	1	1

Animals were given a single oral dose of 5,000 mg GSPE/kg. See Materials and Methods section for details.

**TABLE 2. Acute Dermal Toxicity Study of GSPE in Albino Rats: Summary of Clinical Findings: Total Occurrence/No. of Animals**

Group	Male		Female	
	Day 0 to Day 14	1	Day 0 to Day 14	1
<b>Acutes</b>				
-Appeared Normal	76	5	76	5
-wet urogenital staining	4	2	6	2
-Dried red material around nose	4	3	4	2
-Dried red material around mouth	1	1	0	0
<b>Dermal Observations</b>				
-Scored, not remarkable	34	5	11	3
-No erythema	8	5	19	4
-Erythema (Very slight)	21	5	35	5
-Erythema (slight)	7	3	5	2
-No edema	32	5	59	5
-Edema (Very slight)	4	1	0	0
-Desquamation	21	5	43	5

Animals were given a single dose 2,000 mg GSPE/kg. See Materials and Methods section for details.

vesicles, skin, spleen, stomach, thymus gland, thyroid glands, trachea, urinary bladder and uterus of the dead animal. No other deaths were observed during the study. Seven animals each had wet and/or dried brown and/or yellow urogenital staining and mucoid feces. Matting/material around the mouth (clear wet, dried yellow), hypoactivity and clear ocular discharge were noted for single animals. There were no other clinical findings. Table 1 summarizes clinical findings in these animals. All animals appeared normal by day 3 or earlier and throughout the remainder of the study. No remarkable changes in body weights occurred. Also, no significant changes were observed in the adrenal glands, application site, brain, intestine, esophagus, eyes, heart, kidneys, liver, mesenteric lymph node, lungs, mammary gland, ovaries, pancreas, pituitary, salivary glands, seminal vesicles, skin, spleen, stomach, thymus gland, thyroid glands, trachea, urinary bladder and uterus in these animals.

Gross necropsy noted for the animal that died included brown material adhering to the gastric mucosa and red matting on the tail. No other gross necropsy findings were observed for this animal. There were no gross findings for any examined tissues at the scheduled necropsy. The LD<sub>50</sub> of GSPE was found to be greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats.

*Dermal Toxicity:* The objectives of this study were to evaluate potential systemic toxicity and local irritative effects of GSPE when applied once to the skin of albino rats. The animals were observed at approximately 1, 3 and 4 h after application of 2000 mg/kg on the day of treatment and twice daily (morning and afternoon) thereafter for 14 days both for mortality and clinical observations. The application sites were examined for erythema, edema and other dermal findings beginning approximately 30-60 min after bandage removal and daily thereafter for the next 13 days. The areas of application were clipped free of hair to facilitate dermal observations on study days 7 and 14. Body weights were obtained and recorded on study days 0 (the day of initiation), 7 and 14 (termination). Upon termination, the rats were euthanized by carbon dioxide asphyxiation. For necropsy, the major organ systems of the cranial, thoracic and abdominal cavities were examined for all animals. Urinary bladder, lymph node and organs including adrenal glands, application site, brain, intestine, epididymides,

**TABLE 3. Acute Dermal Toxicity Study of GSPE in Albino Rats: Individual Dermal Observations**

Study Day	A group of five male and female albino rats were administered single doses of GSPE at a dose level of 2,000 mg/kg.											
	M	M	M	M	M	M	ERYTHEMA*, EDEMA*	OTHER FINDINGS	F	F	F	F
1	2,0	1,0	1,0	SNR	1,0	SNR	1,0	1,0	1,0	1,0	1,0	1,0
2	2,0	2,0	1,0	SNR	1,0	1,0	1,0	1,0	1,0	1,0	1,0	2,0
3	2,0	1,0	1,0,d	SNR	1,0	1,0	1,0	1,0	1,0	1,0	1,0	2,0
4	1,0,d	1,0	2,0,d	SNR	1,0,d	2,0	1,0,d	1,0,d	1,0,d	1,0,d	2,0,d	2,0,d
5	1,0,d	1,0	1,0,d	SNR	1,0,d	2,0,d	1,0,d	1,0,d	1,0,d	1,0,d	2,0,d	2,0,d
6	0,0,d	0,0,d	1,0,d	SNR	0,0,d	2,1,d	1,0,d	1,0,d	1,0,d	1,0,d	1,0,d	1,0,d
7	0,0,d	0,0,d	0,0,d	SNR	0,0,d	1,1,d	1,0,d	1,0	1,0,d	1,0,d	1,0,d	1,0,d
8	SNR	0,0,d	SNR	SNR	1,0,d	SNR	1,1,d	1,0,d	SNR	1,0,d	1,0,d	1,0,d
9	SNR	SNR	SNR	SNR	1,1,d	SNR	1,1,d	1,0,d	SNR	1,0,d	1,0,d	1,0,d
10	SNR	SNR	SNR	SNR	0,0,d	SNR	0,0,d	0,0,d	SNR	1,0,d	0,0,d	0,0,d
11	SNR	SNR	SNR	SNR	SNR	SNR	0,0,d	0,0,d	SNR	0,0,d	0,0,d	0,0,d
12	SNR	SNR	SNR	SNR	SNR	SNR	0,0,d	0,0,d	SNR	0,0,d	0,0,d	0,0,d
13	SNR	SNR	SNR	SNR	SNR	SNR	0,0,d	0,0,d	SNR	SNR	0,0,d	0,0,d
14	SNR	SNR	SNR	SNR	SNR	SNR	0,0,d	0,0,d	SNR	0,0,d	SNR	0,0,d

\* Refers to Draize Scale for Dermal Scoring Criteria. See Materials and Methods section for details.

Sex Code: M = Male, F = Female

d = desquamation

SNR = Scored, Not Remarkable

TABLE 4. Acute Dermal Toxicity of GSPE in Albino Rats: Individual Gross Description of Organs

Sex	Organ-Findings	Grade	Organ: No significant findings	Sex	Organ-Findings	Grade	Organ: No significant findings
M	Urinary Bladder Gross: Red Fluid Content	P	Adrenal glands, Application site, Brain, Intestine, Epididymides, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Pancreas, Pituitary, Prostate, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Testes, Thymus gland, Thyroid glands, Trachea	F	None	NA	Adrenal glands, Application site, Brain, Intestine, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Ovaries, Pancreas, Pituitary, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Thyroid gland, Thyroid glands, Trachea, Urinary bladder, Uterus
M	Lymph Node Gross: Cervical-Reddened Bilateral	P	Adrenal glands, Application site, Brain, Intestine, Epididymides, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Pancreas, Pituitary, Prostate, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Testes, Thymus gland, Thyroid glands, Trachea, Urinary bladder	F	None	NA	Adrenal glands, Application site, Brain, Intestine, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Ovaries, Pancreas, Pituitary, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Thyroid gland, Thyroid glands, Trachea, Urinary bladder, Uterus
M	Lymph Node Gross: Cervical-Reddened Right	P	Adrenal glands, Application site, Brain, Intestine, Epididymides, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Pancreas, Pituitary, Prostate, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Testes, Thymus gland, Thyroid glands, Trachea, Urinary bladder	F	None	NA	Adrenal glands, Application site, Brain, Intestine, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Ovaries, Pancreas, Pituitary, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Thyroid gland, Thyroid glands, Trachea, Urinary bladder, Uterus
M	Lymph Node Gross: Cervical-Reddened Right	P	Adrenal glands, Application site, Brain, Intestine, Epididymides, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Pancreas, Pituitary, Prostate, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Testes, Thymus gland, Thyroid glands, Trachea, Urinary bladder	F	None	NA	Adrenal glands, Application site, Brain, Intestine, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Ovaries, Pancreas, Pituitary, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Thyroid gland, Thyroid glands, Trachea, Urinary bladder, Uterus
M	Lymph Node Gross: Cervical-Reddened Bilateral	P	Adrenal glands, Application site, Brain, Intestine, Epididymides, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Pancreas, Pituitary, Prostate, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Testes, Thymus gland, Thyroid glands, Trachea, Urinary bladder	F	None	NA	Adrenal glands, Application site, Brain, Intestine, Esophagus, Eyes, Heart, Kidneys, Liver, Lymph node, Me., Lungs, Mammary gland, Ovaries, Pancreas, Pituitary, Salivary glands, Seminal vesicles, Skin, Spleen, Stomach, Thyroid gland, Thyroid glands, Trachea, Urinary bladder, Uterus

1-Slight, 2-Moderate, 3-Marked, P-Present. A gross grade code describing the observed organs. See Materials and Methods section for details.

esophagus, eyes, heart, kidneys, liver, mesenteric lymph node, lungs, mammary glands, lungs, mammary gland, pancreas, pituitary prostate, salivary glands, seminal vesicles, skin, spleen, stomach, testes, thymus gland, thyroid glands and trachea, were carefully examined.

There were no deaths, test material-related clinical findings, remarkable body weight changes or gross necropsy findings. Table 2 summarizes the clinical observations. Clinical observations included dried red material around the nose and/or mouth for five rats and wet yellow urogenital staining for four rats. These findings are typically noted in association with the bandage/collar application procedures and are not related to test material application. There were no other clinical findings. With the exception of a spontaneous finding of dried material around the mouth for one rat on day 3, all animals appeared normal by day 2 or earlier and throughout the remainder of the study.

Individual dermal observations (Table 3) demonstrated very slight to slight erythema and desquamation on all animals. One male rat had edema from day 6 through day 9. There were no other dermal findings. With the exception of desquamation noted on three animals, all dermal responses completely subsided by day 12 or earlier.

**TABLE 5. Acute Dermal Toxicity Study of GSPE in Albino Rats: Gross Necropsy Observations Incidence Summary**

Group	Male	Female
Number of animals in dose group	5	5
Number of animals terminally euthanized	5	5
Urinary Bladder		
-Red fluid conents	1	0
Lymph Node		
-Cervical-reddened	4	0
No significant changes observed	0	5

Effect of single dose of 2,000 mg GSPE/kg was assessed. See Materials and Methods section for details.

The gross necropsy findings (Table 4) show that four male rats had reddened cervical lymph node(s) and one male rat had red fluid contents in the urinary bladder. These findings are commonly observed in laboratory rats and were not attributed to test material application. Table 5 summarizes and demonstrates that there were no other gross necropsy findings for all examined tissues.

The LD<sub>50</sub> of GSPE was found to be greater than 2000 mg/kg when administered once for 25 h to the clipped, intact skin of male and female rats. In addition, 2000 mg/kg was found to be a no-observed-effect level (NOEL) for systemic toxicity under conditions of the study.

*Dermal Irritation:* The objective was to determine the irritative potential of GSPE following a single exposure to the skin of albino rabbits. The rabbits were observed twice daily (morning and afternoon) for mortality for the duration of the study. The application sites were observed for erythema, edema and other dermal findings approximately 30-60 min and 24, 48 and 72 h after patch removal and once daily thereafter through day 12 id irritation persisted. Dermal irritation was graded in accordance with the method of Draize (Draize, 1965). In order to facilitate dermal observations, the areas of application were clipped free of hair approximately one h prior to collecting 72 h dermal scores. The primary dermal irritation index was calculated from the scores recorded at 30-60 min, 24, 48 and 72 h after patch removal. The mean scores for erythema and edema were calculated separately to the nearest tenth and added together. Body weights were obtained and recorded on study day 0 (initiation) and at the termination of the study. Upon termination, the rabbits were euthanized by intravenous injection of sodium phenobarbital solution and discarded.

There were no deaths or remarkable body weight changes during the study period. All rabbits had slight to severe erythema, very slight to slight edema and desquamation. Erythema completely subsided by day 8 or earlier. Edema completely subsided by day 6 or earlier. One and two animals had exfoliation and eschar, respectively. There were no other dermal findings. All dermal irritation completely subsided by day 12 or earlier. Tables 6 and 7 demonstrate the individual erythema and edema scores following

TABLE 6. Primary Dermal Irritation Study of GSPE in Albino Rabbits-Individual Erythema Scores

Sex	0.5-l h	24 h	48 h	72 h	4 D	5 D	6 D	7 D	8 D	9 D	10 D	11 D	12 D
M	1	1	2	2d	2d	1d	0d	0d	0d	0d	0d	-	-
M	1	1	1	2	2d	1d	0d	0d	0d	0d	0d	-	-
M	1	1	2	3	3d	2d	1d	1d	0d	0d	0d	-	-
F	1	1	1	2d	2d	1d	0d	0d	0d	0d	0d	0	-
F	1	2	3	2dc	4dc	2d	1d	1d	0d	0d	0d	0d	0
F	2	2	4e	4dc	4dex	2d	0d	0d	0d	0d	0d	0	-

d = desquamation e = eschar x = exfoliation

TABLE 7. Primary Dermal Irritation Study of GSPE in Albino Rabbits-Individual Edema Scores

Sex	0.5-l h	24 h	48 h	72 h	4 D	5 D	6 D	7 D	8 D	9 D	10 D	11 D	12 D
M	0	1	1	1	0	0	0	0	0	0	0	0	-
M	0	1	0	0	0	0	0	0	0	0	0	-	-
M	0	1	1	1	1	1	0	0	0	0	0	0	-
F	0	1	1	0	0	0	0	0	0	0	0	0	-
F	0	1	1	2	1	0	0	0	0	0	0	0	0
F	0	2	2	2	1	0	0	0	0	0	0	0	-

TABLE 8. Primary Eye Irritation Study of GSPE in Albino Rabbits-Individual Ocular Irritation Scores

Sex	Tissue	1h	24h	48h	72h*	4d	7d	14d
F	Cornea (O-A)	0 0	0 0	0 0	0 0	0 0	0 0	0 0
	Iris	0 1	0 1	0 0	0 0	0 0	0 0	0 0
	Conjunctiva (R-C-D)	2 2 2	2 2 2	2 1 1	2 1 1	1 1 0	1 0 0	0 0 0
F	Cornea (O-A)	0 0	1 1	1 1	1 1	1 1	0 0	0 0
	Iris	0 1	1 1	0 0	0 0	0 0	0 0	0 0
	Conjunctiva (R-C-D)	2 3 1	2 3 2	2 3 2	2 2 2	2 2 0	2 1 0	0 0 0
F	Cornea (O-A)	0 0	1 0	0 0	1 1	1 1	1 1	0 0
	Iris	0 1	0 1	0 0	0 0	0 0	0 0	0 0
	Conjunctiva (R-C-D)	2 2 2	3 2 1	2 2 1	2 1 0	2 1 0	1 0 0	0 0 0
F	Cornea (O-A)	0 0	0 0	0 0	0 0	0 0	0 0	0 0
	Iris	0 0	0 0	0 0	0 0	0 0	0 0	0 0
	Conjunctiva (R-C-D)	2 3 1	2 2 0	2 2 0	2 1 0	2 1 0	1 1 0	0 0 0
F	Cornea (O-A)	0 0	0 0	0 0	0 0	0 0	0 0	0 0
	Iris	0 0	0 0	0 0	0 0	0 0	0 0	0 0
	Conjunctiva (R-C-D)	2 3 2	2 1 0	2 1 1	1 0 0	1 0 0	1 0 0	0 0 0
F	Cornea (O-A)	0 0	1 1	1 1	1 1	1 1	0 0	0 0
	Iris	0 1	1 1	0 0	0 0	0 0	0 0	0 0
	Conjunctiva (R-C-D)	2 2 1	3 3 3	3 2 2	2 2 0	2 1 0	1 0 0	0 0 0

\* = Fluorescein Solution Applied   O = Opacity   A = Area   R = Redness   C = Chemosis   D = Discharge

application of GSPE, respectively. The primary irritation index was calculated to be 2.7. GSPE received a descriptive rating classification of moderately irritating.

*Eye Irritation:* The rabbits were observed twice daily (morning and afternoon) for mortality for the entire duration of the study. Both eyes of the rabbits were examined for ocular abnormalities prior to initiation of dosing. The pre-initiation examination included the use of sodium fluorescein and ultraviolet light for detection of corneal abnormalities. Rabbits assigned to the study had no pre-existing ocular abnormalities. Both eyes of the rabbits were examined macroscopically for ocular irritation using a hand-held penlight in accordance with the method of Draize at approximately 1, 24, 48 and 72 h after dosing and on days 4, 7 and 14 (Draize, 1965). In addition, both eyes were further examined at 72 h and on days 7 and 14 with sodium fluorescein and ultraviolet light. Body weights were obtained and recorded on study day 0 (initiation) and on day 14 (termination). Upon termination, the rabbits were euthanized by intravenous injection of sodium phenobarbital solution and discarded.

No deaths or remarkable changes or differences were observed in body weights during the study. None of the rabbits vocalized upon instillation of GSPE. The left (control) eyes were free of evidence of ocular irritation and other findings for the duration of the study. Positive conjunctival irritation was noted in the untreated eye of all six rabbits. Four and three animals had iridal and corneal reactions, respectively, and the Maximum Average Score (MAS) for GSPE was 16.7 at 24 h post-instillation. All irritation was reversible and completely subsided by day 14. Tables 8 and 9 show the individual ocular irritation scores as well as the scale used for scoring the ocular irritation and the method for score calculation, respectively.

*Effect of Chronic Administration of 100 mg GSPE/kg/day for Twelve Months to Mice:* In order to assess the chronic effect of GSPE administration, mice were fed GSPE (100 mg/kg/day) mixed in laboratory chow. Changes in serum chemistry, histopathology and integrity of hepatic genomic DNA were considered key for this battery of experiments.

TABLE 9. Primary Eye Irritation Study of GSPE in Albino Rabbits-Scale for Scoring Ocular Irritation\*

I.	Cornea		
	[A] Opacity-degree of density (area most dense taken for reading)		
	No ulceration or opacity	0	
	Dulling of normal luster, details of iris clearly visible.....	1*	
	Easily discernable translucent area, details of iris slightly obscured....	2*	
	Nacreous areas, no details of iris visible, size of pupil barely discernable.....	3*	
	Opaque cornea, iris not discernible through the opacity.....	4*	
	[B] Area of cornea involved		
	No ulceration or opacity	0	
	One quarter or less than but not zero	1*	
	Greater than one quarter, but less than half.....	2*	
	Greater than half, but less than three quarters.....	3*	
	Greater than three quarters, up to whole area.....	4*	
	Score equals A x B x 5      Total maximum = .....	80	
II.	Iris		
	[A]		
	Normal.....	0	
	Markedly deepened rugae, congestion, swelling circumcorneal injection (any or all of these or combination of any thereof), iris still reacting to light (sluggish reaction is positive).....	1*	
	No reaction to light, hemorrhage, gross destruction (any or all of these).....	2*	
	Score equals A x 5      Total maximum = .....	10	
III.	Conjunctivae		
	[A] Redness (refers to palpebral and culcar conjunctivae excluding cornea and iris)		
	Blood vessels normal.....	0	
	Some blood vessels definitely hyperemic (injected above) normal.....	1	
	Diffuse, deeper crimson color, individual vessels not easily discernible.....	2*	
	Diffuse beefy red.....	3*	
	[B] Chemosis: lids and/or nictitating membranes		
	No Swelling.....	0	
	Any swelling above normal (includes nictitating membrane).....	1	
	Obvious swelling with partial eversion of lids.....	2*	
	Swelling with lids about half closed.....	3*	
	Swelling with lids more than half closed.....	4*	
	[C] Discharge		
	No Discharge.....	0	
	Any amount different from normal (does not include small amounts observed in inner canthus of normal animals).....	1	
	Discharge with moistening of the lids and hairs just adjacent to the lids.....	2	
	Discharge with moistening of the lids and hair, and considerable area under the eye.....	3	
	Score equals (A + B + C) x 2      Total maximum = ..... Total maximum score possible = ....	20	110

\*Draize scale for scoring ocular lesions, as published in the guidelines in Subsection F. Hazard evaluation: Human and Domestic Animals distributed in 1982 and the OECD Guidelines for Testing Chemicals distributed in 1987.

\*Starred figures indicate positive effect.

The animals were routinely observed 4-6 times per day during this entire one-year period. No significant changes in the body weight or physical appearance were observed during this entire study, nor were any unusual deaths observed. Following completion of 3, 6, 9 or 12 mon, the mice were sacrificed and the seven vital organs were carefully investigated to determine any macroscopic changes. Overall, all the organs appeared normal and identical to control organs.

Blood urea nitrogen (BUN) level was assessed as a biomarker of kidney function. Figure 1 demonstrates the serum BUN levels in control and GSPE-fed animals at 3, 6, 9 and 12 mon of treatment. No significant changes in serum BUN levels were observed following daily feeding of these animals with GSPE.

Serum alanine aminotransferase (ALT) activity was assessed as a biomarker of liver function or hepatotoxicity. Figure 2 shows the serum ALT activity in control and GSPE-fed mice at 3, 6, 9 and 12 months of treatment. No significant changes in serum ALT activities were observed following daily feeding of these animals with GSPE.

Serum creatine kinase (CK) activity is considered as a biomarker of tissue necrosis and cardiac function. Figure 3 exhibits the serum CK activity in control and GSPE-fed animals at 3, 6, 9 and 12 months of treatment. No significant changes in serum CK levels were observed following as the result of daily feeding of these animals with GSPE.

**Table 10. Time-Dependent Effect of GSPE Feeding on Hepatic Genomic DNA Fragmentation in Mice**

Time (Months)	DNA Fragmentation	
	Control	GSPE
3 months	3.02 ± 0.27 (100%)	3.02 ± 0.16 (100%)
6 months	5.26 ± 0.23 (100%)	4.94 ± 0.17 (94%)
9 months	5.25 ± 0.12 (100%)	5.77 ± 0.19 (110%)
12 months	6.08 ± 0.10 (100%)	5.51 ± 0.33 (90%)

All values are reported as means ± S.E. from three to four samples. See Materials and Methods section for details.

Hepatic genomic DNA fragmentation was monitored as an index of oxidative DNA damage. Table 10 demonstrates the hepatic genomic DNA fragmentation in control and GSPE-fed mice at 3, 6, 9 and 12 months of treatment. No significant changes in hepatic genomic DNA fragmentation were observed following the daily feeding of GSPE to these animals for up to 12 months.

Seven organs of both control and GSPE-fed animal tissues were examined at different magnification levels. Figures 4-17 demonstrate representative histopathological photomicrographs of control and GSPE-fed animals after 3, 6, 9 and 12 months of treatment. No usual histopathological changes were observed in these seven vital organs of GSPE-fed mice.

*Dose-dependent Effects of Chronic GSPE Exposure on Eight Organs in Mice for Six Months:* In order to assess the chronic effect of GSPE administration, mice were fed GSPE (0, 100, 250 or 500 mg/kg/day) in the diet for 6 months. Changes in serum chemistry and histopathology were examined at the conclusion of the study.

The animals were routinely observed several times per day during this entire six-month period. No significant changes in the body weight or physical appearance were observed during the study. No unusual deaths were observed. Following completion of six months, the animals were sacrificed and eight organs were examined to determine any macroscopic changes. Overall, all the organs appeared normal and identical to control organs.

Blood urea nitrogen (BUN) level was assessed as a biomarker of kidney function. Figure 18 demonstrates the serum BUN level in control and GSPE-fed animals after six months of treatment at the three doses. No significant changes in serum BUN levels were observed following daily feeding of these mice with GSPE.

Serum alanine aminotransferase (ALT) activity was used as a biomarker of liver function or hepatotoxicity. Figure 19 shows the serum ALT activity in control and GSPE-fed mice after six months of treatment. No significant changes in serum ALT activities were observed as the result of daily feeding of these animals with the three levels of GSPE.

**FIGURE LEGENDS**

- Figure 1. Time-dependent effect of GSPE feeding on blood urea nitrogen (BUN) level in mice. Mice were fed GSPE (ADI 100 mg/kg/day) mixed in laboratory chow for 12 months. See Materials and Methods section for details.
- Figure 2. Time-dependent effect of GSPE feeding on serum alanine aminotransferase (ALT) activity in mice. Mice were fed GSPE (ADI 100 mg/kg/day) mixed in laboratory chow for 12 months. See Materials and Methods section for details.
- Figure 3. Time-dependent effect of GSPE feeding on serum creatine kinase (CK) activity in mice. Mice were fed GSPE (ADI 100 mg/kg/day) mixed in laboratory chow for 12 months. See Materials and Methods section for details.
- Figure 4. Light micrographs of paraffin-embedded mouse brain.  
*Top left.* Representative mouse brain section (X 400) from control animals following 3 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left.* Representative mouse brain section (X 400) from control animals following 6 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).
- Figure 5. Light micrographs of paraffin-embedded mouse brain  
*Top left.* Representative mouse brain section (X 400) from control animals following 9 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left.* Representative mouse brain section (X 400) from control animals following 12 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).
- Figure 6. Light micrographs of paraffin-embedded mouse heart  
*Top left.* Representative mouse heart section (X 400) from control animals following 3 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left.* Representative mouse heart section (X 400) from control animals following 6 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).
- Figure 7. Light micrographs of paraffin-embedded mouse heart.  
*Top left.* Representative mouse heart section (X 400) from control animals following 9 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left.* Representative mouse heart section (X 400) from control animals

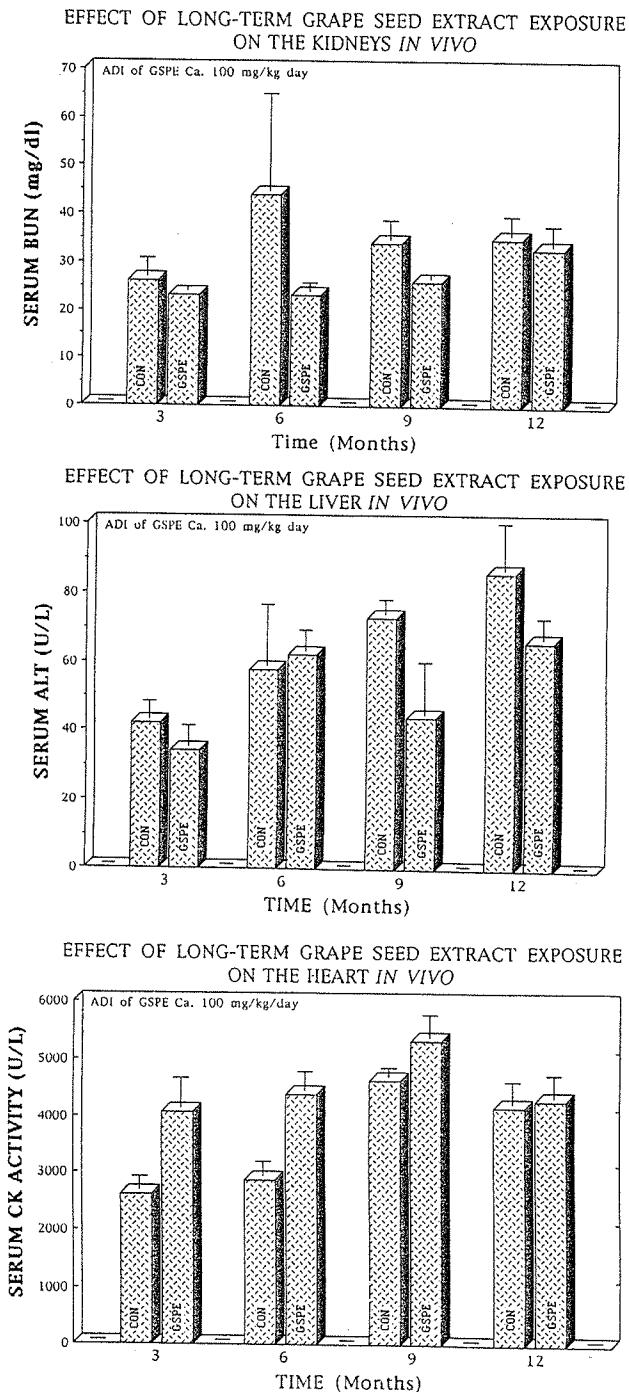


FIGURE 1 (TOP); FIGURE 2 (MIDDLE); FIGURE 3 (BOTTOM)

following 12 months of the study; *Bottom right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).

Figure 8. Light micrographs of paraffin-embedded mouse intestine  
*Top left*. Representative mouse intestine section (X 400) from control animals following 3 months of the study; *Top right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left*. Representative mouse intestine section (X 400) from control animals following 6 months of the study; *Bottom right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).

Figure 9. Light micrographs of paraffin-embedded mouse intestine  
*Top left*. Representative mouse intestine section (X 400) from control animals following 9 months of the study; *Top right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left*. Representative mouse intestine section (X 400) from control animals following 12 months of the study; *Bottom right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).

Figure 10. Light micrographs of paraffin-embedded mouse kidney  
*Top left*. Representative mouse kidney section (X 400) from control animals following 3 months of the study; *Top right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left*. Representative mouse kidney section (X 400) from control animals following 6 months of the study; *Bottom right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).

Figure 11. Light micrographs of paraffin-embedded mouse kidney  
*Top left*. Representative mouse kidney section (X 400) from control animals following 9 months of the study; *Top right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left*. Representative mouse kidney section (X 400) from control animals following 12 months of the study; *Bottom right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).

Figure 12. Light micrographs of paraffin-embedded mouse liver  
*Top left*. Representative mouse liver section (X 400) from control animals following 3 months of the study; *Top right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left*. Representative mouse liver section (X 400) from control animals following 6 months of the study; *Bottom right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).

- Figure 13 Light micrographs of paraffin-embedded mouse liver  
*Top left.* Representative mouse liver section (X 400) from control animals following 9 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left.* Representative mouse liver section (X 400) from control animals following 12 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).
- Figure 14 Light micrographs of paraffin-embedded mouse lung  
*Top left.* Representative mouse lung section (X 400) from control animals following 3 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left.* Representative mouse lung section (X 400) from control animals following 6 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).
- Figure 15 Light micrographs of paraffin-embedded mouse lung  
*Top left.* Representative mouse lung section (X 400) from control animals following 9 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left.* Representative mouse lung section (X 400) from control animals following 12 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).
- Figure 16 Light micrographs of paraffin-embedded mouse spleen  
*Top left.* Representative mouse spleen section (X 400) from control animals following 3 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 3 months); *Bottom left.* Representative mouse spleen section (X 400) from control animals following 6 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months).
- Figure 17 Light micrographs of paraffin-embedded mouse spleen  
*Top left.* Representative mouse spleen section (X 400) from control animals following 9 months of the study; *Top right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 9 months); *Bottom left.* Representative mouse spleen section (X 400) from control animals following 12 months of the study; *Bottom right.* Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 12 months).
- Figure 18 Dose- and time-dependent effect of GSPE feeding on blood urea nitrogen (BUN) level in mice. Mice were fed GSPE (ADI 0, 100, 250 or 500

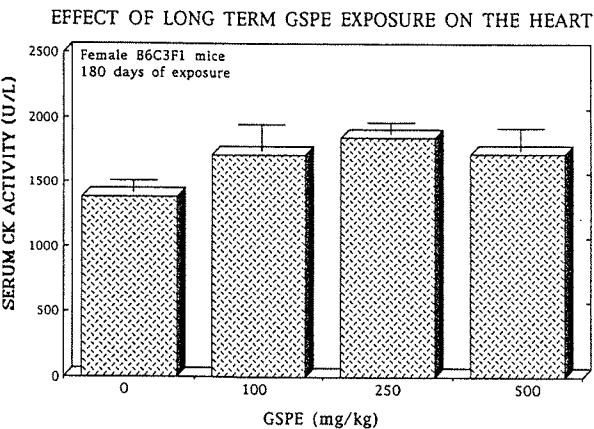
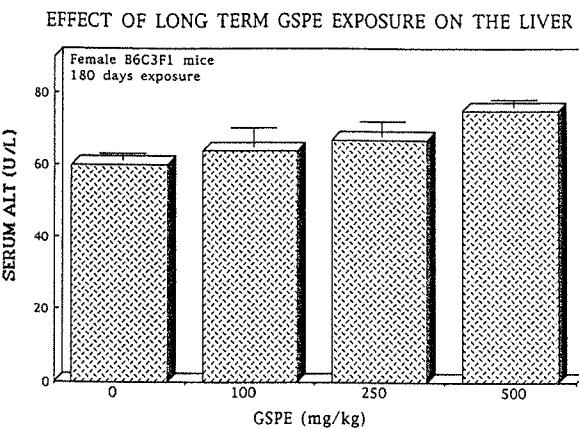
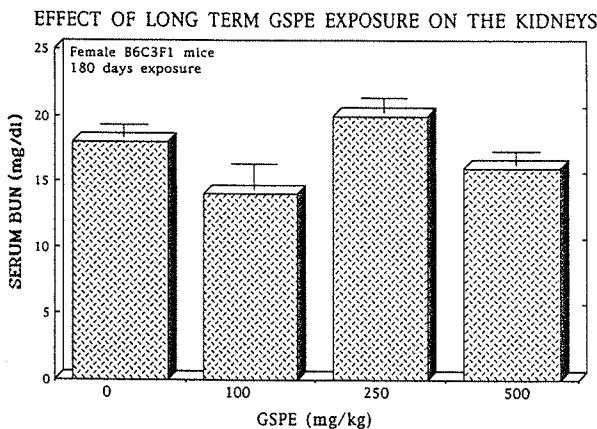


FIGURE 18 (TOP); FIGURE 19 (MIDDLE); FIGURE 20 (BOTTOM)

mg/kg/day) mixed in laboratory chow for 6 months. See Materials and Methods section for details.

Figure 19. Time-dependent effect of GSPE feeding on serum alanine aminotransferase (ALT) activity in mice. Mice were fed GSPE (ADI 0, 100, 250 or 500 mg/kg/day) mixed in laboratory chow for 6 months. See Materials and Methods section for details.

Figure 20. Time-dependent effect of GSPE feeding on serum creatine kinase (CK) activity in mice. Mice were fed GSPE (ADI 0, 100, 250 or 500 mg/kg/day) mixed in laboratory chow for 6 months. See Materials and Methods section for details.

Figure 21. Light micrographs of paraffin-embedded mouse brain. Effects of 6 months GSPE exposure on the brain.  
*Top left.* Representative mouse brain section (X100) from control animals; *Top right.* Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left.* Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right.* Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 22. Light micrographs of paraffin-embedded mouse heart. Effects of 6 months GSPE exposure on the heart.  
*Top left.* Representative mouse heart section (X100) from control animals; *Top right.* Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left.* Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right.* Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 23. Light micrographs of paraffin-embedded mouse duodenum. Effects of 6 months GSPE exposure on the duodenum.  
*Top left.* Representative mouse duodenum section (X100) from control animals; *Top right.* Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left.* Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right.* Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 24. Light micrographs of paraffin-embedded mouse pancreas. Effects of 6 months GSPE exposure on the pancreas.  
*Top left.* Representative mouse pancreas section (X100) from control animals; *Top right.* Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left.* Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months).

months); *Bottom right*. Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 25. Light micrographs of paraffin-embedded mouse kidney. Effects of 6 months GSPE exposure on the kidney.

*Top left*. Representative mouse kidney section (X100) from control animals; *Top right*. Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left*. Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right*. Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 26. Light micrographs of paraffin-embedded mouse liver. Effects of 6 months GSPE exposure on the liver.

*Top left*. Representative mouse liver section (X100) from control animals; *Top right*. Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left*. Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right*. Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 27. Light micrographs of paraffin-embedded mouse lung. Effects of 6 months GSPE exposure on the lung.

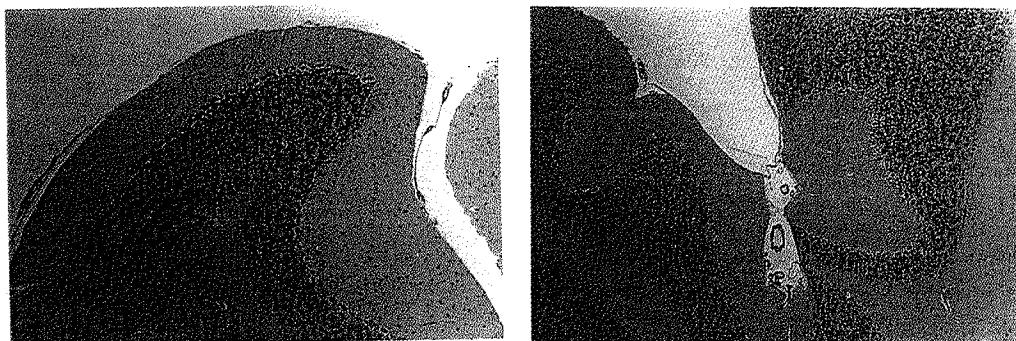
*Top left*. Representative mouse lung section (X400) from control animals; *Top right*. Representative section (X 400) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left*. Representative section (X400) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right*. Representative section (X400) from mouse treated with GSPE (500 mg/kg/day for 6 months).

Figure 28. Light micrographs of paraffin-embedded mouse spleen. Effects of 6 months GSPE exposure on the spleen.

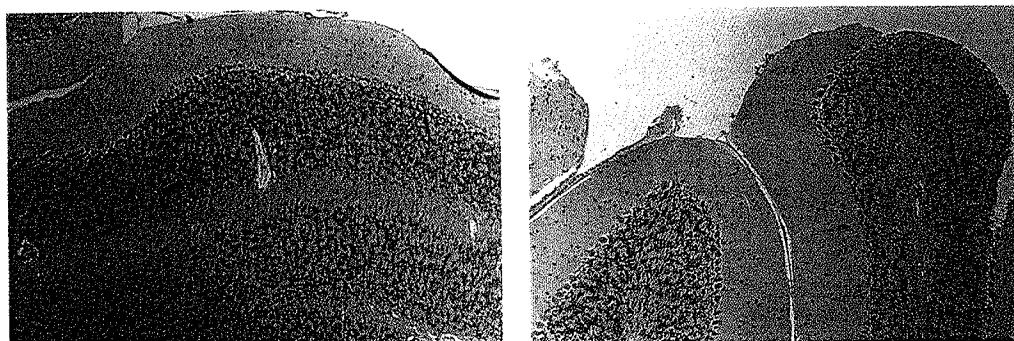
*Top left*. Representative mouse spleen section (X100) from control animals; *Top right*. Representative section (X100) from mouse treated with GSPE (100 mg/kg/day for 6 months); *Bottom left*. Representative section (X100) from mouse treated with GSPE (250 mg/kg/day for 6 months); *Bottom right*. Representative section (X100) from mouse treated with GSPE (500 mg/kg/day for 6 months).

EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE BRAIN

Figure 4

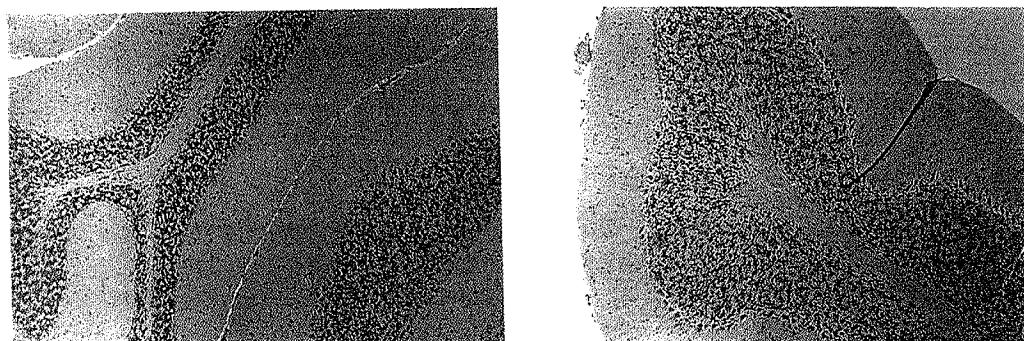


EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE BRAIN

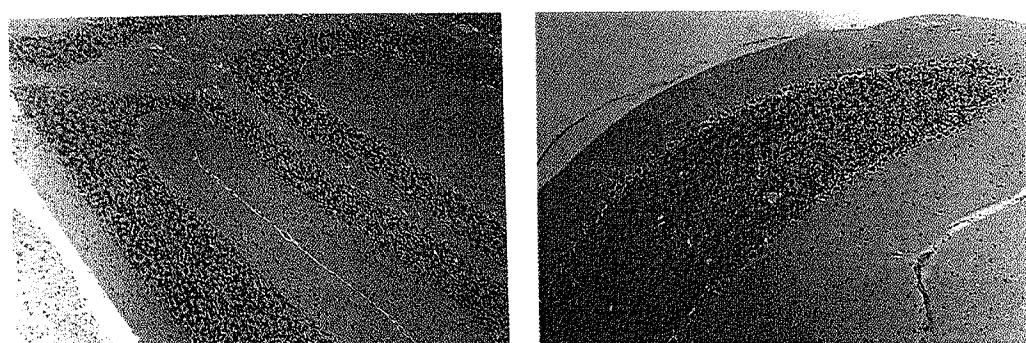


EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE BRAIN

Figure 5

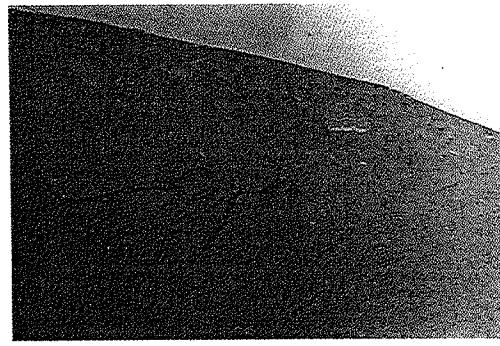
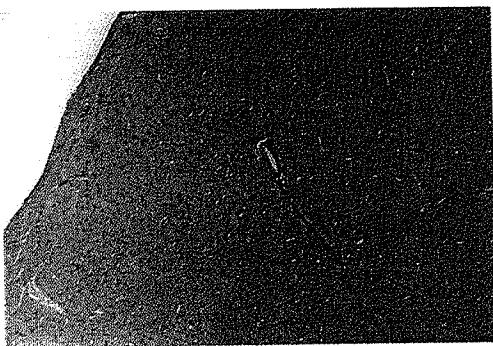


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE BRAIN

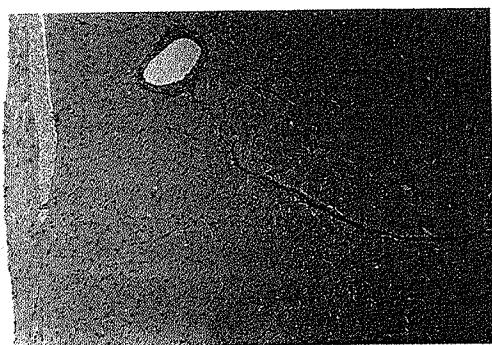


EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE HEART

Figure 6

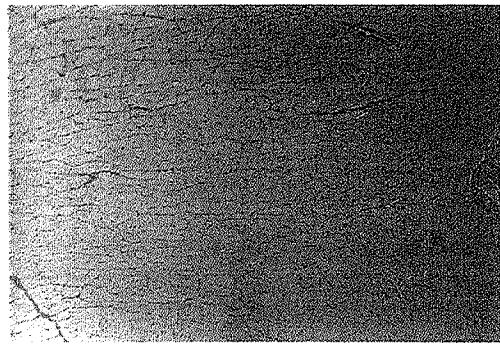
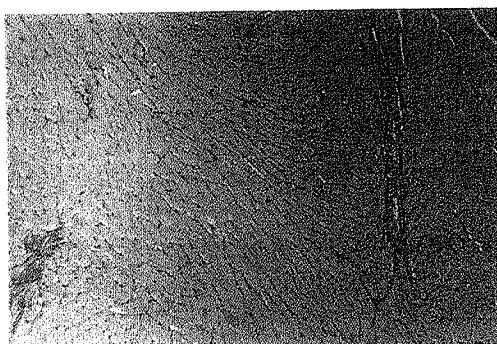


EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE HEART

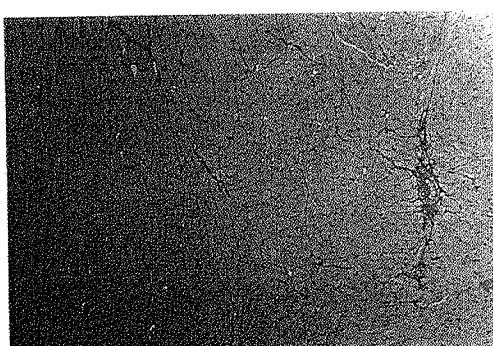


EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE HEART

Figure 7

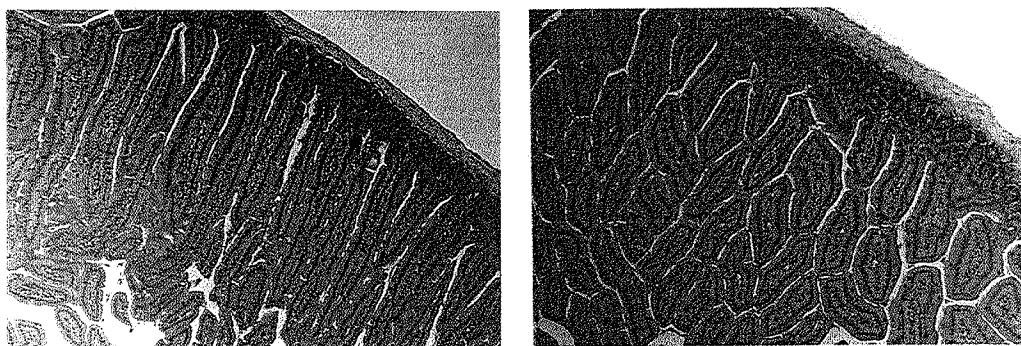


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE HEART

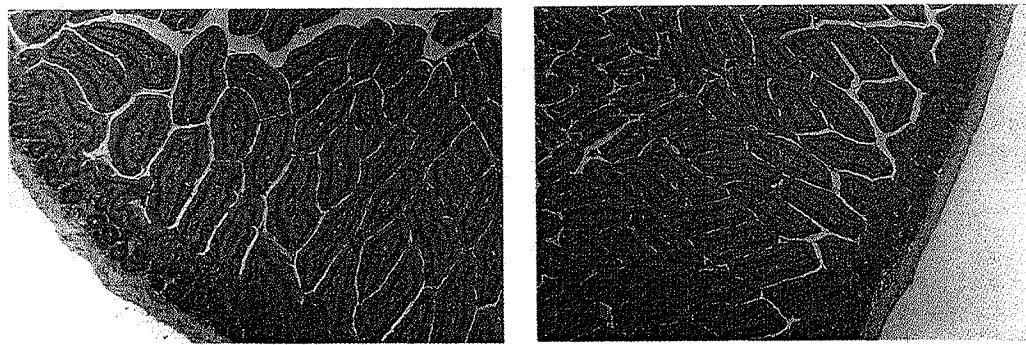


EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE INTESTINE

Figure 8

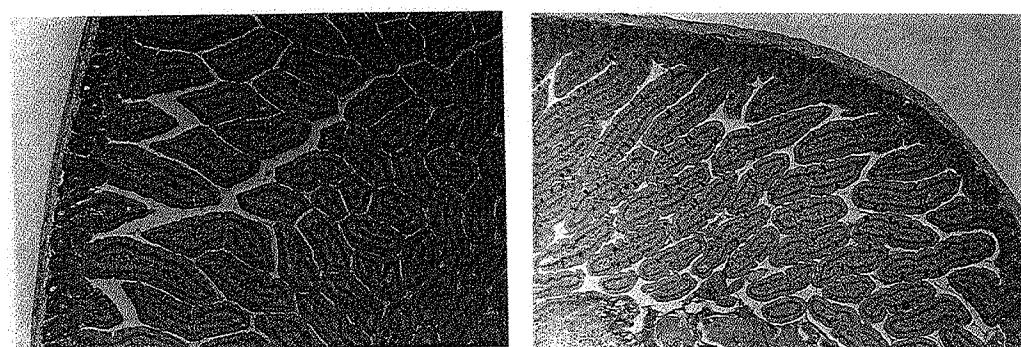


EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE INTESTINE

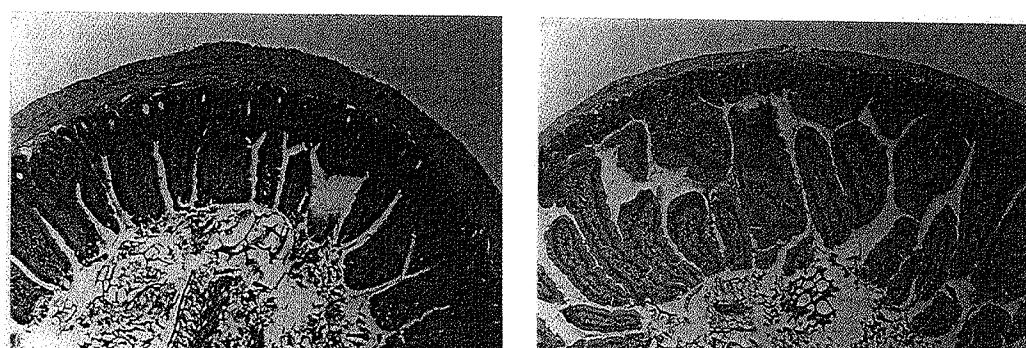


EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE INTESTINE

Figure 9

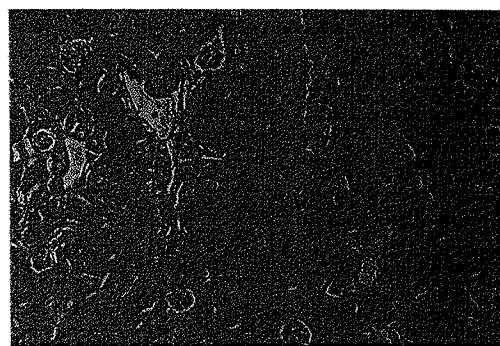
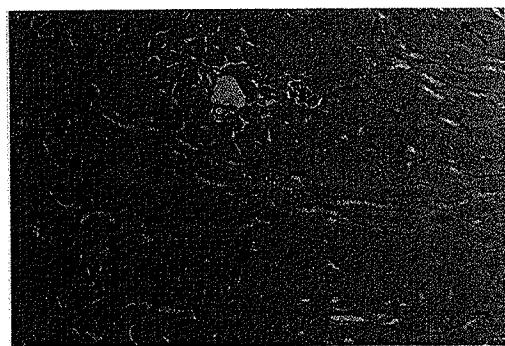


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE INTESTINE

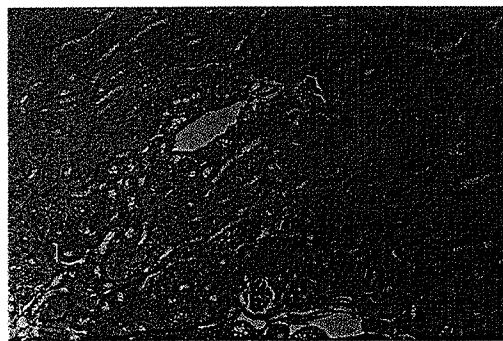


EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE KIDNEY

Figure 10



EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE KIDNEY

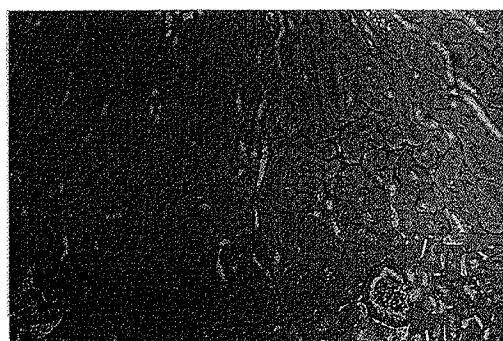


EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE KIDNEY

Figure 11

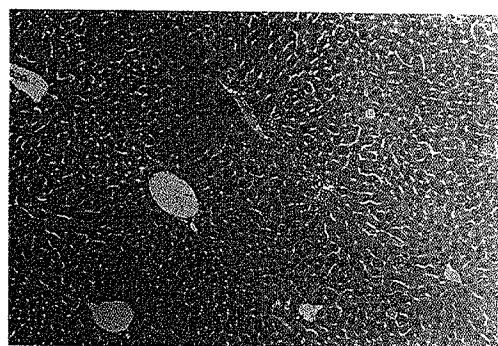
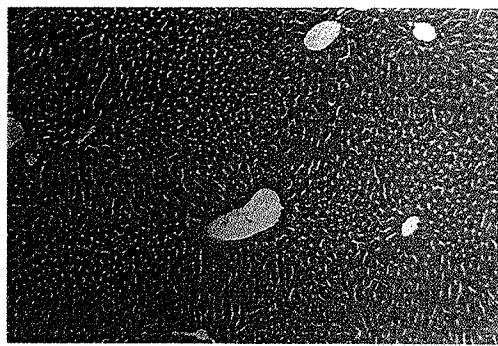


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE KIDNEY

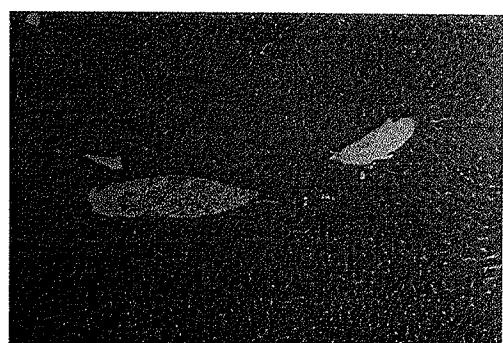
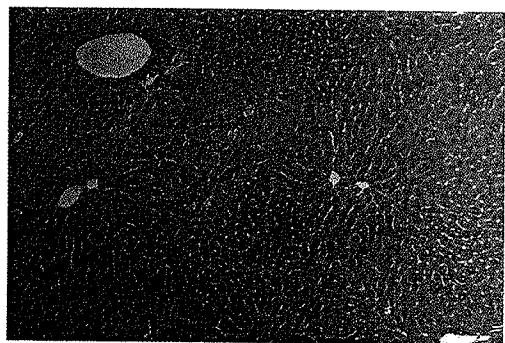


EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE LIVER

Figure 12

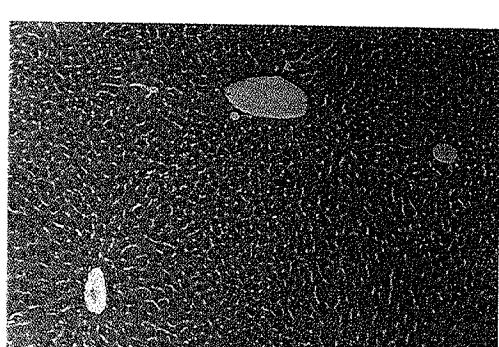
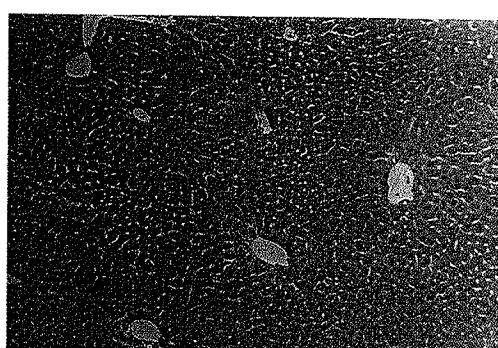


EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE LIVER

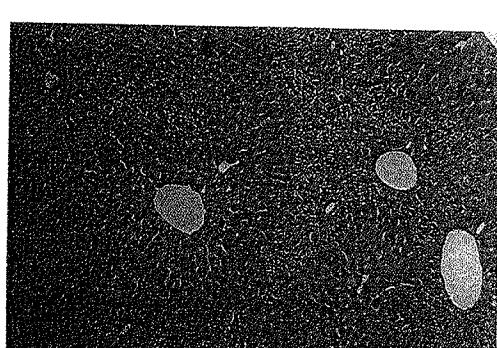


EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE LIVER

Figure 13

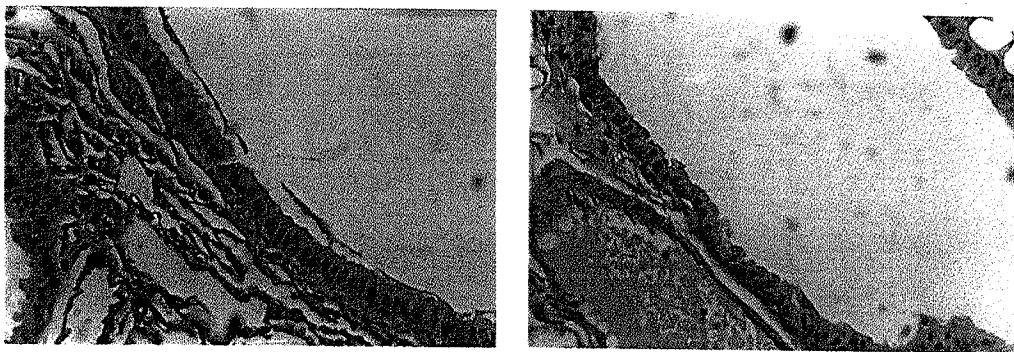


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE LIVER

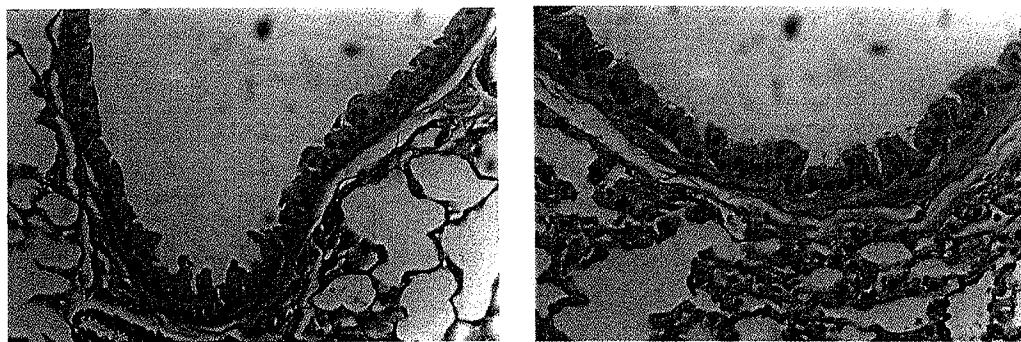


EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE LUNG

Figure 14

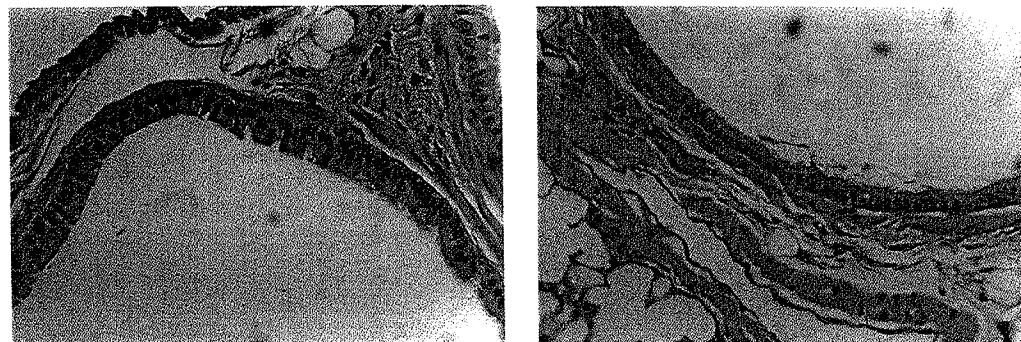


EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE LUNG



EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE LUNG

Figure 15

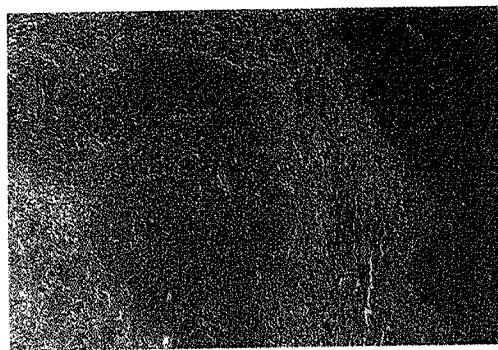


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE LUNG

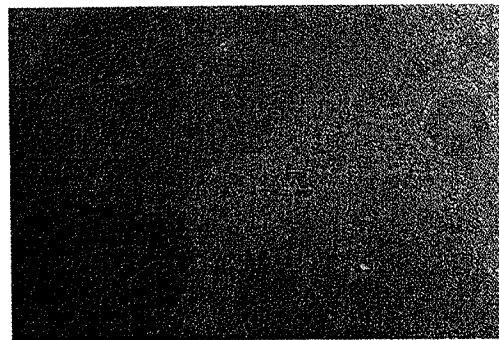


EFFECT OF 3 MONTHS GSPE EXPOSURE ON THE SPLEEN

Figure 16

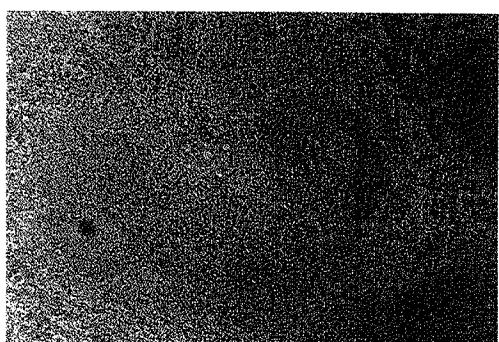
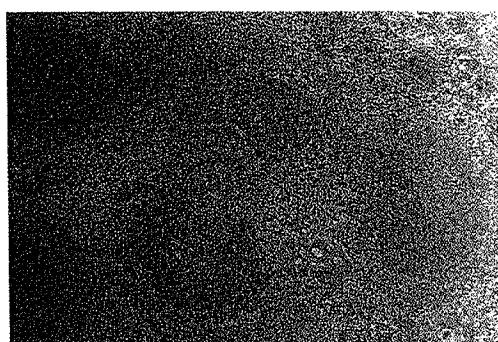


EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE SPLEEN

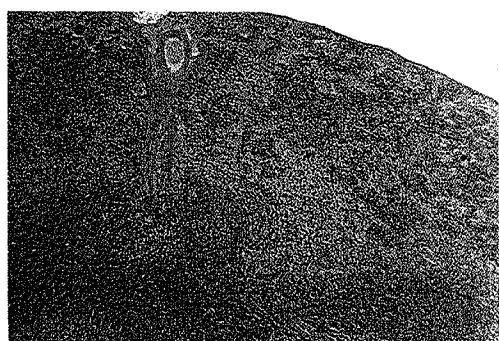
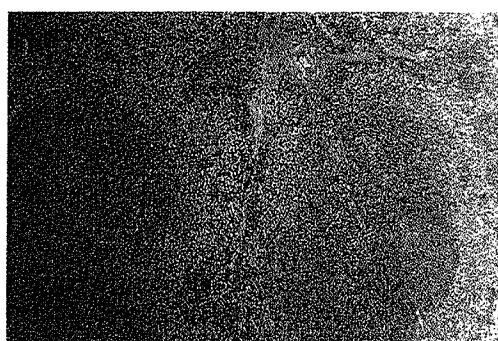


EFFECT OF 9 MONTHS GSPE EXPOSURE ON THE SPLEEN

Figure 17

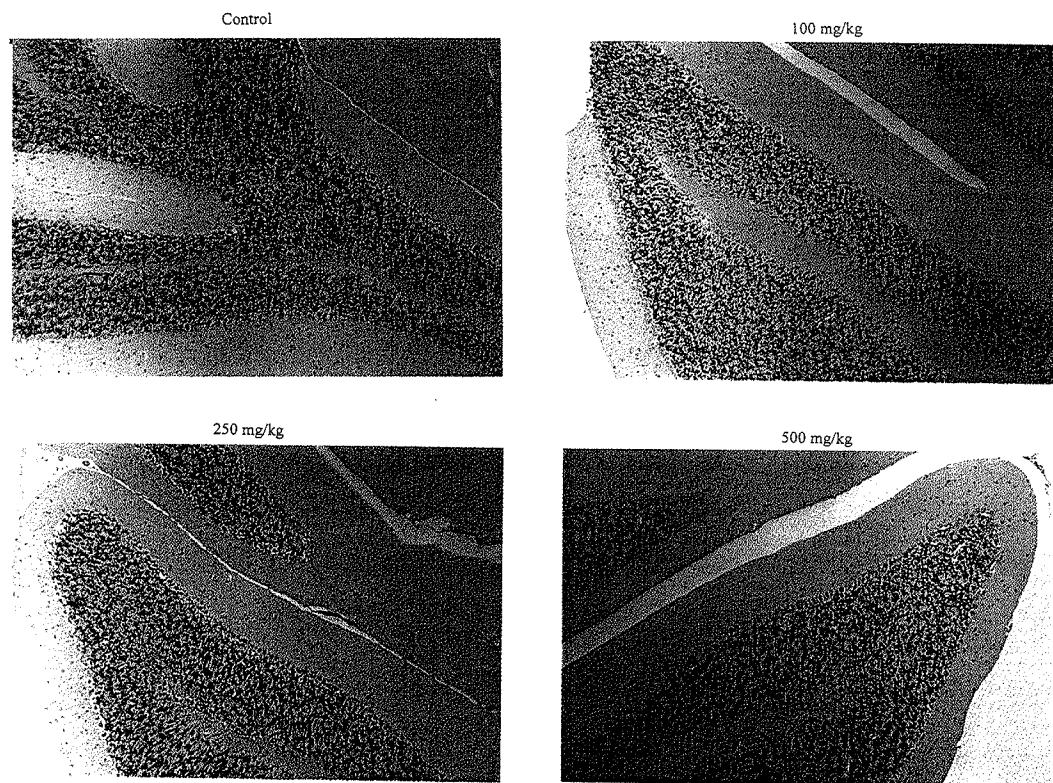


EFFECT OF 12 MONTHS GSPE EXPOSURE ON THE SPLEEN



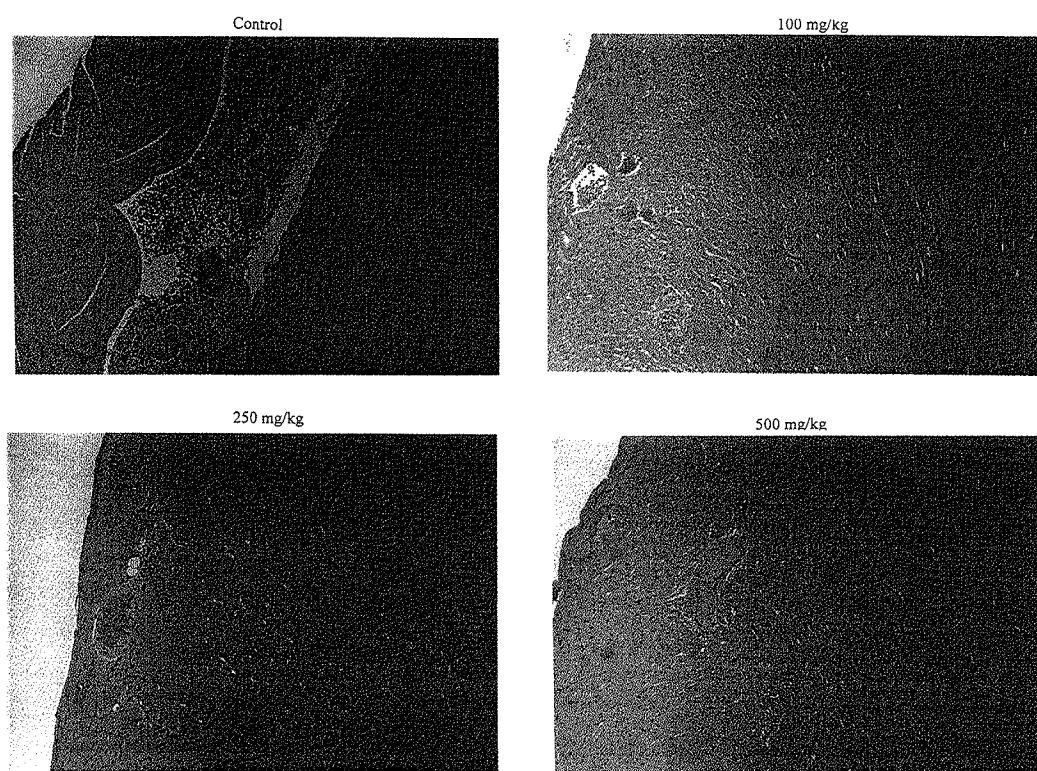
EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE BRAIN

Figure 21



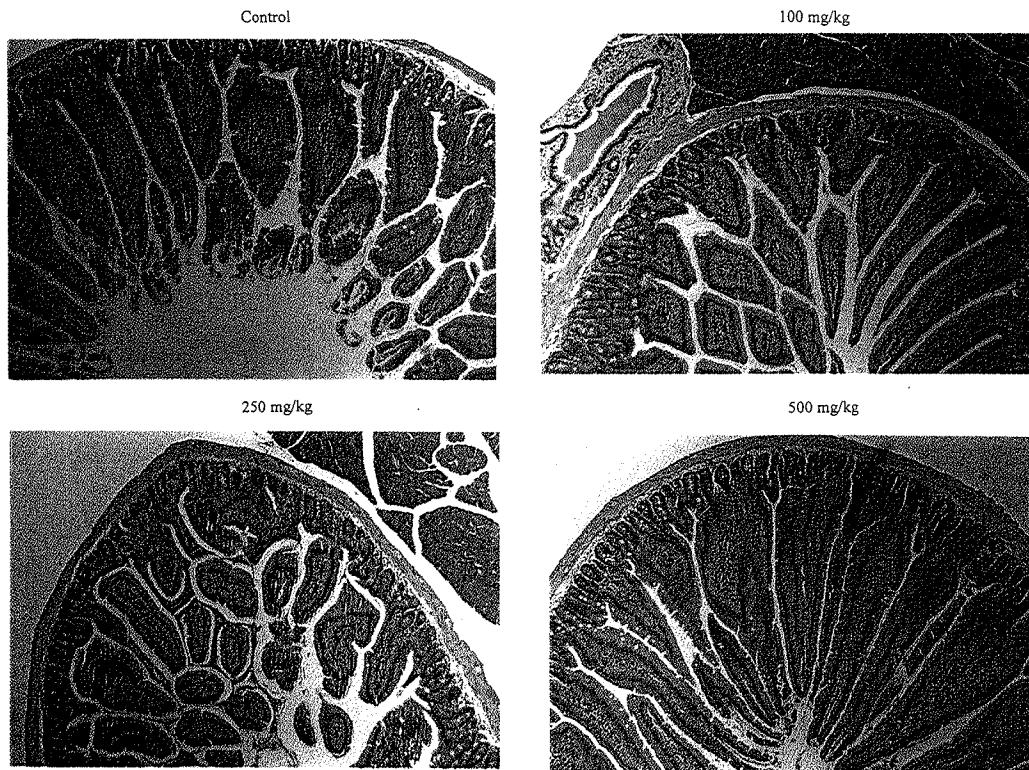
EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE HEART

Figure 22



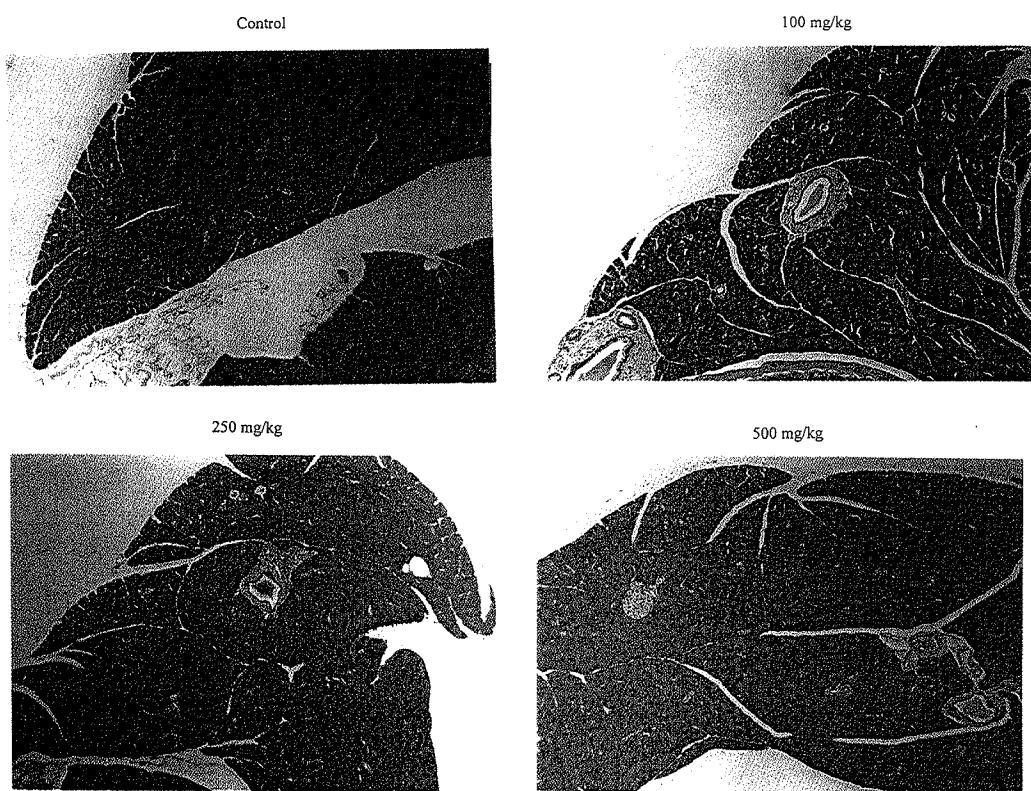
**EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE DUODENUM**

Figure 23



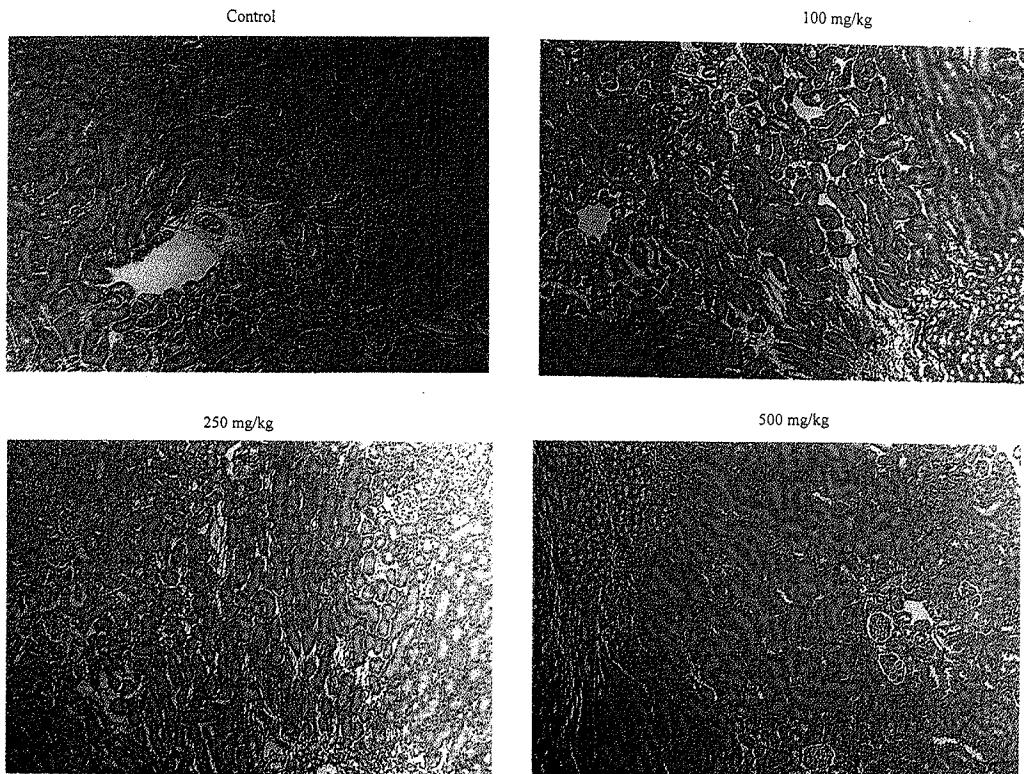
**EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE PANCREAS**

Figure 24



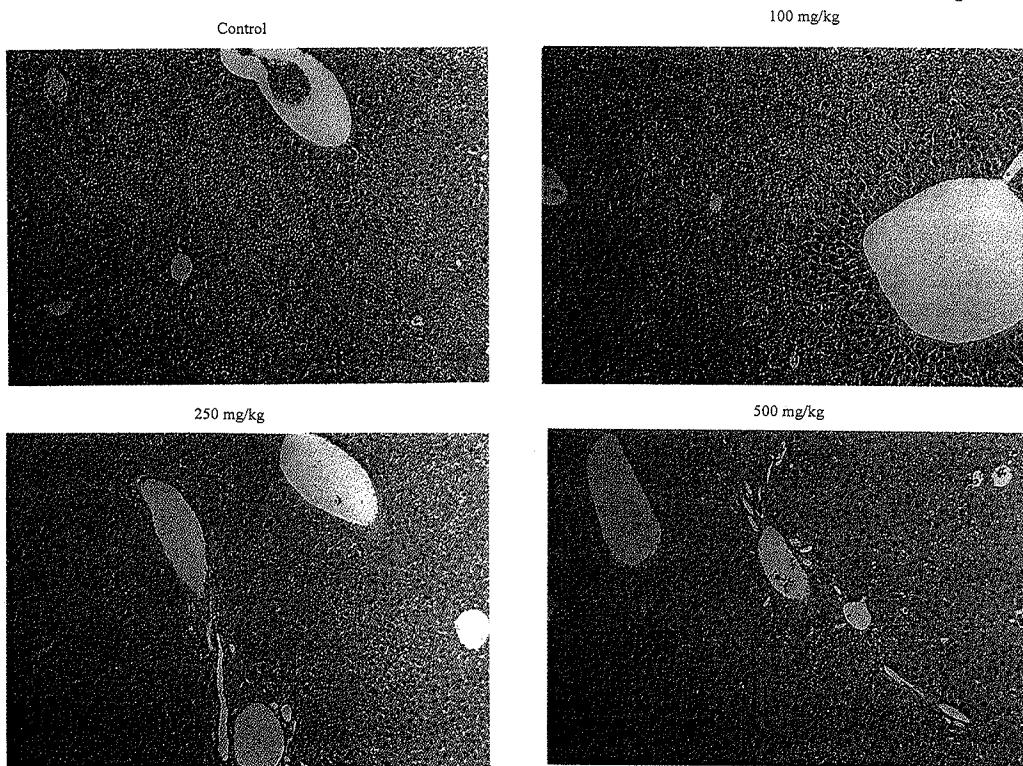
**EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE KIDNEY**

**Figure 25**



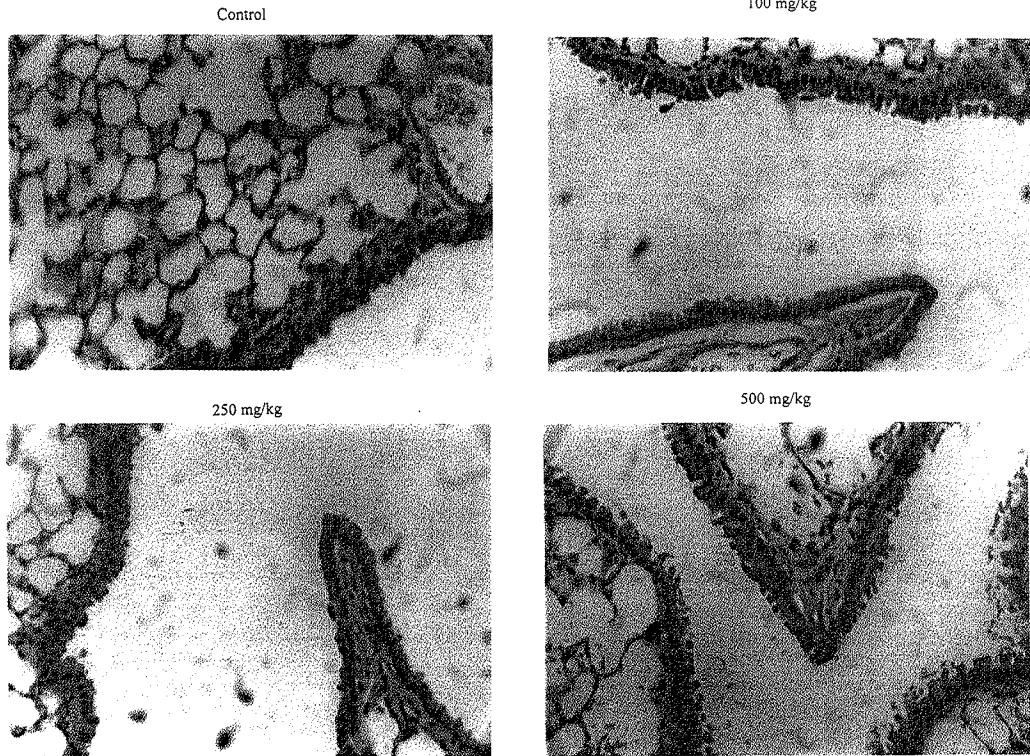
**EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE LIVER**

**Figure 26**



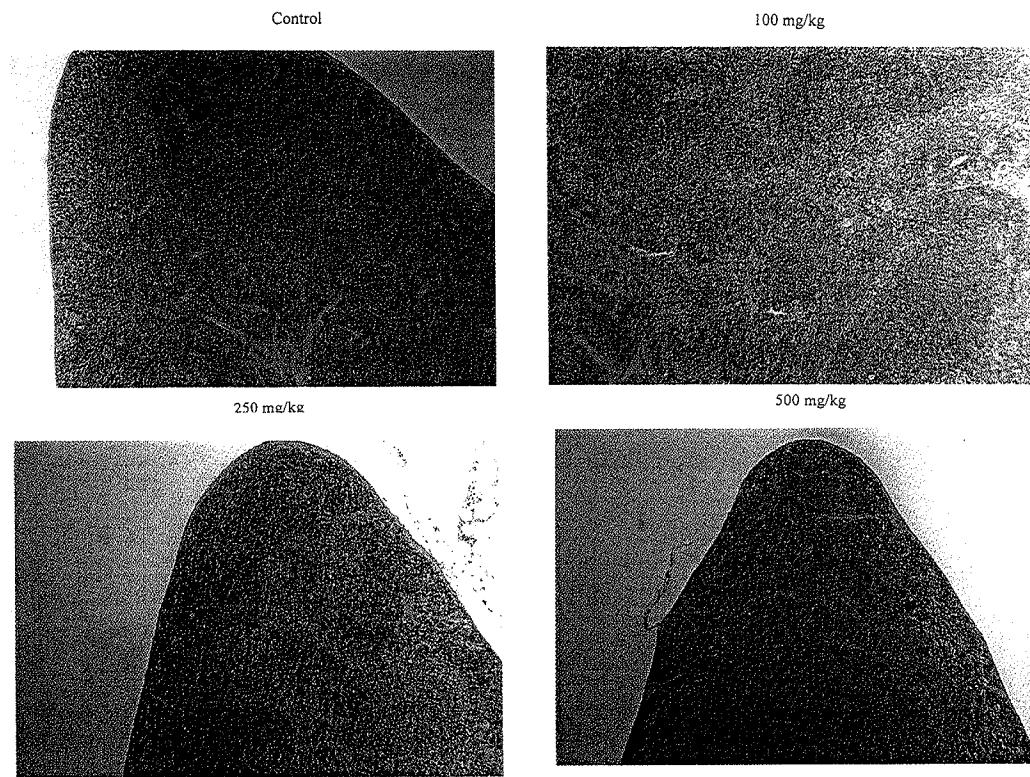
### EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE LUNG

Figure 27



### EFFECT OF 6 MONTHS GSPE EXPOSURE ON THE SPLEEN

Figure 28



Serum creatine kinase (CK) activity was used as a biomarker of tissue necrosis and cardiac function. Figure 20 exhibits the serum CK activity in control and GSPE-fed animals at six months of treatment. No significant changes in serum CK levels were observed following regular feeding of these animals with the three levels of GSPE.

*Histopathological Evaluation:* Eight organs of both control and GSPE-fed animal tissues were examined at various magnification levels. Figures 21-28 demonstrate representative histopathological photomicrographs of tissues from control and GSPE-fed mice after six months of treatment at three different doses (100, 250 and 500 mg/kg/day). No unusual histopathological changes were observed in these eight organs of GSPE-fed mice.

#### *Discussion*

Experiments over the past decade have demonstrated the beneficial effects of a variety of phytopharmaceuticals which are now sold as over-the-counter herbal supplements. According to an estimate of the World Health Organization, nearly 80 percent of the population of developing countries (over 4 billion people) rely on non-traditional medicines, mostly plant drugs, for their primary health care needs. In the United States alone, herbal product sales have exceeded the billion-dollar mark in the last couple of years. A major gap remains in the popularity between conventional allopathic medicines and these plant-derived remedies. Many of the experiments performed have determined the potential beneficial effects of these plant-derived products but there are very few studies that have determined the safety of these traditional medicines. Some of the natural products have extensive research on health benefits, but with very little or no safety studies. More evaluative studies must be done to determine the safety of herbal products consumed by the human population. The series of experiments presented here were specifically designed to test the effects of acute and chronic administration of a novel IH636 grape seed proanthocyanidin extract (GSPE) on multiple models.

A number of *in vitro*, *in vivo* and human clinical studies have been conducted (Bagchi *et al.*, 2000) in our laboratories employing GSPE used in this investigation. GSPE has proven to be an excellent free radical scavenger in both *in vitro* and *in vivo* models, and exhibits significantly superior efficacy as compared to vitamins C, E and  $\beta$ -carotene (Bagchi *et al.*, 1997, 1998a, 1999a; Sato *et al.*, 1999). Oxidative stress

associated programmed and unprogrammed cell deaths induced by smokeless tobacco in normal human oral keratinocytes (Bagchi *et al.*, 1999a) and human liver cell toxicity induced by anticancer drugs were significantly attenuated by GSPE (Joshi *et al.*, 1999). It was also apparent from these studies that GSPE was a better cytoprotective agent as compared to vitamins C and E, singly and in combination, and  $\beta$ -carotene.

GSPE exhibited selective cytotoxicity towards human MCF-7 breast cancer, A-427 lung cancer and CRL-1739 human gastric cancer cells, while enhancing the growth and viability of normal human gastric mucosal cells and murine macrophage J774A.1 cells (Ye *et al.*, 1999). GSPE also protected against myocardial ischemic reperfusion injury and myocardial infarction in Sprague Dawley rats (Sato *et al.*, 1999). Acute and chronic stress-induced oxidative gastrointestinal injury including gastric and intestinal mucosal lipid peroxidation, DNA fragmentation and membrane microviscosity changes were significantly prevented by GSPE (Bagchi *et al.*, 1999b).

Although numerous studies have employed proanthocyanidins in *in vitro* and *in vivo* model systems, no investigation has systematically evaluated the long-term safety of GSPE. The present series of experiments provides much needed information on the safety and chronic toxicity of this novel IH636 grape seed proanthocyanidin extract (GSPE).

The oral toxicity of GSPE (5000 mg/kg) was determined in rats and there were no remarkable body weight changes. Although one female animal died on day 1, there were no other deaths during the entire study. Mucoid feces and various urogenital staining were noted for seven rats each. Single animals were noted with matting/material around the mouth, hypoactivity and clear ocular discharge. There were no other clinical findings indicating that GSPE has a very low or high level of toxicity. All animals appeared normal by day 3 or earlier and throughout the remainder of the study. Gross necropsy findings noted for the animal found dead included brown material adhered to the gastric mucosa and red matting on the tail. There were no other significant changes observed for the rat that died during the study. There were no gross findings upon necropsy. The LD<sub>50</sub> of GSPE was found herein to be greater than 5000 mg/kg when administered once orally via gastric intubation to fasted male and female albino rats. Thus, the data indicates that GSPE has a wide margin of safety.

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Dermal toxicity was also determined in albino rats with a 2000 mg/kg application. No deaths, test material-related clinical findings, remarkable body weight changes or gross necropsy findings were observed. All animals appeared to have very slight to slight erythema and desquamation and one rat had edema. There were no other dermal findings. With the exception of desquamation noted on three animals, all dermal responses completely subsided by day 12 or earlier. The LD<sub>50</sub> of GSPE is therefore greater than 2000 mg/kg when administered once for 24 hr to the clipped, intact skin of male and female albino rats. In addition, 2000 mg/kg is a no-observed-effect level (NOEL) for systemic toxicity under the conditions of the study.

Slight to severe erythema, very slight to slight edema and desquamation were noted on all animals when determining dermal irritation in rabbits. Two rabbits exhibited eschar, and exfoliation was observed on a single animal. There were no other dermal findings. All dermal irritation completely subsided by day 12 or earlier. The Primary Irritation Index (PII) was calculated to be 2.7. As a consequence of these studies, GSPE can be classified as moderately irritating.

The eye irritation study for GSPE in rabbits showed no deaths or remarkable changes in body weights during the study period. Positive conjunctival reactions were noted in the treated eye of all six animals, where four rabbits exhibited iridal irritation and three animals had corneal irritation. All irritation was reversible and completely subsided by day 14.

Chronic oral GSPE administration of 100 mg GSPE/kg/day for 12 months did not cause any toxicity in mouse brain, heart, intestine, kidney, liver, lung, and spleen. Serum chemistry (ALT, BUN and CK) and histopathology of the seven organs remained unchanged and appeared normal. Furthermore, dose-dependent (0, 100, 250 and 500 mg GSPE/kg/day), long-term (6 months) chronic oral GSPE exposure did not cause any toxicity in mouse brain, lung, liver, spleen, heart, kidneys, duodenum and pancreas. Serum chemistry (ALT, BUN and CK) and histopathology of the eight vital organs remained unchanged and appeared close to normal, demonstrating a high degree of safety and lack of toxicity.

In summary, these toxicity and safety studies clearly indicate that acute and chronic long-term GSPE exposure to animals does not appear to adversely influence the

normal physiology or functioning of any of the vital organs, which were examined at the doses and under the conditions used in this study. Thus, the data indicate that GSPE may serve as a safe antioxidant and health-promoting phytopharmaceutical agent.

*Acknowledgment*

The authors acknowledge WIL Research Laboratories, Inc. Ashland, OR for performing the acute toxicity studies as well as Pathology Associates International, Fredrick, MD for the pathology reports.

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