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NTP Technical Report on the Toxicity Study of Chitosan (CASRN 9012-76-4) Administered in Feed to Sprague Dawley [Crl:CD(SD)] Rats

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Foreword

The National Toxicology Program (NTP) is an interagency program within the Public Health Service (PHS) of the Department of Health and Human Services (HHS) and is headquartered at the National Institute of Environmental Health Sciences of the National Institutes of Health (NIEHS/NIH). Three agencies contribute resources to the program: NIEHS/NIH, the National Institute for Occupational Safety and Health of the Centers for Disease Control and Prevention (NIOSH/CDC), and the National Center for Toxicological Research of the Food and Drug Administration (NCTR/FDA). Established in 1978, NTP is charged with coordinating toxicological testing activities, strengthening the science base in toxicology, developing and validating improved testing methods, and providing information about potentially toxic substances to health regulatory and research agencies, scientific and medical communities, and the public.

The Toxicity Study Report series began in 1991. The studies described in the Toxicity Study Report series are designed and conducted to characterize and evaluate the toxicologic potential of selected substances in laboratory animals (usually two species, rats and mice). Substances selected for NTP toxicity studies are chosen primarily on the basis of human exposure, level of production, and chemical structure. The interpretive conclusions presented in the Toxicity Study Reports are based only on the results of these NTP studies. Extrapolation of these results to other species, including characterization of hazards and risks to humans, requires analyses beyond the intent of these reports. Selection per se is not an indicator of a substance's toxic potential.

NTP conducts its studies in compliance with its laboratory health and safety guidelines and FDA Good Laboratory Practice Regulations and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. Studies are subjected to retrospective quality assurance audits before being presented for public review.

NTP Toxicity Study Reports are indexed in the National Center for Biotechnology Information (NCBI) Bookshelf and are available free of charge electronically on the NTP website (http://ntp.niehs.nih.gov). Toxicity data are available through NTP's Chemical Effects in Biological Systems (CEBS) database: https://www.niehs.nih.gov/research/resources/databases/index.cfm.

Table of Contents

Tables

Figures

This report has been reformatted to meet new NTP publishing requirements;
its content has not changed.

About This Report

National Toxicology Program[1]

[1]Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA

Collaborators

K.A. Shipkowski, B.C. Sayers, S.A. Elmore, C.R. Blystone, M.C. Cora, L.M. Fomby, P.M. Foster, M.R. Hejtmancik, M.J. Hooth, A.P. King-Herbert, G.E. Kissling, D.E. Malarkey, B.S. McIntyre, J.T. Painter, T.A. Peace, K.R. Shockley, S.L. Smith-Roe, M.D. Stout, G.S. Travlos, R.W. Tyl, M.K. Vallant, D.Y. Vasconcelos, S. Waidyanatha, N.J. Walker, K.L. Witt

Division of the National Toxicology Program, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Evaluated and interpreted results and reported findings
K.A. Shipkowski, Ph.D., Co-Study Scientist
B.C. Sayers, Ph.D., Co-Study Scientist
S.A. Elmore, D.V.M., Study Pathologist
C.R. Blystone, Ph.D.
M.C. Cora, D.V.M.
P.M. Foster, Ph.D.
M.J. Hooth, Ph.D.
A.P. King-Herbert, D.V.M.
G.E. Kissling, Ph.D.
D.E. Malarkey, D.V.M., Ph.D.
B.S. McIntyre, Ph.D.
K.R. Shockley, Ph.D.
S.L. Smith-Roe, Ph.D.
M.D. Stout, Ph.D.
G.S. Travlos, D.V.M.
M.K. Vallant, M.S., MT
S. Waidyanatha, Ph.D.
N.J. Walker, Ph.D.
K.L. Witt, M.S.

Battelle Columbus Operations, Columbus, Ohio, USA
Conducted study and evaluated pathology findings
M.R. Hejtmancik, Ph.D., Principal Investigator
L.M. Fomby, D.V.M., Ph.D.
T.A. Peace, D.V.M.
D.Y. Vasconcelos, D.V.M., Ph.D.

ILS, Inc., Research Triangle Park, North Carolina, USA
Coordinated NTP Pathology Peer Review (December 19, 2008)
J.T. Painter, D.V.M., Ph.D.

RTI International, Research Triangle Park, North Carolina, USA
Provided sperm parameter data
R.W. Tyl, Ph.D., Principal Investigator

Contributors

NTP Pathology Peer Review, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina, USA
Participated in NTP Pathology Peer Review (December 19, 2008)
S.A. Elmore, D.V.M., National Toxicology Program

Experimental Pathology Laboratories, Inc., Research Triangle Park, North Carolina, USA
Supervised pathology review
M.H. Hamlin, II, D.V.M., Principal Investigator

RTI International, Research Triangle Park, North Carolina, USA
Supported sperm parameter data collection
K. Vick, B.S.

Dynamac Corporation, Research Triangle Park, North Carolina, USA
Prepared quality assessment audits
S. Brecher, Ph.D., Principal Investigator
S. Iyer, B.S.
V.S. Tharakan, D.V.M.

Social & Scientific Systems, Inc., Research Triangle Park, North Carolina, USA
Provided statistical analyses
M.V. Smith, Ph.D., Principal Investigator
L.J. Betz, M.S.
S.F. Harris, B.S.
J.D. Krause, Ph.D.
C.G. Leach, M.S.

Biotechnical Services, Inc., Little Rock, Arkansas, USA
Prepared Toxicity Study Report
S.R. Gunnels, M.A., Principal Investigator
L.M. Harper, B.S.
T.S. Kumpe, M.A.
E.S. Rathman, M.S.
D.C. Serbus, Ph.D.

Peer Review

The draft *NTP Technical Report on the Toxicity Study of Chitosan (CASRN 9012-76-4) Administered in Feed to Sprague Dawley [Crl:CD(SD)] Rats* was evaluated by the reviewers listed below. These reviewers served as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, reviewers determined if the design and conditions of this NTP study were appropriate and ensured that this Toxicity Study Report presents the experimental results and conclusions fully and clearly.

Peer Reviewers

Diane Birt, Ph.D.
Iowa State University (Retired)
Ames, Iowa, USA

Melissa G. Rhodes, Ph.D.
Roivant Sciences, Inc.
Durham, North Carolina, USA

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Abstract

Chitosan is a cationic carbohydrate polymer that is commercially derived from the deacetylation of chitin obtained from seafood shells. The most widespread route of human exposure to chitosan is as a dietary supplement for body weight reduction. Chitosan was nominated by the National Cancer Institute for mechanistic studies designed to measure the potential for vitamin E depletion and osteoporosis following ingestion. Male and female Sprague Dawley rats were exposed to chitosan (86.5% deacetylated, with an average molecular weight of approximately 82 kilodaltons and estimated to be approximately 94% pure) in feed for 6 months.

In this 6-month study, groups of 10 male and 10 female core study rats (Group A) were fed control diets (AIN-93M) or diets containing chitosan at concentrations of 1%, 3%, or 9%, for up to 25 weeks. Two additional groups of 10 male and 10 female rats (Groups B and C) were given the same dietary concentrations for up to 26 weeks. All male and female Group A rats survived to the end of the study. Mean body weights and feed consumption of exposed Group A groups were similar to those of the control groups. Dietary concentrations of 1%, 3%, and 9% resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight per day to males and 650, 1,800, and 6,000 mg/kg per day to females. There were no treatment-related clinical findings in core study animals.

The 9% male and female rats had significantly decreased cholesterol values (26% to 48%), compared to the controls, at all time points. Triglycerides were significantly decreased in 9% male and female rats, but not at every time point. Phosphorus levels were significantly decreased in 9% male rats at weeks 13, 19, and 25; a decrease also occurred in 3% males at week 13. Phosphorus levels were significantly decreased in 3% and 9% females at weeks 13 and 25.

Compared to those of the controls, serum vitamin A concentrations were significantly decreased (approximately 30%) at weeks 13, 19, and 26 in 9% males, at weeks 13 and 26 in 3% males (approximately 15%), and at weeks 19 and 26 in 9% females (approximately 20%). Serum vitamin E concentrations were significantly decreased at all time points in 3% (33% to 42%) and 9% (79% to 82%) males, in 1% (17%) males at week 13, and in 9% (62% to 65%) females at all time points. Hepatic vitamin E concentrations were significantly decreased at week 26 in 3% (48%) and 9% (87%) males and 9% (80%) females. Serum concentrations of 1,25 $(OH)_2$ vitamin D were significantly increased in 9% (105% to 142%) males and (100% to 180%) females at weeks 7, 19, and 26.

Compared to the control groups, percent fat digested was significantly decreased during week 6 in 9% males and females, during week 12 in 3% and 9% males, during week 18 in 9% males and females, and during week 24 in all exposed groups of males and females. Calcium absorption was significantly increased in 9% females during weeks 12 and 24. Fecal weight was significantly increased in 3% and 9% males and females during each collection period, and in 1% females during weeks 12, 18, and 24. Fecal moisture was significantly increased in 9% males (up to 170%) and 9% females at all time points, in 3% males during week 6, and in 3% females during weeks 12 and 18.

Results of this study did not support chitosan as a cause of bone resorption. Significant elevation of parathyroid hormone levels occurred occasionally and inconsistently, while calcium levels remained relatively stable. Bone calcium, bone length, and the histology findings did not indicate calcium loss from the bone following chitosan exposure.

The absolute and relative liver weights of 9% males and females and the absolute and relative thymus weights of 3% males and 9% males and females were significantly less than those of the control groups.

There was a treatment-related decrease in the incidence of periportal fatty change in the liver of 9% females relative to the control group. A decreased incidence of periportal fatty change was observed in the liver of 9% males relative to the control group as well, but this decrease was not significant, and it was the same as that observed in 1% males. The appearance of periportal fatty change was similar in both males and females and in both exposed and control groups.

Under the conditions of the 6-month feed study of chitosan, male and female rats fed 3% and 9% chitosan in the diet had significantly decreased levels of serum vitamin A and serum and hepatic vitamin E and increased levels of serum 1,25 $(OH)_2$ vitamin D. Consumption of high levels of chitosan decreased percentage fat digestion and increased fecal weight and moisture, as well as reduced levels of phosphorous, cholesterol, and triglycerides. Female rats exposed to 9% chitosan also had significant liver weight and histologic changes. Based on the above results, the lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats.

Synonyms: 2-Amino-2-deoxy-beta-D-glucosamine; deacetylated chitin; poliglusam; poly (D-glucosamine)

Trade names: Celox, Chicol, Chitopearl, CTFA 04299, Flonac N, Kytex H, Sea Cure F

Summary of Findings Considered to be Toxicologically Relevant in Sprague Dawley Rats Exposed to Chitosan in Feed for Six Months

	Male Rats	Female Rats
Concentrations in feed	0%, 1%, 3%, 9%	0%, 1%, 3%, 9%
Survival rates	Group A: 10/10, 10/10, 10/10, 10/10 Group B: 9/10, 10/10, 10/10, 8/10 Group C: 10/10, 10/10, 10/10, 10/10	Group A: 10/10, 10/10, 10/10, 10/10 Group B: 10/10, 10/10, 9/10, 10/10 Group C: 10/10, 9/10, 10/10, 10/10
Body weights	Exposed groups similar to the control group	Exposed groups similar to the control group
Clinical findings	None	None
Clinical pathology	↓ Phosphorus ↓ Cholesterol ↓ Triglycerides	↓ Phosphorus ↓ Cholesterol ↓ Triglycerides
Vitamin concentrations	↓ Serum vitamin A ↑ Serum 1,25 $(OH)_2$ vitamin D ↓ Serum vitamin E ↓ Hepatic vitamin E	↓ Serum vitamin A ↑ Serum 1,25 $(OH)_2$ vitamin D ↓ Serum vitamin E ↓ Hepatic vitamin E
Digestive parameters	↓ Percent fat digested ↑ Fecal weight ↑ Fecal moisture	↓ Percent fat digested ↑ Fecal weight ↑ Fecal moisture ↑ Calcium absorbed
Bone parameters	None	None
Reproductive toxicity	None	Not determined
Organ weights	↓ Absolute and relative liver weights ↓ Absolute and relative thymus weights	↓ Absolute and relative liver weights ↓ Absolute and relative thymus weights
Nonneoplastic effects	None	Liver: periportal, fatty change (7/10, 4/10, 4/10, 0/10)

Introduction

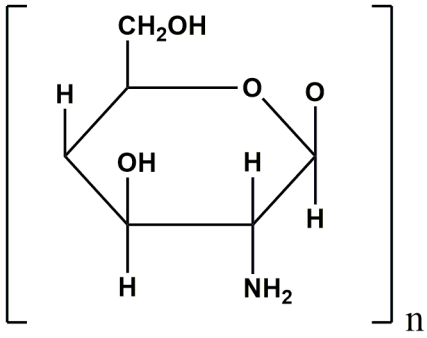

Figure 1. Chitosan (CASRN 9012-76-4; Chemical Formula: $[C_6H_{11}NO_4]_n$)

Synonyms: 2-Amino-2-deoxy-beta-D-glucosamine; deacetylated chitin; poliglusam; poly. (D-glucosamine).
Trade names: Celox, Chicol, Chitopearl, CTFA 04299, Flonac N, Kytex H, Sea Cure F.

Chemical and Physical Properties

Chitosan is a cationic carbohydrate polymer that is commercially derived from the deacetylation of chitin. The primary unit of the chitosan polymer is D-glucosamine. Chitosan exists in multiple forms that can differ in molecular weight [3 to 3,600 kilodaltons (kDa)] and in the degree of deacetylation (40% to 100%)[1]. Chitosan is defined as chitin that is sufficiently deacetylated to form soluble amine salts. Solubility in aqueous, acidic media occurs when deacetylation of chitin reaches approximately 50%[2]. In addition to the degree of deacetylation, chitosan solubility is also dependent on the molecular weight and the distribution of the remaining acetyl groups on the polymer[3]. Chitosan is insoluble in alkaline solutions at pH levels above 6.5. Chitosan products are highly viscous, resembling natural gums[4].

Production, Use, and Human Exposure

Chitin, from which chitosan is derived, is a naturally occurring carbohydrate polymer second only to cellulose in abundance. Chitin is a structural component found in the exoskeleton of arthropods and in the cell walls of fungi and yeast[2]. The primary unit of chitin, *N*-acetyl-D-glucosamine, forms the polymeric structure via 1 → 4 glycosidic bonds. Discarded crab and shrimp shells from the seafood industry are the primary source material of chitin for the commercial production of chitosan[5]. For chitosan production, seafood shells are deproteinized by treatment with an aqueous 3% to 5% sodium hydroxide (NaOH) solution. The resulting product is neutralized and calcium is removed by treatment with an aqueous 3% to 5% hydrochloric acid (HCl) solution at room temperature resulting in a white or slightly pink precipitate of chitin. The *N*-deacetylation of chitin is done by treatment with an aqueous 40% to 45% NaOH solution, and the precipitate is washed with water. The precipitate is then dissolved in aqueous 2% acetic acid and the insoluble material is removed. The resulting clear supernatant solution is neutralized with aqueous NaOH solution producing chitosan as a white precipitate.

Chitosan is used in a wide range of products including use as a flocculating agent for water and waste treatment and as a chelating agent for removal of traces of heavy metals from aqueous

solutions[4]. In agriculture, chitosan is used as a plant growth regulator through foliar application and as an antimicrobial agent and a time-release reservoir for fertilizers in soil amendments.

Chitosan has several current or proposed biomedical applications. Chitosan is considered to be hemostatic due to its cationic nature. As such, wound dressings manufactured from chitosan are available for clinical use[6]. Several drug delivery systems based on chitosan nanoparticles are currently being investigated. Chitosan nanoparticles are capable of permeating the blood brain barrier, and the mucoadhesive properties of chitosan have been shown to enhance drug absorption[2; 7]. Chitosan has also been evaluated for the manufacture of ocular bandage lenses and biodegradable surgical and dental implants[8].

In cosmetics, chitosan is used in a variety of hair and skin products, including hair and body washes, coloring shampoos, and agents for skin cleaning and protection[9]. Chitosan has also been evaluated for use as an additive to toothpaste for prevention of enamel erosion[10].

As a dietary supplement, chitosan is marketed and sold in weight-loss products, but the mechanism behind chitosan-induced inhibition of fat digestion is not well understood. It has been proposed that chitosan acts as a weak anion exchanger and decreases intestinal cholesterol absorption while also increasing the excretion of bile acids[11-13]. Another possible mechanism is that chitosan traps fat in the intestines by increasing the viscosity of the intestinal contents and preventing the hydrolysis of triglycerides[13-15]. The manufacturer-recommended consumption of chitosan as a weight-loss product in humans typically averages 1,000 mg per day, or approximately 14.3 mg/kg per day (based on a 70 kg adult)[16; 17]. There are no available dose or prevalence data for human consumption of chitosan as a dietary supplement.

Absorption, Distribution, Metabolism, Excretion, and Toxicokinetics

The systemic absorption and distribution of chitosan following oral exposure are likely influenced by the molecular weight of the polymer. The effect of molecular weight on chitosan absorption has been evaluated in male Sprague Dawley rats. Oral gavage administration of chitosan with molecular weights of 3.8, 7.5, 13, 22, or 230 kDa resulted in maximum plasma chitosan concentrations (C_{max}) of 20.23, 9.30, 5.86, 4.32, or less than 0.5 µg/mL, respectively[18]. The results of this study suggest that the absorption of chitosan from the gastrointestinal tract following oral exposure is inversely related to chitosan molecular weight, as there is likely low bioavalability associated with the higher molecular weight chitosan polymers.

The biodegradation of chitosan influences absorption and distribution because both are dependent on molecular weight. The biodegradation of chitosan in vivo is dependent on the degree of deacetylation[19]. Enzymatic degradation of chitosan depends on the ability to hydrolyze glucosamine-glucosamine, glucosamine-*N*-acetyl-glucosamine and *N*-acetyl-glucosamine-*N*-acetyl-glucosamine linkages[1]. Degradation of chitosan in vertebrates is thought to occur predominantly by lysozymes and bacterial enzymes in the colon[1]. While eight human chitinases have been identified with three showing enzymatic activity, their capacity to degrade chitosan has not been investigated[1; 20].

Toxicity

Experimental Animals

The acute toxicities of chitosan and chitosan oligomers prepared by enzymatic depolymerization of chitosan have been evaluated. Hirano[5] reported the oral LD_{50} for chitosan as 16 g/kg body weight in mice. No clinical signs of toxicity were observed following a single oral administration of chitosan oligomers up to 10 g/kg in male and female Kunming strain mice[21].

No significant differences in weight gain were observed between exposed male Charles River albino rats and the controls in a 4-week study with 1% or 5% dietary chitosan[22]. In male Wistar rats, no significant differences in growth, feed intake, liver weight, or dried fecal weight were observed between control and chitosan-fed (2% or 5%) animals after 21 days[23]. In male Sprague Dawley rats fed chitosan in the diet for 8 weeks, no toxicity was observed in animals at concentrations up to 5%, progressive growth reductions and clinical pathology disturbances occurred at 10% and 15%, and enlargement of the liver and kidneys was observed at 15%[24].

In female BALB/c mice fed a 5% (4.4 ± 0.7 g/day per animal) chitosan diet for 4 weeks, body weight reduction correlated with significantly decreased feed consumption and alterations in normal gut flora[25].

In a study to evaluate mineral and fat-soluble vitamin status in male Charles River Japan Sprague Dawley rats, exposure to a diet containing 5% chitosan for 2 weeks caused a decrease in mineral absorption and bone mineral content[26]. Decreased serum vitamin E was observed in rats fed 5% chitosan with ascorbic acid supplementation in the diet. Serum vitamin E depletion was not observed in rats given glucosamine instead of chitosan.

Depletion of fat-soluble vitamins has been associated with a variety of neurologic and metabolic disorders. Male C57BL/6 mice fed a vitamin E-deficient diet showed signs of cognitive decline after 3 months of exposure and had increased lipid peroxidation products in brain tissue after 6 months of exposure[27]. Male rats fed a vitamin A-deficient diet for 3 months had lower levels of serum cholesterol, HDL-cholesterol, and triacylglycerol, as well as decreased synthesis of liver fatty acids[28].

The toxicity of glucosamine oligomers has been evaluated in male and female Charles River Japan F344 rats fed 0%, 0.04%, 0.2%, or 1% oligoglucosamine in the diet for 90 days[29]. Glucosamine oligomers are prepared by hydrolysis of chitosan and, similar to the chitosan utilized in this 6-month study, are considered low molecular weight chitosan. In the 1% (653.1 mg/kg per day in males, 719.8 mg/kg per day in females) group, erythema and edema in the snout and on the forelimbs and loss of fur on the forelimbs were observed in both male and female rats. Neutrophilic infiltration in the nasal cavity was also observed in both sexes in the 1% group. These findings were considered to be caused by topical exposure to glucosamine oligomers during feeding and grooming. Decreased feed consumption and body weight gain were also observed in animals in the 1% group in this study and were thought to be the result of feeding difficulty due to the snout and forelimb lesions described above. Rats receiving 1% oligoglucosamine also displayed lower weights of the uterus, ovary, seminal vesicles, and testes (with fewer germ cells).

The intravenous administration of chitosan has been investigated due to the development of chitosan formulations for drug delivery. No adverse effects were reported in rabbits up to 60 days following intravenous administration of chitosan oligosaccharides (prepared by oxidative depolymerization of chitosan) at doses up to 8.6 mg/kg daily for 5 consecutive days[30]. In this study, increased lysozyme activity was observed in rabbit serum collected the day after the last intravenous injection. Chemical modifications and nanoparticle suspensions of chitosan are currently being investigated for drug delivery[1]. As such, modifications made to chitosan could alter the toxicity of the unmodified chitosan polymer.

No adverse effects of chitosan were reported in eye or skin irritation tests in rabbits or guinea pigs, respectively[31].

Humans

Studies designed to evaluate the effectiveness of chitosan as a weight-loss supplement suggest that chitosan is well tolerated in humans. No adverse effects were reported in male (4.5 g chitosan per day) or female (2.5 g per day) volunteers following oral chitosan administration for 12 days[32; 33]. Additionally, no adverse effects were reported following oral administration of chitosan at up to 6.75 g per day for 8 weeks in male and female volunteers[34].

Carcinogenicity

No 2-year carcinogenicity studies of chitosan were identified in the available literature.

Carcinogenicity and chronic toxicity have been evaluated for *N*-acetyl-D-glucosamine, a monomeric constituent of chitosan. F344 rats administered *N*-acetyl-D-glucosamine at concentrations up to 5% in the diet (1,935 mg/kg per day in males and 2,244 mg/kg per day in females) for 104 weeks had no associated increases in tumor response[35]. In a second study in F344 rats, administration of *N*-acetyl-D-glucosamine in feed at concentrations up to 5% in the diet (2,323 mg/kg per day in males and 2,545 mg/kg per day in females) for 52 weeks did not induce an increase in tumor response[35].

Developmental and Reproductive Toxicity

A limited number of developmental and reproductive toxicity studies were identified in the literature.

In a multigenerational prenatal and postnatal assessment of high molecular weight chitosan (HMWCS), F_0 time-mated ICR mice were administered 0, 125, 500, or 2,000 mg/kg HMWCS via a single intraperitoneal injection on gestational day 6 (GD 6) and subjected to a laparotomy or allowed to litter[36]. F_1 offspring (1 mouse/sex per litter) from the same exposure group were mated and females similarly subjected to either a laparotomy or allowed to litter to produce an F_2 generation. F_0 dams in the 2,000 mg/kg group exhibited signs of maternal toxicity (mortality and diarrhea). F_0 dams in the 500 and 2,000 mg/kg groups displayed dose-dependent increases in vaginal bleeding, postimplantation loss, and lower spleen weights. Fetal weights for both generations were lower in the 2,000 mg/kg group. There were no external, visceral, or skeletal malformations attributed to chitosan administration. F_0 dams allowed to litter displayed a dose-related reduction in litter size. F_1 mice exposed in utero to 2,000 mg/kg HMWCS and examined on postnatal day 21 (PND 21) exhibited higher uterus, ovary, and thymus weights. Female F_1

mice exposed in utero to 2,000 mg/kg HMWCS displayed lower thymus weights on PND 56. F_2 mice exposed in utero to 2,000 mg/kg HMWCS displayed lower testis and ovary weights on PNDs 21 and 56.

Chitosan oligomers did not induce morphologic sperm abnormalities in male mice following oral gavage daily for 5 days with up to 5,000 mg/kg[21].

The effects of chitosan nanoparticles (spherical; 200 ± 6 nm or 340 ± 10 nm diameter) have been examined in zebrafish (Danio rerio) embryos. Embryos exposed 4 to 5 hours after fertilization to 0, 5, 10, 20, 30, or 40 μg/mL (200 nm particles) or 0, 10, 20, or 40 μg/mL (340 nm particles) displayed concentration-dependent decreases in hatching rates and increases in mortality 96 hours after exposure[37]. Increased rates of cell death and reactive oxygen species production were observed in all exposure groups. Exposure to 200 nm, but not 340 nm, chitosan nanoparticles induced developmental malformations in embryos, including bent spines, pericardial edema, and opaque yolks.

Genetic Toxicity

No in vitro or in vivo studies evaluating chitosan for mutagenic effects were identified in the available literature.

Chitosan oligomers were negative at concentrations up to 5,000 μg/plate in Salmonella typhimurium strains TA97, TA98, TA100, and TA102 with and without rat liver S9 metabolic activation enzymes, and they were negative for micronucleus induction in mouse bone marrow following oral gavage for 2 days at up to 5,000 mg/kg[21].

Study Rationale

Chitosan was nominated for study by the National Cancer Institute due to widespread human exposure, especially through use as a dietary supplement for body weight reduction, and for concerns regarding potential vitamin E and bone mineral depletion following ingestion. NTP conducted a 6-month study evaluated the effects of dietary chitosan on the development of osteopenia/osteoporosis, fat and calcium absorption, fat-soluble vitamin depletion, and general toxicity effects in Charles River Sprague Dawley rats.

Materials and Methods

Procurement and Characterization of Chitosan

Chitosan was obtained from Vanson HaloSource, Inc. (Redmond, WA), in one lot (02-ASSF-0715), which was used in the 6-month study. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (MRI) (Kansas City, MO) and by the study laboratory at Battelle Columbus Operations (Columbus, OH) (Appendix F). Reports on analyses performed in support of the chitosan studies are on file at the National Institute of Environmental Health Sciences.

The test article, an off-white powder, was identified using infrared and proton nuclear magnetic resonance (NMR) spectroscopy. The percentage of deacetylation of the test article, determined by proton NMR, ranged from 85.97% to 87.17%, with an average of 86.5%. All spectra were consistent with the literature spectra[38; 39], and with the Sadtler spectral database.

The moisture content for lot 02-ASSF-0715 was determined using weight loss on drying, the inorganic content was determined on the dried test article by ashing, viscosity was determined using a Brookfield viscometer, and the most abundant molecular weight was determined using gel permeation chromatography (GPC) with refractive index (RI) detection.

Moisture content was 4.50% water, the average inorganic content was 2.13%, and viscosity was 81.3 centipoise. GPC/RI indicated one major peak and the determined molecular weight of the bulk chemical ranged from 62,755 to 87,343 daltons (Da). This resulted in an average molecular weight of 81,644 g/mol, or approximately 82 kDa, classifying the test article as a low molecular weight chitosan (LMWCS). A sample of lot 02-ASSF-0715 was submitted to Covance Laboratories, Inc. (Madison, WI), for nutritional and contaminant testing using standard methods. Levels of organochlorine and organophosphorous pesticides, nitrosamines, and aflatoxins were below the detection limits of the analytical methods. The purity of lot 02-ASSF-0715 was estimated to be approximately 94% based on the analysis of moisture and inorganic content. Taken together, these data indicated that the test article was chitosan.

To ensure stability, the test article was stored in sealed amber glass vials at room temperature. Reanalysis of the test article was performed during the study using GPC/RI and no degradation of the test article was detected.

Preparation and Analysis of Dose Formulations

The dose formulations were prepared approximately monthly by mixing chitosan with feed. Dose formulations were stored in lined plastic buckets sealed with lids and stored at −30°C to −15°C for up to 42 days.

Homogeneity studies of approximately 0.5% and 9% formulations (5,046 and 90,049 µg/g, respectively) and stability studies of an approximately 0.5% (5,046 µg/g) formulation were performed by the analytical chemistry laboratory using GPC/RI. Two peaks were attributed to chitosan with retention times of approximately 6.9 minutes and 12.1 minutes, respectively. Chitosan quantitation was based on the larger polymeric components of the first peak only because vehicle components co-eluted with the later oligomeric peak. Homogeneity studies of

1% and 9% (10 and 90 mg/g in feed, respectively) dose formulations were performed by the study laboratory using GPC/RI. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in lined plastic buckets sealed with lids at temperatures up to room temperature and for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of chitosan were performed by the study laboratory using GPC/RI. Of the dose formulations analyzed, all nine were within 10% of the target concentrations (Table F-3). Animal room samples were also analyzed; all three were within 10% of the target concentrations.

Animal Source

Male and female Sprague Dawley [Crl:CD(SD)] rats were obtained from Charles River Laboratories (Portage, MI) for use in the 6-month study.

Animal Welfare

Animal care and use are in accordance with the Public Health Service Policy on Humane Care and Use of Animals. All animal studies were conducted in an animal facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. Studies were approved by the Battelle Columbus Operations Animal Care and Use Committee and conducted in accordance with all relevant NIH and NTP animal care and use policies and applicable federal, state, and local regulations and guidelines.

Six-month Study

The rats were 5 to 6 weeks old upon receipt. Rats were quarantined for 12 to 15 days and were 7 to 9 weeks old on the first day of the study. Before the study began, five male and five female rats were randomly selected for parasite evaluation and gross observation for evidence of disease. The health of the animals was monitored during the study according to the protocols of the NTP Sentinel Animal Program (Appendix I). A positive test result for parvovirus occurred in one animal at the 4-week timepoint. Additional testing of serum from this animal and other sentinel animals via other testing methodologies deemed the original positive result to be a false positive. All other test results were negative for rodent pathogens.

The animals in this study were split into three groups, the core group, Group A, and two special study groups, Groups B (vitamin and bone analysis) and C (fat digestion, hematology, clinical chemistry, and urinalysis). Different parameters were evaluated in each group, which allowed for the collection of extensive endpoints (Table 1). Groups of 10 male and 10 female rats were examined per endpoint and there was no crossover of analyses between any of the groups. Group A rats were fed diets containing 0%, 1%, 3%, or 9% chitosan for 25 weeks. Groups B and C rats were fed diets containing the same concentrations for up to 26 weeks. Feed and water were available ad libitum. The AIN-93M diet was used for this study instead of the NTP-2000 diet because of the high levels of fat-soluble vitamins and higher total fat content found in the NTP-2000 diet. The NTP-2000 feed contains almost double the amount of required fat-soluble vitamins and has a higher fat content (7% to 8%) than the AIN-93M feed (4%)[40-42]. One of the primary rationales for this chitosan study was the potential for decreases in fat-soluble vitamin

concentrations, and therefore, utilizing a diet with lower levels of preexisting vitamins and a lower fat content was ideal to avoid confounding potential results. Rats were housed individually. Feed consumption was recorded weekly for core study rats. Core study rats were weighed and clinical findings were recorded initially, on day 8, weekly thereafter, and at the end of the study. Details of the study design and animal maintenance are summarized in Table 2.

Table 1. Distribution of Evaluated Parameters

	Group		
Parameter	**A**	**B**	**C**
Feed consumption	X	–	–
Body weights	X	–	–
Clinical findings	X	–	–
SMVCE	X	–	–
Bone histomorphometry	X	–	–
Gross lesions and histopathology	X	–	–
Vitamin A (serum and liver)	–	X	–
Vitamin E (serum and liver)	–	X	–
1,25 $(OH)_2$ Vitamin D (serum)	–	X	–
Bone calcium, ash, and moisture	–	X	–
Hematology	–	–	X
Clinical chemistry	–	–	X
Vitamin K_1 (plasma and liver)	–	–	X
Feed and fecal analysis	–	–	X
Urinalysis	–	–	X

On the first day of weeks 7, 13, 19, and 26, blood was collected from all Group B rats via the retroorbital plexus under CO_2/O_2 anesthesia for determination of vitamins A, E, and 1,25 $(OH)_2$ vitamin D concentrations. Blood was collected into tubes, allowed to clot, and centrifuged. Sera were stored at approximately −70°C until analysis. Blood samples for vitamin K_1 concentrations in Group C rats, collected into tubes containing EDTA at the same time as hematology collections, were centrifuged; the plasma was harvested, snap frozen, and stored at −70°C protected from light. At study termination (week 26), liver samples were collected from surviving Group B and C rats, processed, and stored frozen for determination of vitamins A and E (Group B) or vitamin K_1 (Group C) concentrations. Blood and liver samples were analyzed by high performance liquid chromatography for vitamins A and E (Covance Laboratories, Inc.), by competitive enzyme immunoassay for 1,25 $(OH)_2$ vitamin D (Antech Diagnostics, Morrisville, NC), or by gas chromatography/mass spectrometry for vitamin K_1 (Analytics, Inc., Gaithersburg, MD). Because most values for vitamin K_1 were below the limit of quantitation, the results are not presented in this Toxicity Study Report.

For 8 days beginning during weeks 6, 12, 18, and 24, Group C rats were placed in metabolism cages (Nalgene Company, Rochester, NY) for fecal and urine collection. During collection periods, rats were allowed control or dosed feed and water ad libitum, and feed samples were collected. Feces were collected for a period of 8 days, with each day's collection being combined with previous days' collection and stored at approximately −20°C. Feces were stored at −70°C after each collection period until shipping to Covance Laboratories, Inc., on dry ice for analyses of calcium, fat, and moisture; the feed samples were also sent for analysis. Fat content in feed and feces was determined gravimetrically by Soxhlet extraction. Feed consumption, fat intake [(total feed consumed per interval) × (% fat in feed/100)], and fat excretion [fecal weight × (% fecal fat/100)], were calculated to estimate fat digestion: {[(fat intake − fat excreted in feces)/fat intake] × 100}. Calcium concentrations in feed and feces were determined using inductively coupled plasma emission spectrometry. Moisture was determined by weight loss upon drying. Urine was collected on ice for each Group C rat over a 24-hour period during the last day in the metabolism cage and coincided with the last day of fecal collection. Total urine collected was transferred to centrifuge tubes and the volume was recorded. Urine creatinine was measured using a Hitachi 911™ chemistry analyzer (Roche Diagnostics, Indianapolis, IN), and deoxypyridinoline was measured using a Metra Total DPD Enzyme Immunoassay Kit (Quidel, San Diego, CA).

On the last day in the metabolism cage, at the beginning of weeks 7, 13, 19, and 25, blood was collected from all Group C rats via the retroorbital plexus under CO_2/O_2 anesthesia for hematology (week 25 only) and clinical chemistry. Blood samples for hematology were collected in tubes containing EDTA as an anticoagulant. Hematology parameters were determined using an Advia 120 hematology analyzer (Bayer Diagnostics Division, Tarrytown, NY). Blood for clinical chemistry determinations was collected in tubes without anticoagulant, allowed to clot, and centrifuged and then the serum was harvested. Except as noted, clinical chemistry parameters were determined using a Hitachi 911™ chemistry analyzer (Roche Diagnostics). For osteocalcin and parathyroid hormone, serum was stored frozen at −20°C until analysis. Serum osteocalcin was measured using a Rat-MID™ Osteocalcin ELISA (Nordic Bioscience Diagnostics, Herlev, Denmark). Serum parathyroid hormone was measured using an Intact PTH Enzyme Immunoassay Kit (ALPCO Diagnostics, Salem, NH).

At study termination (week 26), right and left femurs were collected from the Group B rats for determination of calcium, ash, and moisture. Covance Laboratories, Inc., determined bone moisture by measuring weight loss upon drying, calcium by inductively coupled plasma emission spectrometry, and ash gravimetrically.

At the end of the study (week 25), samples were collected for sperm motility and vaginal cytology evaluations on Group A rats. The parameters evaluated are listed in Table 2. Due to inconsistent sample collection and slide staining, an assessment of estrous cyclicity could not be made. Male animals were evaluated for sperm count and motility. The left testis and left epididymis were isolated and weighed. The tail of the epididymis (cauda epididymis) was then removed from the epididymal body (corpus epididymis) and weighed. Test yolk was applied to slides and a small incision was made at the distal border of the cauda epididymis. The sperm effluxing from the incision were dispersed in the buffer on the slides, and the numbers of motile and nonmotile spermatozoa were counted for five fields per slide by two observers. Following completion of sperm motility estimates, each left cauda epididymis was placed in buffered saline solution. Caudae were finely minced, and the tissue was incubated in the saline solution and then

heat fixed at 65°C. Sperm density was then determined microscopically with the aid of a hemacytometer. To quantify spermatogenesis, the testicular spermatid head count was determined by removing the tunica albuginea and homogenizing the left testis in phosphate-buffered saline containing 10% dimethyl sulfoxide. Homogenization-resistant spermatid nuclei were counted with a hemacytometer.

Necropsies were performed on all Group A animals at study termination (week 25). The heart, right kidney, liver, lung, right ovary, parathyroid gland, right testis, thymus, thyroid gland and parathyroid gland together, and uterus were weighed. Both tibias and both femurs were collected; the lengths of both tibias and the left femur were measured. The right tibia and femur were dehydrated in ethanol (70% to 100%) and infiltrated with glycol methacrylate. Tissues for microscopic examination were fixed and preserved in 10% neutral buffered formalin (except eyes were first fixed in Davidson's solution), processed and trimmed, embedded in paraffin, sectioned to a thickness of 4 to 6 µm, and stained with hematoxylin and eosin. Complete histopathologic examinations were performed by the study laboratory pathologist on 0% and 9% rats. The kidney and liver of males and females and the parathyroid gland and prostate gland of males were examined in all exposure groups. Table 2 lists the tissues and organs routinely examined.

After a review of the laboratory reports and selected histopathology slides by a quality assessment (QA) pathologist, the findings and reviewed slides were submitted to an NTP Pathology Peer Review (PPR) coordinator for a second independent review. Any inconsistencies in the diagnoses made by the study laboratory and QA pathologists were resolved by the NTP pathology peer review process. Final diagnoses for reviewed lesions represent a consensus of the PPR or a consensus between the study laboratory pathologist, NTP pathologist, QA pathologist(s), and the PPR coordinator. Details of these review procedures have been described, in part, by Maronpot and Boorman[43] and Boorman et al.[44].

Table 2. Experimental Design and Materials and Methods in the Six-month Feed Study of Chitosan

Six-month Studies
Study Laboratory
Battelle Columbus Operations (Columbus, OH)
Strain and Species
Charles River Sprague Dawley [Crl:CD(SD)] rats
Animal Source
Charles River Laboratories (Portage, MI)
Time Held Before Study
Group A (core study): 14 (males) or 15 (females) days Groups B and C (special studies): 12 (males) or 13 (females) days
Average Age When Study Began
7 to 8 weeks (Group A males and Groups B and C males and females) 8 to 9 weeks (Group A females)
Date of First Exposure
Group A: August 31 (males) or September 1 (females), 2006 Groups B and C: August 29 (males) or 30 (females), 2006
Duration of Exposure
Group A: 25 weeks Groups B and C: 26 weeks
Date of Last Exposure
Group A: February 15 (males) or 16 (females), 2007 Groups B and C: February 20 (males) or 21 (females), 2007
Necropsy Dates
Group A: February 15 (males) or 16 (females), 2007 Groups B and C: February 20 (males) or 21 (females), 2007
Average Age at Necropsy
32 to 33 weeks (Group A females and Groups B and C males and females) 31 to 32 weeks (Group A males)
Size of Study Groups
10 males and 10 females
Method of Distribution
Animals were distributed randomly into groups of approximately equal initial mean body weights.
Animals per Cage
1
Method of Animal Identification
Tail tattoo
Diet
AIN-93M maintenance purified meal diet (Purina TestDiet, Richmond, IN), available ad libitum, changed twice weekly
Water

Six-month Studies

Tap water (Columbus, OH municipal supply) via automatic watering system (Edstrom Industries, Inc. Waterford, WI), available ad libitum

Cages

Polycarbonate solid-bottom (Lab Products, Inc., Seaford, DE), changed weekly, rotated in rack every 2 weeks

Bedding

Irradiated hardwood bedding chips (P.J. Murphy Forest Products Corporation, Montville, NJ), changed weekly

Rack Filters

Spun-bonded polyester (Snow Filtration Company, Cincinnati, OH), changed every 2 weeks

Racks

Stainless steel (Lab Products, Inc), changed and rotated every 2 weeks

Animal Room Environment

Temperature: 72° ± 3°F
Relative humidity: 50% ± 15%
Room fluorescent light: 12 hours/day
Room air changes: 10/hour

Exposure Concentrations

0%, 1%, 3%, and 9% in feed, available ad libitum

Type and Frequency of Observation

Observed twice daily; Group A rats were weighed and clinical findings were recorded initially, on day 8, weekly thereafter, and at the end of the study. Feed consumption was recorded weekly for Group A rats and during fecal collection periods for Group C rats.

Method of Euthanasia

100% Carbon dioxide

Necropsy

Necropsies were performed on all Group A rats at the end of the study (week 25). Organs weighed were heart, right kidney, liver, lung, right ovary, parathyroid gland, right testis, thymus, thyroid gland and parathyroid gland together, and uterus. Lengths of both tibias and the left femur were measured.

Clinical Pathology

Blood was collected via the retroorbital plexus from all Group C rats on the first day of weeks 7, 13, 19, and 25 for hematology (week 25 only) and clinical chemistry. Urine was collected from Group C rats for 24 hours beginning the last day of weeks 6, 12, 18, and 24.
Hematology: hematocrit (auto and manual); hemoglobin concentration; erythrocyte, reticulocyte, and platelet counts; mean cell volume; mean cell hemoglobin; mean cell hemoglobin concentration; and leukocyte count and differentials
Clinical chemistry: urea nitrogen, creatinine, calcium, phosphorous, total protein, albumin, cholesterol, triglycerides, alanine aminotransferase, alkaline phosphatase, creatine kinase, sorbitol dehydrogenase, bile acids, total osteocalcin, and parathyroid hormone
Urinalysis: creatinine, volume, and deoxypyridinoline

Histopathology

Six-month Studies

Histopathology was performed on 0% and 9% Group A rats. In addition to gross lesions and tissue masses, the following tissues were examined: adrenal gland, bone (left femur and tibia) with marrow, brain, clitoral gland, esophagus, eye, Harderian gland, heart and aorta, large intestine (cecum, colon, rectum), small intestine (duodenum, jejunum, ileum), kidney, liver, lung (with mainstem bronchus), lymph nodes (mandibular and mesenteric), mammary gland, nose, ovary, pancreas, parathyroid gland, pituitary gland, preputial gland, prostate gland, salivary gland, seminal vesicle, skin, spleen, stomach (forestomach and glandular), testis (with epididymis), thymus, thyroid gland, trachea, urinary bladder, and uterus. The kidney and liver of males and females and the parathyroid gland and prostate gland of males were also examined in the 1% and 3% groups.

Sperm Motility

At the end of the study, sperm samples were collected from male Group A rats for sperm count and motility evaluations. The following parameters were evaluated: spermatid heads per gram testis and per testis, spermatid heads per gram cauda and per cauda, and epididymal spermatozoal motility. The left cauda, left epididymis, and left testis were weighed.

Digestion Studies

Feces were collected from Group C rats for 8 days beginning weeks 6, 12, 18, and 24 and analyzed for calcium, fat, and moisture. Fecal calcium and fat content were compared to that in feed samples collected during the same time period to produce values for fat digested and calcium absorbed.

Serum and Hepatic Vitamins

Blood was collected from the retroorbital plexus of Groups B and C rats on the first day of weeks 7, 13, 19, and 25 (Group C), and 26 (Group B). At study termination (week 26), liver samples were collected from Groups B and C rats. Blood and liver samples were analyzed for vitamins A, E, 1,25 $(OH)_2$ D, and/or K_1.

Bone Analysis

At study termination (week 26), right and left femurs were collected from Group B rats, and calcium, ash, and moisture levels were measured.

Statistical Methods

Calculation and Analysis of Lesion Incidences

The incidences of lesions are presented in Appendix A as the numbers of animals bearing such lesions at a specific anatomic site and the numbers of animals with that site examined microscopically. The Fisher exact test[45], a procedure based on the overall proportion of affected animals, was used to determine significance.

Analysis of Continuous Variables

Two approaches were employed to assess the significance of pairwise comparisons between dosed and control groups in the analysis of continuous variables. Organ and body weight data, which historically have approximately normal distributions, were analyzed with the parametric multiple comparison procedures of Dunnet[46] and Williams[47; 48]. Hematology, clinical chemistry, urinalysis, serum and liver vitamin concentrations, digestive and bone parameters, spermatid, and epididymal spermatozoal data, which have typically skewed distributions, were analyzed using the nonparametric multiple comparison methods of Shirley[49] (as modified by Williams[50]) and Dunn[51]. Jonckheere's test[52] was used to assess the significance of the dose-related trends and to determine whether a trend-sensitive test (Williams' or Shirley's test) was more appropriate for pairwise comparisons than a test that does not assume a monotonic dose-related trend (Dunnett's or Dunn's test). Prior to statistical analysis, extreme values identified by the outlier test of Dixon

and Massey[53] were examined by NTP personnel, and implausible values were eliminated from the analysis.

Quality Assurance Methods

The 6-month study was conducted in compliance with Food and Drug Administration Good Laboratory Practice Regulations[54]. In addition, as records from the 6-month study were submitted to the NTP Archives, this study was audited retrospectively by an independent QA contractor. Separate audits covered completeness and accuracy of the pathology data, pathology specimens, final pathology tables, and a draft of this NTP Toxicity Study Report. Audit procedures and findings are presented in the reports and are on file at NIEHS. The audit findings were reviewed and assessed by NTP staff, and all comments were resolved or otherwise addressed during the preparation of this Toxicity Study Report.

Results

Six-month Study

All male and female Group A rats survived to the end of the study (Table 3); however, five rats from Groups B and C died, often after seizures that occurred near the time of blood collection, with the cause of death undetermined. There were no treatment-related clinical findings in Group A animals, although 13 animals from Groups B and C (10 from the 9% group, one from the 3% group, and two from the 1% group) were observed with seizures either during or after the 18-week blood collections. Seizures were not noted at any other time point. Body weights and feed consumption were measured in Group A rats, and mean body weights of exposed males and females were not significantly different from those of the control groups (Table 3 and Figure 2). Feed consumption by 3% and 9% Group A males was greater than that by the controls, but the increase may not be accurate due to observed food spillage possibly due to poor palatability resulting in feed being wasted (Table G-1). Dietary concentrations of 1%, 3%, and 9% resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight per day to males and 650, 1,800, and 6,000 mg/kg per day to females, respectively.

Table 3. Survival, Body Weights, and Feed Consumption of Group A Rats in the Six-month Feed Study of Chitosan[a]

Concentration	Survival[b]	Initial Body Weight (g)	Final Body Weight (g)	Change in Body Weight (g)	Final Weight Relative to Controls (%)	Feed Consumption Week 1	Feed Consumption Week 25
Male							
0%	10/10	238 ± 5	669 ± 20	432 ± 18		22.2	21.2
1%	10/10	243 ± 6	702 ± 21	459 ± 17	105	23.8	20.4
3%	10/10	242 ± 6	687 ± 23	445 ± 21	103	23.6	24.7
9%	10/10	243 ± 6	612 ± 17	369 ± 17	91	21.4	27.3
Female							
0%	10/10	175 ± 3	338 ± 11	162 ± 12		17.7	16.4
1%	10/10	173 ± 2	335 ± 13	162 ± 12	99	22.3	20.3
3%	10/10	177 ± 4	328 ± 11	151 ± 9	97	17.3	17.1
9%	10/10	177 ± 2	301 ± 13	124 ± 12	89	16.9	18.8

[a]Weights and weight changes are given as mean ± standard error. Feed consumption is expressed as grams per animal per day. Differences in weights and weight changes from the control group are not significant by Dunnett's test.
[b]Number of animals surviving at 25 weeks/number initially in group.

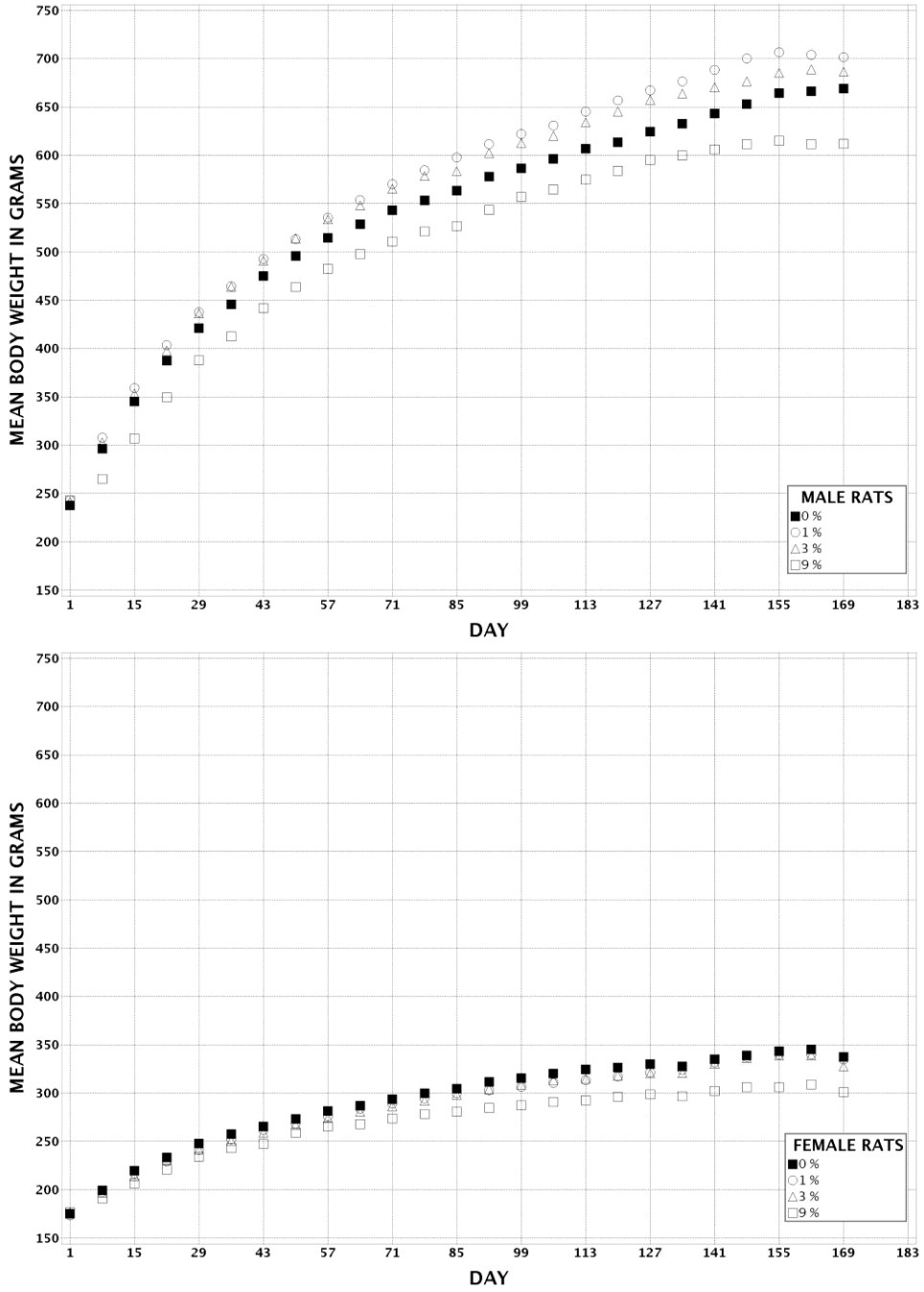

Figure 2. Growth Curves for Group A Rats Exposed to Chitosan in Feed for Six Months

Hematology data for Group C rats are listed in Table B-1. Compared to the control group, mild significant increases (4% to 6%) in automated hematocrit, hemoglobin concentration, mean cell volume, and mean cell hemoglobin were observed in 9% males; manual hematocrit and erythrocyte count were similar to those of the controls. These changes may be due to biological variability and are likely not toxicologically relevant. All other differences from control values in the male and female hematology data were mild or sporadic and not considered toxicologically significant.

Clinical chemistry data for Group C rats are listed in Table 4 and Table B-1. Both the 9% male and female rats had significantly decreased cholesterol values (26% to 48%), compared to the controls, at all time points. Triglycerides values were also significantly decreased in the 9% male (47% to 57%) and female (30%) rats, but not at every time point. Phosphorus levels were significantly decreased in the 9% male rats at weeks 13, 19, and 25 (12% to 18%); a decrease also occurred in the 3% males at week 13 (14%). Similarly, phosphorus levels were significantly decreased in the 3% and 9% females at weeks 13 (20% and 16%, respectively) and 25 (9% and 19%, respectively). A mild, but statistically significant, decrease (4%) in calcium concentration was observed in 9% males at weeks 19 and 25. Alanine aminotransferase (ALT) activity, a marker of hepatocellular injury, was mildly but significantly elevated at week 25 in the 9% male rats (104%) and in the 3% and 9% female rats (28% and 88%, respectively). However, sorbitol dehydrogenase (another marker of hepatocellular injury) was not significantly increased relative to the controls, and hepatocellular changes associated with increases in ALT were not observed microscopically. Thus, the toxicologic significance of the increases in ALT is uncertain. Urea nitrogen was mildly increased in the 9% males (23%) and females (15%) at week 25. Minimal to mild significant alterations were also observed in several other parameters. These alterations were inconsistent or within the range of biological variability.

Total osteocalcin (a marker of bone turnover) and parathyroid hormone levels were analyzed in Group C rats and were occasionally elevated throughout the study. Total osteocalcin was significantly elevated in the 9% males (38%) at week 25, while parathyroid hormone levels were significantly elevated in 9% males (96%) at Week 19 and in 9% females (56%) at week 25 (Table 4 and Table B-1).

Urine deoxypyridinoline/creatinine ratios were calculated at weeks 7, 13, 19, and 25 for both males and females in Group C and were mostly unchanged (Table 4 and Table B-1). A significant increase, compared to the control group, occurred at week 25 in the 9% males (28%). In females, minimal increases and decreases occurred inconsistently across all time points with a significant increase at week 7 in the 9% group (42%) and significant decreases at weeks 13 (26%) and 19 (20%) in the 1% group compared to controls.

To calculate the deoxypyridinoline/creatinine ratios, urine volume, urine creatinine concentrations, and urine deoxypyridinoline concentrations were measured at weeks 7, 13, 19, and 25. Urine volume was significantly decreased in various male and female exposure groups throughout the study, but most consistently in the 9% chitosan group (approximately 40% to 60%). Increases in urine creatinine concentration tended to parallel the decreases in urine volume indicating proper kidney function.

Table 4. Selected Clinical Chemistry and Urinalysis Data for Group C Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
Clinical Chemistry				
n	10	10	10	10
Calcium (mg/dL)				
Week 13	12.6 ± 0.1	12.5 ± 0.1	12.3 ± 0.2	12.4 ± 0.2
Week 19	12.5 ± 0.1	12.3 ± 0.2	12.3 ± 0.1	12.0 ± 0.1*
Week 25	12.1 ± 0.1	12.1 ± 0.2	12.0 ± 0.1	11.6 ± 0.1*
Phosphorus (mg/dL)				
Week 13	8.4 ± 0.3	8.1 ± 0.3	7.2 ± 0.3**	7.4 ± 0.4*
Week 19	8.2 ± 0.4	7.7 ± 0.2	7.4 ± 0.3	6.7 ± 0.2**
Week 25	6.9 ± 0.3	6.8 ± 0.2	6.7 ± 0.1	5.8 ± 0.3**
Cholesterol (mg/dL)				
Week 7	82 ± 5	75 ± 8	80 ± 6	53 ± 3**
Week 13	95 ± 7	84 ± 8	90 ± 7	53 ± 2**
Week 19	101 ± 6	87 ± 10	94 ± 8	59 ± 4**
Week 25	95 ± 6	81 ± 8	90 ± 6	49 ± 4**
Triglycerides (mg/dL)				
Week 7	202 ± 28	234 ± 43	226 ± 30	88 ± 15*
Week 13	198 ± 33	202 ± 38	195 ± 24	86 ± 8**
Week 19	180 ± 26	218 ± 43	210 ± 29	95 ± 13*
Week 25	173 ± 18	207 ± 30	218 ± 24	109 ± 13
Alanine aminotransferase (IU/L)				
Week 25	28 ± 3	29 ± 2	29 ± 1	57 ± 2**
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	15 ± 1	14 ± 1
Total osteocalcin (ng/mL)				
Week 7	445.7 ± 17.2	439.8 ± 15.8	441.8 ± 18.2	520.4 ± 22.6
Week 13	306.2 ± 13.0	289.7 ± 28.6	245.4 ± 37.9	372.6 ± 23.4
Week 19	239.4 ± 12.4	225.7 ± 10.6	181.6 ± 26.8	269.2 ± 20.9
Week 25	158.3 ± 10.0	168.1 ± 11.6	145.9 ± 22.7	218.3 ± 14.6*
Parathyroid hormone (ng/mL)				
Week 7	1.882 ± 0.137	1.643 ± 0.449	1.838 ± 0.348	1.521 ± 0.368
Week 13	2.343 ± 0.350	2.763 ± 0.479	3.215 ± 0.537	2.433 ± 0.222
Week 19	1.879 ± 0.186	3.101 ± 0.475	2.710 ± 0.365	3.679 ± 0.361**
Week 25	2.668 ± 0.475	2.924 ± 0.276	3.981 ± 0.349	2.848 ± 0.506

	0%	1%	3%	9%
Urinalysis				
n				
Week 7	10	9	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	10	10	10
Volume (mL)				
Week 7	8.3 ± 0.8	7.5 ± 1.5	6.4 ± 0.8	5.0 ± 1.1*
Week 13	7.9 ± 1.0	4.6 ± 0.3**	5.1 ± 0.4*	4.5 ± 0.5**
Week 19	10.7 ± 1.6	4.0 ± 0.4**	5.3 ± 0.7*	5.6 ± 0.6
Week 25	8.6 ± 1.2	5.4 ± 0.6*	6.1 ± 0.8*	5.1 ± 0.6**
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.810 ± 0.135	1.889 ± 0.148	1.810 ± 0.159	1.920 ± 0.160
Week 13	0.910 ± 0.035	0.890 ± 0.031	0.930 ± 0.040	0.960 ± 0.078
Week 19	0.530 ± 0.050	0.550 ± 0.034	0.570 ± 0.042	0.660 ± 0.048
Week 25	0.430 ± 0.030	0.470 ± 0.030	0.480 ± 0.020	0.550 ± 0.027**
Female				
Clinical Chemistry				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	10
Calcium (mg/dL)				
Week 13	12.9 ± 0.2	13.1 ± 0.1	12.7 ± 0.2	12.5 ± 0.2
Week 19	12.9 ± 0.1	13.1 ± 0.2	12.8 ± 0.1	12.5 ± 0.1
Week 25	12.7 ± 0.3	12.8 ± 0.1	12.7 ± 0.2	12.3 ± 0.2
Phosphorus (mg/dL)				
Week 13	8.1 ± 0.5	7.4 ± 0.4	6.5 ± 0.4**	6.8 ± 0.3*
Week 19	8.4 ± 0.4	8.2 ± 0.5	8.1 ± 0.3	7.4 ± 0.5
Week 25	6.8 ± 0.2	6.4 ± 0.2	6.2 ± 0.3*	5.5 ± 0.3**
Cholesterol (mg/dL)				
Week 7	80 ± 6	81 ± 8	67 ± 4	59 ± 4**
Week 13	92 ± 8	86 ± 7	73 ± 5	58 ± 4**
Week 19	107 ± 7	105 ± 9	91 ± 8	67 ± 5**
Week 25	94 ± 7	108 ± 5	96 ± 8	63 ± 4**

	0%	1%	3%	9%
Triglycerides (mg/dL)				
Week 7	88 ± 12	130 ± 48	81 ± 8	86 ± 14
Week 13	125 ± 10	163 ± 30	140 ± 23	88 ± 23*
Week 19	143 ± 15	181 ± 32	137 ± 18	90 ± 13
Week 25	188 ± 31	231 ± 44	245 ± 31	158 ± 35
Alanine aminotransferase (IU/L)				
Week 25	25 ± 3	28 ± 3	32 ± 2**	47 ± 4**
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	19 ± 2	16 ± 1
Total osteocalcin (ng/mL)				
Week 7	293.6 ± 19.4	287.5 ± 21.2	282.1 ± 34.7	316.7 ± 23.5
Week 13	197.9 ± 22.6	202.3 ± 15.4	184.4 ± 19.4	234.2 ± 14.5
Week 19	158.1 ± 18.3	184.8 ± 13.2	166.7 ± 24.7	210.1 ± 16.0
Week 25	107.9 ± 18.6	97.1 ± 7.1	96.0 ± 16.2	148.8 ± 15.1
Parathyroid hormone (ng/mL)				
Week 7	0.995 ± 0.150[b]	1.156 ± 0.176	1.092 ± 0.182	1.023 ± 0.146
Week 13	1.506 ± 0.203	1.734 ± 0.194	1.925 ± 0.306	1.767 ± 0.212
Week 19	1.406 ± 0.232	1.994 ± 0.353	1.845 ± 0.418	1.673 ± 0.223
Week 25	1.471 ± 0.189[b]	1.628 ± 0.220	1.818 ± 0.224	2.301 ± 0.212*
Urinalysis				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	9
Volume (mL)				
Week 7	8.2 ± 1.2	8.5 ± 1.0	5.4 ± 0.8	3.4 ± 0.3**
Week 13	6.4 ± 0.7	6.5 ± 0.6	4.1 ± 0.8*	2.9 ± 0.5**
Week 19	7.7 ± 1.2	8.1 ± 1.4	5.0 ± 0.8	3.4 ± 0.5**
Week 25	8.2 ± 1.5	9.1 ± 1.5	5.8 ± 0.9	3.7 ± 0.5**
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.620 ± 0.128	1.240 ± 0.129	1.940 ± 0.229	2.300 ± 0.182*
Week 13	0.580 ± 0.039	0.430 ± 0.037**	0.540 ± 0.034	0.570 ± 0.042
Week 19	0.450 ± 0.017	0.360 ± 0.016*	0.440 ± 0.016	0.520 ± 0.020
Week 25	0.340 ± 0.043	0.222 ± 0.022	0.340 ± 0.027	0.411 ± 0.026

*Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test.
**$P \leq 0.01$.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 9.

Serum and hepatic vitamin concentrations were measured in Group B rats (Table 5 and Table C-1). Exposure concentration-dependent decreases were observed in serum vitamin A concentrations starting at week 13 in the male rats. The decreases reached statistical significance at weeks 13 (27%), 19 (26%), and 26 (29%) in 9% males and at weeks 13 (15%) and 26 (16%) in 3% males. Females were less affected with significant decreases observed in the 9% group at weeks 19 (18%) and 26 (21%). Exposure concentration-dependent decreases were also observed in serum vitamin E concentrations in male rats at all time points. The decreases were statistically significant at all time points in 3% (33% to 42%) and 9% males (79% to 82%) and in 1% males at week 13 (17%), with the 9% group measuring between 18% to 21% that of control values throughout the study. Females were less affected with significant decreases in serum vitamin E levels observed in the 9% group (approximately 60%) only (all time points). Hepatic vitamin E concentrations were significantly decreased at week 26 in 3% and 9% males (48% and 87%, respectively) and 9% females (80%). In the 9% group, levels of hepatic vitamin E measured only 13% and 20% of control values in the males and females, respectively. Serum concentrations of 1,25 $(OH)_2$ vitamin D were significantly increased in 9% males (105% to 142%) and females (100% to 180%) at weeks 7, 19, and 26 compared to the control groups. Results of plasma hepatic vitamin K concentrations in Group C rats are not discussed or presented, as many samples were below the level of quantification.

Table 5. Serum and Hepatic Vitamin Concentration Data for Group B Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
n				
Week 7	9	10	10	10
Week 13	9	10	10	10
Week 19	9	10	10	10
Week 26	9	10	10	8
Serum vitamin A (µg/mL)				
Week 7	0.532 ± 0.021	0.506 ± 0.033	0.513 ± 0.026	0.453 ± 0.018
Week 13	0.561 ± 0.024	0.499 ± 0.019	0.476 ± 0.022*	0.410 ± 0.009**
Week 19	0.533 ± 0.028	0.506 ± 0.031	0.475 ± 0.019	0.392 ± 0.014**
Week 26	0.476 ± 0.019	0.444 ± 0.024	0.398 ± 0.017**	0.336 ± 0.026**
Serum 1,25 $(OH)_2$ vitamin D (pg/mL)				
Week 7	124.4 ± 19.6	163.3 ± 21.7	183.2 ± 26.9	297.4 ± 41.0**
Week 13	70.1 ± 7.3	57.4 ± 5.3	77.3 ± 4.4	86.1 ± 8.5
Week 19	20.6 ± 2.8	21.7 ± 6.1	22.9 ± 2.2	42.3 ± 3.1**[b]
Week 26	27.7 ± 3.4[c]	28.0 ± 4.3	36.1 ± 4.6[b]	66.9 ± 11.9**
Serum vitamin E (µg/mL)				
Week 7	19.33 ± 1.43	15.38 ± 1.29	12.92 ± 0.48**	4.14 ± 0.23**
Week 13	21.08 ± 1.61	17.45 ± 1.06*	12.27 ± 0.86**	4.33 ± 0.27**

	0%	1%	3%	9%
Week 19	20.59 ± 1.61	16.19 ± 0.96	12.86 ± 0.42**	4.07 ± 0.32**
Week 26	19.66 ± 1.66	17.35 ± 1.37	12.35 ± 0.61**	3.59 ± 0.65**
Liver vitamin E (µg/g)				
Week 26	66.8 ± 16.2	55.0 ± 6.8	34.6 ± 2.2**	8.5 ± 0.8**
Female				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 26	10	10	9	10
Serum vitamin A (µg/mL)				
Week 7	0.272 ± 0.011	0.253 ± 0.007	0.260 ± 0.012	0.266 ± 0.012
Week 13	0.308 ± 0.020	0.295 ± 0.011	0.309 ± 0.019	0.281 ± 0.018
Week 19	0.283 ± 0.014	0.271 ± 0.015	0.291 ± 0.012	0.231 ± 0.010*
Week 26	0.316 ± 0.015	0.302 ± 0.014	0.294 ± 0.018	0.249 ± 0.010**
Serum 1,25 $(OH)_2$ vitamin D (pg/mL)				
Week 7	104.0 ± 15.1	96.7 ± 10.9	111.0 ± 8.7	208.1 ± 18.2**
Week 13	60.6 ± 7.5	60.7 ± 7.9	69.3 ± 11.0	110.1 ± 16.9
Week 19	11.6 ± 1.6	12.6 ± 1.7	15.8 ± 1.4	31.4 ± 3.2**
Week 26	19.2 ± 2.2	20.7 ± 4.2	28.6 ± 6.5	53.7 ± 5.8**
Serum vitamin E (µg/mL)				
Week 7	18.65 ± 0.71	20.08 ± 0.87	18.38 ± 0.85	6.99 ± 0.58**
Week 13	19.81 ± 1.41	20.85 ± 1.06	20.19 ± 1.20	7.48 ± 0.38**
Week 19	21.02 ± 1.76	19.74 ± 1.75	19.86 ± 1.08	7.37 ± 0.57**
Week 26	20.94 ± 1.56	23.43 ± 1.66	22.23 ± 1.75	7.28 ± 0.64**
Liver vitamin E (µg/g)				
Week 26	84.5 ± 8.9	97.1 ± 10.1	82.0 ± 11.8	17.2 ± 3.2**

*Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test.
**Significantly different ($P \leq 0.01$) from the control group by Shirley's test.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 9.
[c]n = 7.

Digestive parameters were calculated for Group C rats and are listed in Table 6. Compared to the control groups, percent fat digested was significantly decreased at week 6 in 9% males (28%) and females (14%), during week 12 in 3% and 9% males (8% and 33%, respectively), during week 18 in 9% males (20%) and females (10%), and during week 24 in all exposed groups of males and females (up to 32%). Calcium absorption was significantly increased in 9% females during weeks 12 (55%) and 24 (154%). Fecal weight was significantly increased in 3% and 9% males (up to 170%) and females (up to 126%) during each collection period and in 1% females

during weeks 12, 18, and 24 (18% to 29%). Fecal moisture was significantly increased in 9% males and females at all time points (10% to 15%), in 3% males (4%) at week 6, and in 3% females (7%) at weeks 12 and 18.

Male rats did not display any changes in testis or epididymis weights or sperm parameters, indicating that chitosan did not exhibit the potential to be a reproductive toxicant in male rats (Table E-1).

Table 6. Digestive Data for Group C Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
n				
Weeks 6–7	10	10	10	10
Weeks 12–13	10	10	10	10
Weeks 18–19	10	10	10	9
Weeks 24–25	10	10	10	10
Fat digested (%)				
Weeks 6–7	97.04 ± 0.40	97.55 ± 0.22	94.37 ± 0.84	69.55 ± 3.01**
Weeks 12–13	94.79 ± 0.46	93.36 ± 0.83	87.08 ± 0.68**	63.50 ± 2.40**
Weeks 18–19	97.56 ± 0.58	98.48 ± 0.19	95.87 ± 0.70	77.59 ± 1.83**
Weeks 24–25	97.01 ± 0.19	95.61 ± 0.32**	92.14 ± 0.87**	66.18 ± 3.24**
Calcium absorbed (%)				
Weeks 6–7	31.69 ± 1.84	34.57 ± 4.05	27.54 ± 1.83	33.01 ± 1.59
Weeks 12–13	19.81 ± 3.36	14.73 ± 0.76	18.42 ± 3.25	28.01 ± 2.69
Weeks 18–19	13.33 ± 4.33	18.42 ± 5.43	3.64 ± 2.62	11.11 ± 1.35
Weeks 24–25	2.93 ± 1.54	5.14 ± 1.08	0.70 ± 1.57	9.46 ± 1.88
Fecal weight (g)				
Weeks 6–7	21.42 ± 0.68	21.01 ± 1.93	31.33 ± 0.90**	52.39 ± 2.85**
Weeks 12–13	24.32 ± 1.68	27.70 ± 1.37	32.84 ± 1.73**	47.59 ± 4.30**
Weeks 18–19	23.11 ± 1.25	22.67 ± 1.85	33.30 ± 1.72**	62.38 ± 3.67**[b]
Weeks 24–25	26.43 ± 1.12	25.75 ± 0.73	37.17 ± 1.11**	56.35 ± 3.45**
Fecal moisture (%)				
Weeks 6–7	45.0 ± 0.5	42.0 ± 1.6	46.8 ± 0.4*	51.0 ± 0.8**
Weeks 12–13	46.8 ± 2.0	49.0 ± 0.8	48.8 ± 0.6	53.6 ± 0.8**
Weeks 18–19	47.7 ± 1.1	45.3 ± 1.8	49.1 ± 0.7	54.8 ± 1.5**[b]
Weeks 24–25	47.2 ± 0.6	45.7 ± 0.5	49.3 ± 0.7	53.1 ± 0.8**

	0%	1%	3%	9%
Female				
n				
Weeks 6–7	10	10	10	9
Weeks 12–13	10	10	10	10
Weeks 18–19	10	10	10	10
Weeks 24–25	8	9	10	10
Fat digested (%)				
Weeks 6–7	96.47 ± 0.49	95.53 ± 1.30	95.46 ± 0.66	83.23 ± 2.69**
Weeks 12–13	97.12 ± 1.58	98.54 ± 0.99	97.27 ± 1.16	91.95 ± 2.70
Weeks 18–19	99.17 ± 0.18	97.52 ± 0.50	97.15 ± 1.24	89.61 ± 2.53**
Weeks 24–25	98.66 ± 0.08	97.68 ± 0.39**	96.79 ± 0.49**	86.73 ± 1.55**
Calcium absorbed (%)				
Weeks 6–7	31.44 ± 2.35	24.42 ± 2.54	24.36 ± 2.50	32.29 ± 1.69
Weeks 12–13	14.84 ± 1.76	17.03 ± 3.11	17.96 ± 1.22	23.02 ± 2.39*
Weeks 18–19	8.96 ± 3.00	9.78 ± 1.98	0.47 ± 3.37	13.07 ± 1.65
Weeks 24–25	5.65 ± 2.84	9.23 ± 2.74	8.25 ± 1.59	14.50 ± 1.40*
Fecal weight (g)				
Weeks 6–7	14.37 ± 0.91	15.76 ± 0.60	19.85 ± 1.64**	32.61 ± 1.67**[b]
Weeks 12–13	15.37 ± 0.60	18.41 ± 1.28*	21.11 ± 1.07**	30.83 ± 2.78**
Weeks 18–19	16.30 ± 0.86	19.23 ± 0.97*	25.21 ± 1.42**	36.58 ± 2.41**
Weeks 24–25	16.01 ± 0.92[b]	20.66 ± 1.14**	24.85 ± 1.19**	35.78 ± 2.27**
Fecal moisture (%)				
Weeks 6–7	45.3 ± 1.1	45.3 ± 0.4	47.3 ± 0.8	50.0 ± 0.9**[b]
Weeks 12–13	45.9 ± 0.7	47.5 ± 1.0	49.3 ± 0.5**	52.7 ± 1.0**
Weeks 18–19	46.1 ± 1.1	47.2 ± 0.4	49.5 ± 0.9**	53.0 ± 0.7**
Weeks 24–25	47.2 ± 0.6[b]	48.4 ± 1.4	49.2 ± 0.6	52.6 ± 0.9**

*Significantly different ($P \leq 0.05$) from the control group by Shirley's test.
**$P \leq 0.01$.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 10.

Bone parameters in Groups A and B rats were generally unaffected by chitosan exposure (Table C-2). Bone moisture was significantly increased, relative to the control group, in 9% females (7%).

The absolute and relative liver weights of Group A 9% males and females were significantly less (22% and 21% lower, respectively) than those of the respective control groups (Table 7 and Table D-1). The absolute and relative thymus weights of Group A 3% and 9% males and 9% females were significantly less than those of the controls (Table D-1).

There was a significant decrease in the incidence of periportal fatty change of the liver in Group A female rats in the 9% group compared to the control group and decreases in 1% and 3% females that resulted in a negative trend (Table 7 and Table A-2). In male rats, there were decreases in the incidences of periportal fatty change in the 1% and 9% groups, and the severities were decreased in the 3% and 9% groups (Table 7 and Table A-1). Fatty change was characterized by hepatocytes with clear vacuoles (lipid), mostly located within the periportal region of the liver (zone 1) (Figure 3).

Table 7. Liver Parameter Data for Group A Rats in the Six-month Feed Study of Chitosan

	0%	1%	3%	9%
Male				
n[a]	10	10	10	10
Necropsy body wt	669 ± 20	702 ± 21	687 ± 23	612 ± 17
Liver weight[b]				
Absolute	25.19 ± 0.87	24.87 ± 1.35	23.74 ± 1.51	19.53 ± 0.71[##]
Relative	37.662 ± 0.731	35.321 ± 1.179	34.345 ± 1.411[#]	31.933 ± 0.817[##]
Periportal, Fatty Change[c]	6 (1.7)[d]	3 (1.7)	6 (1.3)	3 (1.0)
Female				
n	10	10	10	10
Necropsy body wt	338 ± 11	335 ± 13	328 ± 11	301 ± 13
Liver weight				
Absolute	12.54 ± 0.82	12.47 ± 0.39	11.85 ± 0.29	9.85 ± 0.20[##]
Relative	36.900 ± 1.502	37.341 ± 0.444	36.346 ± 0.904	33.036 ± 0.910[#]
Periportal, Fatty Change	7 (1.1)	4 (1.0)	4 (1.0)	0**

[#]Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.
[##]Significantly different ($P \leq 0.01$) from the control group by Williams' test.
**Significantly different ($P \leq 0.01$) from the control group by the Fisher exact test.
[a]Number of animals with liver weighed and with liver examined microscopically.
[b]Liver weights (absolute weights) and body weights are given in grams; Liver-weight-to-body-weight ratios (relative weights) are given as mg liver weight/g body weight (mean ± standard error).
[c]Number of animals with lesion.
[d]Average severity grade of lesions in affected animals: 1 = minimal, 2 = mild, 3 = moderate, 4 = marked.

Hepatocytes contained large, well-defined, single round vacuoles (macrovesicular) within each cell that displaced the nuclei and cytoplasm to the cell periphery (Figure 4) and can be compared with a liver lacking fatty change (Figure 5).

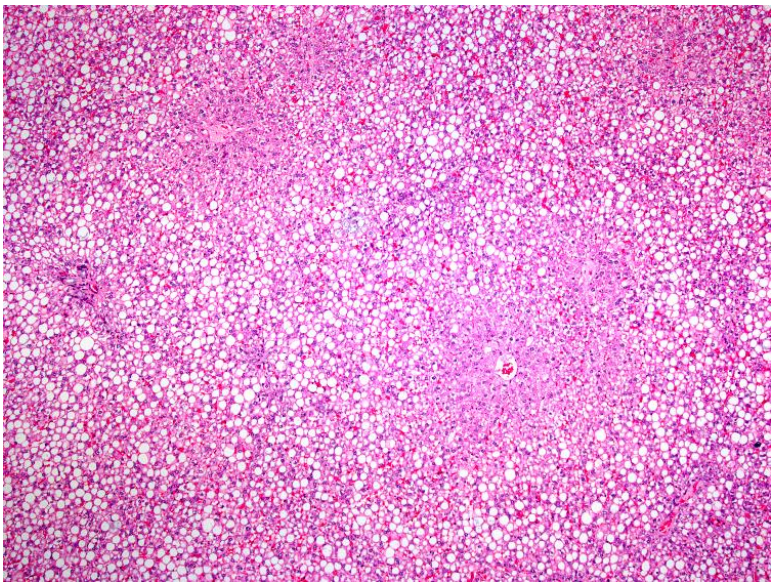

Figure 3. Section of the Liver from a Control Male Sprague Dawley Rat from the Six-month Feed Study of Chitosan with a Moderate Degree of Fatty Change (H&E)

There is a predominant periportal distribution of affected hepatocytes.

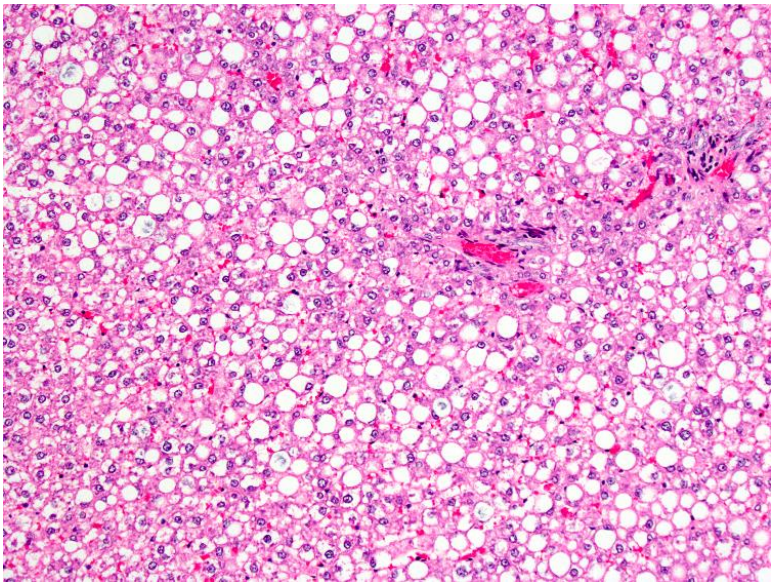

Figure 4. Higher Magnification of Figure 3 (H&E)

The fatty change is characterized by round, discrete vacuoles within hepatocytes that displace the nuclei and cytoplasm to the periphery.

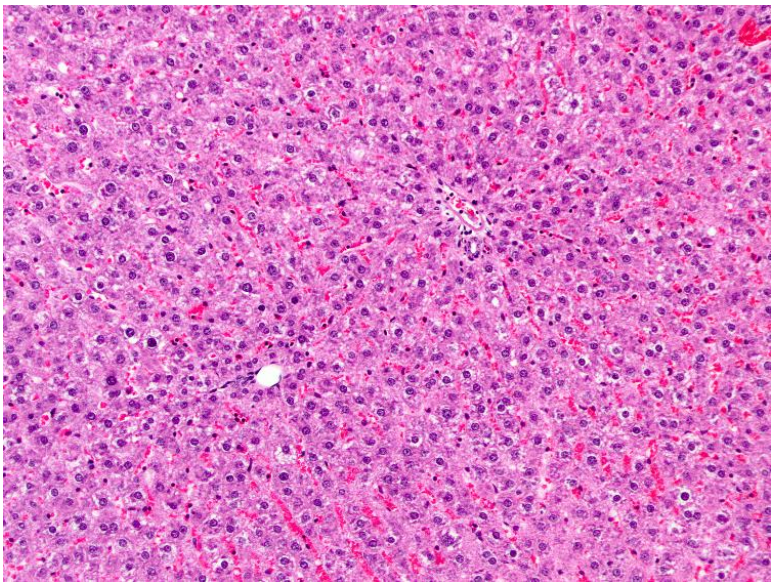

Figure 5. Section of the Liver with a Lack of Fatty Change from a Male Sprague Dawley Rat Exposed to 9% Chitosan in Feed for Six Months (H&E)

There is some minimal vacuolization within many hepatocytes. The vacuoles lack distinct round borders and the nuclei are centrally located, consistent with glycogen accumulation.

Discussion

Human exposure to chitosan occurs primarily through consumption of dietary supplements, as chitosan is marketed as a fiber-like supplement to increase satiation and promote weight loss through inhibition of fat absorption[16]. The acute toxicity of chitosan has previously been examined in human studies (12 days or up to 8 weeks) evaluating the effectiveness of chitosan as a weight-loss supplement, and the results from these studies demonstrated no observable toxicity following oral administration of chitosan[32-34]. However, there is indication of serum vitamin and bone mineral depletion following consumption of chitosan in rats[26]. Therefore, NTP conducted 6-month feed studies to evaluate the effects of dietary chitosan on bone metabolism, fat-soluble vitamin levels, and dietary fat and calcium absorption, as well as general toxicity in Charles River Sprague Dawley rats.

Feed concentrations of 1%, 3%, and 9% chitosan, which resulted in average daily doses of approximately 450, 1,500, and 5,200 mg chitosan/kg body weight per day to males and 650, 1,800, and 6,000 mg/kg per day to females, were selected based on existing data from animal studies[24; 26]. The 9% concentration is higher than the typical 5% NTP concentration limit, but the 9% diet was considered to be nutritionally adequate. The AIN-93M feed was selected for this study over the NTP-2000 feed based on the high levels of fat-soluble vitamins and higher total fat content found in the NTP-2000 feed. The NTP-2000 feed contains almost double the amount of required fat-soluble vitamins and has a higher fat content (7% to 8%) than the AIN-93M feed (4%)[41; 42]. One of the primary rationales for this chitosan study was the potential for decreases in fat-soluble vitamin concentrations, and therefore utilizing a diet with lower levels of preexisting vitamins and a lower fat content was ideal to avoid confounding potential results.

The animals used in this study were split into three groups, the core group, Group A, and two special study groups, Groups B and C. Different parameters were evaluated in each group, which, while allowing for the collection of extensive endpoints, meant that only 10 animals were examined per endpoint instead of 30, as there was no crossover of analyses between the groups.

Multiple endpoints were evaluated at multiple time points (6, 12, 18, and 24 weeks) in Group C rats to determine effects on fat absorption. Treatment-related decreases in percentage fat digestion of 20% to 33% in males and 5% to 14% in females relative to control, were consistently observed in the 9% group with effects also noted in males in the 3% group (decreases of 2% to 8%). Stronger responses were observed in males relative to females. Additionally, fecal weight was significantly increased in 1% females at weeks 12, 18, and 24 (19%, 18%, and 29%, respectively), and in 3% (35% to 56%) and 9% (96% to 170%) males and females relative to controls at all time points. These data suggest that consumption of chitosan reduced the absorption of fat in the feed, resulting in increased fecal weight due to fat being excreted. Similar results have been observed in other studies. Deuchi et al.[55] reported that rats fed deacetylated chitosan had decreased fat digestion; as the degree of deacetylation increased, fat digestibility decreased. The chitosan used by Deuchi et al.[55] was 70% to 90% deacetylated, which is a level very similar to the chitosan (86.5% deacetylated) used in the current study. Gallaher et al.[12] demonstrated that male Wistar rats exposed to 10% chitosan in AIN-93 feed had increased fecal fat excretion and dry fecal weight and decreased cholesterol absorption relative to control rats, similar to what was observed in the current study.

Due to the high percentage of chitosan in the feed of the 9% group, it is possible that the observed decreases in percentage fat digested were due to bulk chitosan in the feces confounding the amount of fat actually being excreted. Misrepresented fecal weights would alter the calculated amount of fat excreted in the feces, which would subsequently affect the calculation of percentage fat digested. The observed increases in fecal weight could also be attributed to an increase in the percentage fecal moisture, which was significantly increased in both males and females in the 3% and 9% groups. In Group A, there were decreases, albeit not significant, in mean body weights of 9% males and females (decreases of 9% and 11%, respectively), but overall there were no significant changes in the body weights of rats exposed to chitosan; the mean body weights of exposed animals were similar to those of control animals. Considering the large decrease in percent fat digested, combined with the significant increase in fecal weight observed in 9% males and females, it would be expected that mean body weights would significantly decrease due to more fat being excreted than digested. The slight mean body weight decrease observed in this study could be due in part to excretion of bulk chitosan, but regardless, the magnitude of increase in fecal fat excretion as well as the decrease in hepatic periportal fatty change still indicates a treatment-related response.

Consistent significant decreases in cholesterol levels were observed in 9% male and female rats; triglycerides levels were also affected but not as consistently as cholesterol. Decreases in cholesterol were consistent with many other studies and not an unexpected finding, as chitosan is well known to have a cholesterol lowering effect in rats[14; 56-59]. The mechanism by which chitosan lowers cholesterol is still controversial, but recent studies indicate that chitosan, acting as a weak anion exchange resin, reduces cholesterol by causing a decrease in its absorption in the small intestine and by inducing increases in bile acid excretion[11-13]. With bile acid excretion, plasma or liver cholesterol is utilized to maintain the bile acid pool[12]. Alternatively, the cholesterol lowering effects of chitosan may be related to an increase in viscosity of intestinal contents, which entrap fat and prevent lipolysis, or this mechanism may be in addition to chitosan's ability to bind bile acids[13-15].

Along with an inhibition in dietary fat absorption and decreases in serum lipids there were also treatment-related decreases in the levels of fat-soluble vitamins A and E. Serum and liver vitamin E levels were substantially affected, being 62% to 87% lower in the 9% males and females. These findings are similar to those of Deuchi et al.[26] where decreases in serum and liver vitamin E levels were observed after 14 days of consuming a 5% chitosan feed. In this same study, liver vitamin A levels were decreased, but vitamin A serum levels were unchanged. Bile and lipids are needed for the absorption of dietary vitamins A and E, as both must be incorporated into intestinal micelles for their absorption[60]. Thus, it is highly plausible that the decrease in dietary fat absorption, including cholesterol, led to the decreases in serum and liver concentrations of these vitamins. It is also possible that, by some unknown mechanism, chitosan may enhance vitamin A or E requirements in the peripheral tissues.

There were no histologic changes associated with the observed decreases in vitamin levels; however, the decreases were significant enough to suggest nutritional inadequacies. The long-term effects of vitamin A and vitamin E deficiencies are well-known[60-63], and it is unknown what deficiency-related effects would have been observed had these decreased levels been maintained for a longer period of time. When circulating levels of vitamin E, specifically α-tocopherol, are depleted, tissue damage can occur. Vitamin E depletion in humans has subsequently been correlated with anemia, disruption of normal growth, decreased responses to infection, and

pregnancy concerns[62]. Vitamin A is essential in numerous biological processes and pathways, including growth, vision development, immune function, and metabolism. Severe vitamin A deficiency (VAD) results in disruption of normal tissue function and is associated with childhood blindness, anemia, and depressed responses to infection; VAD during a severe infection may result in death[61-63]. While the long-term effects of vitamin deficiency in rodents are not as well understood, the available literature on human deficiencies suggests that the decreases in vitamin A and E observed in this study may be detrimental over time.

In contrast to decreases in vitamins A and E, 1,25 $(OH)_2$ vitamin D (bioactive vitamin D) levels were significantly elevated in 9% male and female rats. Vitamin D's main function is to help maintain normal calcium and phosphorus levels by regulating the intestinal absorption of these minerals from the diet. In addition to the increased 1,25 $(OH)_2$ vitamin D levels, significant decreases in serum phosphorus were also seen in male and female rats. Although intestinal absorption of phosphorus was not measured in this study, chitosan has been observed by others to cause a significant reduction in intestinal phosphorus absorption[64]. Low phosphorus concentrations stimulate 1,25 $(OH)_2$ vitamin D production by the kidney, therefore the increased levels of 1,25 $(OH)_2$ vitamin D observed in this study may be the result of the low phosphorus levels. Increased levels of 1,25 $(OH)_2$ vitamin D can cause an increase in intestinal absorption of calcium regardless of serum calcium levels. Significant elevation in intestinal absorption of calcium was observed sporadically in the female rats, but serum calcium levels were relatively stable. This effect is most likely due to a loss of calcium through the urine, which has been observed in other chitosan feed studies[64; 65] and is known to occur in cases of hypophosphatemia-induced elevations in 1,25 $(OH)_2$ vitamin D due to Fanconi's syndrome[66]. The reported urinary calcium loss in chitosan feed studies may be compensatory or directly induced by the chitosan.

Significant decreases in urine volume were observed in various male and female groups, but most consistently in the 9% group where decreases of 40% to 58% of the control group volume were observed. As the urine volumes decreased, urine creatinine concentrations were seen to increase significantly. This is consistent with proper renal function. The most likely cause of the decrease in urine volume is decreased consumption of water, although water consumption was not measured, so this cannot be certain. However, the mild increases in urea nitrogen in the 9% male and female rats at 25 weeks (the only time point measured) supports decreased water consumption (i.e., mild dehydration). Water retention in the intestine may have contributed to the decreases in urine volume, as fecal moisture was mildly increased in some of the treatment groups, although it is highly unlikely this would be the primary cause and no diarrhea was observed.

There was a significant decrease in the occurrence of periportal fatty change, or lipid accumulation, in the livers of 9% females relative to the controls, and this negative trend was maintained in both 1% and 3% females, although not significantly. In male rats, the incidences of periportal fatty change were decreased in both 1% and 9% groups and the severities were decreased in both the 3% and 9% groups. The decrease in lipid accumulation was inconsistent between male and female rats in the 9% exposure groups, as a more severe decrease was observed in the 9% female rats (100% lower) compared to the 9% male rats (50% lower) relative to the respective controls. The morphologic features observed during this study (periportal hepatocytes with large, single, well-defined intracytoplasmic vacuoles displacing the nucleus), were consistent with the intracytoplasmic lipid accumulation that is associated with fatty change[67]. During normal function, fatty acids circulate between the liver and adipose tissue,

which maintains a balance of triglycerides between the two locations. When this balance becomes skewed, hepatic fatty acids can accumulate as small vacuoles in the hepatocytes and progress over time into larger globules[67; 68].

Lipid accumulation in the liver can occur via multiple mechanisms, including 1) increased synthesis of fatty acids, 2) increased uptake of fatty acids from adipose tissue and/or the diet, 3) improper removal of fatty acids from the liver, or 4) decreased oxidation of fatty acids[69]. Diet and nutritional status can also influence lipid accumulation[68; 70]. Singh et al.[71] demonstrated that albino rats administered vitamin A orally for 2 days had increased hepatic lipid accumulation. In the present study, there were treatment-related decreases in hepatic vitamin A and E in both male and female rats, which could have contributed to the loss of periportal lipid accumulation observed in the animals fed 9% chitosan. Lipid accumulation in the liver can also occur due to imbalanced uptake of lipids from the blood and secretion of lipoproteins from the hepatocytes[72]. In this chitosan study, the fatty change (lipid accumulation) observed was periportal, or in Zone 1. Zone 1 is closest to the incoming vasculature and receives the majority of oxygenated blood, and Zone 1 hepatocytes are generally resistant to the effects of nutritional deficiencies[73]. Therefore, the decrease in fatty change observed in rats fed 9% chitosan could be an adaptive response to the vitamin and mineral depletion noted in this study.

The incidences and severities of fatty change in both male and female control animals was particularly high (6/10, males; 7/10, females; average severity 1.7 and 1.1, respectively), suggesting that the Charles River Sprague Dawley rats used in this study may have a normally high level of hepatic periportal lipid accumulation. Figure 3, Figure 4, and Figure 5, included in this report, are well representative of the observations made in this study, as the increased severity of periportal fatty change in control animals was a strong response.

Absolute and relative liver weights of male and female rats were significantly decreased in animals fed 9% chitosan relative to control animals. As described above, there were decreases in the incidence of periportal fatty change in all exposed animals, particularly in the female rats fed 9% chitosan. The decrease in liver weights observed in the 9% animals could be due to the loss of fat accumulation in the livers, which would alter the weight of the organs.

The absolute and relative thymus weights of 3% and 9% males and 9% females were also significantly decreased relative to those of control groups. The thymus is extremely sensitive to toxic compounds and similar stressors, and alterations in thymus weight can be an indicator of apoptosis and organ atrophy in response to a toxic insult. Nutritional status can cause a decrease in thymus weight, in particular vitamin, mineral, and fatty acid deficiencies[74]. In the current study, male and female rats fed 9% chitosan had depleted levels of serum vitamin A and E, liver vitamin E, and serum cholesterol and triglycerides, indicating nutritional inadequacies. The observations from this chitosan study, combined with what is known about the thymus, suggest that exposure to chitosan may have induced reductions in thymus weight secondary to nutritional deficiencies.

Results of this study did not support chitosan as a cause of bone resorption. Significant elevation of parathyroid hormone levels occurred occasionally and inconsistently, while calcium levels were relatively stable. Calcium was mildly, but significantly, decreased at only two time points in male groups by no more than 4%. Additionally, serum total osteocalcin and urinary deoxypyridinoline level, both biomarkers of bone turnover, while occasionally significantly

elevated, lacked any consistent increases over time or between sexes. In fact, deoxypyridinoline was significantly decreased at some time points. Lastly, bone calcium, bone length, and the histology findings of this study did not support calcium loss from the bone.

Although bone parameters were unaffected by chitosan exposure, a limitation of this study may be that the time frame of the study was not extensive enough to adequately evaluate bone loss. Rats are generally not considered skeletally mature until 10 months of age, and the long bones in rats can continue to grow until 30 months of age, making it difficult to observe any loss of bone before that point[75]. In a study of female Charles River Sprague Dawley rats, Wronski et al.[76] observed closed growth plates in the tibias of 15-month-old animals. In a separate study, Fukuda and Iida[77] noted that natural decreases in bone mineral density did not begin until 15 months of age in female Wistar rats. Also, standard osteoporosis studies using rat models commonly utilize ovariectomized animals, which mimics the conditions of menopause and generally increases rates of bone remodeling and bone loss. Ovariectomized SHRSP rats fed 10% chitosan alongside a low calcium diet exhibited decreased bone mineral density and increased femur stiffness[64]. Following ovariectomy, bone loss in the femurs, specifically the femoral neck, is still not observed until a minimum of 30 days postprocedure[75]. Therefore, given the time frame of the study there was reduced likelihood of observing any osteologic changes possibly induced by chitosan exposure.

There were no treatment-related clinical findings in the core, Group A animals, but there were instances of seizures in Groups B and C animals. Thirteen animals from Groups B and C (two 1%, one 3%, and ten 9%) were observed with seizures either during or after the 18-week blood collection. Seizures were not noted at any other time point. Similarly, there was no treatment-related mortality in the Group A animals, but five animals from Groups B and C died, often after seizures, near the time of blood collection. Cause of death was undetermined for these animals. While there was no clear connection between chitosan treatment and the incidence of seizures, there was an exposure concentration-related increase in the occurrence of seizures. Therefore, it is possible that chitosan exposure may have induced the increased rate of seizures observed in this study.

Under the conditions of the 6-month feed study of chitosan, male and female rats fed 3% and 9% chitosan in the diet had significantly decreased levels of serum vitamin A and serum and hepatic vitamin E and increased levels of serum 1,25 $(OH)_2$ vitamin D. Consumption of high levels of chitosan decreased percentage fat digestion and increased fecal weight and moisture, as well as reduced levels of phosphorous, cholesterol, and triglycerides. Female rats exposed to 9% chitosan also had significant liver weight and histologic changes. Based on the above results, the lowest-observed-effect level for chitosan exposure was 1% (approximately equivalent to 450 mg/kg) in male and 9% (approximately equivalent to 6,000 mg/kg) in female rats.

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Appendix A. Summary of Lesions in Rats in the Six-month Feed Study of Chitosan

Tables

Table A-1. Summary of the Incidence of Nonneoplastic Lesions in Group A Male Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Disposition Summary				
Animals initially in study	10	10	10	10
Survivors				
Terminal euthanasia	10	10	10	10
Animals examined microscopically	10	10	10	10
Alimentary System				
Liver	(10)	(10)	(10)	(10)
Degeneration, cystic	0	0	0	1 (10%)
Hematopoietic cell proliferation	2 (20%)	3 (30%)	3 (30%)	6 (60%)
Inflammation, chronic active	10 (100%)	10 (100%)	10 (100%)	9 (90%)
Periportal, fatty change	6 (60%)	3 (30%)	6 (60%)	3 (30%)
Pancreas	(10)	(0)	(0)	(10)
Basophilic focus	1 (10%)	–	–	0
Inflammation	2 (20%)	–	–	1 (10%)
Stomach, forestomach	(10)	(0)	(0)	(10)
Epithelium, hyperplasia	3 (30%)	–	–	1 (10%)
Cardiovascular System				
Blood vessel	(10)	(0)	(0)	(10)
Inflammation	0	–	–	1 (10%)
Heart	(10)	(0)	(0)	(10)
Cardiomyopathy	5 (50%)	–	–	3 (30%)
Mineralization	0	–	–	1 (10%)
Endocrine System				
Adrenal cortex	(10)	(1)	(0)	(10)
Vacuolization cytoplasmic	0	0	–	1 (10%)
Parathyroid gland	(10)	(10)	(10)	(10)
Hyperplasia	1 (10%)	0	0	0
Pituitary gland	(10)	(0)	(0)	(10)
Cyst	1 (10%)	–	–	0
Thyroid gland	(10)	(0)	(0)	(10)
C-cell, hyperplasia	0	–	–	1 (10%)
General Body System				
None	–	–	–	–

	0%	1%	3%	9%
Genital System				
Preputial gland	(10)	(0)	(0)	(10)
Inflammation	0	–	–	1 (10%)
Inflammation, chronic active	0	–	–	2 (20%)
Prostate	(10)	(10)	(10)	(10)
Inflammation	8 (80%)	9 (90%)	10 (100%)	10 (100%)
Testes	(10)	(0)	(0)	(10)
Mineralization	0	–	–	1 (10%)
Hematopoietic System				
Lymph node, mandibular	(10)	(0)	(0)	(10)
Infiltration cellular, plasma cell	1 (10%)	–	–	2 (20%)
Spleen	(10)	(0)	(0)	(10)
Hematopoietic cell proliferation	5 (50%)	–	–	2 (20%)
Thymus	(10)	(0)	(0)	(10)
Atrophy	1 (10%)	–	–	0
Integumentary System				
Skin	(10)	(0)	(0)	(10)
Hemorrhage	0	–	–	1 (10%)
Mineralization	0	–	–	1 (10%)
Ulcer	0	–	–	1 (10%)
Musculoskeletal System				
Skeletal muscle	(0)	(0)	(0)	(1)
Inflammation, granulomatous	–	–	–	1 (100%)
Nervous System				
None	–	–	–	–
Respiratory System				
Lung	(10)	(0)	(0)	(10)
Hemorrhage	2 (20%)	–	–	0
Inflammation, chronic active	2 (20%)	–	–	4 (40%)
Nose	(10)	(0)	(0)	(10)
Inflammation	1 (10%)	–	–	0
Goblet cell, hyperplasia	0	–	–	1 (10%)
Special Senses System	–	–	–	
Eye	(10)	(1)	(0)	(10)
Choroid, fibrosis	0	1 (100%)	–	0
Lens, cataract	0	1 (100%)	–	0

	0%	1%	3%	9%
Harderian gland	(10)	(0)	(0)	(10)
Hyperplasia	0	–	–	1 (10%)
Infiltration cellular, lymphocyte	2 (20%)	–	–	1 (10%)
Urinary System				
Kidney	(10)	(10)	(10)	(10)
Infarct	0	0	1 (10%)	0
Mineralization	2 (20%)	4 (40%)	3 (30%)	5 (50%)
Nephropathy	9 (90%)	9 (90%)	9 (90%)	9 (90%)
Cortex, cyst	1 (10%)	0	0	0
Pelvis, dilatation	2 (20%)	0	1 (10%)	0
Pelvis, inflammation	1 (10%)	0	0	0
Urinary bladder	(10)	(0)	(0)	(10)
Transitional epithelium, hyperplasia	0	–	–	1 (10%)

[a]Number of animals examined microscopically at the site and the number of animals with lesion.

Table A-2. Summary of the Incidence of Neoplasms and Nonneoplastic Lesions in Group A Female Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Disposition Summary				
Animals initially in study	10	10	10	10
Survivors				
Terminal euthanasia	10	10	10	10
Animals examined microscopically	10	10	10	10
Alimentary System				
Liver	(10)	(10)	(10)	(10)
Hematopoietic cell proliferation	1 (10%)	1 (10%)	2 (20%)	1 (10%)
Inflammation, chronic active	9 (90%)	9 (90%)	9 (90%)	10 (100%)
Periportal, fatty change	7 (70%)	4 (40%)	4 (40%)	0
Pancreas	(10)	(0)	(0)	(10)
Atrophy	0	–	–	1 (10%)
Inflammation	1 (10%)	–	–	0
Inflammation, chronic active	0	–	–	1 (10%)
Cardiovascular System				
Heart	(10)	(0)	(0)	(10)
Cardiomyopathy	1 (10%)	–	–	0
Endocrine System				
Pituitary gland	(10)	(0)	(0)	(10)

	0%	1%	3%	9%
Rathke's cleft, hyperplasia	1 (10%)	–	–	0
General Body System				
None	–	–	–	–
Genital System				
Clitoral gland	(10)	(0)	(0)	(10)
Inflammation, chronic active	2 (20%)	–	–	0
Hematopoietic System				
Spleen	(10)	(0)	(0)	(10)
Hematopoietic cell proliferation	1 (10%)	–	–	0
Thymus	(10)	(0)	(0)	(10)
Atrophy	1 (10%)	–	–	0
Integumentary System				
Mammary gland	(10)	(0)	(0)	(10)
Adenoma	0	–	–	1 (10%)
Musculoskeletal System				
None	–	–	–	–
Nervous System				
Brain	(10)	(0)	(0)	(10)
Developmental malformation	1 (10%)	–	–	0
Respiratory System				
Lung	(10)	(0)	(0)	(10)
Mineralization	0	–	–	1 (10%)
Alveolar epithelium, hyperplasia	0	–	–	1 (10%)
Alveolus, infiltration cellular, histiocyte	2 (20%)	–	–	0
Artery, mineralization	1 (10%)	–	–	1 (10%)
Nose	(10)	(0)	(0)	(10)
Goblet cell, hyperplasia	1 (10%)	–	–	0
Special Senses System				
Harderian gland	(10)	(0)	(0)	(10)
Infiltration cellular, lymphocyte	1 (10%)	–	–	1 (10%)
Urinary System				
Kidney	(10)	(10)	(10)	(10)
Mineralization	8 (80%)	8 (80%)	5 (50%)	6 (60%)
Nephropathy	5 (50%)	6 (60%)	5 (50%)	0

[a]Number of animals examined microscopically at the site and the number of animals with lesion.

Appendix B. Clinical Pathology Results

Tables

Table B-1. Hematology, Clinical Chemistry, and Urinalysis Data for Group C Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
Hematology				
n	10	10	10	10
Hematocrit (auto) (%)				
Week 25	45.5 ± 0.4	47.1 ± 0.5	46.3 ± 0.4	47.4 ± 0.6*
Hematocrit (manual) (%)				
Week 25	47.2 ± 0.5[b]	48.2 ± 0.5	47.6 ± 0.5	48.9 ± 0.6
Hemoglobin (g/dL)				
Week 25	14.9 ± 0.2	15.4 ± 0.2	15.2 ± 0.1	15.7 ± 0.2**
Erythrocytes (10^6/μL)				
Week 25	8.44 ± 0.08	8.59 ± 0.12	8.43 ± 0.08	8.45 ± 0.12
Reticulocytes (10^3/μL)				
Week 25	186.0 ± 14.3	138.4 ± 6.3**	157.7 ± 7.2	139.3 ± 9.1**
Mean cell volume (fL)				
Week 25	53.9 ± 0.4	54.9 ± 0.6	54.9 ± 0.2	56.1 ± 0.6*
Mean cell hemoglobin (pg)				
Week 25	17.6 ± 0.2	18.0 ± 0.2	18.0 ± 0.1	18.6 ± 0.2**
Mean cell hemoglobin concentration (g/dL)				
Week 25	32.7 ± 0.2	32.7 ± 0.1	32.9 ± 0.2	33.2 ± 0.2
Platelets (10^3/μL)				
Week 25	916 ± 52	824 ± 26	921 ± 19	973 ± 36
Leukocytes (10^3/μL)				
Week 25	10.62 ± 0.98	9.39 ± 0.94	7.38 ± 0.69	9.54 ± 0.91
Segmented neutrophils (10^3/μL)				
Week 25	2.04 ± 0.38	1.48 ± 0.24	1.06 ± 0.12	1.76 ± 0.39
Lymphocytes (10^3/μL)				
Week 25	8.02 ± 0.64	7.42 ± 0.74	6.01 ± 0.62	7.44 ± 0.65
Monocytes (10^3/μL)				
Week 25	0.31 ± 0.05	0.32 ± 0.05	0.20 ± 0.02	0.19 ± 0.03
Basophils (10^3/μL)				
Week 25	0.04 ± 0.01	0.04 ± 0.01	0.02 ± 0.00	0.03 ± 0.01
Eosinophils (10^3/μL)				
Week 25	0.21 ± 0.05	0.15 ± 0.03	0.09 ± 0.01*	0.11 ± 0.03

	0%	1%	3%	9%
Clinical Chemistry				
n	10	10	10	10
Urea nitrogen (mg/dL)				
Week 25	12.4 ± 0.6	12.1 ± 0.5	12.7 ± 0.5	15.3 ± 0.9**
Creatinine (mg/dL)				
Week 25	0.62 ± 0.01	0.64 ± 0.02	0.62 ± 0.01	0.64 ± 0.02
Calcium (mg/dL)				
Week 13	12.6 ± 0.1	12.5 ± 0.1	12.3 ± 0.2	12.4 ± 0.2
Week 19	12.5 ± 0.1	12.3 ± 0.2	12.3 ± 0.1	12.0 ± 0.1*
Week 25	12.1 ± 0.1	12.1 ± 0.2	12.0 ± 0.1	11.6 ± 0.1*
Phosphorus (mg/dL)				
Week 13	8.4 ± 0.3	8.1 ± 0.3	7.2 ± 0.3**	7.4 ± 0.4*
Week 19	8.2 ± 0.4	7.7 ± 0.2	7.4 ± 0.3	6.7 ± 0.2**
Week 25	6.9 ± 0.3	6.8 ± 0.2	6.7 ± 0.1	5.8 ± 0.3**
Total protein (g/dL)				
Week 25	7.4 ± 0.1	7.2 ± 0.1	7.3 ± 0.1	6.9 ± 0.1*
Albumin (g/dL)				
Week 19	4.8 ± 0.1	4.6 ± 0.1	4.7 ± 0.1	4.5 ± 0.0*
Week 25	4.8 ± 0.1	4.7 ± 0.1	4.8 ± 0.1	4.6 ± 0.0
Cholesterol (mg/dL)				
Week 7	82 ± 5	75 ± 8	80 ± 6	53 ± 3**
Week 13	95 ± 7	84 ± 8	90 ± 7	53 ± 2**
Week 19	101 ± 6	87 ± 10	94 ± 8	59 ± 4**
Week 25	95 ± 6	81 ± 8	90 ± 6	49 ± 4**
Triglycerides (mg/dL)				
Week 7	202 ± 28	234 ± 43	226 ± 30	88 ± 15*
Week 13	198 ± 33	202 ± 38	195 ± 24	86 ± 8**
Week 19	180 ± 26	218 ± 43	210 ± 29	95 ± 13*
Week 25	173 ± 18	207 ± 30	218 ± 24	109 ± 13
Alanine aminotransferase (IU/L)				
Week 25	28 ± 3	29 ± 2	29 ± 1	57 ± 2**
Alkaline phosphatase (IU/L)				
Week 7	134 ± 7	134 ± 7	138 ± 8	137 ± 16
Week 13	100 ± 6	95 ± 6	102 ± 6	82 ± 5
Week 19	91 ± 11	87 ± 7	84 ± 4	72 ± 7
Week 25	85 ± 7	83 ± 7	82 ± 5	64 ± 5*

	0%	1%	3%	9%
Creatine kinase (IU/L)				
Week 25	192 ± 29	205 ± 27	233 ± 23	245 ± 20
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	15 ± 1	14 ± 1
Bile acids (µmol/L)				
Week 25	9.6 ± 2.3	6.4 ± 2.7	2.4 ± 0.2**	4.3 ± 0.8
Total osteocalcin (ng/mL)				
Week 7	445.7 ± 17.2	439.8 ± 15.8	441.8 ± 18.2	520.4 ± 22.6
Week 13	306.2 ± 13.0	289.7 ± 28.6	245.4 ± 37.9	372.6 ± 23.4
Week 19	239.4 ± 12.4	225.7 ± 10.6	181.6 ± 26.8	269.2 ± 20.9
Week 25	158.3 ± 10.0	168.1 ± 11.6	145.9 ± 22.7	218.3 ± 14.6*
Parathyroid hormone (ng/mL)				
Week 7	1.882 ± 0.137	1.643 ± 0.449	1.838 ± 0.348	1.521 ± 0.368
Week 13	2.343 ± 0.350	2.763 ± 0.479	3.215 ± 0.537	2.433 ± 0.222
Week 19	1.879 ± 0.186	3.101 ± 0.475	2.710 ± 0.365	3.679 ± 0.361**
Week 25	2.668 ± 0.475	2.924 ± 0.276	3.981 ± 0.349	2.848 ± 0.506
Urinalysis				
n				
Week 7	10	9	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	10	10	10
Creatinine (mg/dL)				
Week 7	192.5 ± 15.1	227.2 ± 30.7	269.4 ± 33.6	254.9 ± 37.2
Week 13	249.4 ± 25.1	360.7 ± 19.5*	350.3 ± 22.5*	334.0 ± 35.8
Week 19	204.3 ± 20.4	394.2 ± 32.5**	345.1 ± 26.0**	302.5 ± 26.6
Week 25	254.1 ± 27.4	374.8 ± 25.6*	345.9 ± 27.1	325.4 ± 36.0
Volume (mL)				
Week 7	8.3 ± 0.8	7.5 ± 1.5	6.4 ± 0.8	5.0 ± 1.1*
Week 13	7.9 ± 1.0	4.6 ± 0.3**	5.1 ± 0.4*	4.5 ± 0.5**
Week 19	10.7 ± 1.6	4.0 ± 0.4**	5.3 ± 0.7*	5.6 ± 0.6
Week 25	8.6 ± 1.2	5.4 ± 0.6*	6.1 ± 0.8*	5.1 ± 0.6**
Deoxypyridinoline (nmol/L)				
Week 7	3,396.0 ± 268.0	4,210.0 ± 643.0	4,917.0 ± 826.0	4,754.0 ± 761.0
Week 13	2,185.1 ± 188.9	3,197.3 ± 148.3*	3,233.8 ± 218.0*	3,129.1 ± 296.5*
Week 19	1,084.9 ± 158.9	2,209.6 ± 246.3**	1,963.0 ± 200.5*	1,994.9 ± 214.3*

	0%	1%	3%	9%
Week 25	1,083.5 ± 145.9	1,699.3 ± 139.6*	1,658.3 ± 136.7*	1,750.8 ± 167.6*
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.810 ± 0.135	1.889 ± 0.148	1.810 ± 0.159	1.920 ± 0.160
Week 13	0.910 ± 0.035	0.890 ± 0.031	0.930 ± 0.040	0.960 ± 0.078
Week 19	0.530 ± 0.050	0.550 ± 0.034	0.570 ± 0.042	0.660 ± 0.048
Week 25	0.430 ± 0.030	0.470 ± 0.030	0.480 ± 0.020	0.550 ± 0.027**
Female				
Hematology				
n	10	9	10	10
Hematocrit (auto) (%)				
Week 25	45.5 ± 1.0	44.9 ± 0.9	44.5 ± 0.8	45.2 ± 0.9
Hematocrit (manual) (%)				
Week 25	47.4 ± 1.1	46.9 ± 1.0	46.5 ± 0.8	46.6 ± 0.9
Hemoglobin (g/dL)				
Week 25	15.2 ± 0.4	15.0 ± 0.3	15.0 ± 0.3	15.1 ± 0.3
Erythrocytes (10^6/μL)				
Week 25	8.16 ± 0.19	8.17 ± 0.18	8.01 ± 0.11	8.10 ± 0.15
Reticulocytes (10^3/μL)				
Week 25	135.2 ± 14.6	109.2 ± 6.1	109.6 ± 7.7	129.5 ± 14.6
Mean cell volume (fL)				
Week 25	55.8 ± 0.6	55.0 ± 0.3	55.6 ± 0.3	55.8 ± 0.8
Mean cell hemoglobin (pg)				
Week 25	18.7 ± 0.2	18.4 ± 0.1	18.7 ± 0.1	18.7 ± 0.3
Mean cell hemoglobin concentration (g/dL)				
Week 25	33.5 ± 0.1	33.4 ± 0.2	33.6 ± 0.2	33.4 ± 0.2
Platelets (10^3/μL)				
Week 25	791 ± 43	798 ± 40	848 ± 38	1,024 ± 51**
Leukocytes (10^3/μL)				
Week 25	6.62 ± 0.92	3.66 ± 0.49*	5.72 ± 0.87	4.92 ± 0.58
Segmented neutrophils (10^3/μL)				
Week 25	1.15 ± 0.24	0.53 ± 0.10*	0.67 ± 0.11	0.67 ± 0.11
Lymphocytes (10^3/μL)				
Week 25	5.09 ± 0.79	2.93 ± 0.41	4.78 ± 0.77	4.06 ± 0.49
Monocytes (10^3/μL)				
Week 25	0.24 ± 0.04	0.13 ± 0.02	0.18 ± 0.03	0.11 ± 0.02*

	0%	1%	3%	9%
Basophils ($10^3/\mu L$)				
Week 25	0.02 ± 0.00	0.01 ± 0.00*	0.02 ± 0.01	0.01 ± 0.00
Eosinophils ($10^3/\mu L$)				
Week 25	0.12 ± 0.02	0.06 ± 0.01	0.08 ± 0.01	0.07 ± 0.02
Clinical Chemistry				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	10
Urea nitrogen (mg/dL)				
Week 25	14.2 ± 1.4	14.1 ± 0.7	15.2 ± 0.7	16.3 ± 0.7*
Creatinine (mg/dL)				
Week 25	0.65 ± 0.02	0.68 ± 0.01	0.70 ± 0.01	0.69 ± 0.02
Calcium (mg/dL)				
Week 13	12.9 ± 0.2	13.1 ± 0.1	12.7 ± 0.2	12.5 ± 0.2
Week 19	12.9 ± 0.1	13.1 ± 0.2	12.8 ± 0.1	12.5 ± 0.1
Week 25	12.7 ± 0.3	12.8 ± 0.1	12.7 ± 0.2	12.3 ± 0.2
Phosphorus (mg/dL)				
Week 13	8.1 ± 0.5	7.4 ± 0.4	6.5 ± 0.4**	6.8 ± 0.3*
Week 19	8.4 ± 0.4	8.2 ± 0.5	8.1 ± 0.3	7.4 ± 0.5
Week 25	6.8 ± 0.2	6.4 ± 0.2	6.2 ± 0.3*	5.5 ± 0.3**
Total protein (g/dL)				
Week 25	8.2 ± 0.2	9.0 ± 0.1**	8.6 ± 0.2	8.4 ± 0.2
Albumin (g/dL)				
Week 19	5.9 ± 0.2	6.2 ± 0.1	5.9 ± 0.2	5.7 ± 0.1
Week 25	5.8 ± 0.2	6.5 ± 0.2*	6.2 ± 0.1	6.2 ± 0.1
Cholesterol (mg/dL)				
Week 7	80 ± 6	81 ± 8	67 ± 4	59 ± 4**
Week 13	92 ± 8	86 ± 7	73 ± 5	58 ± 4**
Week 19	107 ± 7	105 ± 9	91 ± 8	67 ± 5**
Week 25	94 ± 7	108 ± 5	96 ± 8	63 ± 4**
Triglycerides (mg/dL)				
Week 7	88 ± 12	130 ± 48	81 ± 8	86 ± 14
Week 13	125 ± 10	163 ± 30	140 ± 23	88 ± 23*
Week 19	143 ± 15	181 ± 32	137 ± 18	90 ± 13

	0%	1%	3%	9%
Week 25	188 ± 31	231 ± 44	245 ± 31	158 ± 35
Alanine aminotransferase (IU/L)				
Week 25	25 ± 3	28 ± 3	32 ± 2**	47 ± 4**
Alkaline phosphatase (IU/L)				
Week 7	102 ± 7	99 ± 5	99 ± 7	95 ± 10
Week 13	57 ± 4	63 ± 4	71 ± 7	59 ± 5
Week 19	49 ± 5	53 ± 3	55 ± 6	46 ± 6
Week 25	46 ± 4	44 ± 2	51 ± 6	44 ± 7
Creatine kinase (IU/L)				
Week 25	258 ± 44	193 ± 46	210 ± 50	225 ± 26
Sorbitol dehydrogenase (IU/L)				
Week 25	17 ± 3	17 ± 2	19 ± 2	16 ± 1
Bile acids (µmol/L)				
Week 25	10.7 ± 1.8	8.2 ± 1.1	32.0 ± 14.1	10.8 ± 1.1
Total osteocalcin (ng/mL)				
Week 7	293.6 ± 19.4	287.5 ± 21.2	282.1 ± 34.7	316.7 ± 23.5
Week 13	197.9 ± 22.6	202.3 ± 15.4	184.4 ± 19.4	234.2 ± 14.5
Week 19	158.1 ± 18.3	184.8 ± 13.2	166.7 ± 24.7	210.1 ± 16.0
Week 25	107.9 ± 18.6	97.1 ± 7.1	96.0 ± 16.2	148.8 ± 15.1
Parathyroid hormone (ng/mL)				
Week 7	0.995 ± 0.150[b]	1.156 ± 0.176	1.092 ± 0.182	1.023 ± 0.146
Week 13	1.506 ± 0.203	1.734 ± 0.194	1.925 ± 0.306	1.767 ± 0.212
Week 19	1.406 ± 0.232	1.994 ± 0.353	1.845 ± 0.418	1.673 ± 0.223
Week 25	1.471 ± 0.189[b]	1.628 ± 0.220	1.818 ± 0.224	2.301 ± 0.212*
Urinalysis				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 25	10	9	10	9
Creatinine (mg/dL)				
Week 7	98.2 ± 14.7	107.1 ± 12.3	206.3 ± 55.2*	192.0 ± 8.3**
Week 13	144.7 ± 14.8	139.5 ± 15.9	247.5 ± 30.9*	241.6 ± 29.0**
Week 19	142.0 ± 19.7	137.7 ± 19.4	196.3 ± 23.6	230.3 ± 19.2**
Week 25	179.8 ± 59.7	120.3 ± 24.1	184.5 ± 20.5	217.9 ± 23.4*

	0%	1%	3%	9%
Volume (mL)				
Week 7	8.2 ± 1.2	8.5 ± 1.0	5.4 ± 0.8	3.4 ± 0.3**
Week 13	6.4 ± 0.7	6.5 ± 0.6	4.1 ± 0.8*	2.9 ± 0.5**
Week 19	7.7 ± 1.2	8.1 ± 1.4	5.0 ± 0.8	3.4 ± 0.5**
Week 25	8.2 ± 1.5	9.1 ± 1.5	5.8 ± 0.9	3.7 ± 0.5**
Deoxypyridinoline (nmol/L)				
Week 7	1,622.0 ± 328.0	1,378.0 ± 295.0	4,130.0 ± 1,109.0*	4,423.0 ± 355.0**
Week 13	875.5 ± 129.6	587.0 ± 68.1	1,364.0 ± 215.9	1,421.6 ± 267.0
Week 19	666.3 ± 106.9	487.7 ± 68.5	894.9 ± 122.1	1,212.3 ± 107.4**
Week 25	565.7 ± 178.2	250.4 ± 47.1	625.1 ± 83.7	891.5 ± 114.1*
Deoxypyridinoline/creatinine (nmol/mg)				
Week 7	1.620 ± 0.128	1.240 ± 0.129	1.940 ± 0.229	2.300 ± 0.182*
Week 13	0.580 ± 0.039	0.430 ± 0.037**	0.540 ± 0.034	0.570 ± 0.042
Week 19	0.450 ± 0.017	0.360 ± 0.016*	0.440 ± 0.016	0.520 ± 0.020
Week 25	0.340 ± 0.043	0.222 ± 0.022	0.340 ± 0.027	0.411 ± 0.026

*Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test.
**$P \leq 0.01$.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 9.

Appendix C. Vitamin Concentration and Bone Parameter Results

Tables

Table C-1. Serum and Hepatic Vitamin Concentration Data for Group B Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
Male				
n				
Week 7	9	10	10	10
Week 13	9	10	10	10
Week 19	9	10	10	10
Week 26	9	10	10	8
Serum vitamin A (µg/mL)				
Week 7	0.532 ± 0.021	0.506 ± 0.033	0.513 ± 0.026	0.453 ± 0.018
Week 13	0.561 ± 0.024	0.499 ± 0.019	0.476 ± 0.022*	0.410 ± 0.009**
Week 19	0.533 ± 0.028	0.506 ± 0.031	0.475 ± 0.019	0.392 ± 0.014**
Week 26	0.476 ± 0.019	0.444 ± 0.024	0.398 ± 0.017**	0.336 ± 0.026**
Serum 1,25 $(OH)_2$ vitamin D (pg/mL)				
Week 7	124.4 ± 19.6	163.3 ± 21.7	183.2 ± 26.9	297.4 ± 41.0**
Week 13	70.1 ± 7.3	57.4 ± 5.3	77.3 ± 4.4	86.1 ± 8.5
Week 19	20.6 ± 2.8	21.7 ± 6.1	22.9 ± 2.2	42.3 ± 3.1**[b]
Week 26	27.7 ± 3.4[c]	28.0 ± 4.3	36.1 ± 4.6[b]	66.9 ± 11.9**
Serum vitamin E (µg/mL)				
Week 7	19.33 ± 1.43	15.38 ± 1.29	12.92 ± 0.48**	4.14 ± 0.23**
Week 13	21.08 ± 1.61	17.45 ± 1.06*	12.27 ± 0.86**	4.33 ± 0.27**
Week 19	20.59 ± 1.61	16.19 ± 0.96	12.86 ± 0.42**	4.07 ± 0.32**
Week 26	19.66 ± 1.66	17.35 ± 1.37	12.35 ± 0.61**	3.59 ± 0.65**
Liver vitamin A (µg/g)				
Week 26	57.4 ± 17.6	29.9 ± 2.5	39.6 ± 3.1	31.4 ± 3.7
Liver vitamin E (µg/g)				
Week 26	66.8 ± 16.2	55.0 ± 6.8	34.6 ± 2.2**	8.5 ± 0.8**
Female				
n				
Week 7	10	10	10	10
Week 13	10	10	10	10
Week 19	10	10	10	10
Week 26	10	10	9	10
Serum vitamin A (µg/mL)				
Week 7	0.272 ± 0.011	0.253 ± 0.007	0.260 ± 0.012	0.266 ± 0.012
Week 13	0.308 ± 0.020	0.295 ± 0.011	0.309 ± 0.019	0.281 ± 0.018
Week 19	0.283 ± 0.014	0.271 ± 0.015	0.291 ± 0.012	0.231 ± 0.010*
Week 26	0.316 ± 0.015	0.302 ± 0.014	0.294 ± 0.018	0.249 ± 0.010**

	0%	1%	3%	9%
Serum 1,25 $(OH)_2$ vitamin D (pg/mL)				
Week 7	104.0 ± 15.1	96.7 ± 10.9	111.0 ± 8.7	208.1 ± 18.2**
Week 13	60.6 ± 7.5	60.7 ± 7.9	69.3 ± 11.0	110.1 ± 16.9
Week 19	11.6 ± 1.6	12.6 ± 1.7	15.8 ± 1.4	31.4 ± 3.2**
Week 26	19.2 ± 2.2	20.7 ± 4.2	28.6 ± 6.5	53.7 ± 5.8**
Serum vitamin E (µg/mL)				
Week 7	18.65 ± 0.71	20.08 ± 0.87	18.38 ± 0.85	6.99 ± 0.58**
Week 13	19.81 ± 1.41	20.85 ± 1.06	20.19 ± 1.20	7.48 ± 0.38**
Week 19	21.02 ± 1.76	19.74 ± 1.75	19.86 ± 1.08	7.37 ± 0.57**
Week 26	20.94 ± 1.56	23.43 ± 1.66	22.23 ± 1.75	7.28 ± 0.64**
Liver vitamin A (µg/g)				
Week 26	65.2 ± 5.4	58.9 ± 5.0	62.3 ± 6.3	60.3 ± 4.8
Liver vitamin E (µg/g)				
Week 26	84.5 ± 8.9	97.1 ± 10.1	82.0 ± 11.8	17.2 ± 3.2**

*Significantly different ($P \leq 0.05$) from the control group by Dunn's or Shirley's test.
**Significantly different ($P \leq 0.01$) from the control group by Shirley's test.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data.
[b]n = 9.
[c]n = 7.

Table C-2. Bone Data for Groups A and B Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
n	10	10	10	10
Male				
Bone calcium (%)	23.79 ± 0.21[b]	23.95 ± 0.22	23.92 ± 0.30	23.74 ± 0.11[c]
Bone ash (%)	45.33 ± 0.79[b]	45.24 ± 0.67	45.83 ± 0.52	43.46 ± 0.62[c]
Bone moisture (%)	29.90 ± 0.49[b]	30.30 ± 0.44	29.72 ± 0.36	31.79 ± 0.62[c]
Left femur length (mm)	43.96 ± 0.34	44.33 ± 0.30	44.10 ± 0.30	43.42 ± 0.37
Left tibia length (mm)	48.00 ± 0.37	48.27 ± 0.36	47.95 ± 0.37	47.57 ± 0.41
Right tibia length (mm)	48.06 ± 0.32	48.41 ± 0.41	47.95 ± 0.33	47.57 ± 0.43
Female				
Bone calcium (%)	24.65 ± 0.17	24.96 ± 0.20	24.77 ± 0.23[b]	24.84 ± 0.12
Bone ash (%)	47.07 ± 0.58	47.14 ± 0.57	47.44 ± 0.46[b]	45.87 ± 0.44
Bone moisture (%)	28.40 ± 0.54	28.45 ± 0.45	28.53 ± 0.49[b]	30.37 ± 0.37**
Left femur length (mm)	36.65 ± 0.21	36.75 ± 0.17	36.73 ± 0.28	36.37 ± 0.26
Left tibia length (mm)	40.56 ± 0.28	40.25 ± 0.23	40.62 ± 0.40	40.10 ± 0.24
Right tibia length (mm)	40.53 ± 0.30	40.42 ± 0.24	40.74 ± 0.42	40.12 ± 0.21

**Significantly different ($P \leq 0.01$) from the control group by Shirley's test.
[a]Data are presented as mean ± standard error. Statistical tests were performed on unrounded data. Bone content data are from Group B rats at week 26 and bone lengths are from Group A rats at week 25.
[b]n = 9.
[c]n = 8.

Appendix D. Organ Weights and Organ-Weight-to-Body-Weight Ratios

Tables

Table D-1. Organ Weights and Organ-Weight-to-Body-Weight Ratios for Group A Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
n	10	10	10	10
Male				
Necropsy body wt	669 ± 20	702 ± 21	687 ± 23	612 ± 17
Heart				
Absolute	1.82 ± 0.07	1.81 ± 0.06	1.86 ± 0.08	1.77 ± 0.06
Relative	2.723 ± 0.089	2.589 ± 0.070	2.710 ± 0.091	2.904 ± 0.085
R. Kidney				
Absolute	2.04 ± 0.04	2.04 ± 0.04	2.11 ± 0.06	1.88 ± 0.04*
Relative	3.068 ± 0.088	2.920 ± 0.047	3.093 ± 0.088	3.093 ± 0.094
Liver				
Absolute	25.19 ± 0.87	24.87 ± 1.35	23.74 ± 1.51	19.53 ± 0.71**
Relative	37.662 ± 0.731	35.321 ± 1.179	34.345 ± 1.411*	31.933 ± 0.817**
Lung				
Absolute	2.49 ± 0.11	2.77 ± 0.09	2.62 ± 0.08	2.53 ± 0.14
Relative	3.738 ± 0.163	3.949 ± 0.095	3.841 ± 0.138	4.120 ± 0.160
R. Testis				
Absolute	1.696 ± 0.054	1.778 ± 0.046	1.726 ± 0.062	1.750 ± 0.028
Relative	2.555 ± 0.108	2.546 ± 0.078	2.534 ± 0.107	2.883 ± 0.104
Thymus				
Absolute	0.763 ± 0.045	0.727 ± 0.065	0.606 ± 0.063*	0.489 ± 0.032**
Relative	1.147 ± 0.071	1.030 ± 0.077	0.888 ± 0.091*	0.797 ± 0.045**
Thyroid gland and parathyroid gland				
Absolute	0.033 ± 0.003	0.034 ± 0.002	0.034 ± 0.002	0.031 ± 0.002
Relative	0.049 ± 0.004	0.048 ± 0.003	0.050 ± 0.003	0.051 ± 0.003
Parathyroid gland				
Absolute	0.0012 ± 0.0001	0.0010 ± 0.0001	0.0011 ± 0.0001	0.0011 ± 0.0001
Relative	0.002 ± 0.000	0.001 ± 0.000	0.002 ± 0.000	0.002 ± 0.000
Female				
Necropsy body wt	338 ± 11	335 ± 13	328 ± 11	301 ± 13
Heart				
Absolute	1.14 ± 0.03	1.09 ± 0.02	1.15 ± 0.03	1.03 ± 0.02**
Relative	3.393 ± 0.121	3.295 ± 0.094	3.515 ± 0.100	3.473 ± 0.134
R. Kidney				
Absolute	1.12 ± 0.04	1.10 ± 0.02	1.13 ± 0.03	1.01 ± 0.03

	0%	1%	3%	9%
Relative	3.311 ± 0.085	3.311 ± 0.095	3.465 ± 0.108	3.399 ± 0.104
Liver				
Absolute	12.54 ± 0.82	12.47 ± 0.39	11.85 ± 0.29	9.85 ± 0.20**
Relative	36.900 ± 1.502	37.341 ± 0.444	36.346 ± 0.904	33.036 ± 0.910*
Lung				
Absolute	1.83 ± 0.06	1.80 ± 0.08	1.81 ± 0.05	1.65 ± 0.05
Relative	5.463 ± 0.181	5.396 ± 0.170	5.552 ± 0.202	5.557 ± 0.281
R. Ovary				
Absolute	0.054 ± 0.005	0.049 ± 0.005	0.057 ± 0.005	0.056 ± 0.007
Relative	0.161 ± 0.015	0.147 ± 0.015	0.179 ± 0.021	0.190 ± 0.026
Thymus				
Absolute	0.436 ± 0.033	0.400 ± 0.036	0.383 ± 0.023	0.302 ± 0.021**
Relative	1.284 ± 0.081	1.188 ± 0.083	1.169 ± 0.062	1.000 ± 0.047**
Thyroid gland and parathyroid gland				
Absolute	0.028 ± 0.002	0.027 ± 0.002	0.035 ± 0.002	0.031 ± 0.002
Relative	0.084 ± 0.005	0.082 ± 0.007	0.106 ± 0.007	0.104 ± 0.008
Parathyroid gland				
Absolute	0.0007 ± 0.0001	0.0009 ± 0.0001	0.0008 ± 0.0001	0.0008 ± 0.0001
Relative	0.002 ± 0.000	0.003 ± 0.000*	0.002 ± 0.000	0.003 ± 0.000*
Uterus				
Absolute	0.657 ± 0.052	0.744 ± 0.060	0.714 ± 0.038	0.789 ± 0.096
Relative	1.980 ± 0.186	2.252 ± 0.191	2.184 ± 0.104	2.650 ± 0.329

*Significantly different ($P \leq 0.05$) from the control group by Williams' or Dunnett's test.
**$P \leq 0.01$.
[a]Organ weights (absolute weights) and body weights are given in grams; organ-weight-to-body-weight ratios (relative weights) are given as mg organ weight/g body weight (mean ± standard error).

Appendix E. Reproductive Tissue Evaluations

Tables

Table E-1. Summary of Reproductive Tissue Evaluations for Group A Male Rats in the Six-month Feed Study of Chitosan[a]

	0%	1%	3%	9%
n	10	10	10	10
Weights (g)				
Necropsy body wt	669 ± 20	702 ± 21	687 ± 23	612 ± 17
L. Cauda epididymis	0.2013 ± 0.0073	0.2134 ± 0.0079	0.2281 ± 0.0167	0.2072 ± 0.0103
L. Epididymis	0.6874 ± 0.0184	0.7047 ± 0.0274	0.7398 ± 0.0175	0.6402 ± 0.0165
L. Testis	1.7349 ± 0.0423	1.8209 ± 0.0478	1.7922 ± 0.0619	1.7900 ± 0.0333
Spermatid measurements				
Spermatid heads (10^6/testis)	207.79 ± 18.44	183.39 ± 9.19	238.70 ± 20.45	175.57 ± 8.43
Spermatid heads (10^6/g testis)	120.38 ± 11.23	101.50 ± 5.84	135.54 ± 14.11	98.05 ± 4.29
Epididymal spermatozoal measurements				
Sperm motility (%)	86.0 ± 0.37	86.1 ± 0.46	85.9 ± 0.46	85.8 ± 0.47
Sperm (10^6/cauda epididymis)	169.25 ± 14.82	182.38 ± 8.81	160.75 ± 12.63	157.63 ± 12.41
Sperm (10^6/g cauda epididymis)	833 ± 52	856 ± 33	711 ± 33	760 ± 46

[a]Data are presented as mean ± standard error. Differences from the control group are not significant by Dunnett's test (body and tissue weights) or Dunn's test (spermatid and epididymal spermatozoal measurements).

Appendix F. Chemical Characterization and Dose Formulation Studies

Table of Contents

Tables

Figures

F.1. Procurement and Characterization of Chitosan

Chitosan was obtained from Vanson HaloSource, Inc. (Redmond, WA), in one lot (02-ASSF-0715), which was used in the 6-month study. Identity, purity, and stability analyses were conducted by the analytical chemistry laboratory at Midwest Research Institute (MRI) (Kansas City, MO) and by the study laboratory at Battelle Columbus Operations (Columbus, OH). Reports on analyses performed in support of the chitosan studies are on file at the National Institute of Environmental Health Sciences.

The test article, an off-white powder, was identified as chitosan by the analytical chemistry laboratory using infrared (IR) and proton nuclear magnetic resonance (NMR) spectroscopy and by the study laboratory using IR spectroscopy. The percentage of deacetylation of the test article, determined by proton NMR, ranged from 85.97% to 87.17%, with an average of 86.5%. All spectra were consistent with the literature spectra[38; 39], and with the Sadtler spectral database. Representative IR and NMR spectra are presented in Figure F-1 and Figure F-2, respectively.

The moisture content for lot 02-ASSF-0715 was determined by the analytical chemistry laboratory using weight loss on drying in a 110°C oven for 24 hours; the inorganic content was determined on the dried test article by ashing at 500°C for 4 hours. Viscosity was determined at approximately 22.5°C using a Brookfield viscometer fitted with an SC4-18/R13 spindle at a speed of 30 rpm. Lot 02-ASSF-0715 was characterized by the analytical chemistry laboratory using gel permeation chromatography (GPC) with refractive index (RI) detection using system A (Table F-1) to find the most abundant molecular weight. Samples were prepared by transferring approximately 75 mg of the test article into a vial, and adding a 25 mL aliquot of diluent; vials were sealed with Teflon®-lined septa and crimp caps, allowed to stand for 2 hours at ambient temperature, swirled by hand, and placed on a rotary shaker for at least 1 hour. Standards containing a total of six molecular weight dextran markers with known peak molecular weights (Mp) (4,400, 21,400, 43,500, 196,000, 277,000, and 3,900,000 Mp) were prepared; approximately 10 mg of each marker (3 mg of 3,900,000 Mp marker) and 10 mL of diluent were pipetted into vials, sealed with Teflon®-lined septa and crimp caps, allowed to stand for a least 2 hours (the 3,900,000 marker was allowed to stand overnight) at ambient temperature to dissolve the standards, then swirled to mix prior to analysis.

For lot 02-ASSF-0715, weight loss on drying indicated 4.50% water, the average inorganic content by ashing was determined to be 2.13%, and viscosity was 81.3 centipoise. GPC/RI indicated one major peak and the determined molecular weight of the bulk chemical ranged from 62,755 to 87,343 daltons (Da). This resulted in an average molecular weight of 81,644 g/mol, or approximately 82 kDa, classifying the test article as a low molecular weight chitosan (LMWCS). A sample of chitosan was submitted to Covance Laboratories, Inc. (Madison, WI), for nutritional and contaminant testing using standard methods. For lot 02-ASSF-0715, levels of organochlorine and organophosphorous pesticides, nitrosamines, and aflatoxins were below the detection limits of the analytical methods. The purity of lot 02-ASSF-0715 was estimated to be approximately 94% based on the analysis of moisture and inorganic content. Taken together, these data indicated that the test article was chitosan.

To ensure stability, the test article was stored in sealed amber glass vials at room temperature. Reanalysis of the test article was performed during the study by the study laboratory using GPC/RI by system B, and no degradation of the test article was detected.

F.2. Preparation and Analysis of Dose Formulations

The dose formulations were prepared approximately monthly by mixing chitosan with feed (Table F-2). Dose formulations were stored in lined plastic buckets sealed with lids and stored at −30°C to −15°C for up to 42 days.

Homogeneity studies of approximately 0.5% and 9% formulations (5,046 and 90,049 µg/g, respectively) and stability studies of an approximately 0.5% (5,046 µg/g) formulation were performed by the analytical chemistry laboratory using GPC/RI by system C (Table F-1). Two peaks were attributed to chitosan with retention times of approximately 6.9 minutes and 12.1 minutes, respectively. Chitosan quantitation was based on the larger polymeric components of the first peak only because vehicle components co-eluted with the later oligomeric peak. Homogeneity studies of 1% and 9% dose formulations (10 mg/g and 90 mg/g in feed, respectively) were performed by the study laboratory using GPC/RI by system B. Homogeneity was confirmed, and stability was confirmed for at least 42 days for dose formulations stored in lined plastic buckets sealed with lids at temperatures up to room temperature and for at least 7 days under simulated animal room conditions.

Periodic analyses of the dose formulations of chitosan were performed by the study laboratory using GPC/RI by system B. Of the dose formulations analyzed, all nine were within 10% of the target concentrations (Table F-3). Animal room samples of dose formulations were also analyzed; all three were within 10% of the target concentrations.

Table F-1. Gel Permeation Chromatography Systems Used in the Six-month Feed Study of Chitosan[a]

Detection System	Column	Solvent System
System A		
Refractive index	In series: NOVEMA 10,000 Å, 300 mm × 8 mm, 10 µm and NOVEMA 3,000 Å, 50 mm × 8 mm (guard) and NOVEMA 3,000 Å, 300 mm × 8 mm, 10 µm (Polymer Standards Service GmbH, Mainz, Germany)	0.25% Trifluoroacetic acid, isocratic, flow rate 1.0 mL/minute
System B		
Refractive index	In series: BioSep-SEC-S2000 145 Å, 300 mm × 4.6 mm, 5 µm and BioSep-SEC-S3000 290 Å, 300 mm × 4.6 mm, 5 µm (Phenomenex, Torrance, CA)	1% Trifluoroacetic acid, isocratic, flow rate 0.35 mL/minute
System C		
Refractive index	In series: Alltech® Macrosphere 100 Å, 250 mm × 4.6 mm, 7 µm and Alltech® Macrosphere 300 Å, 250 mm × 4.6 mm, 7 µm (Grace, Columbia, MD)	1% Trifluoroacetic acid, isocratic, flow rate 0.5 mL/minute

[a]The liquid chromatographs were manufactured by Waters Corporation (Milford, MA) (System A), Agilent (Palo Alto, CA) (System B), or Perkin Elmer (Boston, MA) (System C).

Table F-2. Preparation and Storage of Dose Formulations in the Six-month Feed Study of Chitosan

Six-month Feed Study
Preparation
The appropriate amounts of chitosan and AIN-93M feed (87 kg for 1% and 3% formulations and 79 kg for the 9% formulation) were weighed in tared stainless steel buckets and layered into a Patterson-Kelly twin-shell blender. The chitosan beaker was rinsed twice with portions of the blank feed, added to the blender, and the formulation was mixed for 15 minutes. The dose formulations were prepared approximately monthly.
Chemical Lot Number
02-ASSF-0715
Maximum Storage Time
42 days
Storage Conditions
Stored in plastic-lined 5 gallon plastic buckets sealed with lids at −30° to −15°C
Study Laboratory
Battelle Columbus Operations (Columbus, OH)

Table F-3. Results of Analyses of Dose Formulations Administered to Rats in the Six-month Feed Study of Chitosan

Date Prepared	Date Analyzed	Target Concentration[a] (mg/g)	Determined Concentration[b] (mg/g)	Difference from Target (%)
August 15, 2006	August 17–18, 2006	10	9.1	−10
		30	27.3	−9
		90	83.5	−7
	October 2–3, 2006[c]	10	9.99	0
		30	30.3	+1
		90	92.4	+3
October 10, 2006	October 11–12, 2006	10	9.5	−5
		30	27.0	−10
		90	94.2	+5
January 2, 2007	January 2–3, 2007	10	10.6	+6
		30	29.5	−2
		90	94.3	+5

[a]10, 30, and 90 mg/g are equivalent to 1%, 3%, and 9% chitosan concentrations, respectively.
[b]Results of duplicate analyses.
[c]Animal room samples.

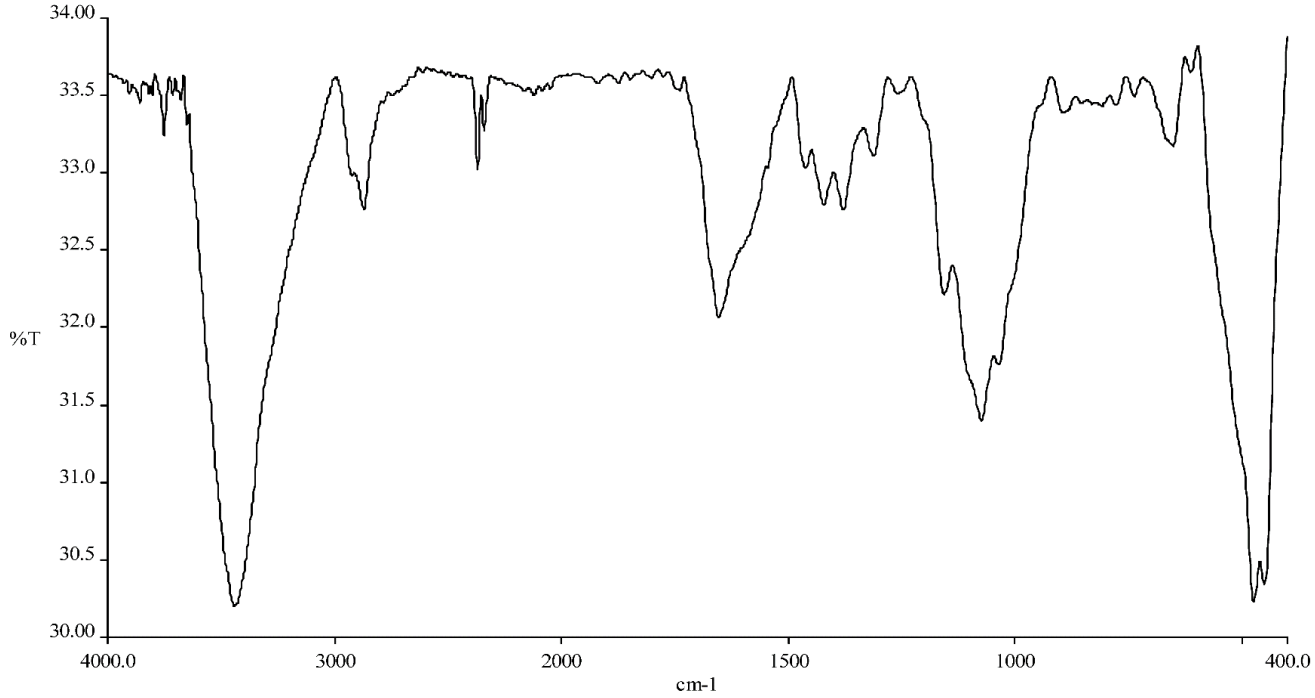

Figure F-1. Infrared Absorption Spectrum of Chitosan

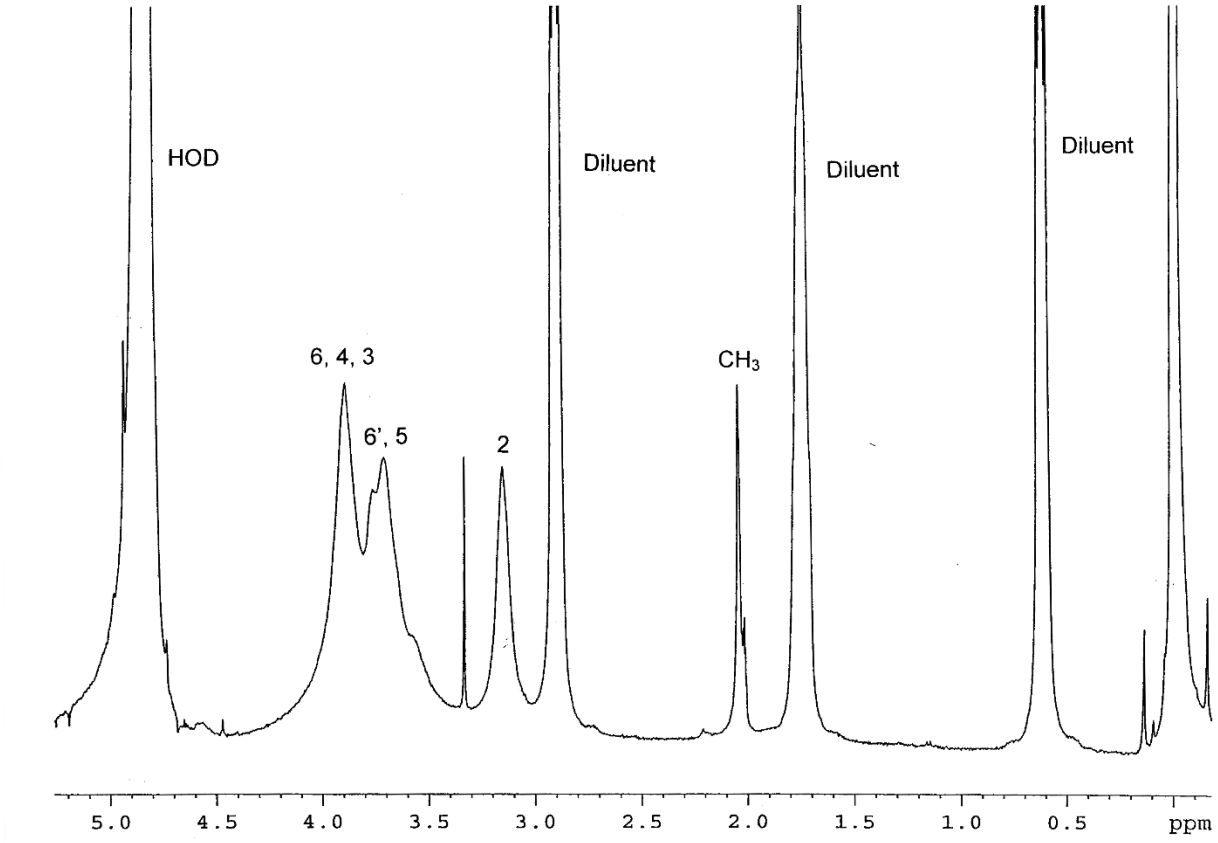

Figure F-2. Proton Nuclear Magnetic Resonance Spectrum of Chitosan

Appendix G. Feed and Compound Consumption in the Six-month Feed Study of Chitosan

Tables

Table G-1. Feed and Compound Consumption by Group A Male Rats in the Six-month Feed Study of Chitosan

Week	0%		1%			3%			9%		
	Feed[a] (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	22.2	238	23.8	243	980	23.6	242	2,929	21.4	243	7,931
2	21.6	297	23.1	308	750	23.0	303	2,278	26.9	265	9,137
3	22.8	346	24.0	359	668	25.4	354	2,156	27.3	307	8,002
4	22.6	388	23.5	404	582	24.9	398	1,877	26.7	350	6,872
5	22.3	421	23.5	438	537	25.8	436	1,774	27.4	388	6,355
6	21.5	446	21.7	465	467	24.6	464	1,591	26.4	413	5,759
7	23.1	475	23.5	493	477	25.7	491	1,570	27.8	442	5,662
8	22.3	496	24.1	513	470	25.2	514	1,471	27.1	464	5,259
9	22.3	514	24.1	535	450	25.1	534	1,411	26.7	483	4,980
10	21.8	529	23.2	554	419	25.1	548	1,373	26.2	498	4,735
11	21.8	543	23.3	570	409	25.5	566	1,353	25.5	511	4,493
12	21.6	554	23.1	585	395	24.5	579	1,270	26.4	521	4,557
13	22.3	563	22.6	598	378	24.8	584	1,274	26.9	527	4,595
14	21.5	578	22.9	612	374	25.7	602	1,280	26.4	544	4,371
15	21.7	587	22.7	622	365	25.5	613	1,249	26.7	557	4,315
16	22.2	597	22.7	631	360	27.0	620	1,306	28.5	565	4,542
17	23.4	607	23.7	645	367	28.0	634	1,325	29.2	575	4,569
18	23.6	614	24.6	657	375	28.4	646	1,320	29.0	584	4,468
19	22.3	624	23.0	667	345	26.6	657	1,214	27.8	595	4,202
20	23.6	633	23.2	677	343	25.4	664	1,148	27.4	600	4,112
21	23.8	643	23.4	689	340	24.8	670	1,110	28.5	606	4,230
22	24.1	653	23.1	700	330	25.7	677	1,139	26.9	612	3,959
23	22.6	665	21.3	707	301	25.7	686	1,125	25.4	615	3,715
24	21.5	666	19.6	704	278	24.9	689	1,084	25.4	612	3,738
25	21.2		20.4			24.7			27.3		
Mean for Weeks											
1–13	22.2	447	23.3	466	537	24.9	462	1,717	26.4	416	6,026
14–24	22.8	624	22.7	665	343	26.2	651	1,209	27.4	588	4,202

[a]Grams of feed consumed per animal per day.
[b]Milligrams of chitosan consumed per kilogram body weight per day.

Table G-2. Feed and Compound Consumption by Group A Female Rats in the Six-month Feed Study of Chitosan

Week	0%		1%			3%			9%		
	Feed[a] (g/day)	Body Weight (g)	Feed (g/day)	Body Weight (g)	Dose[b] (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)	Feed (g/day)	Body Weight (g)	Dose (mg/kg)
1	17.7	175	22.3	173	1,286	17.3	177	2,940	16.9	177	8,606
2	15.1	199	15.4	198	779	15.6	197	2,381	17.6	191	8,298
3	15.8	220	16.2	217	747	15.0	214	2,102	16.9	206	7,371
4	16.2	233	16.7	229	728	15.6	231	2,023	17.3	221	7,044
5	16.0	248	17.4	241	722	15.9	242	1,974	17.3	234	6,649
6	14.7	258	17.5	252	694	14.7	251	1,759	16.4	243	6,067
7	15.9	266	17.9	262	683	15.2	259	1,759	16.7	248	6,069
8	15.9	274	17.3	268	645	15.5	267	1,739	16.7	259	5,810
9	15.9	281	17.7	276	641	15.9	274	1,740	16.7	266	5,656
10	15.9	287	18.2	284	641	15.7	281	1,678	16.6	268	5,577
11	15.2	294	17.4	289	601	15.5	286	1,624	16.2	274	5,329
12	15.8	300	17.5	295	594	15.9	292	1,632	15.7	279	5,071
13	15.3	305	16.1	300	537	16.9	298	1,699	16.2	281	5,192
14	15.3	312	16.8	303	555	16.3	304	1,609	16.5	285	5,218
15	14.7	316	16.2	307	528	16.7	309	1,623	16.8	288	5,258
16	16.2	320	19.1	311	615	17.7	314	1,694	19.2	291	5,940
17	15.9	325	18.2	314	581	16.9	315	1,611	18.0	293	5,537
18	16.4	327	19.5	317	615	17.8	318	1,679	19.1	296	5,803
19	17.8	330	19.6	321	610	18.9	321	1,768	18.6	299	5,607
20	17.3	328	21.7	324	670	18.8	321	1,757	19.2	297	5,819
21	18.2	335	21.1	332	636	19.0	330	1,725	19.1	302	5,688
22	18.5	339	19.7	337	584	19.2	336	1,712	18.9	306	5,554
23	17.4	343	17.5	340	515	17.3	339	1,532	17.7	306	5,201
24	16.1	345	17.2	340	506	15.6	339	1,381	16.7	309	4,863
25	16.4		20.3			17.1			18.8		
Mean for Weeks											
1–13	15.8	257	17.5	253	715	15.7	251	1,927	16.7	242	6,364
14–24	16.7	329	18.8	322	583	17.7	322	1,645	18.2	297	5,499

[a]Grams of feed consumed per animal per day.
[b]Milligrams of chitosan consumed per kilogram body weight per day.

Appendix H. Ingredients and Nutrient Composition in AIN-93M Maintenance Purified Diet

Tables

Table H-1. Ingredients of AIN-93M Maintenance Purified Rodent Diet

Ingredients	Percent by Weight
Corn starch	46.5692
Dextrin	15.5000
Casein (vitamin free)	14.0000
Sucrose	10.0000
Powdered cellulose	5.0000
Soybean oil	4.0000
AIN-93M mineral mix	3.5000
AIN-93M vitamin mix	1.0000
Choline bitartrate	0.2500
L-Cystine	0.1800
t-Butylhydroquinone	0.0008

Table H-2. Vitamins, Minerals, and Nutrient Composition of AIN-93M Maintenance Purified Rodent Diet

	Amount
Vitamins	
A	4.00 IU/g
D_3 (added)	1.00 IU/g
E	78.80 IU/g
K (as menadione)	0.75 ppm
Thiamine hydrochloride	6.00 ppm
Riboflavin	6.50 ppm
Niacin	30.00 ppm
Pantothenic acid	16.00 ppm
Folic acid	2.10 ppm
Pyridoxine	5.80 ppm
Biotin	0.20 ppm
B_{12}	28.00 mcg/kg
Choline chloride	1,250.00 ppm
Ascorbic acid	0.00 ppm
Minerals	
Calcium	0.50%
Phosphorus	0.31%
Potassium	0.36%
Magnesium	0.05%

	Amount
Sodium	0.13%
Chlorine	0.20%
Fluorine	1.00 ppm
Iron	39.00 ppm
Zinc	35.00 ppm
Manganese	11.00 ppm
Copper	6.00 ppm
Cobalt	0.00 ppm
Iodine	0.21 ppm
Chromium	1.00 ppm
Molybdenum	0.14 ppm
Selenium	0.22 ppm
Typical Analysis	
Protein	13.06%
Fat	4.00%
Fiber	5.00%
Carbohydrate	73.80%
Metabolizable energy	3.83%

Appendix I. Sentinel Animal Program

Table of Contents

Tables

I.1. Methods

Rodents used in the National Toxicology Program are produced in optimally clean facilities to eliminate potential pathogens that may affect study results. The Sentinel Animal Program is part of the periodic monitoring of animal health that occurs during the toxicological evaluation of test compounds. Under this program, the disease state of the rodents is monitored via sera from extra (sentinel) animals in the study rooms. The sentinel animals and the study animals are subject to identical environmental conditions. Furthermore, the sentinel animals come from the same production source and weanling groups as the animals used for the studies of test compounds.

Blood samples were collected from each rat and allowed to clot and the serum was separated. All samples were processed appropriately and tested for the presence of pathogens at BioReliance Corporation (Rockville, MD) or the Research Animal Diagnostic Laboratory (RADIL), University of Missouri, Columbia, MO. The laboratory methods and agents for which testing was performed are tabulated below; the times at which samples were collected during the studies are also listed.

Blood was collected from five rats per sex per time point, except at study termination when blood was collected from four males and five females.

Table I-1. Laboratory Methods and Agents Tested for in the Sentinel Animal Program

Method and Test	Time of Collection
ELISA	
Kilham rat virus (KRV)	4 weeks
Pneumonia virus of mice (PVM)	End of quarantine, 4 weeks, study termination
Rat coronavirus/sialodacryoadenitis virus (RCV/SDA)	End of quarantine, 4 weeks, study termination
Rat parvovirus (RPV)	4 weeks
Sendai	End of quarantine, 4 weeks, study termination
Toolan's H-1 virus (H-1)	4 weeks
Immunofluorescence Assay	
H-1	4 weeks
KRV	4 weeks
Parvovirus	End of quarantine, 4 weeks, 6 weeks, study termination
RCV/SDA	End of quarantine
RPV	4 weeks
Multiplex Fluorescent Immunoassay	
H-1	6 weeks
KRV	6 weeks
Parvo NS-1	6 weeks
Rat minute virus	6 weeks
RPV	6 weeks

I.2. Results

A positive test result for parvovirus occurred in one animal at the 4-week timepoint; additional testing of serum from this animal and other sentinel animals via other testing methodologies deemed the original positive result to be a false positive. All other test results were negative for rodent pathogens.

National Toxicology Program

NTP Central Data Management, MD EC-03
National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709

http://ntp.niehs.nih.gov

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